ACTA MICROBIOLOGICA

ACADEMIAE SCIENTIARUM HUNGARICAE

ADIUVANTIBUS

E. FARKAS, J. HORVÁTH, S. KOTLÁN, R. MANNINGER,
A. PELC, K. RAUSS, J. SZIRMAI, J. WEISSFEILER

REDIGIT

G. IVÁNOVICS

TOMUS VIII FASCICULUS 1

AKADÉMIAI KIADÓ
BUDAPEST
1961

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STUDIES ON THE OLIGOSACCHARIDE-DECOMPOSING ACTIVITY OF YEASTS

By

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(Received October 31, 1959)

Summary. The first step of raffinose, lactose, saccharose, maltose fermentation, *i. e.* the cleavage of these oligosaccharides have been studied in *Saccharomyces carlsbergensis* and *Candida pseudotropicalis*. Live or acetone-treated cells and cell-free homogenates were used for these studies. The fermentation process was followed by paper chromatography.

It was stated that *S. carlsbergensis* cleaved raffinose and saccharose extracellularly and only the monosaccharides were taken up by the living cells. The cleavage of raffinose appeared to be a stepwise procedure as the cleavage of melibiose started only after the total raffinose available had been used up. We failed in demonstrating the extracellular cleavage of maltose. Such a process was observed only in acetone-treated *S. carlsbergensis* cells. Lactose was readily taken up by *Candida pseudotropicalis* and cleavage was only present in acetonized or homogenized cells.

Pre-fermentation hydrolysis of oligosaccharides has been demonstrated in the experiments and suggestions are made as to the possible cellular localization of this process.

The examination of the sugar fermentation of yeasts is of both taxonomic and physiologic importance, since the fermentation of certain mono- and oligosaccharides allows taxonomic conclusions to be drawn [1], while from a physiological point of view it supplies information about the permeation, decomposition and utilization as carbon and energy sources of the same substances [2]. Thus the former registers only the final result, while the latter examines the mechanisms as well.

In physiological studies, after having discovered the mechanism one may attempt to influence the processes both negatively and positively and one may examine the factors enhancing or inhibiting the metabolism of yeast cells.

In our laboratory we have studied the possible role of certain chronically administered drugs in provoking endogenous mycoses or in increasing the severity of an actual mycotic process by enhancing the metabolism of pathogenic yeasts, particularly *Candida albicans*. As a first step of this work the influence of the above substances on the metabolism of fungi, particularly the sugar fermentation of yeasts was examined [3]. In order to specify the site of action in the sequence of reactions, we started with the examination of the first step of oligosaccharide fermentation, *i. e.* their cleavage to monosaccharides. The present paper summarizes our general observations.

Materials and methods

The examinations were performed with the tri- and disaccharides raffinose, lactose, saccharose and maltose. The decomposition of raffinose, saccharose and maltose was examined with *Saccharomyces carlsbergensis*, that of lactose with *Candida pseudotropicalis*. The experiments were performed both with live and acetone-treated cells, and cell-free homogenates. The changes taking place during utilization of the sugars were followed by paper chromatography in every case.

Fungus strains were cultivated in Roux flasks on the molasses agar of CSILLAG [4], incubating for 4 days at room temperature. Details of further treatment of the cultures are given in Fig. 1. Centrifugation was performed at 4000 rpm. for 30 minutes. For acetone treatment cold (+4° C) acetone was used. The cell sediment was resuspended by a stepwise addition of an equal volume of acetone. The acetone treatment lasted 10 minutes on every occasion. For homogenization centrifuged cells cooled to +4° C were ground with a double volume of quartz sand for 10 minutes; both the grinder and the sand were cooled. Live cells, acetone powder and homogenates were suspended in the same volume of phosphate buffer each. M/15 pH 7.2 phosphate buffer was used throughout. The homogenate was centrifuged at about 6000 rpm. for 30 minutes in order to facilitate G—5 filtration. The material was incubated in a 1 per cent sugar solution except for raffinose which was used in a 2 per cent concentration; samples were taken at certain intervals. The quantity of the inoculum was changed by diluting the material in order to accelerate or slow down the process. The actual quantities of yeast used in the single experiments are given in the legend to the Figures. Incubation volume was 3 ml each, in Widal tubes. At certain intervals an aliquot part was removed and fermentation stopped by exposure to 100° C for 2 minutes in a water bath. The material was centrifuged and from the supernatant 20 μl samples were transferred by means of a micropipette to the chromatography paper. The scheme of the procedure is shown in Fig. 1.

Paper chromatography was performed according to our own method [5] using a solvent mixture generally applied for the separation of organic acids (butanol-glacial acetic acid-water = = 4 : 1 : 1). The ascending technique was applied for 24 hours using an unspecified lot of medium tight filtre paper. Developing was carried out after a benzidine spray in furfurol vapour without heating [5].

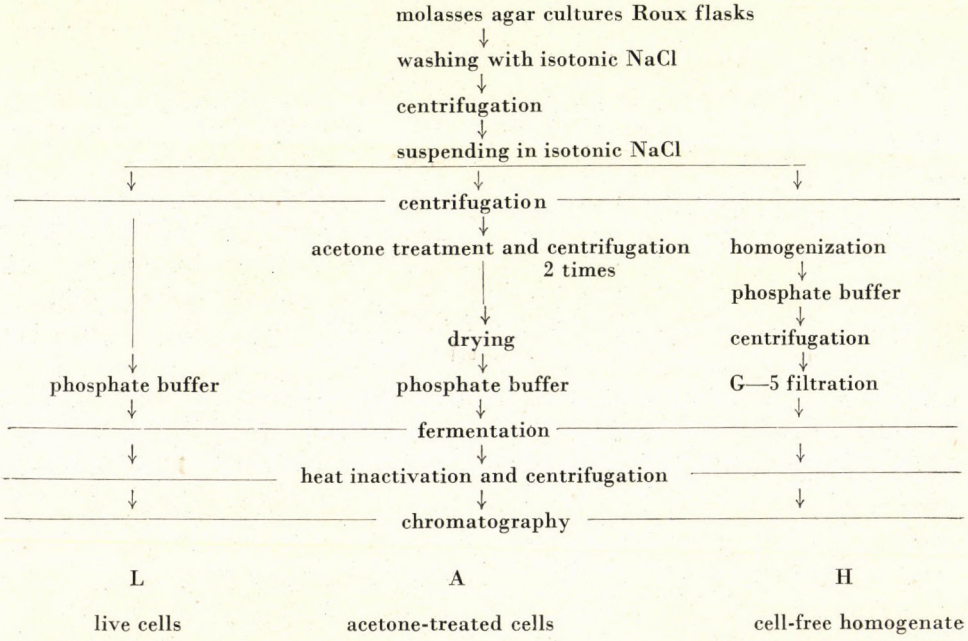

Fig. 1. Procedure

Experimental

The results of the experiments are presented in the chromatograms.

Raffinose decomposition of the live cells is presented on the chromatograms in Figs. 2*a*, *b*, *c*.

The chromatograms show the extracellular and stepwise character of raffinose decomposition. Fructose, the split product of raffinose decomposition did not appear on the chromatograms, it was probably immediately taken up by the cells. By using 3 different sizes of inoculum the 3 phases of the process could be demonstrated separately. The first step was the decomposition of raffinose to melibiose and the third the utilization of galactose or glucose. Every step was preceded by the consumption of the substrate available from the previous phase of the process.

The raffinose decomposing activity of the acetone-treated cells is shown in Fig. 3*a*, *b*.

Raffinose decomposition was stepwise in acetone-treated cells as well. In this case it was possible to demonstrate fructose. Moreover, by applying a heavy inoculum (Fig. 3*b*) the synthesis of a sugar-like material of low R_f value was observed. The stretched-out spots in the middle of the chromatogram suggested a simultaneous presence of both glucose and galactose.

The results achieved with the cell-free homogenates are presented in Fig. 4*a*, *b*.

The stepwise cleavage was obvious here, too, and the synthesis of the sugar-like material could also be observed.

Lactose utilization of the live cells is presented on Fig. 5.

Fig. 5 demonstrates the fact that lactose is taken up by the cells without cleavage.

The lactose decomposing activity of the acetone-treated cells is demonstrated on Fig. 6.

After acetone treatment the lactose decomposing activity became extracellular. The chromatogram suggests a re-synthesis of lactose.

The lactose decomposing activity of the cell-free homogenate is demonstrated on Fig. 7.

In the cell-free homogenate again a re-synthesis of lactose and synthesis of a sugar-like material is observed.

Sucrose decomposition by live cells is presented on Fig. 8*a*, *b*.

The saccharose inversion by live cells is extracellular.

The sucrose-decomposing activity of acetone-treated cells is demonstrated on Figs. 9*a*, *b*. 14 and 15.

Acetone treatment had no influence on sucrose cleavage. In experiments with a heavy inoculum (Fig. 9*b*) the synthesis of a sugar-like material ensued also under these conditions. After 72 hours (and partly also after 48 hours) glucose and fructose appeared as split products of the newly-synthetized sugar.

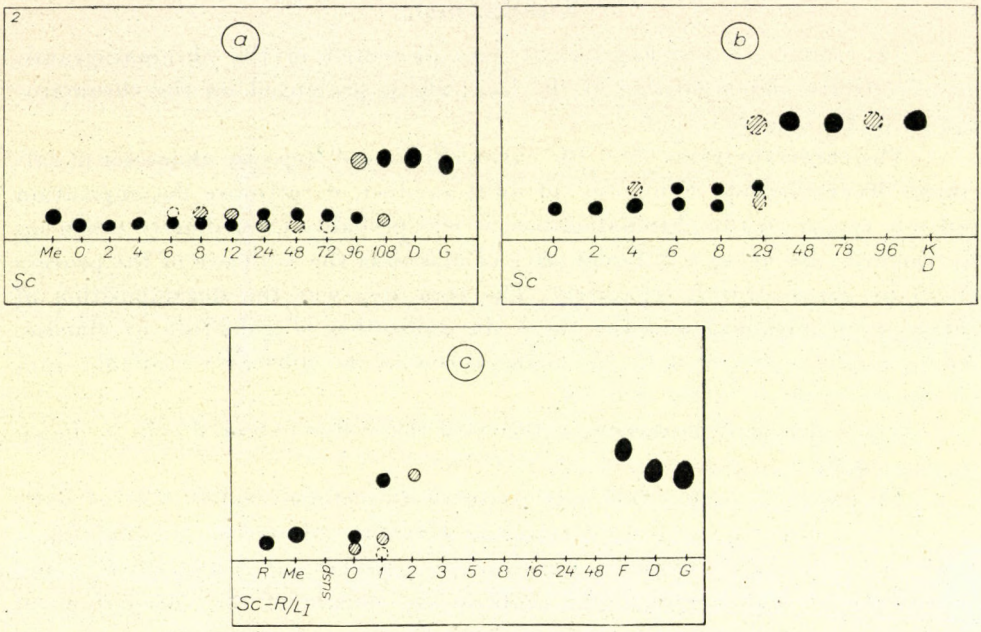

Fig. 2a, b, c. Paper chromatogram of raffinose decomposition by live *Saccharomyces carlsbergensis* cells (drawing from the original)

a) Controls: left melibiose, right glucose and galactose. The figures on the start line demonstrate the sampling intervals in hours following inoculation. Inoculum: 5 mg of live cells (wet weight)
b) Control: glucose, right. The figures on the start line demonstrate the sampling intervals in hours following inoculation. Inoculum: 50 mg of live cells (wet weight)
c) Left: raffinose, melibiose and cell suspension controls. Right: fructose, glucose and galactose controls. The figures on the start line demonstrate the sampling intervals in hours following inoculation. Inoculum: 2 g of live cells (wet weight)

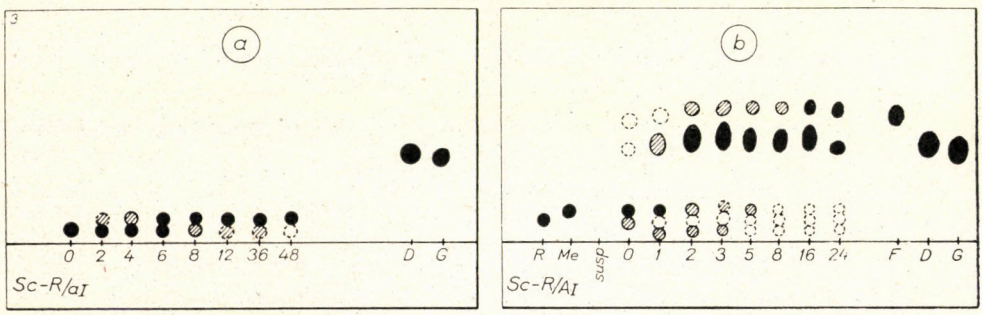

Fig. 3a, b. Paper chromatogram of raffinose decomposition by acetone-treated *Saccharomyces carlsbergensis* cells (drawing from the original)

a) Right: glucose and galactose controls. The figures on the start line demonstrate the sampling intervals in hours following inoculation (0 to 48 hours). Inoculum: acetone-treated cells (50 mg live wet weight)
b) Left: raffinose, melibiose and cell suspension controls. Right: fructose, glucose and galactose controls. The figures on the start line demonstrate the sampling intervals in hours following inoculation. Inoculum: acetone-treated cells (2 g live wet weight)

The sucrose-decomposing activity of the cell-free homogenate is demonstrated on Fig. 10*a*, *b*.

Sucrose cleavage was accomplished accordingly also by a cell-free homogenate.

Heavy inocula were passed through a Seitz filtre instead of a G-5 filtre and consequently — as observed under the microscope — certain cell particles passed into the filtrate, though no presence of undamaged cells could be de-

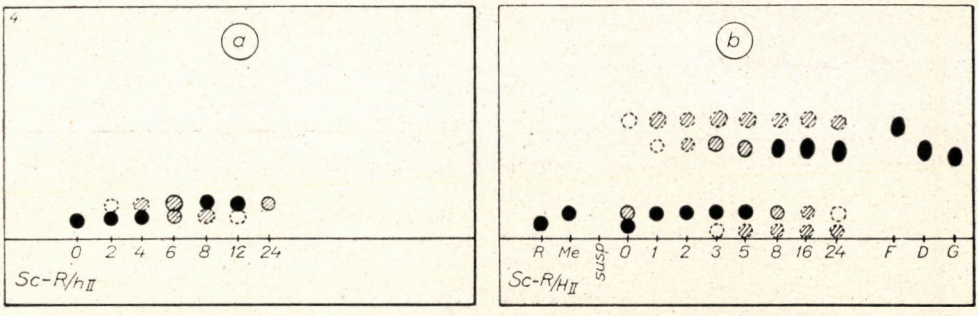

Fig. 4a, b. Paper chromatogram of raffinose decomposition by a cell-free homogenate of *Saccharomyces carlsbergensis* (drawing from the original)

a) The figures on the start line demonstrate the sampling intervals following inoculation from 0 to 24 hours. Inoculum: homogenized cells (50 mg live wet weight)

b) Left: raffinose, melibiose and cell suspension controls. Right: fructose, glucose and galactose controls. The figures on the start line demonstrate the sampling intervals in hours following inoculation. Inoculum: homogenized cells (2 g live wet weight)

tected. The presence of these cell particles accounted for the disappearance of monosaccharide components from the solution. The synthesis of a sugar was also encountered in this case, which was however utilized later.

Maltose-decomposing activity of live cells is demonstrated on Fig. 11*a*, *b*.

The maltose uptake of live cells is a slow process and free glucose could be demonstrated in only one case with a heavy inoculum.

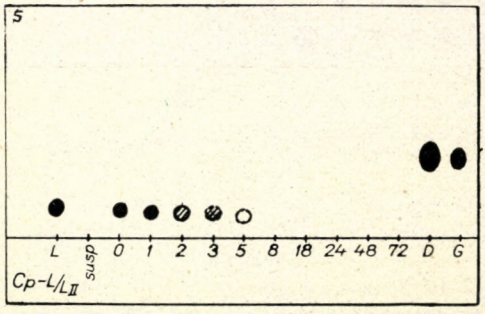

Fig. 5. Lactose decomposition by live *Candida pseudotropicalis* cells (drawing from the original) Left: lactose and cell suspension controls. Right: glucose and galactose controls. The figures on the start line demonstrate the sampling intervals in hours following inoculation from 0 to 72 hours. Inoculum: 2 g live cells (wet weight)

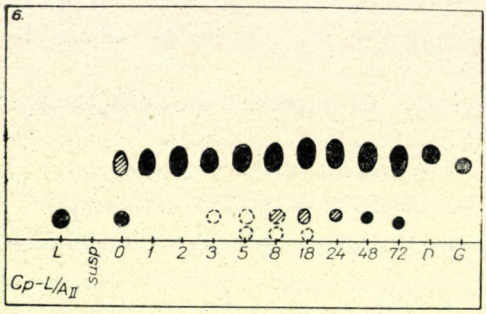

Fig. 6. Lactose decomposition by acetone-treated *Candida pseudotropicalis* cells (drawing from the original)

Left: lactose and cell suspension controls. Right: glucose and galactose controls. The figures on the start line demonstrate the sampling intervals in hours following inoculation. Inoculum: acetone-treated cells (2 g live wet weight)

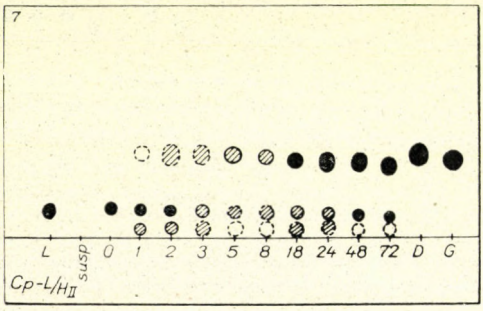

Fig. 7. Lactose decomposition by a cell-free homogenate of *Candida pseudotropicalis* (drawing from the original)

Left: lactose and cell suspension controls. Right: glucose and galactose controls. The figures on the start line demonstrate the sampling intervals in hours following inoculation. Inoculum: homogenized cells (2 g live wet weight)

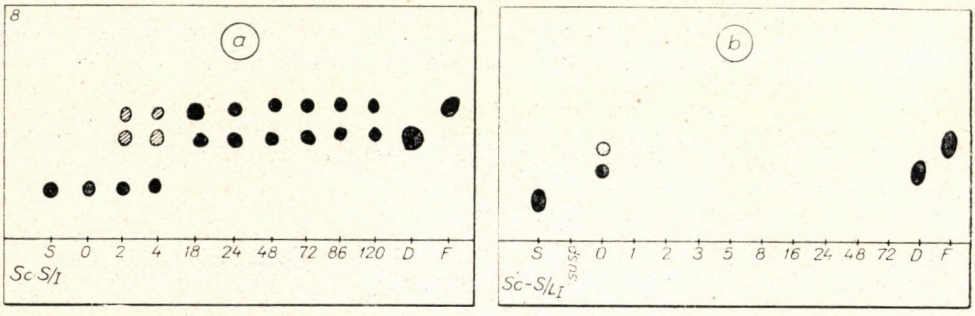

Fig. 8a, b. Sucrose decomposition by live *Saccharomyces carlsbergensis* cells (drawing from the original)

a) Left: sucrose control. Right: glucose and fructose controls. The figures on the start line demonstrate the sampling intervals in hours following inoculation. Inoculum: 50 mg live cells (wet weight)

b) Left: sucrose and cell suspension controls. Right: glucose and fructose controls. The figures on the start line demonstrate the sampling intervals in hours following inoculation (0 to 72 hours). Inoculum: 2 g live cells (wet weight)

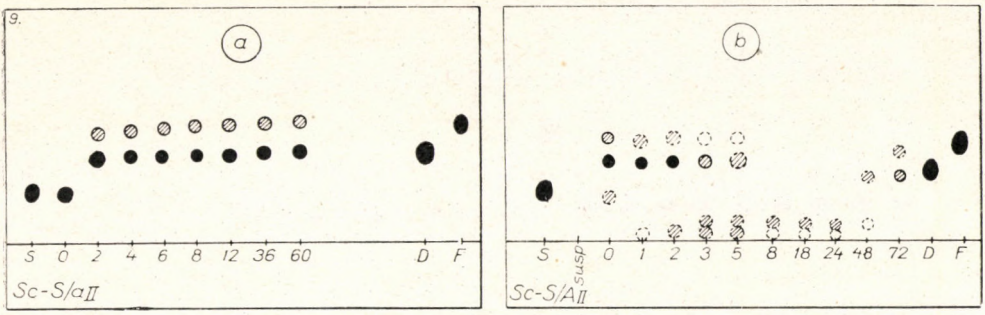

Fig. 9a, b. sucrose decomposition by acetone-treated *Saccharomyces carlsbergensis* cells (drawing from the original)

a) Left: sucrose control. Right: glucose and fructose controls. The figures on the start line demonstrate the sampling intervals in hours following inoculation (0 to 60 hours). Inoculum: acetone-treated cells (50 mg live wet weight)

b) Left: sucrose and cell suspension controls. Right: glucose and fructose controls. The figures on the start line demonstrate the sampling intervals in hours following inoculation. Inoculum: acetone-treated cells (2 g live wet weight)

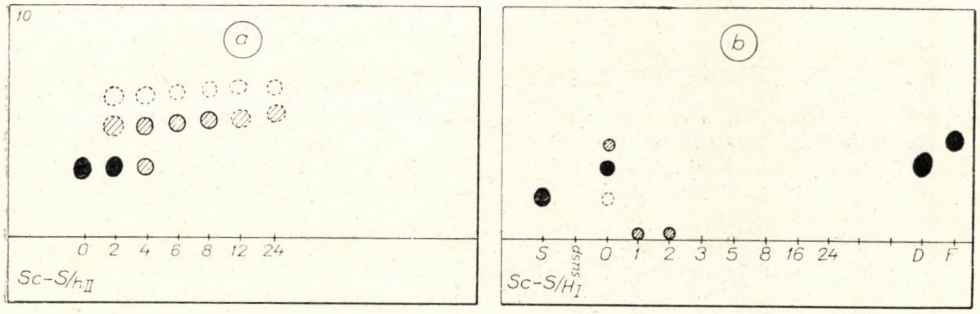

Fig. 10a, b. Sucrose decomposition by a cell-free homogenate of *Saccharomyces carlsbergensis* (drawing from the original)

a) The figures on the start line demonstrate the sampling intervals in hours following inoculation (0 to 24 hours). Inoculum: homogenized cells (50 mg live wet weight)

b) Left: sucrose and cell suspension controls. Right: glucose and fructose controls. The figures on the start line demonstrate the sampling intervals in hours following inoculation (0 to 24 hours). Inoculum: homogenized cells (2 g live wet weight). Instead of G-5, Seitz filtre was used

The maltose-decomposing activity of acetone-treated cells is shown on Fig. 12*a, b*.

The cleavage of maltose to glucose could be demonstrated by applying a heavy inoculum. The synthesis of a sugar-like material was again observed.

The maltose-decomposing activity of a cell-free inoculum is demonstrated on Fig. 13.

In the homogenate passed through G-5 and Seitz filtres no maltose cleavage could be demonstrated.

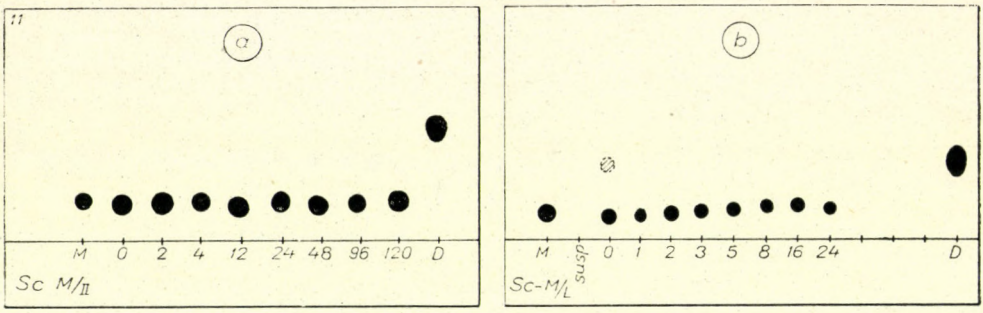

Fig. 11a, b. Maltose decomposition by live *Saccharomyces carlsbergensis* cells (drawing from the original)

a) Left: maltose control. Right: glucose control. The figures on the start line demonstrate the sampling interval in hours following inoculation. Inoculum: 50 mg live cells (wet weight)
b) Left: maltose and cell suspension controls. Right: glucose controls. The figures on the start line demonstrate the sampling intervals in hours following inoculation. Inoculum: 2 g live cells (wet weight)

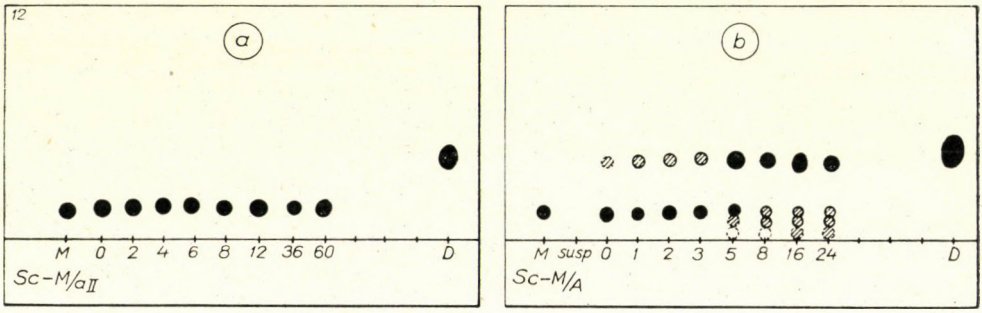

Fig. 12a, b. Maltose decomposition by acetone-treated *Saccharomyces carlsbergensis* cells (drawing from the original)

a) Left: maltose control. Right: glucose control. The figures on the start line demonstrate the sampling intervals in hours following inoculation (0 to 60 hours). Inoculum: acetone-treated cells (50 mg live wet weight)
b) Left: maltose and cell suspension controls. Right: glucose control. The figures on the start line demonstrate the sampling intervals in hours following inoculation. Inoculum: acetone-treated cells (2 g live wet weight)

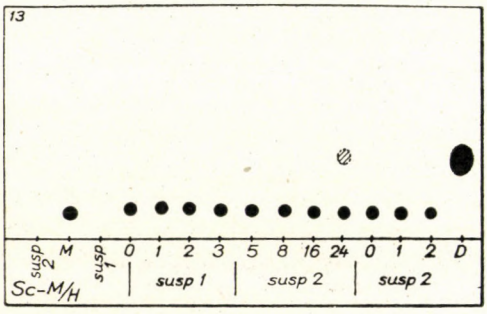

Fig. 13. Maltose decomposition by a cell-free homogenate of *Saccharomyces carlsbergensis* (drawing from the original)
Left: suspension 2, maltose and suspension 1. controls. Right: glucose controls. The figures on the start line demonstrate the sampling intervals in hours following inoculation. Inoculum: homogenized cells (2 g live wet weight).
Suspension 1: G—5 filtered homogenate
Suspension 2: Seitz-filtered homogenate

Discussion

The present findings concerning raffinose decomposition supported our earlier results. In the course of developing our chromatography method for the quick examination of the fermentation quotient we already observed that the 3/3 raffinose fermentation was a stepwise process [5]. The first split products were melibiose and fructose. After the utilization of fructose, melibiose was hydrolized to glucose and galactose. Of these products glucose was fermented first.

The stepwise character of raffinose decomposition was also obvious with acetone-treated cells and the cell-free extracts of homogenates.

The stepwise nature of decomposition can be explained only on an enzyme-biological basis. The possibility of an adaptive enzyme synthesis in the sequence of the appearance of substrates has been excluded by the results of experiments carried out with acetone-treated cells and cell-free homogenates. Though the stepwise character of decomposition was demonstrated also in these systems, the single steps seemed to be too short to permit any enzyme synthesis. According to the genetic examinations by LOSADA [7] and WINGE and ROBERTS [8] strains with *e. g.* gen g_s (slow galactozymase synthetizer) may need as much as some days to accomplish galactose fermentation. Consequently, it is the enzyme synthesis and not the enzymatic reaction that proceeds some orders of magnitude slower. In our experiments the original presence of the enzymes was clearly demonstrated by the results obtained with treated cells. Nevertheless, the undamaged structure has a certain role in the regulation of enzymatic reactions, as the same quantity of live material required more time for the process with undamaged cells than with acetone-treated or homogenized cells.

It was remarkable that the coordination of the individual enzymatic reactions corresponded to the stepwise sequence of the raffinose decomposition process of the individual types, *i. e.* 3/3 yeasts exhibited first a behaviour as 1/3 ones and next as 2/3 ones. The above observation has suggested the correspondence of this line to the sequence of evolution of the strain. For the time being there is no possibility to decide whether the sequence of strain evolution follows a 1/3 to 3/3 line or vice versa. We may, however, consider the progressive process as the more probable one, as an 1/3 fermentation may be initiated by the presence of invertase, an enzyme frequently present in yeasts.

As to other organisms, raffinose decomposition does not inevitably start with an invertase effect, *e. g. Microsporum gypseum* and *M. canis* are fermenting raffinose but not sucrose [9]. The raffinose-decomposing activity of certain *E. coli* strains is also enhanced by α-galactosidase [10], thus leaving the sucrose intact and utilizing only galactose. At present only one

yeast is known to cleave raffinose without invertase by α-galactosidase (melibiase) activity [11]. The split-product galactose is chiefly utilized by oxidation.

The sugar synthesis by acetone-treated cells and cell-free homogenates is not surprising in the light of the data on oligosaccharide-synthesis by extracts with hydrolyzing activity [12], and the Taka diastase produced from moulds is also able to exhibit transfructosidation by transferring the fructose component of raffinose to another sugar, *e. g.* glucose [13].

Lactose is taken up by live cells without extracellular hydrolysis. Hydrolysis may, however, be demonstrated in the fermentation fluid of acetone-treated cells and cell-free homogenates. Accordingly, lactose decomposition may occur either when passing through the cell wall or within the cell. Acetone treatment either demasques cell-wall enzymes, or — supposing an intracellular hydrolysis — enhances a re-flow of products through the damaged cell wall into the fermentation fluid. In this case the cleavage occurs either actually within the cell or by an enzyme loosely bound to the cell wall, which enzyme passes into the solution after the removal of cell debris following homogenization. Up to now there has been no explanation why the split products do not disappear from the fermentation fluid of acetone-treated cells and cell-free homogenates. One might suppose the presence of a correlation between the appearance of the splitting activity and the persistence of the split products in the medium.

As to the mechanism of lactose fermentation, opinions still differ. Hydrolysis and direct fermentation by phosphorolysis or phosphorilation both have their own experimental proofs. The fact that certain yeasts are fermenting lactose quicker than the building-stone monosaccharides [15] is no clear evidence of direct fermentation as the complex process may be influenced by permeability. More suggestive are the findings by Rogosa [16], Kluyver and Custers [17] and Willstätter and Oppenheimer [18]. According to Rogosa [16], lactose was fermented by a yeast strain which did not ferment galactose in a galactose-glucose mixture; nevertheless it is still to be decided whether the galactose was permeating (a glucose-adapted strain being used) or rather the lactose had done so owing to its glucose component. It is well-known, however, that sugars enter the cells by active transport [18]. Cluyver and Custers [17] found that *Blastodendrium intermedium* was not respirating on lactose, only on a glucose substrate. Accordingly there is no free glucose formed in the case of lactose fermentation, as the yeast does not consume oxygen. Willstätter and Oppenheimer [18] stated that the lactase content of yeasts does not suffice to ensure a higher fermentation rate for lactose than for monosaccharides, thus fermentation does not seem to start with hydrolysis. In bacteria *(Streptococcus lactis)* Novikova [20, 21] found some proof of phosphorolysis, namely lactose decomposition required phosphate, the labile

organic phosphate content increased and glucose-1-P was also utilized if added as substrate.

The opposed view is supported by the data of ROGOSA [16], who observed that a strain adapted to lactose, became simultaneously adapted to galactose as well, though the latter sugar had never been used for nutrition of the strain in question. In FISCHER's [22, 23, 24] view the fermentation of polysaccharides is generally preceded by hydrolysis. In the manufacturing of dairy products lactose is usually rendered digestible by yeast lactase hydrolysis [25, 26, 27]. This procedure is performed by intact cells, though the zymase complex is inactivated by heating or cooling in order to prevent the loss of sugar.

The possibility of polysaccharide synthesis has already been discussed in connection with raffinose.

The extracellular inversion of sucrose takes place quickly and live cells take up the split products glucose and fructose at an equal speed. Both acetone-treated cells and cell-free extracts are inverting sucrose rapidly nevertheless they seem to ferment fructose still quicker. This supports the view that the utilization of sugar by live cells is determined by the permeation of glucose and fructose; while in acetone-treated cells and cell-free extracts the permeability factor is abolished and only their turnover rate controls the velocity of utilization.

As to sucrose fermentation, the results of KOSIKOV, GELYMAN and RAEVSKAYA [28] are of great interest. They observed that even species deprived of invertase fermented saccharose, and demonstrated that in these cases the role of invertase (β-fructosidase) was taken over adaptively by maltase (α-glucosidase). According to DOUDOROFF, KAPLAN and HASSID [29] *Pseudomonas saccharophila* cleaves sucrose by phosphorolysis.

In the course of maltose fermentation by live cells usually not free glucose can be demonstrated although with the use of heavy inocula free glucose is present in a short early phase. We think that in live cells the cleavage of sugar may take place during permeation of the cell wall. Glucose was,however, demonstrated when using acetone-treated cells and this fact supports the view that a cleavage might be localized in the cell wall. This is further proved by the results obtained with cell-free homogenates not decomposing maltose. This makes it highly probable that the maltase had been removed together with the cell wall debris during the extraction procedure.

In the case of maltose it is generally supposed that the intact molecule is permeating the cells [2]. It has been observed, however, that maltose fermentation might be stimulated by minute amounts of glucose, fructose and traces of an unidentified substance [30]. Nevertheless, no exact data are available as to the possible role of the above substances.

Generally, the extracellular localization of cleavage is considered more probable, as the speed, *i. e.* the rate of cleavage, is the same in both live and

acetone-treated cells. Had on the contrary the monosaccharide content of the medium originated from re-permeation this process should have been greatly enhanced by acetone treatment.

The chromatograms have demonstrated also the fact that live cells, acetone-treated cells and cell-free extracts equally synthetize one or more sugar-like compounds out of which one component mostly proved to be the sugar decomposed at the start. Nevertheless, for the time being we do not wish to enter into a detailed discussion of this problem.

In the case of live cells the possibility of a re-permeation of monosaccharides from the cells is contradicted by the results of the lactose-decomposition experiment in the course of which free monosaccharides could only be demonstrated in the fermentation fluid containing acetone-treated cells. The extracellular activity of invertase in yeasts is a known fact [2]. The 0-time samples obtained from experiments carried out with heavy inocula (live cells used with raffinose and sucrose) also support the view that the permeation of the sugar and the re-permeation of the components after cleavage can be avoided. According to the chromatograms, 0-time samples exhibited a remarkable degree of cleavage. This greatly supports the probability of an extracellular cleavage.

Our experimental results have generally suggested the occurrence of cleavage previous to fermentation; this process, however, appears to be intracellular in some and extracellular in other cases.

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AETIOLOGICAL AND EPIDEMIOLOGICAL STUDIES OF POLIOMYELITIS IN SZEGED, 1956—1959

By

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Summary. Enterovirus isolations carried out in Szeged in the years 1956—1959 are summarized. One hundred and ninety strains, including 116 strains of poliovirus, were isolated. Data on the incidence of neutralizing antibodies against the three types of poliovirus in the sera of inhabitants of Szeged are presented. Factors influencing the course of the country-wide epidemics in the years 1957 and 1959 are discussed.

In Hungary the first attempts to demonstrate poliovirus by laboratory methods were made in 1952 [1]. Since tissue culture methods had become available, FÖLDES [2, 3, 4], PINTÉR et al. [5, 6], MOLNÁR and FORNOSI [7, 8] and MOLNÁR [9] have reported on the isolation and investigation of enteroviruses including poliovirus strains.

In the present report the results of the diagnostic examinations for enteroviruses carried out in this Institute from 1956 to 1959 are summarized. Most of the specimens examined derived from patients with poliomyelitis, the rest, from patients with diarrhoea or less definable minor illnesses. Furthermore the incidence of neutralizing antibodies against poliovirus in the sera of inhabitants of the town Szeged and its surroundings are presented.

Materials and methods

Tissue cultures. Human embryonic cell cultures; HeLa cells prepared as published previously [10]; human amnion cell cultures by a method [11] essentially the same as described by ZITCER et al. [12].

Faecal samples were stored at —15°C, then suspended 1 : 10 Hanks' solution containing 500 I. U. penicillin and 500 μg streptomycin per ml. The suspensions were centrifuged at 3000 r. p. m. for 25 minutes. The supernatants were centrifuged again, at 10 000 r. p. m. for 30 minutes. Tissue cultures were inoculated with 0.2 ml of the second supernatants. Throat swabs were placed in 2 to 3 ml Hanks' solution, and the extracts were inoculated into tissue cultures, 0.2 ml per tube.

Suckling mice one or two days of age were subcutaneously inoculated with 0.03 ml faecal suspension.

Neutralization tests. To a virus suspension containing about 100 TCD 50 per 0.1 ml the same volume of typing serum was added, and the serum-virus mixture was incubated at 37° C for 1 hour. Tissue cultures were inoculated with 0.2 ml of the mixture. Hyperimmune sera against each of the three types of poliovirus were prepared in rabbits: ECHO reference sera were kindly supplied by the *Virus Laboratory of Sheffield University*.

Human sera were inactivated at 56° C for 30 minutes, diluted 1 : 4 and tested against 100 TCD_{50} of each of the three types of poliovirus (Mahoney, MEF_1 and Saukett).

Results

One hundred and ninety virus strains were isolated from faecal specimens obtained from different parts of Hungary during the period 1956—1959. Of the isolated strains 116 proved to be poliovirus.

In 1956 nine strains were isolated, all from faecal samples. Seven of them were poliovirus, *viz.* 3 type 1, 3 type 2 and 1 type 3 virus.

A poliomyelitis epidemic occurred in Hungary in 1957. The incidence rate was higher than ever before [13]; for the town Szeged it was 27 per 100 000 inhabitants.

It was attempted to classify the cases yielding virus according to the clinical picture. Though the grouping was to some extent arbitrary, it essentially agreed with the principles of classification generally accepted. "Typical", "abortive" and "questionable" cases of poliomyelitis, "non-polio" and "abacterial meningitis" were distinguished. Cases with permanent paralysis, needing after-care, were regarded clinically "typical"; patients showing paresis but soon completely recovered were classified with the group of "abortive" cases; neurological cases with uncertain symptoms not characteristic of poliomyelitis were regarded as "questionable". The distribution of the cases yielding virus according to the clinical picture are presented in Table I.

Table I

Distribution of the virus strains isolated in 1957, according to the clinical picture

Virus	Clinical picture				Total
	typical	abortive	non polio	abacterial meningitis	
Poliovirus 1	18	4			22
Poliovirus 2	1				1
Poliovirus 3	2				2
ECHO		5	2		7
Unidentified cytopathogenic agent		2	1		3
Coxsackie				11	11
Total:	21	11	3	11	46

Table I shows that the great majority of the strains derived from "typical" cases in various parts of Hungary were type 1 poliovirus strains. The same was found by MOLNÁR [9]. This fact proves the predominance of this type of virus in the 1957 epidemic. From cases of abacterial meningitis occurring with a particular high frequency in September, 1957, Coxsackie A strains were recovered. Of the seven ECHO strains in Table I, three belonged to type 1, two to type 8, and two to type 9.

During this epidemic, the frequency of neutralizing antibodies against the three types of poliovirus in the sera of inhabitants of Szeged was also studied. Most of the sera tested were obtained from children. The sera were sent in from May to mid-July, 1957.

Table II

Incidence of neutralizing antibodies against poliovirus in the sera of persons of different age

Age group, years	Number of sera tested	Sera neutralizing poliovirus					
		type 1		type 2		type 3	
		Number	Per cent	Number	Per cent	Number	Per cent
1/2—2	30	21	70	5	17	6	20
3—7	8	6	75	4	50	4	50
8—15	29	19	65	16	55	17	59
16 and above	14	12	86	9	64	9	64

As shown in Table II, the incidence rates of type 2 and 3 antibodies in the different age groups approximately agreed with the rates published by other authors, whereas type 1 antibody under 7 years of age was remarkably more frequent than expected. The high incidence might be attributed to the widespread frequency of poliomyelitis infection during the epidemic.

In 1958, when the incidence of poliomyelitis was unusually low (1.7 per 100 000 population), few faecal specimens were examined. Ten strains were isolated: five type 1, one type 3 strain, and four unidentified strains being neither polio nor Coxsackie virus. Of the six cases yielding poliovirus only two were "typical".

1959 was another epidemical year. The morbidity rate for Szeged was 13 per 100 000 population. During this epidemic 78 poliovirus strains were isolated, 75 of these belonging to type 1 and 3 to type 3. Besides, 46 Coxsackie B strains, and an unidentified strain, were isolated. The strains obtained were classified according to the clinical symptoms (Table III).

The high incidence of poliovirus in the faeces of non-poliomyelitis cases was remarkable. Six such patients harboured poliovirus in their throats as well.

In the same year faecal specimens of children admitted to the *Department of Paediatrics, University Medical School, Szeged*, with enteric symptoms, chiefly diarrhoea, were also examined. Coxsackie B virus was isolated from most of these faeces. It was remarkable that the frequency of poliovirus increased continuously from April to August and declined suddenly thereafter. Unlike this, the incidence of Coxsackie B strains was the highest in August and September.

Table III

Distribution of the virus strains isolated in 1959, according to the clinical picture

Virus	Clinical picture					Total
	typical	abortive	questionable	non polio	abacterial meningitis	
Poliovirus 1	46	4	2	17+6*		75
Poliovirus 2						
Poliovirus 3				3		3
Coxsackie B.....		3		32+10*	1	46
Unidentified cytopathogenic agent		1				1
Total:	46	8	2	52+16*	1	125

* Isolated from throat swab.

After the 1959 epidemic, in December, we tested 42 sera for neutralizing antibodies against the three types of poliovirus. The results are summarized in Table IV.

Table IV

Incidence of neutralizing antibodies against poliovirus in the sera of persons of different age

Age group, years	Number of sera tested	Sera neutralizing					
		type 1 poliovirus		type 2 poliovirus		type 3 poliovirus	
		Number	Per cent	Number	Per cent	Number	Per cent
3—7	31	25	81	17	55	13	42
Over 16	11	9	82	6	54	7	64

Although the number of sera tested is too small for drawing definite conclusions, the high incidence of type 1 antibodies in the age group from 3 to 7 years is remarkable and cannot be attributed to the preceding Salk vaccination, as antibodies against type 2 and type 3 were considerably less frequent. Immune response to type 2 virus was generally the best as demonstrated by other authors [14].

Discussion

The period 1956—1959 was of great importance in the history of poliomyelitis and of enterovirus infections in Hungary. The country-wide epidemics in the years 1957 and 1959 exceeded all the poliomyelitis epidemics registered

in Hungary until then. The type distribution of the few poliovirus strains isolated in this laboratory does not indicate the predominance of type 1 in 1956. Nevertheless, taking into consideration the high proportion of type 1 strains in MOLNÁR's material, the limited outbreak in 1956 was probably the precursor of the country-wide epidemic in 1957.

According to PETRILLA [13], the 1957 epidemic came to an end at an earlier season than poliomyelitis epidemics usually do in Hungary. He tends to attribute this phenomenon to the Salk vaccination campaign in face of the epidemic.

The low incidence of poliomyelitis in 1958 — based on the studies of DÖMÖK et al. [15] — might be explained at least partly by the interfering effect of the Coxsackie B-3 virus, which caused a country-wide Bornholm epidemic in the summer of the same year. Our observations in Szeged in 1959 were similar. After the poliomyelitis epidemic had come to an end Coxsackie B-3 strains were chiefly isolated.

It should be noted that in 1959 HeLa cells were exclusively used. This might explain why neither ECHO nor Coxsackie A strains were isolated in that year.

We think that the current vaccination campaign with SABIN's live attenuated vaccine will greatly alter the epidemiological character of poliomyelitis in Hungary. The investigations presented here might be useful for evaluating the postvaccination changes in the epidemiology of poliomyelitis.

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A SIMPLE AND PRACTICAL METHOD FOR THE PURIFICATION OF DIPHTHERIA TOXOID

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Summary. A practical procedure has been worked out for the purification of diphtheria toxoid. The formalin treatment of high value toxin, obtained from standing cultures grown on digested meat media and harvested at optimum time, is performed with a calculated amount of formalin at an adequate temperature. The toxoid thus produced is precipitated with trichloracetic acid in the presence of sodium trichloracetate ions, at a pH of approximately 4.0. The material is immediately redissolved and then precipitated again with trichloracetic acid in the presence of potassium rhodanate. The precipitate is then dissolved, subjected to short dialysis, centrifuged, perfectly detoxicated and sterilized by mild formalin treatment. The antigen thus obtained is of short Kf value, and, in general, of 1000 to 1300 Lf/mg/N purity. From good quality crude toxoid at least 90 per cent of the antigen can be recovered. The concentrated purified toxoid can be stored for over a year without loss of value. Adsorbed to precipitated aluminium phosphate, such purified toxoid produced adequate immunity both in experimental animal and in humans. Owing to its purity, the vaccine prepared from this material causes at most a mild vaccination reaction.

The widespread use of combined precipitated vaccines and the endeavours at diminished inoculation reactions call for routine production of purified antigens, including commonly applied diphtheria toxoid.

Our purpose was to develop a procedure ensuring purity of 1000 Lf/mg/N or even higher of the diphtheria toxoid without impairing its immunogenic action or causing a significant loss of antigen. This degree of purity has been found acceptable, because in large groups of children inoculated with combined precipitated diphtheria pertussis vaccine, prepared from toxoid of such purity exerted good immunogenicity but caused no, or only very mild, reaction [1].

In the present work, the modifying effect of conversion of toxin into toxoid and acid precipitation in the presence of various salts or ions has been studied.

Materials and methods

Culture media and strains. Diphtheria toxin was produced from the strain P. W. 8 Weissensee on POPE's [2] tryptic digest medium. The concentrated toxoids obtained from the Institute "Human" for Serobacteriological Production and Research, Budapest, were prepared on LINGOOD's medium [3].

Production and detoxication of toxin. Toxin was prepared in standing cultures in Roux flasks; Lf and Kf values were checked in samples taken from several flasks from the third or

fourth day of cultivation. When the rise in toxin production was slowed down and associated with low Kf values, usually on the fifth to seventh day of cultivation, the toxin was harvested and detoxicated with formalin, as described below.

Titration of toxin and toxoid. Toxin and toxoid were titrated by RAMON's technique [4] with our standard serum equivalent to the international standard preparation. Kf values are given with reference to 25 Lf.

Reagents. A 50 per cent (w/w) solution of trichloracetic acid was used for precipitation. Sodium trichloracetate was prepared by w/w mixture of 10 *N* NaOH solution and trichloracetic acid. Potassium rhodanate was employed as a 25 per cent stock solution.

Determination of pH. A pH measuring apparatus supplied with a glass electrode was used.

Purity of the antigen. Purity was calculated as Lf/mg/N. SCHULEK—FÓTI's micro-method [5] was employed for the determination of nitrogen.

Electrophoresis and chromatography. Electrophoresis was made on Whatman's paper No. 4, with 1500 V, in pyridine buffer, for one hour. Descending chromatography was performed at right angles, in butanol-acetic acid-water medium for 24 hours. The chromatograms were dried by heat, developed with 0.2 per cent ninhydrine, and fixed with copper nitrate.

Tests of active immunization. Groups of 5 guinea pigs each were immunized with precipitated vaccine of 30 Lf/ml prepared from purified toxoid and administered in 0.5 ml doses of a twofold and fifteenfold dilution on two occasions at an interval of four weeks. On the 14th day following the second inoculation, samples of blood were taken and antitoxin content was determined in a mixture containing equal amounts of the sera. Children half to one year of age were given 0.5 ml doses of vaccine at an interval of four weeks. Blood was withdrawn fourteen days and six months after the second inoculation; the antitoxin content was determined by individual titration.

The sera obtained from both the immunized animals and children were tested by the method of JENSEN [6]. With guinea pig sera, titration was carried out at the level of Lr/300, in the case of sera from inoculated children at Lr/3000.

The data for the inoculated children were analysed by statistical methods, examining significant differences in the two arithmetic means by the "*t*" test in the case of samples composed of few elements.

Results

A great number of crude diphtheria toxoid samples were tested for trichloracetic acid concentration of the antigen in the presence of sodium trichloracetate. The results of a characteristic test are presented in Table I.

Table I

Concentration of diphtheria toxoid with 50 per cent trichloracetic acid at pH 4.0 in the presence of various amounts of sodium trichloracetate

Crude toxoid: Lf/ml = 84, Kf = 40 min.

Sodium trichloracetate %	Lf recovery %	Kf minutes	Lf/mg N
	after concentration		
0	81	36	460
0.01—0.02	88	30	555
0.02—0.04	87	29	520
0.04—0.08	87	31	510
0.16—0.32	89	40	480

The addition of a certain amount of sodium trichloracetic acid to crude toxoid before precipitation with trichloracetic acid resulted in higher recovery,

greater purity, and shortening of the Kf. Increase of the sodium trichloracetate concentration presented no advantage. Under our experimental conditions sodium trichloracetate values of 0.01 to 0.02 per cent were found optimal.

In subsequent experiments the pH optimum was determined for the precipitation of diphtheria toxoid in the presence of small amounts of sodium trichloracetate. The results are presented in Table II.

Table II

Concentration of diphtheria toxoid with 50 per cent trichloracetic acid in the presence of 0.015 per cent of sodium trichloracetate at various pH values
Crude toxoid: Lf/ml = 84, Kf = 40 min.

pH	Lf recovery %	Kf minutes	Lf/mg N
	after concentration		
3.5	100	40	483
3.8	95	34	510
4.0	93	33	550
4.2	92	28	600
4.5	82	25	620

With sodium trichloracetate + trichloracetic acid precipitation at pH 4.0—4.2 the antigen was recovered in at least 90 per cent. It had a low Kf value and showed a satisfactorily high purity. A rise in pH caused only slight improvement; in that case, however, the antigen recovery decreased. Thus, under the conditions applied, pH 4.0—4.2 was regarded as optimal.

In order to obtain the formolizing procedure most suitable for purification, the following experiments were performed. Toxin samples from larger batches were detoxicated with different concentrations of formalin at different temperatures for different times. The formol toxoids were concentrated after various periods of incubation with 0.015 per cent sodium trichloracetate + trichloracetic acid at pH 4.0. The most important data are shown in Table III.

According to Table III, the result of concentration is considerably influenced by the mode of detoxication. The best results as to the recovery of antigen, the Kf and purity were obtained with 0.3 per cent formalin at 37° C generally on the 12th to 15th day, with 0.6 per cent formalin at 20° C after the third week. Concentrations performed at other times were disadvantageous for the Kf value and purity.

According to an earlier observation of MANDULA [7], in the presence of a high concentration of KSCN the albumin loses its heat-coagulability and can be fractionated by acid precipitation. Taking this finding into consideration, it was thought that the addition of KSCN to a toxoid which had already

Table III

Result of concentration of diphtheria toxoids obtained by different modes of detoxication
Crude toxin: Lf/ml = 116, Kf = 9 min.
Concentrated with 50 per cent trichloracetic acid at pH 4.0 in the presence of 0.012 per cent sodium trichloracetate

Batch	Formalin treatment			Lf recovery %	Kf minutes	Lf/mg N
	%	days	z °C	after concentration		
76/1	0.3	8	35	86	33	650
76/2	0.3	12	35	85	25	780
76/3	0.3	20	35	90	22	725
76/4	0.6	8	20	78	34	600
76/5	0.6	12	20	89	25	750
76/6	0.6	20	20	96	22	800

been concentrated with trichloracetic acid would make it possible to separate the antigen from at least a considerable part of nitrogen-containing impurities. Unlike the above-described experiments, these studies were carried out with batch 43/II toxoid, prepared and concentrated by the Institute "Human" for Serobacterial Production and Research. After the addition of KSCN, the antigen was precipitated with trichloracetic acid. It was found that diphtheria toxoid which had previously been treated with trichloracetic acid could again be precipitated in the presence of KSCN at pH 4.0 to 5.0. After uniting the fractions, the antigen was recovered without loss. The purity of the preparation obtained at pH 4.0 was nearly identical with that precipitated at pH 5.0. Accordingly, in subsequent experiments the KSCN-concentrated diphtheria toxoids were precipitated at pH 4.0.

For the determination of the optimum quantity of KSCN, to concentrated toxoids various amounts of KSCN were added and the precipitation with trichloracetic acid was repeated. The results are shown in Table IV.

From Table IV it is seen that after the second precipitation with trichloracetic acid, independently of the KSCN concentration, the purity of the various antigens was nearly identical. The result of purification was remarkable and the antigen was always recovered without loss.

The actual protein concentration is well-known to play an important part in purification technique. Table V presents data as to the influence of antigen concentration on purity.

From Table V it is evident that at higher antigen concentrations higher purities were obtained. Examination of high level antigens revealed that the optimum concentration of the antigen was between 500 and 1000 Lf/ml.

In the following experiments the effect of repeated KSCN-trichloracetic acid precipitations was studied. The question whether these procedures result

Table IV

Trichloracetic acid repurification of diphtheria toxoid in the presence of various amounts of potassium rhodanate

Toxoid: Batch 43/II Human, Lf/mg N = 313, Kf = 30 min.
Recovery: 100 per cent in every experiment

KSCN %	Lf/mg N	Kf minutes
10.0	674	47
7.5	660	40
5.0	653	40
2.5	706	38
2.0	717	76
1.0	793	55
0.5	777	54

Table V

Trichloracetic acid repurification of diphtheria toxoid at various concentrations of antigen

KSCN = 0.5 per cent. Precipitation at pH 4.0.
Toxoid: Batch 43/II Human, Lf/mg N = 313, Kf = 30 min.

Lf/ml	Lf/mg N	Kf minutes
500	720	73
250	648	55
125	568	45
63.5	442	35

Table VI

Potassium rhodanate repurification of various trichloracetic acid concentrates of toxoid

Batch	Lf recovery %	Kf, minutes		Lf/mg N	
		Before precipitation	After precipitation	Before precipitation	After precipitation
25	97	62	90	313	632
42/1	76	40	56	443	704
42/3	87	30	43	329	530
43/II	100	30	54	313	777
43/IV	84	35	52	332	770
45/1	82	39	57	710	904
46/2	85	45	119	320	424
47/1	96	41	56	224	372
48/5	97	34	39	412	780
49/1	88	28	40	781	830
50/1	99	30	40	602	917

in further purification was regarded important since in the presence of KSCN the antigen could be precipitated without loss. However, repeated precipitations brought no further improvement.

In these investigations batch 43/II Human toxoid was used. Results with other batches which had been produced on LINGOOD's medium [3] are presented in Table VI.

From Table VI it is clear that the indicated trichloracetic acid-concentrated toxoids could be further purified by re-precipitation with 0.5 per cent KSCN + trichloracetic acid. Depending on the quality of the antigen, as much as 400 to 900 Lf/mg N was obtained. These stable diphtheria toxoid concentrates were prepared with a slight or no loss of antigen.

A similar good result was obtained with toxoids which had been produced in POPE's medium [2], harvested at the optimal time, slightly formolized and concentrated with sodium trichloracetate + trichloracetic acid. The results are shown in Table VII.

Table VII

Concentration and repurification with 1 per cent potassium rhodanate-trichloracetic acid of diphtheria toxoids

Batch	Crude toxin			Concentrated toxoid*			Purified toxoid		
	Lf/ml	Total Lf	Kf min.	Lf recovery %	Kf min.	Lf/mg N	Lf recovery %	Kf min.	Lf/mg N
76/a**	114	460.000	9	86	16	890	100	18	1275
Mix. III	90	2,700.000	10	98	20	670	100	22	1000
Mix. IV	65	4,500.000	11	96	30	480	95	40	1050

* Concentration in the presence of 0.012 per cent sodium trichloracetate at pH 4.0 with 50 per cent trichloracetic acid.

** Batch 76/a was prepared in POPE's beef medium which had been diluted before inoculation with distilled water to 4 : 1. After cultivation for 6 days 0.3 cent per formalin was added and left to stand at 37° C for 20 days.

Depending on the quality of the crude toxoid, a purity corresponding to 1000 to 1300 Lf/mg N was obtained with a slight loss. Short Kf values showed the good quality of the antigen. As the example of "diphtheria toxoid mixture No. IV" shows, the procedure can adequately be applied in case of toxoids of medium level and also for semi-plant scale or industrial preparation.

Since Kf is regarded by some authors [8, 9] as the indicator of the quality of antigen, examinations were performed with diphtheria toxoid No. IV as to the relationship between Kf and Lf at the purified toxoid — standard antitoxin equivalence point. The data are presented in Table VIII.

When evaluating the above findings, the paper of LEVINE et al. [8] was consulted, who described a similar connection, showing that only toxoids with

Table VIII

Relationship between the flocculation time (Kf) and titre (Lf) of purified toxoid at the toxoid—antitoxin equivalence point

Lf value observed	Kf values	
	Calculated	Observed
37	26	28
33	33	33
28	40	40
20	64	57
15	95	90

* Calculation of Kf: $\log Kf = 3.66 - 1.42 \log Lf$

a linear logarithmic relationship between Kf and Lf are exerting an effective protective action. According to PRIGGE's theory [10], the potency of vaccines expressed in protective units (PU) and the Lf value show the following relationship

$$PU = c\sqrt{Lf \cdot A}$$

where c is a constant depending on the quality of the adsorbent, and A is the weight of the adsorbent. With such toxoids a standard antigenic effect may be expected when equal quantities of Lf are inoculated. Of course, the quality of the purified antigen depends to a large extent on the basic crude material. The purification of good quality diphtheria toxoids by the described technique yielded an antigen of very high standard without any sign referring to the harmful effect of the procedure.

On the basis of the present investigations, the steps of the purification procedure can be summarized as follows.

Preparation and purification of diphtheria toxoid

1. During incubation the toxin production is under constant control. The toxin is harvested when Kf is the lowest (generally after 5 to 7 days).

2. The toxin is treated with 0.3 per cent formalin at 35° C for 12 to 15 days or with 0.6 per cent formalin at 18° C to 22° C for 21 days.

3. To the practically totally detoxicated antigen 0.015 per cent sodium trichloracetate is added, then it is precipitated with 50 per cent trichloracetic acid at pH 4.0. After complete precipitation, the mixture is immediately centrifuged.

4. The precipitate is dissolved in distilled water with the addition of N NaOH so as to obtain an approximately neutral reaction and a calculated Lf concentration of 500 to 1000 per ml.

5. To the solution 1 per cent KSCN is added, then the antigen is precipitated again with 50 per cent trichloracetic acid at pH 4.0. After complete precipitation the mixture is immediately centrifuged.

6. The precipitate is dissolved in the smallest possible amount of distilled water and *N* NaOH so as to give an approximately neutral reaction. The highly

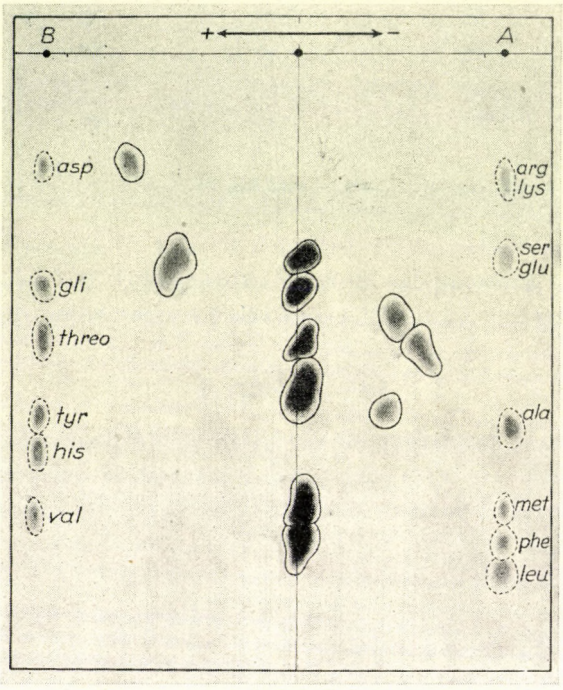

Fig. 1. Electrophoretic and chromatographic pattern of supernatant remaining after precipitation of diphtheria toxoid with KSCN + trichloracetic acid

concentrated antigen solution is dialysed against dilute sodium bicarbonate, then against distilled water until no trace of KSCN remains.

7. In order to remove floating corpuscles, the dialysed antigen is centrifuged for 30 minutes, the volume of the solution is measured, the pH is adjusted to 7.0—7.5 and aliquots are taken for the examination of Lf, Kf and N. Then the antigen is placed into a sterile flask. After the addition of 0.3 per cent formalin the flask is closed with a sterile stopper and the fluid is vigorously shaken.

8. The formolized antigen is kept at 35° C for one week then at room temperature for 30 days. Then aliquots are taken for toxicity, sterility and antigenicity tests. When in exceptional cases detoxication is not complete

further 0.3 per cent formalin should be added. The repeated addition of formalin exhibits no harmful effect.

For vaccine preparation, the purified toxoid is mixed to an adequate quantity of pH 5.5 $AlPO_4$ made according to HOLT's procedure [11]. After the addition of the antigen, the mixture is adjusted to pH 3.5 with a 10 per cent sterile $AlCl_3$ solution. Previous investigations [12] have shown that by this method an improved antigen can be produced, as the adsorption of the antigen becomes stronger and more selective. The reaction of the mixture should be ascertained by means of an electric pH meter throughout the procedure; when the pH indicated is reached, a sterile 15.75 per cent Na_3PO_4 solution is immediately added until the reaction has been adjusted to pH 6.0—6.3. Finally, the vaccine is preserved with HOLT's acetate-merthiolate [11].

In order to study the mechanism of KSCN + trichloracetic acid purification, it was necessary to determine the nitrogen-containing substances present in the supernatant which had been separated from the antigen.

After precipitating the antigen with KSCN + trichloracetic acid, the supernatant was concentrated and subjected to electrophoresis. This revealed the presence of acid, neutral and alkaline amino acids. The amino acids obtained by electrophoresis were further separated by descending chromatography. The two-dimensional chromatograms are shown in Fig. 1.

Accordingly, the supernatant contained various amino acids but no higher proteins. Similar analyses showed no free amino acids in the KSCN-purified antigen. Trichloracetic acid-concentrated diphtheria toxoid which had not been treated with KSCN gave the chromatogram presented in Fig. 2.

According to Fig. 2 the toxoid concentrate not subjected to KSCN-purification contained acid, neutral and alkaline amino acids. These corresponded to free amino acids, as on prolonged dialysis they were removed. By electrophoresis and chromatography this dialysed antigen was shown to be devoid of amino acids. Accordingly, its purity defined as Lf/mg N, increased.

These examinations have revealed the mechanism of KSCN purification. On precipitation with trichloracetic acid considerable amounts of amino acids are probably adsorbed on the precipitate. This adsorption seems to be prevented by KSCN, as KSCN is more effectively adsorbed on the surface of the precipitate than amino acids. That the phenomenon was really due to ionic adsorption was proven by precipitation with KBr, NaBr and KI. These compounds acted similarly to KSCN. The lyotropic property of Br^- and I^- ions is closely related to that of SCN^- ions; thus it may be assumed that the mechanism of the purification method is connected with the lyotropic property of KSCN.

The present method is a practical purification procedure allowing the preparation of a toxoid of high purity along with a minimum loss of antigen. The immunizing capacity and the ability for the preparation of vaccines of

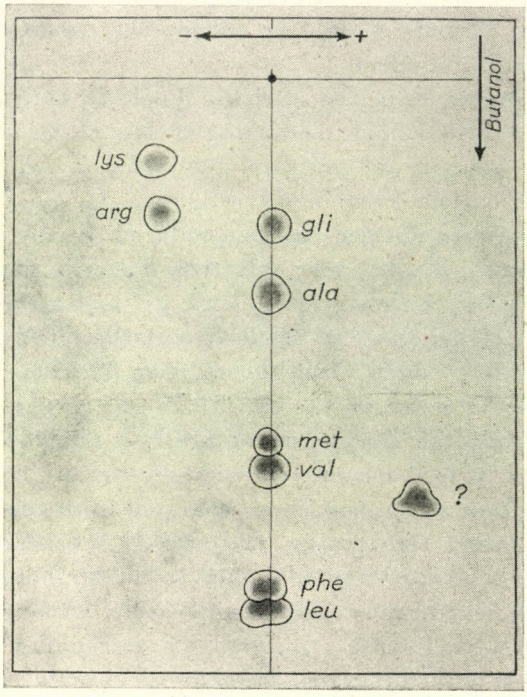

Fig. 2. Electrophoretic and chromatographic pattern of diphtheria toxoid concentrated by trichloracetic acid precipitation

the diphtheria toxoid obtained by our method were considered in further experiments.

Batch 43/II Human toxoid concentrate was further purified by KSCN + + trichloracetic acid treatment and used for the preparation of $AlPO_4$-precipitated combined vaccines.

The immunizing potency of the vaccines was examined at the Serum and Vaccine Control Department of this Institute. The results are shown in Tables IX and X.

In Table IX the effective immunizing potency of the KSCN-purified diphtheria toxoid in animal experiments is shown.

Table X summarizes the result of immunization of children of half to one year of age. In the examinations two combined diphtheria-pertussis-tetanus vaccines were compared. One of them was batch No. 3744/a of *Berna* (Switzerland), the other, batch OKI 7 was our own preparation from diphtheria toxoid purified by reprecipitation with KSCN + trichloracetic acid.

According to Tables IX and X, the immunizing potency of batch OKI 7 was satisfactory in all respects. The antibody content of the blood serum of both the guinea pigs and children showed that purification with KSCN of the toxoid had not reduced its antigenic potency.

Table IX

The immunizing potencies for guinea pigs of various diphtheria-pertussis-tetanus combined vaccines prepared from KSCN + trichloracetic acid precipitated diphtheria toxoid Batch 43/II Human

Batch	Toxoid Lf/ml	Diphtheria A.U./ml in sera of guinea pigs			
		after first immunizaton		after second immunization	
		I	II	I	II
OKI 3	7.5	1.0—2.0	0.05	4—8	1—2
OKI 4	15	0.5—1.0	0.4—0.5	2—4	16
OKI 5	30	0.5—1.0	0.05—0.1	1—2	1—2
OKI 6	30	0.05—0.1	1—2	16	16
OKI 7	30	0.5—1.0	0.5—1.0	2—6	16
OKI 8	60	0.5	0.1—0.5	4—8	8—16
OKI 10	30	2.0	0.05—0.1	4—8	8—16
OKI 13	30	0.5—1.0	0.5	4—8	16
OKI 14*	30	0.1—0.5	0.5—1.0	16	16
Human 32	30	0.1—0.5	0.5—1.0	16	16
Berna 3744/a	.	4—8	1—2	4—8	32—64

I = Immunization with 1/2 ml vaccine
II = Immunization with 1/15 ml vaccine
* Antigen not purified with KSCN

Table X

The immunizing potency for children of diphtheria-pertussis-tetanus combined vaccine prepared from KSCN + trichloracetic acid precipitated diphtheria toxoid Batch 43/II Human
Age of children, 1/2 to 1 year

Designation of vaccine	Distribution of sera according to A.U./ml											No. of persons		Log. average of A.U./ml	
	I. Two weeks						II. Six months								
	after immunization											I.	II	I	II
	0.05	0.1	0.2	0.5	1.0	2.0	0.001	0.05	0.1	0.2	0.5				
	0.1	0.2	0.5	1.0	2.0	5.0	0.05	0.1	0.2	0.5	1.0				
OKI 7	0	3	7	4	2	5	5	3	6	3	3	21	20	0.60	0.08
Berna 3744/a	4	5	6	1	2	2	10	2	2	2	1	20	17	0.29	0.03

I		II	
t	p%	t	p%
2.33	<5 >2	2.11	<5

Discussion

In recent years a number of investigations have been carried out with respect to the theoretical and practical problems of purification of diphtheria toxoid. First of all the paper of ERICSSON and NEUMÜLLER [13] should be mentioned, in which important data have been reported as to the special role of trichloracetate ions beyond the acid precipitation, and as to the role played by the short formolizing period in the decrease of the formation of formol-protein impurities. These substances, due to their similar physicochemical properties, are very difficult to separate from the toxoid. In the present experiments care has been taken to employ simple procedures. The "forced adsorption" of POPE and STEVENS [14] requiring ultrafiltration, submerged cultivation and the application of a special charcoal, and other methods requiring the use of semisynthetic media [11, 15, 16], although ensuring extreme purity, are difficult to carry out and have not yet been routinely employed in this country.

In our very simple procedure of purifying the toxoid produced in a medium prepared from tryptic digest of beef, the following conditions are considered necessary. (i) The application of a strain producing high levels of toxin within a short incubation period. In this way the ratio of protein impurities formed during cultivation can be decreased. (ii) Optimal circumstances in formalin treatment (quantity, temperature, time) to inhibit production of aspecific formol-proteins. (iii) The application of optimal quantities of trichloracetate in the trichloracetic acid concentration of the crude toxoid, in order to increase the difference between the precipitability of antigen and protein impurities. (iv) The addition of potassium rhodanate allows the re-precipitation of the antigen with trichloracetic acid without loss and the separation of the contaminating low molecular weight nitrogen containing substances.

By the method described, starting from the filtrate of flask cultures, we were able to obtain preparations having a purity of 1000 to 1300 Lf/mg N. The antigen was recovered in at least 85 to 90 per cent. The vaccines prepared from this antigen had a very effective immunizing potency and caused very slight inoculation reactions.

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SALMONELLA-UNTERSUCHUNGEN IN BUDAPEST IN DEN JAHREN 1956—1958

Von

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(Eingegangen am 19. März 1960)

Zusammenfassung. 1. Es wird über die im Verlauf von 3 Jahren (1956—1958) im Bakteriologischen Laboratorium der Budapester Station für Gesundheitswesen und Epidemiologie durchgeführten Salmonella-Untersuchungen berichtet. Während der 3 Jahre wurden aus Stuhlproben, die aus Berufsreihenuntersuchungen und bei Erkrankungen vorgenommenen Untersuchungen stammten, 1847 Salmonella-Stämme isoliert.

2. Die Durchseuchung der gesunden Bevölkerung machte im Durchschnitt der 3 Jahre 0,32% aus.

3. Das Vorkommen der Salmonella-Fälle in den Jahren 1956, 1957 und 1958 wird besprochen.

4. Unter den 1847 Stämmen waren 15 verschiedene Typen anzutreffen. Bei der Hälfte der Stämme handelte es sich um *Salmonella saint paul*.

5. Von den Erkrankten waren 43% jünger als 2 Jahre.

6. Die Ausscheidung des Krankheitserregers hörte in 80% der Fälle innerhalb eines Monats auf.

Die Behandlung der Salmonella-Frage nimmt in der medizinischen und veterinärmedizinischen Literatur von Jahr zu Jahr mehr Raum ein. In der ganzen Welt hat man beobachtet, dass die Zahl der Salmonella-Fälle in den letzten Jahren stark zunahm [1—14]. Auch in Ungarn ist ihre Zahl wesentlich höher als in früheren Jahren und Jahrzehnten. Diese Tatsache erfordert, dass wir uns mit der Frage der Salmonella-Infektionen — als sehr akeinem tuellen hygienischen und epidemiologischen Problem — eingehender beschäftigen.

Nachfolgend berichten wir über das dreijährige Salmonellen-Material des Bakteriologischen Laboratoriums der Budapester Station für Gesundheitswesen und Epidemiologie. Dieses Material stammt aus zwei streng abgegrenzten Gebieten: 1. Von Stuhl-Reihenuntersuchungen der Werktätigen im Bereich der Lebensmittelversorgung und der Kinderkollektiven in Gross-Budapest. 2. Von bei Erkrankungen vorgenommenen Stuhluntersuchungen in einzelnen Bezirken von Budapest.

Material und Methoden

Verarbeitung der Untersuchungsmaterialien. Bei den Berufs- und anderen Reihenuntersuchungen wurden mit den Stuhlproben Wismuthsulfit-Agar- (LOVREKOVICH) und Desoxycholat-Zitrat-Agarplatten beimpft; den von Kranken stammenden Stuhl verimpften wir nicht nur auf obige Nährböden, sondern auch auf Brillantgrün-Agarplatten und Natrium-Selenit-Anreicherungsböden. Vom Anreicherungsnährboden erfolgte die Abimpfung nach 24stündiger Inkubation auf Wismuthsulfit-Agar und Brillantgrün-Agar. Bei jedem Material geschah die Ablesung zweimal, zuerst nach 24stündiger Inkubation bei 37° C, sodann nach Stehen bei Zimmertemperatur während weiterer 24 Stunden. Die Identifizierung der verdächtigen Kolonien erfolgte nach den üblichen biochemischen und serologischen Methoden.

Ergebnisse und Besprechung

Vom 1. März 1956 bis zum 1. Januar 1959 wurden im Rahmen der Berufsreihenuntersuchungen 205 000, im Zusammenhang mit Erkrankungen 74 000, insgesamt also nahezu 280 000 Salmonella-Untersuchungen durchgeführt. Aus diesem Material ergaben sich 653 bzw. 1194, insgesamt also 1847 positive Salmonella-Befunde. Diese Zahlen umfassen weder die wiederholt positiven Salmonella-Resultate, noch die Typhus- bzw. Paratyphus A-, Paratyphus B- und Paratyphus C-Fälle (Tabelle I).

Bei der Bewertung der Zahlenangaben muss berücksichtigt werden, dass die Anzahl der mit Laboratoriumsuntersuchungen verifizierten positiven Fälle nicht mit der Anzahl der effektiven Infektionen identisch ist. Ein gewisser Prozentsatz der positiven Fälle ergibt sich aus Forschungsuntersuchungen; in diesem Material kommen aber sicher auch Rekonvaleszenten vor. Gewiss gibt es jedoch erheblich mehr effektive Salmonella-Erkrankungen als die Fälle, in denen die bakteriologische Untersuchung vorgenommen wird.

Tabelle I

Die Ergebnisse der Salmonella-Untersuchungen

Untersuchungsmaterial	Zahl	Salmonella-positiv
Berufsreihenuntersuchungen	205 000	653
Mit Erkrankungen zusammenhängende Untersuchungen	74 000	1194
Insgesamt	279 000	1847

Da es sich bei dem Salmonellosen-Problem um weitverzweigte Fragen handelt, sollen aus dem uns zur Verfügung stehenden Material nur einige wichtige herausgegriffen werden, und zwar die Infektivität der gesunden Bevölkerung, das zahlenmässige Vorkommen der Salmonella-Fälle in den einzelnen Jahren, die Aufteilung der beobachteten Salmonellen nach Typen, das Alter der Erkrankten und das Problem der Ausscheidungsdauer.

Die Infektivität der gesunden Bevölkerung. Auf die Frage, wie häufig Salmonella-Ausscheidung unter der gesunden Bevölkerung vorkommt, geben die Reihenuntersuchungen Anhaltspunkte. Über diese Frage stehen folgende ungarische Angaben zur Verfügung. Rauss hat in seiner 1949 erschienenen Mitteilung [15] das Material der Jahre 1936—1944 aufgearbeitet. In diesen 9 Jahren ergaben 800 000 Untersuchungen lediglich 46 Salmonella-Fälle, d. h. 0,006% (ohne Ty, Paty A, B und C). Lányi und Hamar [16] teilten die diesbezüglichen Angaben der Bakteriologischen Abteilung des Staatlichen Hygiene-Instituts aus dem Jahre 1953 mit. In nahezu 100 000 Reihenuntersuchungen fanden sie 83 = 0,085% positiv. Wir stellten in den letzten 3 Jahren bei 205 000

Berufsreihenuntersuchungen 653 = 0,32% Salmonella-Träger fest. Während also in den Jahren 1936—1944 in Ungarn auf 16 000 Menschen 1 Ausscheider entfiel, kam 1953 bereits 1 auf 1000 und heutzutage 1 auf 300. Diese Tatsache beweist, dass die Zahl der Salmonellosen auch in Ungarn stark gestiegen ist.

Im Jahre 1957 war im Juni und Juli in Budapest eine Salmonella-Epidemie aufgetreten. Die Angaben dieses Jahres bieten daher die Möglichkeit festzustellen, wie sich das Verhältnis der Ausscheider zur Zeit einer Epidemie gegenüber den epidemiefreien Perioden verändert. Bei den 78 000 Reihenuntersuchungen im Jahre 1957 betrug die Zahl der Salmonella-Positiven 273, d. h. 0,35%. Wenn wir die Verhältniszahl der Positiven in den Monaten mit und ohne Epidemie des Jahres gesondert errechnen, so finden wir, dass sie in der epidemiefreien Periode auf 0,16% sinkt und in der Epidemieperiode auf 1% ansteigt. Dementsprechende Angaben gewannen wir auch bei der diesbezüglichen Analyse des Materials vom Jahre 1958, als in den Sommermonaten zwar die Zahl der Erkrankungen gestiegen, aber keine epidemieartige Vermehrung der Fälle eingetreten war. Der Jahresdurchschnitt der Salmonella-Ausscheider war 0,15%, d. h. er stimmte mit dem in der epidemiefreien Periode des Jahres 1957 festgestellten Prozentsatz überein.

Das Vorkommen der Salmonella-Fälle in den einzelnen Jahren. Aus den Zahlenangaben unseres mit Erkrankungen zusammenhängenden Untersuchungsmaterials seien folgende hervorgehoben: Im Jahre 1956 wurden 177, im Jahre 1957 563 und 1958 454 positive Fälle beobachtet (Abb. 1).

Bei der niedrigen Zahl des Jahres 1956 handelt es sich um eine relative Ziffer. Einerseits befasste sich das Laboratorium 1956 nur 8 Monate mit diesen Untersuchungen, anderseits bleibt 1956 die Verhältniszahl der mit Erkrankungen zusammenhängenden Untersuchungen (29,6%) im Vergleich zu sämtlichen Untersuchungen noch bedeutend hinter den Verhältniszahlen der Jahre 1957—1958 (37,7% bzw. 39%) zurück. Es muss bemerkt werden, dass dies wahrscheinlich nicht auf einer niedrigeren Erkrankungszahl beruhte. Wir fanden nämlich in den Sommermonaten 0,6% gesunde Ausscheider, woraus geschlossen werden darf, dass in diesen Monaten dementsprechend mehr Erkrankungen vorgekommen sind. Die niedrigere Zahl der bei Erkrankungen vorgenommenen Untersuchungen lässt sich darauf zurückführen, dass die einschlägige Arbeit unseres Laboratoriums im Jahre 1956 noch nicht allgemein bekannt war und die Ärzte bei enteralen Erkrankungen nur selten an Salmonellose dachten.

Im Jahre 1957 kamen 563 positive Salmonella-Fälle in unserem Krankenmaterial vor. Hiervon stammten 351 aus der in den Sommermonaten aufgetretenen Epidemie. Im Juni und Juli hat die Zahl der Salmonella-Kranken und -Ausscheider im Vergleich zu den ersten 5 Monaten des Jahres mächtig zugenommen. Bis Juni wurden im Monatsdruchschnitt 20 Fälle beobachtet, im Juni und Juli sprang die Zahl der Positiven auf 215 bzw. 236. In den

entsprechenden Monaten des Jahres 1956 war die Zahl der Salmonella-Fälle gleichfalls gestiegen, aber 1957 erreichte die Anzahl der positiven Fälle dieser beiden Monate die Zahl der in 8 Monaten des Jahres 1956 gezüchteten Stämme. Im August sank die Zahl der positiven Fälle, und im September entsprach sie bereits dem Monatsdurchschnitt und zeigte

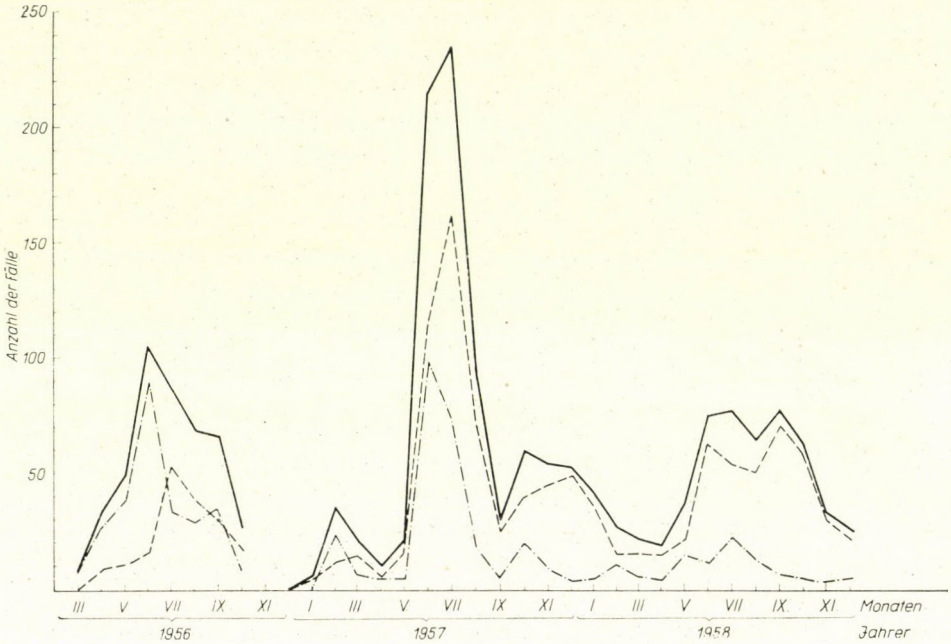

Abb. 1. Monatliche Verteilung der in den Jahren 1956—1958 gezüchteten Salmonella-Stämme
Zeichenerklärung:
— — — — — Aus Krankenmaterial gezüchtete Stämme;
—.—.—.—.— von Berufsreihenuntersuchungen gezüchtete Stämme;
———————— insgesamt

damit das Abklingen der sommerlichen Salmonella-Epidemie an. In der überwiegenden Mehrzahl, zu 76%, waren die Erkrankungen von dem Stamm *Salmonella saint paul* verursacht worden, und in derselben Zeitspanne zählten auch 61% der von Berufsreihenuntersuchungen herrührenden Stämme zu diesem Typus. Angesichts des stürmischen Ansteigens der Erkrankungen hat unser Lebensmittellaboratorium Untersuchungen durchgeführt, um festzustellen, worauf das epidemieartige Erscheinen der Salmonellose beruht. Wie die Untersuchungen ergaben, waren die zu jener Zeit in den Handel gekommenen rohen Fleischsorten (hauptsächlich die unrichtig behandelten tiefgekühlten Rohfleischsorten) und die in den Fleischereien benutzten Gegenstände (Fleischtransportkisten, Hackbänke, Eisschränke usw.) in hohem Prozentsatz mit Bakterien der Salmonella-Gruppe infiziert. Unter den Salmonella-Typen kam im

höchsten Prozentsatz *Salmonella saint paul*, dann *Salmonella heidelberg* und *Salmonella anatum* vor, was mit der Typenverteilung der aus den Stuhlproben gezüchteten Salmonella-Stämme übereinstimmte [17].

Unter den bisher erwähnten Angaben ist die im Februar 1957 mit Massenerkrankungen aufgetretene Lebensmittelvergiftung nicht berücksichtigt, die von den in Ungarn zum erstenmal gezüchteten *Salmonella heidelberg* hervorgerufen worden war. Von den Erkrankten wurde der Erreger in nahezu 100 Fällen gezüchtet. Ungefähr zur gleichen Zeit mit der Lebensmittelvergiftung wurde derselbe Erreger auch aus den aus verschiedenen Teilen der Stadt eingesandten Stuhlproben gewonnen, ebenso war er unter den von Berufsreihenuntersuchungen stammenden Stämmen vertreten.

Im Jahre 1958 kamen unter den mit Erkrankungen zusammenhängenden Untersuchungen 454 positive Salmonella-Fälle vor. In den 5 Monaten zwischen Juni und Oktober war die Zahl der positiven Fälle nahezu doppelt so hoch wie in den übrigen 7 Monaten zusammen, was auf die saisonartige Zunahme der Salmonellose im Sommer deutet. Eine grössere Epidemie war aber in diesem Jahr nicht zu beobachten.

Sofern wir unsere Angaben mit den von RAUSS bzw. von LÁNYI und HAMAR mitgeteilten vergleichen, so finden wir auch hier eine bedeutende Vermehrung der positiven Salmonella-Fälle. In 9 Jahren isolierte RAUSS von Kranken insgesamt 96 *Salmonella typhi murium*-, 26 *Salmonella enteritidis Gärtner*- und 17 andere Salmonella-Stämme. LÁNYI und HAMAR züchteten 1953 nur 58 Salmonella-Stämme. In unserem 3jährigen Krankenmaterial war aber die Zahl der gezüchteten Stämme 1194. Diese Erhöhung dürfte nur zum geringen Teil auf die ständige Entwicklung der bakteriologischen Diagnostik und auf die Tatsache zurückzuführen sein, dass die Ärzte dieses Krankheitsbild häufiger erkennen und bei Enteritiden häufiger Stuhlproben untersuchen lassen.

Die Typen der gezüchteten Salmonella-Stämme. Unter den gezüchteten Stämmen kamen 15 verschiedene Salmonella-Typen vor (Abb. 2).

Etwa 50% der Fälle machte *Salmonella saint paul* aus. Häufig waren *Salmonella anatum*, *Salmonella bareilly*, *Salmonella heidelberg*, *Salmonella typhi murium*, *Salmonella meleagridis* und *Salmonella manhattan* vertreten, und zwar zu 5—11%. *Salmonella bredeney*, *Salmonella kottbus*, *Salmonella abony*, *Salmonella enteritidis* (Gärtner), *Salmonella oranienburg* und *Salmonella tennessee* wurden nur in 0,1—2% der Fälle beobachtet. Der hier seit 3 Jahren in grösster Zahl vorkommende Typ *Salmonella saint paul* wurde 1940 in den Vereinigten Staaten [18] beschrieben und erschien in Deutschland 1954 [19] und in der Ostslowakei 1955 [11]. In Ungarn wurde er zuerst von KUBINYI und LÁNYI [20] 1953 aus dem Stuhl von Kranken und Ausscheidern isoliert. Noch im selben Jahr wurde bei einer umfangreichen, von Sülzwurst hervorgerufenen Fleischvergiftung neben *Salmonella anatum* auch *Salmonella saint paul* als Erreger festgestellt [21]. Seither hat sich *Salmonella saint paul* zum häufigsten

Salmonella-Typ entwickelt und ist in diesen Jahren mindestens so endemisch in Ungarn geworden wie *Salmonella typhi murium* in vielen anderen Ländern. In unserem Material sind ausserdem 5 Typen erschienen, die in Ungarn, zumin-

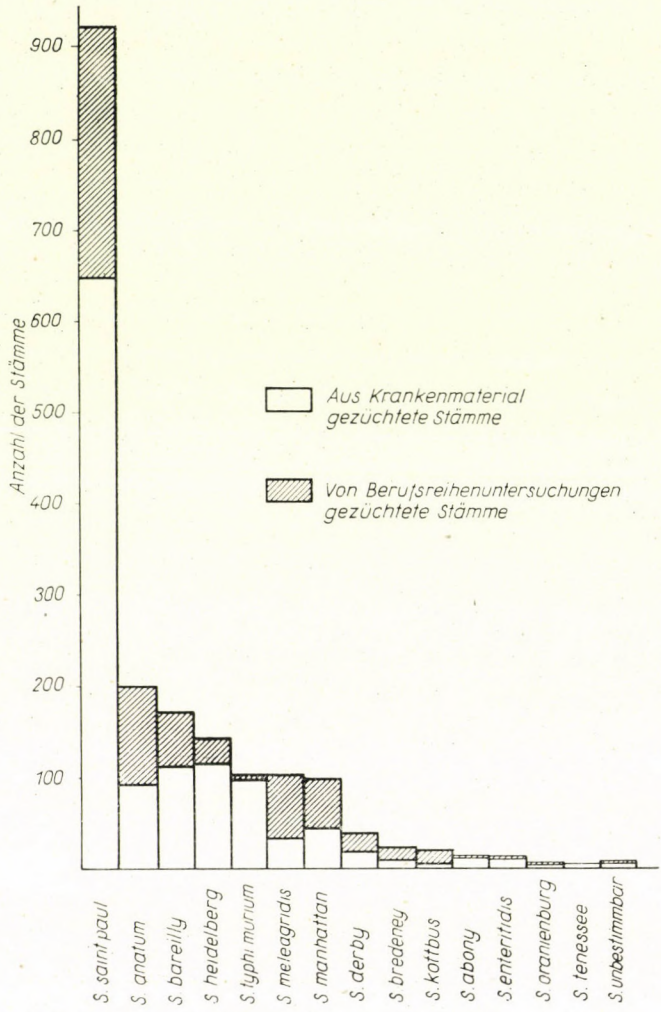

Abb. 2. Vorkommen der Salmonella-Typen in den Jahren 1956—1958

dest bei menschlichen Erkrankungen, bisher nicht beobachtet wurden *(Salmonella heidelberg, Salmonella manhattan, Salmonella bredeney, Salmonella oranienburg* und *Salmonella tennessee)*. Von diesen ist *Salmonella heidelberg*, wie bereits erwähnt wurde, heute eine der häufigsten Typen. Hier sei unsere Beobachtung erwähnt, dass es Stämme gibt, die öfter aus Kranken als anlässlich der Reihenuntersuchungen gezüchtet werden können, z. B. *Salmonella typhi murium, Salmonella heidelberg* und *Salmonella bareilly*. Die bei uns vorkommenden

Typen sind auch in den grossen zusammenfassenden Mitteilungen der ausländischen Literatur als häufigste Typen erwähnt worden [3, 22].

Das Alter der Erkrankten (Tabelle II) lenkt die Aufmerksamkeit auf eine sehr wichtige Tatsache.

Tabelle II

Jahr	—2 Jahre		2—6 Jahre		6—14 Jahre		Über 14 Jahre		Insgesamt	
	Zahl	%	Zahl	%	Zahl	%	Zahl	%	Zahl	%
1956	66	38	16	9	11	6	84	47	177	100
1957	235	42	127	22	26	5	175	31	563	100
1958	211	46	68	15	36	8	139	31	454	100
Insg.	512	43	211	18	73	6	398	33	1194	100

Wie aus den Daten der drei Jahre hervorgeht, handelte es sich bei 61%, d. h. der überwiegenden Mehrzahl der Erkrankten, um Kinder unter 6 Jahren, bei 43% um Kinder von noch nicht 2 Jahren. Bei der Epidemie des Jahres 1957 war dieser Prozentsatz noch etwas erhöht. Das zeigt, dass diese Altersgruppe der Salmonella-Infektion gegenüber am empfindlichsten ist. Im Jahre 1958 verglichen wir die Altersverteilung der an Salmonellose und Dysenterie Erkrankten. Interessanterweise kamen Kinder unter 6 Jahren in beiden Gruppen in nahezu demselben Prozentsatz vor (61% bzw. 57%). Bei der Dysenterie verhielt es sich indessen bei den Gruppen unter 2 Jahren und von 2—6 Jahren umgekehrt: die Mehrzahl der Erkrankungen trat in der Altersgruppe von 2—6 Jahren zutage, und auf die Gruppe der Kinder bis zu 2 Jahren entfielen lediglich 18% sämtlicher Dysenteriefälle. Obwohl die Anzahl unserer Dysenteriefälle im Jahr 1958 etwa das Doppelte der Salmonella-Fälle betrug, war in der Altersgruppe unter 2 Jahren die absolute Zahl der Shigella-Positiven dennoch niedriger als die der Salmonella-Positiven. Für die stärkere Disposition der jüngsten Altersgruppe den Salmonella-Infektionen gegenüber zeugt auch die Tatsache, dass in unserem Material die Hälfte der Kinder in der Altersgruppe unter 2 Jahren das 1. Jahr noch nicht überschritten hatte und 1/3 sogar jünger war als 6 Monate. 1, 2 oder 3 Monate alte Säuglinge waren unter den Kranken auch zu finden. In dieser Altersklasse kann es sich offenbar nur um eine Kontaktinfektion handeln. Das Untersuchungsmaterial aus dieser Altersgruppe war von den Ärzten meistens als dysenterieverdächtig zugesandt worden. Die auf Grund des klinischen Bildes für Dysenterie gehaltenen Fälle haben sich jedoch in der überwiegenden Mehrzahl als Salmonellosen erwiesen. In der Literatur wurde gleichfalls auf die spezielle Empfänglichkeit der Säuglinge und Kleinkinder den Salmonella gegenüber hingewiesen [23—30], weiterhin darauf, dass die Salmonellose in diesem Alter oft nicht im Bilde der Gastroenteritis, sondern als schwere generalisierte Sepsis auftritt.

Die Ausscheidungsdauer. In den Jahren 1957 und 1958 untersuchten wir auch die Ausscheidungsdauer. In diesen beiden Jahren fanden wir 528 Salmonella-Ausscheider, bei denen wir den Erreger im Stuhl zwei- oder mehrmal (bei einzelnen mehr als 30mal) nachzuweisen vermochten. In 80% der Fälle hörte die Ausscheidung innerhalb eines Monats auf, und zwar in 30% der Fälle binnen 1 Woche, in 26% nach 2 Wochen, in 15% nach 3 Wochen und in 9% nach 4 Wochen. Bei den restlichen 20% dauerte die Ausscheidung länger als einen Monat, 2—3—4—5 Monate, in einigen Fällen sogar länger als ein halbes Jahr. Hier muss erwähnt werden, dass wir bereits einige chronische Salmonella-Ausscheider kennen, welche die Bakterien seit mehr als 1—2 Jahren kontinuierlich ausscheiden. Einen Zusammenhang zwischen Ausscheidungsdauer und Salmonella-Typ haben wir nicht feststellen können.

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THE RELATION OF SURFACE PROPERTIES AND ANTIBIOTIC-RESISTANCE IN STAPHYLOCOCCUS AUREUS

III. THE EFFECT OF UNSATURATED FATTY ACIDS ON THE RESISTANCE TO ANTIBIOTICS

By

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Summary. *Staphylococcus aureus* strains of various sensitivity to antibiotics were tested for sensitivity to unsaturated fatty acids, *viz.* oleic acid, linoleic acid and linolenic acid.

Sensitivity to the bacteriostatic effect of the unsaturated fatty acids showed no relation to antibiotic-sensitivity; oleic acid, linolenic acid and linoleic acid, as present in the agar medium, inhibited the growth of the strains tested in concentrations exceeding 0.12, 0.03 and 0.03 per cent, respectively. In fluid medium higher concentrations were required.

The penicillin-sensitivity of the polyresistant and penicillin-resistant strains was enhanced by the unsaturated fatty acids present in concentrations as low as 0.004 per cent.

In the presence of substatic concentrations of penicillin, unsaturated fatty acids reduced the oxygen consumption of the penicillin-resistant cells by 45 per cent; that of the polyresistant cells by 59 per cent. Their effect on sensitive strains proved to be less marked.

Unsaturated fatty acids exerted no direct action on the endopenicillinase enzyme.

The marked synergism demonstrated between penicillin and unsaturated fatty acids in the case of resistant strains appears to be caused by the increased vulnerability of these cells and by the inhibitory effect of fatty acids on penicillinase production.

The diversity in the surface structure of resistant and sensitive cells, and its practical importance are pointed out.

Although the influence of saturated and unsaturated fatty acids on the growth of bacteria had been recognized long ago, certain details of the mechanism of this effect have remained unrevealed [1, 2, 3]. KODICEK [4] suggested that the effect of fatty acids highly depends on the composition of the bacterial surface, and this is in accordance with the fact that Gram-positive and negative bacteria are distinct in their sensitivity to fatty acids. The surface phenomena which are different in antibiotic-sensitive and resistant strains of *St. aureus* were dealt with in our previous reports [5, 6, 7]. In order to obtain further data on these divergencies, it seemed interesting to investigate, how unsaturated fatty acids influenced the sensitivity to various antibiotics of resistant and sensitive *St. pyogenes aureus* strains.

Materials and methods

Bacteria. St. aureus No. 100 (Duncan strain), sensitive to each of the antibiotics penicillin (P), streptomycin (Sm), chloramphenicol (Ch), terramycin (T), aureomycin (A) and erythromycin (E).

St. aureus No. 102, sensitive to P, Sm, Ch, T, A and E.
St. aureus No. 1, resistant to P, sensitive to Sm, Ch, T, A and E.
St. aureus No. 53, resistant to P, Sm, Ch, T, A and E (polyresistant strain).
St. aureus No. 54, resistant to P, Sm, Ch, T, A and E. (polyresistant strain)
B. subtilis No. 60, resistant to P, sensitive to Sm, Ch, T, A and E.
B. megaterium No. 44, sensitive to P, Sm, Ch, T, A and E.

Each of the *St. aureus* strains produced coagulase, liquified gelatine, fermented mannite and produced pigment.

Sensitivity to antibiotics was tested by the paper disc method on solid medium, and by serial dilution in broth. When using the former method, 1 ml of the 1 : 10 diluted 24-hour broth culture of the bacterium to be tested was plated on agar plates. After drying for 90 minutes the paper discs were placed on the plates. The dishes, 8 mm in diameter each, were cut out from Schleicher—Schüll 2043/B filtre paper soaked in antibiotic solution and dried. Each contained 50 μg of one of the antibiotics penicillin, streptomycin, aureomycin or terramycin, or 30 μg of chloramphenicol. A strain was regarded as sensitive if the extinction zone was more than 25 mm, and resistant if it was less than 8 mm, in diameter.

Serial dilutions were prepared in Widal tubes; to 1 ml of each dilution one drop of the 1 : 10 diluted 24-hour broth culture of the bacterium was added. Results were read after 24 hour's incubation at 37° C.

Penicillinase was assayed according to HENRY and HOUSEWRIGHT [8]; the results are expressed in terms of μl CO_2.

Oxygen consumption was determined in the Warburg apparatus in a total volume of 4 ml of nutrient broth inoculated with 10^9 bacteria per ml. The frequency of shaking was 120 per minute.

Results

In a preliminary experiment the sensitivity to fatty acids of antibiotic-sensitive and antibiotic-resistant (in the following, sensitive and resistant) strains were compared. The strains were tested against oleic acid, linoleic acid and linolenic acid in both agar and broth media. The growth of the Staphylococcus strains tested was not influenced by 0.12 per cent oleic acid (or sodium oleate) in the agar medium. Higher concentrations proved to be bacteriostatic. The threshold concentration for linoleic acid and linolenic acid was equally 0.03 per cent. *B. megaterium* and *B. subtilis* proved to be remarkably more sensitive to the fatty acids than *St. aureus*. For the former, concentrations exceeding 0.01 per cent were bacteriostatic. The same fatty acids were less efficient in fluid medium; both sensitive and resistant bacteria were able to grow, at a slow rate, in broth containing even 2 per cent unsaturated fatty acid. As a conclusion of these experiments, sensitive and resistant bacteria could not be distinguished on the basis of their sensitivity to the bacteriostatic effect of fatty acids. On the other hand, the more unsaturated linoleic and linolenic acids generally were more efficient than oleic acid.

Next, the influence of minute amounts of fatty acids on the resistance to antibiotics of Staphylococcus strains was tested, on agar medium. The results are shown in Table I.

The sensitivity of the sensitive strain No. 102 was not influenced by 0.04 per cent sodium oleate, whereas the resistance to penicillin of the penicillin-resistant strain No. 1 and of the polyresistant strain No. 53 had considerably weakened. It should be noted that their resistance to other antibiotics was unchanged and that linoleic acid and linolenic acid behaved similarly.

Table I

Sensitivity of St. aureus strains to antibiotics on normal agar and on agar medium containing 0.04 per cent sodium oleate

Strain	Sensitivity	Medium	Diameters of the inhibition zones in mm					
			P	Sm	Ch	T	A	E
No. 102	sensitive	agar	28	28	25	31	23	29
		oleate-agar	31	33	29	37	23	33
No. 1	penicillin-resistant	agar	0	25	23	29	26	29
		oleate-agar	15	28	26	30	21	31
No. 53	polyresistant	agar	0	0	0	0	0	3
		oleate-agar	11	0	0	0	0	6

In broth 0.004 per cent sodium oleate did not alter the sensitivity of the sensitive strains of *St. aureus* (Table II).

Table II

Effect of 0.004 per cent sodium oleate in the nutrient broth on the penicillin-sensitivity of sensitive and resistant bacteria

Bacterium	Sensitivity to antibiotics	Sensitivity to penicillin = U/ml in the		Increase in sensitivity
		broth	oleate-broth	
St. aureus No. 102	sensitive	0.12	0.12	—
St. aureus No. 1	penicillin-resistant	200.00	6.00	33 : 1
St. aureus No. 53	polyresistant	100.00	2.5	40 : 1
B. megaterium No. 44	sensitive	0.25	0.25	—
B. subtilis No. 60	resistant	50.00	50.00	—

Unlike this, the penicillin-resistant and the polyresistant strains became 33 times and 40 times respectively, more sensitive to penicillin (see also Fig. 1). Practically the same results were obtained in experiments with the other two fatty acids.

Thus, substatic doses of unsaturated fatty acids increased the sensitivity to penicillin of the penicillin-resistant and polyresistant strains, but did not alter the antibiotic-sensitivity of the sensitive strains. This action of the unsaturated fatty acids, which appears to be specific for resistant strains, is to be distinguished from the bacteriostatic effect of these compounds.

In the following experiments the influence of oleic acid on the O_2 consumption by sensitive and resistant strains of *St. aureus* was tested in the Warburg apparatus, in the presence of penicillin (Table III).

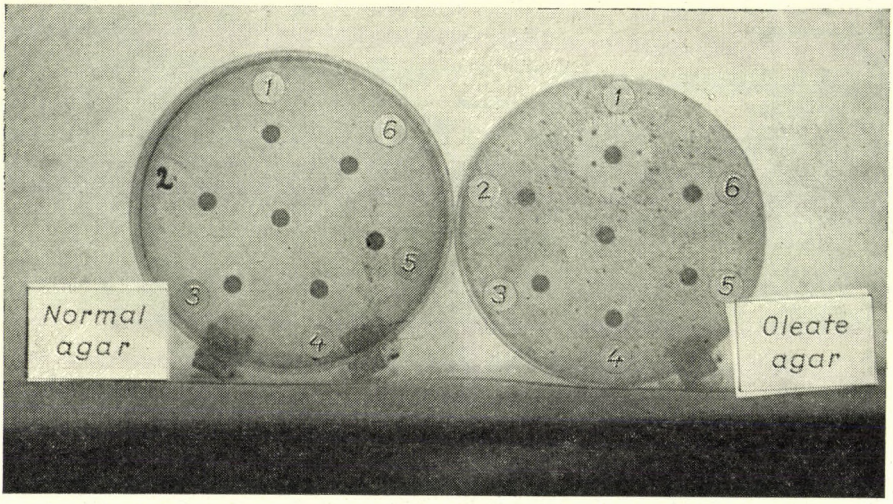


Fig. 1. Sensitivity to antibiotics of the polyresistant *St. pyogenes aureus* strain No. 53 as tested on normal agar and on agar medium containing 0.04 per cent sodium oleate
1. penicillin, 2. streptomycin, 3. chloramphenicol, 4. terramycin, 5. aureomycin, 6. terramycin. Note the difference in the sensitivity to penicillin

Table III

Effect of 0.004 per cent sodium oleate on the O_2 consumption of sensitive and resistant strains of St. aureus in the presence of substatic doses of penicillin

Strain	Sensitivity	O_2 consumption in μl/mg dry bacterium in broth containing				Reduction per cent D—C
		none A	oleate B	penicillin C	oleate + penicillin D	
No. 53	polyresistant	1120	1012	874	362	59
No. 100	sensitive	853	589	555	481	13
No. 1	penicillin-resistant	876	771	766	338	56

As shown in column B of Table III, 0.004 per cent oleic acid slightly reduced O_2 consumption: most significantly that of the sensitive strain, while the respiration of the polyresistant strain was hardly reduced. In column C of Table III the O_2 consumption in the presence of substatic concentrations of penicillin (300 units per ml for the resistant strains, 15 units per ml for the sensitive one) is presented. The reduction in the respiration was remarkable in

each case. Column D of Table III shows the figures obtained in the presence of both sodium oleate (0.004 per cent) and penicillin (see above). The effects as shown in the last column appear to be synergistic in the case of the penicillin-resistant and polyresistant strains (56 and 59 per cent reduction, respectively). A synergistic effect like this could not be demonstrated with the sensitive strain.

Based on the foregoing data, the synergism observed has been ascribed either to a direct action on penicillinase activity, or to the inhibition of the synthesis of this enzyme, or the to increased vulnerability of the resistant cells due to certain features distinguishing the surface of these cells from that of

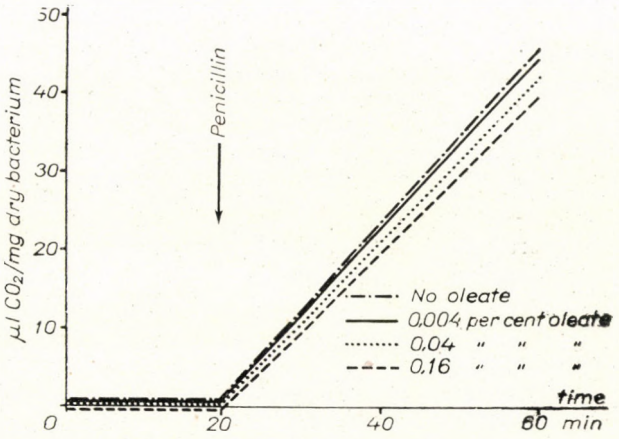


Fig. 2. Effect of sodium oleate on the penicillinase activity of the polyresistant strain No. 53

the sensitive ones. To obtain further evidence, we examined the effect of various concentrations of sodium oleate on the endopenicillinase activity of the polyresistant strain No. 53. The results are presented in Fig. 2.

As seen in Fig. 2, enzyme activity was not altered markedly by concentrations from 0.004 per cent to 0.160 per cent of sodium oleate. In addition, neither of the three unsaturated fatty acids tested proved to be synergistic concerning the sensitivity to penicillin of *B. subtilis*, a bacterium producing exopenicillinase. Thus, a direct action on the enzyme has no, or hardly any, role in the synergistic effect. This is probably due to other factors; namely, inhibition of the enzyme production and surface effects.

In further experiments we examined how the endopenicillinase activity of the polyresistant strains was altered on the addition of 0.04 per cent sodium oleate at various stages of cultivation. The results are shown in Table IV.

Markedly reduced endopenicillinase activities were found in the 24-hour cultures if the unsaturated fatty acid had been added after 6 to 8 hours of cultivation, *i. e.* soon after the logarithmic phase had begun. It should be

Table IV

Endopenicillinase activity of 24-hour shake cultures of a polyresistant strain of St. aureus (Cultivation at 37° C)

Time of adding oleate,* hour of cultivation	CO_2 production in μl/mg dry bacterium per hour
0	75
2	70
4	75
6	78
8	16
No oleate	207

* Sodium oleate, 0.04 per cent

noted that bacterial cells at this stage of cultivation are most sensitive to the bacteriostatic and bactericidal effects of fatty acids.

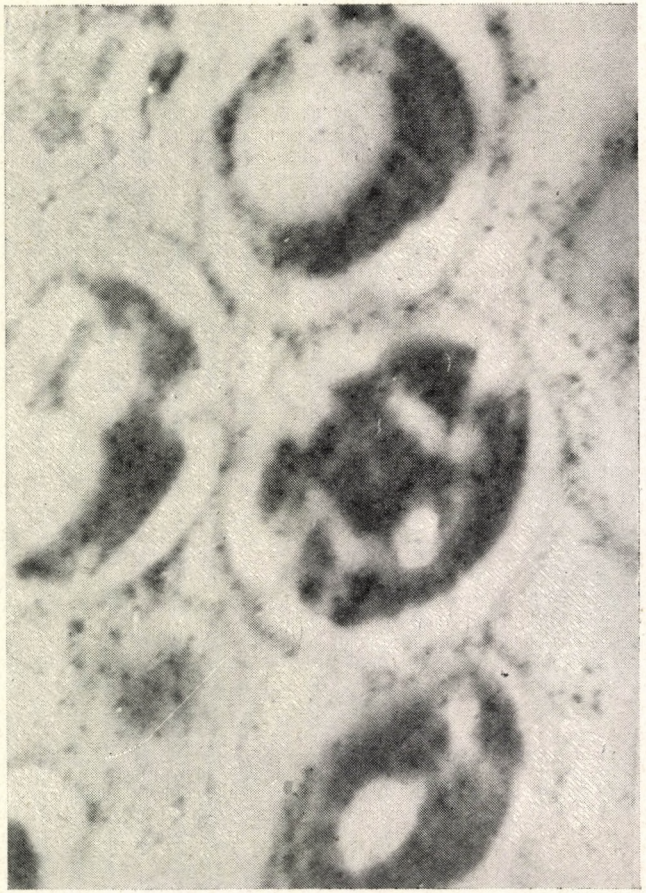
The electronmicrographs show sodium-oleate-treated (Fig. 3 and 4) and normal (Fig. 5) *St. aureus* cells in ultra-thin sections.

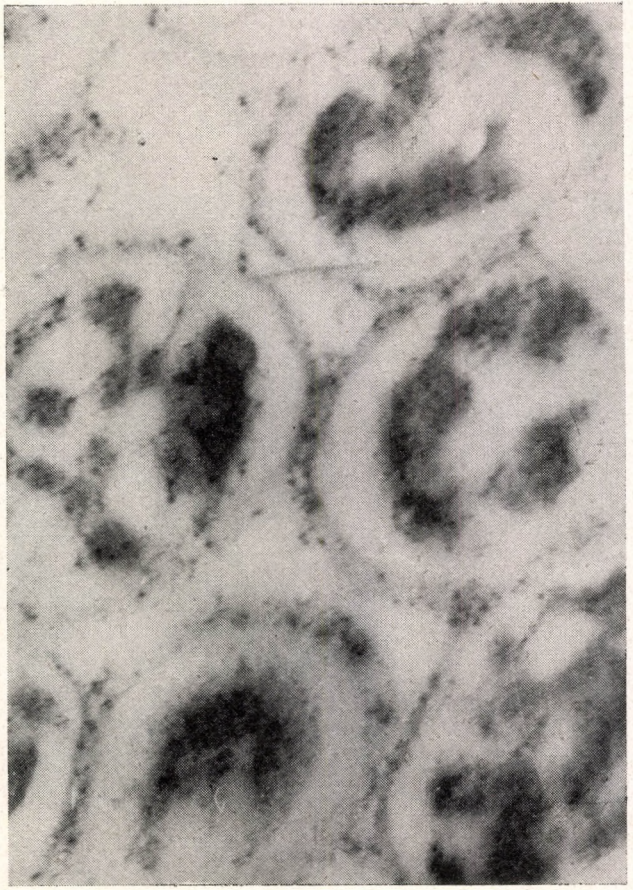
The former appear as swollen cells with reduced electron density, especially in the marginal areas; the cytoplasm being detached the cell wall shows loose structure.

Discussion

Synergism *in vitro* and *in vivo* between certain plant oils and penicillin has been suggested by several authors. As demonstrated by GABRIELLI [9], extracts of *Pinus pumilio* suspended in 2 per cent Tween 80 are highly synergistic on penicillin-sensitive and penicillin-resistant strains of Staphylococcus. MARUZELLA and BLOCH [10], who examined 240 plant oils, reported that the effect of antibiotics, most frequently that of penicillin, was enhanced by the oils in 58 combinations.

Our own findings suggest that this effect of plant oils might be caused by unsaturated fatty acids present in the oil preparations. It is well known that, within certain limits, the more unsaturated a fatty acid and the longer its carbon chain, the more antibacterial it is. Since this effect of fatty acids is dependent on the composition of the cell surface, the synergism demonstrable on resistant strains suggests only that the composition of the cell wall is different in resistant and in sensitive strains of Staphylococcus. Another possible explanation is that unsaturated fatty acids inhibit the production of penicillinase. VÁCZI et al. [11] found the surface of resistant bacteria more abundant in lipids than that of sensitive ones. Differences in lipid composition and metabolism might explain, why synergism was demonstrable also on resistant, but not on sensitive Staphylococcus strains.





Figs. 3 and *4*. Polyresistant *St. aureus* cells treated with sodium oleate. Electronmicrographs of ultra-thin sections. Fixed by 0.3 per cent $KMnO_3$, embedded in methylmetacrylate. × 60 000 (Made in the Central Laboratory of University Medical School, Debrecen)

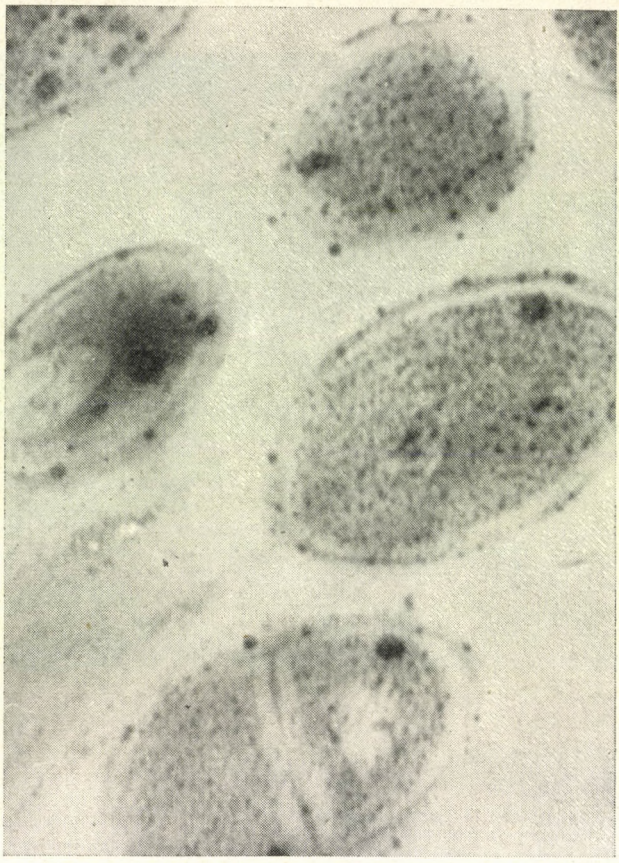


Fig. 5. Control cells

The following data support this conception. (i) Lecithine, being able to be coupled to oleic acid, acts as a detoxicant, thus inhibits or even stops the destructive effect of the latter. (ii) VÁCZI et al. have demonstrated that the increase in the resistance to antibiotics of *E. coli* runs parallel with the rise in the ratio cephalin per lecithine in the cell surface. (iii) According to MARKOV and SAEV [13], the surface of sensitive Staphylococci is poor in monophosphate esters. (iv) GILISSEN and RUDA [14] have demonstrated that the more resistant a Staphylococcus strain, the higher its phosphatase activity.

The data suggest that on the surface of resistant cells the detoxication of fatty acids is inhibited. If so, the fatty acids retain their dual capacity of (i) destroying the cell, and (ii) inhibiting penicillinase production. Penicillin acts by inhibiting cell-wall synthesis, and its effect is most marked during the logarithmic phase when the cells are most sensitive to fatty acids. The resulting twofold damage considerably reduces the viability of the bacterium.

The above results have provided new evidence of relationships between the surface structure and sensitivity to antibiotics of bacteria, particularly of Staphylococci; furthermore, they have pointed to the unusually favourable synergistic effect of unsaturated fatty acids on penicillin-resistant and poly-resistant strains of *St. pyogenes aureus*.

Acknowledgement. We are indebted to Dr. K. BENKŐ for the electronmicrographs.

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STUDIES ON THE NATURE OF PHASE VARIATION OF SH. SONNEI

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Summary. (i) The properties of phase II of *Sh. sonnei* and of R variants of enteric bacteria have been compared.

(ii) The morphological, cultural and physicochemical properties (spontaneous, trypaflavine and acid agglutination, Millon reaction) of phase II are similar to those of enteric R variants.

(iii) Phase II bacteria, as well as the R variants of enteric organisms, are non-virulent and only slightly toxic for mice. The absence of human pathogenicity of these bacteria is shown by the lack of phase II agglutinins and mouse-protecting antibodies in sera of patients who have been suffering from Sonne dysentery.

(iv) The reversion of phase II into phase I is not possible. Observations on this kind of reversion may be explained by the dissociation of mixed cultures.

(v) From phase II an artificial antigenic variant may be obtained.

(vi) The present investigations have shown that the phase variation of *Sh. sonnei* should be regarded as an S—R variation.

The colonial variation of *Sh. sonnei* has been known since the classical observation of SONNE [1]. The serological difference existing between the two types of colonies has also been early realized [2]. The opinions regarding the essence of the variation are not uniform. According to earlier investigators, it corresponds to an S—R variation [3, 4]. This concept is postulated today by KAUFFMANN [5]. Since the work of WAALER [6] and particularly that of WHEELER and MICKLE [7] it has been widely believed that the phenomenon is independent of the S—R variation, and the terms phase I and phase II have come into general use. These authors isolated a third type of colony, which differed from phase II colonies with its more distinct roughness, its tendency of giving spotaneous agglutination and its different antigenic structure. Bacteria forming colonies of this type were regarded by the latter authors as the R variants. These findings were noted also by BAKER et al. [8], who confirmed the concept by the observation that the somatic antigens isolated from phase I and II gave closely similar analytical results, whereas in the R variant no somatic antigen could be demonstrated.

A critical study of the immune-biological properties of phase II, however, makes it questionable whether the phenomenon is rightly regarded as a variation other than S—R. In the present investigations we have attempted to settle

the problem. For the sake of simplicity, in the experimental part of this paper the terms phase I and II and R variant are to be used without reference to our opinion.

Materials and methods

Cultures. In the experiments 10 freshly-isolated and 10 stock cultures of *Sh. sonnei* were used. Several of the strains were pure phase II cultures. The stock cultures had been maintained in this laboratory for 1 to 10 years.

Immune sera. Phase I sera were prepared in rabbits with suspensions obtained from isolated phase I colonies. The colonies were removed with a loop and suspended in saline containing 0.2 per cent formalin. The suspension contained 1000 million bacteria per ml. The rabbits were injected intravenously 4 or 5 times at 4-day intervals. The titres of the sera were 1/1600 to 1/6400. In spite of the careful preparation of the antigen, phase II agglutinins appeared up to 1/5 to 1/10 of the phase I titre of the sera. These were removed by phase II suspensions (10 ml 1/20 diluted serum absorbed by bacteria grown in 2 to 3 Kolle flasks).

Phase II sera were prepared from phase II colonies of strains which had been isolated freshly or years ago. From agar plates the selected colony was transferred into broth, then this culture was streaked again on an agar plate. Provided that the culture was purely in phase II, the selected colony was transferred onto an agar slant (see part entitled "The stability of phase II"). The growth from the slant was suspended in 0.2 per cent NaCl and killed by the addition of 0.2 per cent formalin. Immunization was carried out as described above. The titres of phase II sera were 1/800 to 1/1600. Cross absorption experiments showed that sera prepared from old and freshly-isolated strains were identical.

Agglutination. Serum dilutions were prepared in 0.5 ml volumes. To each tube 1 drop of formolized suspension containing 1000 million organisms was added. In order to avoid spontaneous agglutination, when phase II bacteria were examined, dilution was performed with 0.2 per cent NaCl solution, and the suspensions were prepared with a solution containing 0.2 per cent NaCl and 0.2 per cent formalin. The use of formolized suspensions allows an easier reading than that of living organisms, as in contrast to the powdery aggregation shown by the latter, the formolized bacteria give a coarser type of agglutination on shaking.

Acid agglutination was carried out as recommended by BEGUIN et al. [19] in glycine buffer at different pH values ranging from 1.9 to 3.5. In 1000 ml of distilled water 7.505 g of glycine and 5.58 g of NaCl were dissolved. By mixing different quantities of this solution and of N/10 HCl, a series of tubes containing buffer solution with different pH values were prepared. Into each tube 1 drop of formolized suspension was pipetted. Readings were made after the tubes had been allowed to stand at room temperature for 1 to 2 hours.

Millon's test. The culture from an agar slant was suspended in 3 ml distilled water, washed 3 times by centrifugation, then resuspended in 3 ml of distilled water. To 1 ml of suspension 5 drops of Millon reagent were added. The tube was then kept in boiling water for 5 to 10 minutes. Positive reaction was indicated by a brick-red deposit.

Artificial induction of R variants was attempted by the method of BEGUIN et al. [19] a) in serum broth and b) in hypertonic broth.

a) The organisms vere serially subcultured in broth containing phase II serum in a dilution of 1/100. Altogether 20 transfers were made at 2-day intervals. Then the cultures were streaked on agar plates and the colonies with the morphological properties of R variants were isolated.

b) The transfers described under a) were performed in broth containing 2 per cent NaCl.

Toxicity. Bacteria were dried with acetone and ground flourfine in a mortar. Then suspensions were prepared containing 10 mg/ml of the bacteria. Mice were injected intraperitoneally with graded amounts of the suspensions. Toxicity was estimated from the number of mice killed within 72 hours.

Virulence. Suspension containing various numbers of bacteria were prepared from 24-hour Bacto-peptone water cultures. The density of the suspensions was measured turbidimetrically against a standard. Groups of 4 mice each were injected intraperitoneally with various numbers of bacteria. Virulence was estimated from the number of mice killed within 72 hours. When the mucin technique was employed, the bacteria were suspended in 5 per cent sterile 1701 W mucin.

Mouse-protection potency of sera. Graded quantities of sera were injected intraperitoneally to groups of mice. One hour later the animals were infected intraperitoneally with titrated amounts of peptone water cultures suspended in 5 per cent mucin. For the precise determination of the LD_{50} the corresponding doses of bacteria were simultaneously injected into untreated mice.

Results

The problem of "R variant". In order to isolate the third colonial type, the "R variant" of WAALER and WHEELER, isolated colonies of phase II strains maintained for years in our collection were carefully examined. In some cases among the colonies smaller and rougher colonies were found. These were removed, agglutinated in phase II serum and used for the immunization of rabbits and for Castellani's absorption. The serological examinations showed that the antigenic structure of these morphologically somewhat different colonies was perfectly identical with that of phase II organisms, displaying cross agglutination and mutual absorption of the heterologous sera. Thus the difference between the two types of colony was merely a morphological one. It is known that colonies of R-appearance are not always characterized by an altered immune-biological property, and their appearance is often only a morphological change depending on certain circumstances [9, 10].

In subsequent experiments the artificial induction of R variants was attempted, by means of serial subculturing of the organisms in homologous agglutinating serum and hypertonic broth. The procedure was carried out on 5 phase II strains of the culture collection. When the 20th subculture from the hypertonic broth was streaked on agar plates, one of the strains gave off some rougher colonies. The subculture of one of these colonies was designated as R_1 and used for the preparation of agglutinating serum.

Properties of phase II and of variant R_1. A comparison was made between the properties of phase II, of variant R_1 and of R variants of enteric bacteria.

According to ARKWRIGHT [11, 12, 13], WHITE [14], SCHÜTZE [15], IBRAHIM [16], DE KRUIF [17], SCHOLTENS [18], HEYMAN [9], KRÖGER [10] and BEGUIN [19], the R variants of enteric bacteria are characterized by the following properties: 1) morphological and cultural behaviour; 2) antigenic structure; 3) immune-biological behaviour. The chemical properties of the antigens will be discussed in a separate paper.

1. *Morphological, cultural and physicochemical properties*

The following properties may be regarded as characteristic of the R variants of enteric bacteria.

a) rough colonies;
b) formation of deposit in broth, the supernatant often remains clear;
c) spontaneous agglutination in saline;
d) sensitivity to trypaflavine;
e) acid agglutination between pH 2 and 3 [19];

f) precipitation and staining with Millon's reagent [20].

a) Phase II colonies show the known morphological characters of R variants, with the difference that they are larger and somewhat flatter. Colonies of variant R_1 resemble more closely the typical R variants. The colonial morphology itself is, however, not decisive, as it is a property changing easily according to the cultural circumstances. As shown by the careful studies of HEYMAN [9] and KRÖGER [10], on soft media the colonies become less rough, while with higher concentrations of agar an increased roughness can be observed.

b) Phase II does not produce such a distinct deposit in broth as the R variants do. As it will be discussed in connection with the problem of spontaneous agglutination, this property is obviously connected with the lower salt sensitivity of the former organism. Variant R_1 formed a deposit in broth.

c)—f) Data concerning sodium chloride, trypaflavine and acid agglutination, and data of Millon's reaction, are presented in Table I.

Table I

Comparison of S and R strains of enteric bacteria and Sh. sonnei phase II and its variant R_1

Variant	Strain	Agglutination					Millon's reaction
		0.9 % NaCl		Trypaflavine 0.3 %	pH 2—3.5	pH 4—5	
		living	100° C				
S	*Sh. sonnei* phase I	—	—	—	—	—	—
	Sh. flexneri 1b	—	—	—	—	—	—
	Sh. flexneri 3	—	—	—	—	—	—
R	*Sh. sonnei* 1 phase II	—	+	+	+	—	++
	Sh. sonnei 2 „	—	+	+	+	—	++
	Sh sonnei 3 „	—	+	+	+	—	++
	Sh. sonnei 4 „	—	+	+	+	—	++
	Sh. sonnei 5 „	—	+	+	+	—	++
	Sh. sonnei R_1	+	+	+	+	—	++++
	S. typhi 63 R	+	+	+	+	—	++++

All the reactions were negative with phase I and the control *Sh. flexneri* strains. Similarly to *S. typhi* R form, which was used as a control, phase II and variant R_1 were sensitive to trypaflavine and to pH 2.0—3.5 and gave a positive Millon reaction. The only difference between phase II and *S. typhi* R variant was that the former did not agglutinate in saline. Phase II, however, is agglutinated when the strains are boiled in saline [22]. According to our observations, the salt-sensitivity of the R forms of enteric bacteria is sometimes revealed at boiling only. Variant R_1 gave spontaneous agglutination in saline.

Accordingly, as to morphological, cultural and physicochemical properties, no essential difference can be demonstrated between phase II, the artificial variant and the R variants of enteric bacteria.

2. *Antigenic structure.* As to the antigenic structure, the serological properties of R variants differ from those of the S forms [11, 12]. The serological difference between *Sh. sonnei* phase I and phase II was first observed by ORSKOV and LARSEN [2]. This finding was confirmed later by LEUCHS [23], BRAUN [24], RAUSS [25] and ROELCKE [26]. The antigenic structure of the "R variant" isolated by WHEELER [7] and BAKER [8] differed from that of phase II. GOEBEL and JESAITIS [27] too, found a serological difference between phase II and the phage-resistant form regarded as an R variant.

The present results have confirmed the well-known fact that no antigenic connection exists between phase I and II (Table II). Variant R_1 is agglutinated by lower dilutions of serum phase II. The connection is unilateral, as phase II does not agglutinate in serum R_1. Thus the titre of serum phase II shows some decline when absorbed by R_1; absorption by phase II, however, does not change the titre of serum R_1. The different antigenic structure of phase II and of variant R_1 is further demonstrated by the observation that *Sh. boydii* 6 and *E. coli* 014, which are closely related to phase II [7], are not agglutinated by serum R_1.

Table II

Antigenic structure of Sh. sonnei phase I, phase II and variant R_1

Sera		Agglutination titres				
Unabsorbed	Adsorbed by	phase I	phase II	R_1	*Sh. boydii* 6	*E. coli* 014
Phase I	0	2560	—	—	—	—.
	phase II or R_1	2560	—	—	—	—
Phase II	0	—	2560	320	1280	640
	R_1	—	1280	—	640	640
R_1	0	—	—	640	—	—
	phase II	—	—	640	—	—

Accordingly, phase II, corresponding to the principal criterium of enteric R variants, shows an antigenic structure different from that of phase I. The artificial variant, R_1, differs in antigenic structure also from phase II.

3. *Immune-biological properties.* As regards the immune-biological behaviour of the R variants, ARKWRIGHT [13] and IBRAHIM [16] noted that these had no protecting potency. As they cannot be isolated from patients, the conception that they represent a non-pathogenic and avirulent variant, became generally acknowledged [15, 17]. This opinion found confirmation in the fact

that the R variants of enteric bacteria show decreased toxicity and virulence [15, 17, 28].

As phase II of *Sh. sonnei* can be shown at the first, direct plating of materials, its pathogenicity is certainly a question of importance. In the literature there are several pertaining papers with both positive and negative data [29]. We attempted to approach the question by performing animal experiments and by examining the sera of patients.

The LD_{50} doses presented in Table III show that the toxicity of acetone-killed phase I bacteria is approximately 3 times higher than that of phase II and of variant R_1. BOIVIN found a higher difference between the toxicity of S and R variants [28]. Our results corresponded mostly to more recent investigations, which have pointed out that by a suitable procedure a lipoid-like toxic substance might be isolated from the R variants [30].

As regards the virulence for mice, the difference is even more considerable. For killing 50 per cent of the mice, approximately 200 million phase I bacteria were necessary. The corresponding number for both phase II and variant R_1 was approximately 1000 million. Thus a 5fold difference existed in the LD_{50} between phase I and the latter bacteria. With mucin the virulence of phase I was so increased that about 10 bacteria were sufficient to kill 50 per cent of the animals. Under the same conditions the LD_{50} of phase II was about 10 million times higher (Table III).

Table III

Toxicity and virulence for mice of Sh. sonnei phase I, phase II and variant R_1

Toxicity of acetone-dried bacteria				Virulence of 6-hour peptone water cultures						
				Without mucin				With 5% mucin		
mg	phase I	phase II	R_1	No. of bacteria	Phase I	Phase II	R_1	No. of bacteria	Phase I	Phase II
0.5	3/4	4/4	4/4	100 million	3/4	.	.	10	2/4	.
1.0	2/4	4/4	4/4	200 million	2/4	.	.	100	0/4	.
2.0	0/4	3/4	4/4	400 million	0/4	4/4	4/4			
3.0	0/4	2/4	2/4	600 million	.	3/4	3/4	50 million	.	1/4
4.0	.	1/4	0/4	800 million	.	2/4	2/4	100 million	.	2/4
				1000 million	.	1/4	0/4	200 million	.	0/4

Key: surviving/infected animals.

The great differences in the virulence are shown also by phagocytosis and invasion experiments carried out in mice. The animals were injected intraperitoneally, and 1 hour later the peritoneal exudate was examined. With phase I a high rate of multiplication of bacteria, a slight degree of leukocyte reaction and phagocytosis, with phase II a powerful phagocytic activity and practically

no extracellular bacteria were observed. Two hours after infection hardly any bacteria were seen in the peritoneal cavity of mice which had received phase II, while in the animals which had been given phase I, an enormous number of bacteria and broken up leukocytes were present. As to the invasive ability of phase I and II, there was also a considerable difference. After intraperitoneal injection phase II caused only late and transient bacteriaemia, whereas phase I bacteria were found in the heart-blood as soon as 10 minutes after infection (Table IV).

Table IV

Virulence for mice of Sh. sonnei phase I and II

Phase	No. of bacteria injected i. p.	Result of heart-blood culture at minutes after infection			
		10	30	60	120
I	50 million	++	++++	++++	++++
II	300 million	—	—	+	—

The fact that phase II strains do not cause keratoconjunctivitis in the eye of the guinea-pig, is another sign of the lack of virulence. Phase I bacteria, similarly to other shigellae, are regularly capable of giving rise to keratoconjunctivitis [39].

Thus, as to animal toxicity and virulence, phase II bacteria are similar to the R variants of other enteric bacteria.

The pathogenic role of the examined organisms was most clearly demonstrated by the mouse-protection experiments performed with sera from patients (Table V).

Table V

Agglutinin and mouse-protection titres against Sh. sonnei phase I and II of sera from patients with dysentery

Interval between onset of disease and sampling of blood	Average agglutinin titre		Mouse-protection titre		
	Phase I	Phase II	Doses of immunizing serum ml	Challenging doses	
				Phase I 3750 LD_{50}	Phase II 2.5 LD_{50}
10 days	1 : 80	1 : 20	0.01	2/4	0/4
			0.1	4/4	0/4
20 days	1 : 320	1 : 20	0.01	3/4	0/4
			0.1	4/4	0/4
20 months.....	1 : 20	1 : 20	0.01	0/4	0/4
			0.1	0/4	0/4

Key: surviving/infected animals

Table V presents the average of the mouse-protection potency and agglutinating titres of a pool of the sera from 10 patients, who had been suffering from a *Sh. sonnei* infection. During the course of the disease phase II bacteria could be abundantly isolated from the faeces of these patients. The blood specimens were taken on the 10th and 20th day of the disease and 20 months later.

The average agglutinating titre for phase I was 1/320 at the 20th day of the disease. At the same time the increase of agglutinins for phase II could not be demonstrated. The low titre for phase II may be considered normal since 20 months later, after the immunity had ceased, it was still at the same level.

The mouse-protection titre of the serum was even more demonstrative. The serum taken on the 10th and 20th day of the disease was protective in 0.01 ml amounts for 50 per cent of the mice against a challenging dose of 3750 LD_{50} of phase I, while 0.1 ml serum afforded no protection against 2.5 LD_{50} of phase II bacteria. In accordance with our observations on the duration of immunity after *Sh. flexneri* infection [38], no humoral antibodies were shown in 0.1 ml of the serum pool 20 months after the onset of the disease.

The present experiments, in accordance with the unsuccessful attempts of ROELCKE at infecting man with phase II [29], confirm that phase II is non-pathogenic. Thus in this respect it also corresponds to the behaviour of R variants.

The stability of phase II. The finding that phase II can be reverted in the human body or in animal experiments into phase I [32], has been regarded as one of the evidences for the difference between phase II and the R variant, since a reversion of the R form into the S form has not been demonstrated. Reversion of phase II *in vitro* has also been noted [33, 34]. To elucidate the problem of both theoretical and practical importance the following experiments were performed.

From a primary culture on eosin-methylene blue agar typical phase I colonies were picked and streaked on agar plates. Next day 5 of the morphologically typical phase II colonies, which appeared in addition to the phase I colonies, were suspended in 0.5 ml of saline. The density of the suspension was set to contain 1000 million organisms per ml. Part of the suspension was transferred into broth, other parts, each containing 100 million organisms, were injected intraperitoneally into mice. The broth culture was streaked after 6 hours of incubation on an agar plate; the mice were killed 1 hour after the injection and cultures were made from the heart-blood and peritoneal exudate. One of the experiments is presented in Table VI.

From Table VI it is clear that 3 out of the 5 morphologically typical first generation phase II colonies of one of the examined strains were in reality mixed cultures, which after a single transfer in broth dissociated into phase I

Table VI

Reversion of phase II (strain No. 137)

Proportion of colonies on agar plates inoculated with broth cultures			Result of cultivation 1 hour after the infection of mice			
			Heart-blood		Peritoneal cavity	
Designation of culture	Phase I %	Phase II %	Phase I	Phase II	Phase I	Phase II
1	20	80	++	—	++	+++
2	—	100	sterile	sterile	—	++++
3	5	95	+	—	++	+++
4	10	90	++	—	++	+++
5	—	100	sterile	sterile	—	++++

and phase II colonies. The colonies were examined with slide agglutination in pure phase I and II sera. The dissociation of the same 3 colonies was observed in mice as well. From the peritoneal exudate both phases, from the heart-blood phase I colonies only were recovered. It has already been discussed that the inability of phase II organisms to produce bacteriaemia is an evidence of the lack of virulence. In these experiments 5 colonies of each of 10 freshly isolated strains were examined. In 8 strains 1 to 3 of the 5 colonies consisted of mixed cultures.

Typical phase II colonies from the cultures showing dissociation were subsequently examined by the above-described method. In the second generation the examination of 85 colonies revealed that one or two colonies of 4 strains still consisted of mixed phases. In the third generation only one colony of one strain showed dissociation. In the fourth generation no dissociation occurred. These experiments shed light on the phenomenon that has been believed the reversion of phase II and which, accordingly, may be attributed to the dissociation of mixed colonies. These observations are in agreement with those of SERÉNY [35], who showed that in *Sh. sonnei* cultures colonies with star-like structure are frequently encountered. This light refraction phenomenon is due to the simultaneous occurrence of phase I and II constituents.

Discussion

The problem whether the so-called "phase variation" of *Sh. sonnei* can really be regarded as a phenomenon independent of the S—R variation has been approached from several aspects. The careful investigation of pure phase II strains of the culture collection did not reveal any colonies different in antigenic structure from phase II. Only a drastic procedure (cultivation in hypertonic broth) induced some colonies serologically and morphologically different from phase II.

The properties of phase II and of the artificial variant were compared with the R variants of enteric bacteria. The three kinds of organism showed no essential difference either in morphological, cultural and physicochemical properties or in antigenic structure. *Accordingly, phase II is to be regarded as an R variant.* The results of the experiments concerning virulence for mice and human pathogenicity of phase II have confirmed this opinion. Phase II bacteria are perfectly avirulent for mice, their virulence can hardly be enhanced with mucin, and compared with phase I strains, they are only slightly toxic for mice. The fact that during disease no protective antibodies are produced against phase II, is an indirect proof of the lack of human pathogenicity of these bacteria. In this respect they are similar to the R variants of enteric bacteria.

If phase II corresponds to an R variant, how should the position of the artificially induced variant be explained? It is known from the examinations of WHITE [36] and HENDERSON [37] that variation does not end at the R form, but may proceed and result in ϱ variants (ϱ_1 and ϱ_2). Although the variant is mostly related serologically to the R form, it has an antigenic structure independent of that of the latter. It would appear that variant R_1 is the ϱ_1 variant of *Sh. sonnei*. This seems to be confirmed by the serological behaviour of R_1: it is agglutinated by phase II serum, but the reverse of this reaction cannot be demonstrated. This shows that strain R_1 is a degraded variant of phase II, as in phase II the antigen characterizing R_1 is present, while R_1 lacks the components of phase II and contains only the former antigen, which in phase II is probably more deeply situated. The "third colonial type" of WAALER [6] and WHEELER [7] and BAKER [8] was apparently a ϱ variant, as well as GOEBEL's [27] "R variants" produced by phage action.

The present investigations have settled the long debated question whether phase II may revert to phase I. It has been clearly demonstrated that when a reversion seems to occur, in reality a dissociation of colonies containing both phase I and II is taking place. In our experiments seemingly pure phase II colonies contained phase I bacteria in the second and even in the third generation. In such cultures the appearance of phase I under suitable conditions may be mistaken for an evidence of reversion.

The present investigations have shown that phase II of *Sh. sonnei* corresponds to the so far known characteristic properties of the R variants of enteric bacteria. Accordingly, the so-called phase variation may be regarded as a phenomenon identical with the S—R variation,

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TEMPERATE BACTERIOPHAGES ISOLATED FROM RHIZOBIUM MELILOTI

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Summary. FISK's test was combined with LEDERBERG's replica method and by the resulting test 152 strains of *Rh. meliloti* freshly isolated from nodules were examined in every possible combination. A hundred and three strains proved to be phage carriers or lysogenic. From the unfiltered lysates 63 phage isolates could be recovered; on the basis of the immunity test 44 of these appeared to be temperate, 11 behaved as virulent. Eight phages were unsuitable for the immunity test. Of the 44 temperate phages 8 proved to be polyvalent, 10 and 26 showed medium and low valency, respectively. Three sensitive strains of *Rh. meliloti* were attempted to lysogenize using 40 different phages. Of the lysogenic derivatives thus obtained 13 were examined for changes in their phage pattern. Based on the changes caused in the phage pattern of the bacteria the 13 phages were divided into 7 groups.

The agricultural importance of Rhizobiophages was recognized chiefly by the investigations of RAZUMOVSKAYA [1], KLECZKOWSKA [2] and ALLEN and ALLEN [3] several decades ago. Since then, though general interest has been focussed on lysogeny and the genetic phenomena accompanying it [6], lysogeny in Rhizobia has been scarcely studied and the existence of temperate Rhizobiophages has not been mentioned but in two short communications[4, 5].

In the present investigations a great number of *Rh. meliloti* isolates were studied to obtain lysogenic derivates in sufficient number for our future genetic studies. The existence of numerous temperate Rhizobiophages has been demonstrated.

Materials and methods

Origin of the bacteria. The *Rh. meliloti*, *Rh. trifolii* and *Lotus rhizobia* strains were obtained partly from Budapest, partly from Balatonberény. They were isolated from the nodules of Melilotus, Trifolium and Lotus plants, respectively. The nodules cut off together with small pieces of root were washed with sterile water several times, then treated with 70 per cent ethanol for about half a minute and washed once more with sterile water. This was followed by treatment with 0.1 per cent mercury chloride for 3—4 minutes. After another washing the nodules were burst between two slides each; the expressed juice was diluted with sterile water and plated in LAIRD's M5 agar medium. The purified cultures obtained by repeated platings were numbered in the order of their isolation.

Identification of the isolates. The clonal cultures thus obtained were identified by inoculation into the host plant. For this purpose seedlings, grown on NUTMAN's agar in tubes under sterile conditions were used. The experiments were repeated three times. Seeds were sterilized in the same manner as nodules.

Methods of cultivation. The cultures were maintained on LAIRD's M2 and M5 media as modified by us, and on ASHBY's medium. The composition of the M5 medium was 0.5 g K_2HPO_4; 0.2 g $MgSO_4$; 0.1 g NaCl; 100 ml yeast water; 900 ml H_2O. Medium M2 was prepared by adding

5 g of mannite and 1 g of potato dextrose to 1 litre M5 medium. The stock cultures were maintained on ASHBY's medium composed as follows; 0.2 g K_2HPO_4; 0.2 g $MgSO_4$; 0.2 g $(NH_4)_2SO_4$; 10.0 g mannite; 0.2 g NaCl; 0.1 g $CaSO_4$; 5.0 g $CaCO_3$; 20 g agar; 1000 ml H_2O.

Isolation of Rhizobiophages. FISK's combination test originally applied for Staphylococci was combined with LEDERBERG's replica method [8, 9]. According to FISK's technique every combination of 4 strains is tested on one plate; this method, though effective, proved to be too slow to test a large collection of bacteria; therefore, instead of streaking the indicator organism on the surface of the medium we plated it in the usual double soft agar layer. Using a replicating apparatus, 15 strains could be placed by a single movement on the top of this layer. During incubation, the phage-liberating cultures formed easily recognizable lytic zones in the plate culture of the indicator bacterium. The diameter of the lytic area seemed to depend on the amount of the released phage. In order to isolate the phages, small pieces of agar from the lytic zones were picked and suspended in M2 medium; the phages were isolated by repeated platings. Propagation and titration of phages was based on ADAMS' methodical work [10]. The cultures were filtered through Schott G5 glass filtres.

Soil bacteriophages were isolated by CROSSE and HINGORANI's method [7] as modified by us. Briefly, in Erlenmeyer flasks 50—100 g fresh soil sample was added to 60 ml M2 medium inoculated with a fresh Rhizobium culture. After incubation the cultures were centrifuged and filtered. The lysates were plated and incubated. The phages were isolated from the plaques appearing in the cultures. Of the soil phages thus obtained only one, No. 16-3, was used in experiments.

Lysogenization. One loopful of the filtered lysate was brought onto an indicator plate. If secondary colonies had grown on the lytic area, bacteria were isolated from there, and propagated and purified by clonal isolation. The clones were tested for phage production on indicator bacteria. Their immunity against the homologous phage was demonstrated by dropping on the filtrate of the homologous phage (spot test).

Experimental

Collection of bacteria. On the basis of the literary data it was supposed that a large collection of bacteria was needed to obtain lysogenic strains. For this purpose isolation of nodule bacteria was attempted from 173 *Rh. meliloti* nodules. From these 152 bacterial strains were obtained. Each of these induced nodulation in the experimentally infected seedlings. From *Rh. trifolii* 15, from *Rh. lotus* 12 strains were obtained. Most of the strains were of the mucoid type; one of them, *Rh. meliloti* No. 65, is a pigment-producing strain.

In the identification tests nodulation in host plants grown under sterile conditions became visible after 10 days and the nodules were fully developed within one month. The number of the nodules, *i. e.* the quantitative efficiency of the strains, was not taken into account.

Testing for lysogeny by the combination test. The technique applied allowed to test the 152 *Rh. meliloti* strains in every possible combination. Of these 103 strains proved to be phage carriers or lysogenic. The so-called unfiltered lysates, which had been picked from the lytic zones, and suspended in M2, contained 10^1 to 10^6 infective particles each. From the 96 unfiltered lysates tested 63 phages (66 per cent) were recovered, whereas 33 were lost, probably by inadequate storage.

Demonstration of lysogeny. The virus-carrying bacteria were tested for immunity against the homologous phage. The strains showing immunity were regarded as lysogenic and the liberated phages as temperate [6].

The immunity test consisted of parallel titrations of the filtrates in cultures of both the phage carrier and an appropriate indicator strain. Of the 63 carriers 44 proved to be lysogenic, whereas in 11 cases the phage appeared to be virulent. Eight phages were unsuitable for this test.

Host range studies. Host range was established by testing phages against each of the *Rh. meliloti* strains available and against 5 *Rh. trifolii*, 5 *Rh. lotus* and 4 *Erwinia tumefaciens* strains. The same replica method was used as in the combination tests.

Based on their host-range, the 44 temperate Rhizobiophages were divided into the following groups: (i) polyvalent phages lysing 40—73 host organisms; (ii) phages with medium valency lysing 19—33 organisms; (iii) those with low valency lysing 1—15 strains each. The phages in groups (i), (ii) and (iii) numbered 8, 10 and 26 respectively.

Plaque morphotypes. It is generally accepted that the shape, size and outline of the plaques are characteristic of the phage strain. The plaques brought about by the Rhizobiophages studied by us were rather variable. From pin-point plaques to those with 4—6 mm in diameter every size was found; their appearance varied from turbid to clear. In certain plaques the clear centre was surrounded by a turbid halo. In some cases the plaque was target-like showing concentric zones.

Lysogenization. In order to demonstrate lysogenization by the temperate Rhizobiophages, 3 *Rh. meliloti* strains (Nos. 29, 41 and 69), which had been found susceptible to most of the phage isolates, were plated on agar medium and tested with 40 phage isolates. Bacteria were then picked from the secondary colonies growing in the plaques, and purified by successive platings. Most of the derivates thus obtained proved to be phage producing and immune against the homologous phage. The clones which had been stabilized after repeated isolations were used in the following experiments.

Phage patterns of the lysogenized derivates. In addition to serological relationships the altered phage pattern of the lysogenized bacteria is regarded as the most appropriate basis for grouping bacteriophages and to establish the degree of relationship among them. Host-range studies using only non-lysogenized hosts offer less valuable information.

In Tables I and II the phage patterns of two *Rh. meliloti* strains (Nos. 29 and 41) and of their lysogenized clones are presented.

The phage pattern for 13 of the 40 lysogenized derivatives was established with 41 phage isolates. On the basis of the changes induced in the phage pattern by lysogenization the 13 phages were divided into 7 well-distinguishable groups as shown in Table III.

Furthermore, it should be mentioned that the strain *Rh. meliloti* No. 41, which was immune against the phages No. 57, 62 and 169, became susceptible to the same phages when lysogenized by group A phages (Table I). Elucidation

Table I

Changes in the phage pattern of Rh. meliloti No. 41 by lysogenization

The susceptible clone and its lysogenic derivatives	Indicator phages																										
	36	64	128	132	138	4	16—3	160	161	32	111	72	31	34	38	143	147	157	152	153	154	157	167	170	169	62	57
41	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	o	o	o
41(16—3)	+	+	+	+	+	+	0	0	0	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
(160)	+	+	+	+	+	+	0	0	0	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
(161)	+	+	+	+	+	+	0	0	0	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
41(32)	+	+	+	+	+	0	+	+	+	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
(38)	+	+	+	+	+	+	+	+	+	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
(72)	+	+	+	+	+	+	+	+	+	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
(143)	+	+	+	+	+	+	+	+	+	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
(145)	+	+	+	+	+	+	+	+	+	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
41(111)	+	+	+	+	+	+	+	+	+	+	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
41(147)	+	+	+	+	+	+	+	+	+	+	+	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
41(151)	+	+	+	+	+	+	+	+	+	+	+	0	+	0	0	0	0	0	0	0	0	0	0	0	0	0	0
41(152)	+	+	+	+	+	+	+	+	+	+	0	+	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Explanations: + = lysis by the indicator phage

0 = no lysis

The 16-3 phage was isolated from a soil sample

Table II

Changes in the phage pattern of Rh. meliloti No. 29 by lysogenization

The susceptible clone and its lysogenic derivatives	Indicator phages																							
	37	43	73	79	90	57	96	121	123	164	168	145	147	159	157	166	151	31	32	38	72	111	152	153	169
29	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
29(111)	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	0	0	0	0	0	0	0	0
29(38)	+	+	+	+	+	0	+	+	+	+	+	+	+	+	+	+	0	0	0	0	0	0	0	0	0
29(166)	+	+	+	+	+	+	+	+	+	+	+	+	+	0	0	0	0	0	0	0	0	0	0	0	0
(152)	+	+	+	+	+	+	+	+	+	+	+	+	+	0	0	0	0	0	0	0	0	0	0	0	0
29(145)	+	+	+	+	+	+	+	+	+	+	+	0	0	0	0	0	0	0	0	0	0	0	0	0	0
29(32)	+	+	+	+	+	+	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Explanations : + = lysis by the indicator phage

0 = no lysis

Table III

The 7 groups formed by Rhizobiophages

Group	Phages No.
A	16-3, 160, 161
B	32, 72, 143
C	38, 166
D	147, 151
E	111
F	152
G	145

of this phenomenon needs further investigations. These and the biological characterization of the groups are in progress.

Conclusions

1. In contrast to literary data, specific temperate phages were often found in *Rh. meliloti* cultures and could be isolated therefrom by the combination test. The divergence in the results can be attributed to the following differences in the technique: (i) instead of soil samples we used a great number of freshly isolated bacteria as starting material; (ii) a great number of strains were tested for phage carriership in every possible combination. The mass investigations were made possible by the appropriate technique worked out by us.

2. The plaque morphology of the phages was variable.

3. As regards their host-range, most of the phages appeared specific; polyvalent phages and phages with medium valency occurred less frequently. The relative high frequency of phages with narrow host-range might explain the negative results obtained by others.

4. Attempts were made to infect three susceptible strains of *Rh. meliloti* with 40 distinct Rhizobiophages. A number of lysogenized derivatives, *i. e.* phage-producing clones immune against the homologous phage, were brought about.

5. On the basis of the changes induced by lysogenization in the phage pattern of the bacteria the 13 phages tested so far were divided into 7 groups. Studies on the relationships among the remaining phage derivatives as well as the biological characterization of the related strains are in progress.

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GENETIC RECOMBINATION IN STREPTOMYCES AUREOFACIENS

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Summary. Genetic recombination was observed in *Streptomyces aureofaciens*. By the method applied the colonies of the prototrophic recombinants are directly observable and countable. The average recombinational frequency for single parental complementary auxotrophs was found to be 8.5×10^{-6}. Some relation is suggested to exist between the mechanism of the genetic recombination and the antibiotic activity of the recombinants. Recombinants with increased antibiotic activity were produced more frequently by biochemical mutants deriving from two different descendant lines than by those originating in the same line.

Several authors have reported on genetic recombination in the genus Streptomyces [1—6]. ALIKHANYAN [5] demonstrated that with *Streptomyces rimosus* these genetic interactions might be accompanied by increased antibiotic activity [5].

The present work provides new data on recombination in *Streptomyces aureofaciens* and on the relation of recombination to antibiotic activity.

Materials and methods

The biochemical mutants were prepared from two strains (L. S. 536 [7] and Lederle 8234) isolated at different places. As mutagenic agents UV or X irradiation was applied through several generations (Fig. 1). In this way three lineages of auxotrophs were obtained.

To augment the conidium count, the filtration method was applied after each irradiation. The filtration method was first used by FRIES [8] in studies on Ophiostoma, and BRAENDLE and SZYBALSKI [2] adapted the method to Streptomycetes. The penicillin technique [9], which is extensively applied in similar studies on bacteria, cannot be adapted to Streptomycetes, and efforts to replace penicillin by other antibiotics have been unsuccessful [2].

The conidia to be irradiated, which had been obtained from cultures of the prototrophs grown on the minimal medium (MM), were suspended in distilled water. The composition of the MM was 15.0 g sucrose, 3.5 g $Ca(NO_3)_2$, 1.0 g KH_2PO_4, 0.5 g $MgSO_4 \cdot 7H_2O$, 1.0 g NaCl, 0.05 g $FeSO_4 \cdot 7H_2O$, 1 litre distilled water, 20.0 g agar, with the pH adjusted to 6.8. Usually 1—15 per cent of the conidia had survived irradiation. The irradiated suspensions were inoculated into minimal broth composed of 25.0 g soluble starch, 2.0 g K_2HPO_4, 1.0 g KH_2PO_4, 3.0 g $Ca(NO_3)_2$, 2.0 g KNO_3, 0.5 g $MgSO_4 \cdot 7H_2O$, 1.0 g $CaCO_3$, 10.0 mg $ZnSO_4$, 1.0 mg $MnSO_4$, 1.0 mg $CuSO_4 \cdot 5H_2O$, 1.0 mg $FeSO_4 \cdot 7H_2O$, 1000 ml distilled water; pH was adjusted to 6.8. The medium was distributed in 500 ml Erlenmeyer flasks, 100 ml in each. The inoculated flasks were incubated at 27° C for 16 hours under continuous shaking, then the cultures were passed through G3 filtres. The filtrates contained mainly auxotrophic conidia, for these had not begun to germinate in the minimal broth. Unlike these, most of the prototrophic conidia, those which had germinated, were removed by the filtration. The conidia in the filtrates were plated on synthetic complete medium (CM), and incubated at 27° C for 10—14 days. The composition of the CM was 15.0 sucrose, 3.5 g $Ca(NO_3)_2$, 1.0 g $(NH_4)_2HPO_4$, 1.0 g K_2HPO_4, 1.0 g NaCl, 0.5 g $MgSO_4 \cdot 7H_2O$, 0.05 g $FeSO_4 \cdot 7H_2O$, 1.0 g *l*-asparagine; glycine, *l*-alanine, *l*-valine, *l*-norvaline, *l*-leucine, *l*-isoleucine, *l*-aspartic acid, *l*-glutamic acid, *l*-phenylalanine, *l*-tyrosine, *l*-tryptophan, *l*-proline, *l*-serine, *l*-threonine, *l*-oxyproline, *l*-cysteine, *l*-methionine, *l*-argi-

nine, *l*-ornithine, *l*-citrulline, *l*-lysine and *l*-histidine in the final concentration $0.5 \cdot 10^{-3}$ *M*; Ca pantothenate, nicotinic acid amide, vitamin B_1 and vitamin B_6 in the final concentration 1.0×10^{-4} *M*; vitamin B_2, cytosine, uracil, adenosine, guanine, xanthine and hypoxanthine in the final concentration 0.5×10^{-4} *M*; 20.0 g washed agar and 1000 ml distilled water; pH adjusted to 6.8. Conidia from the colonies grown on the CM were plated on MM and CM simultaneously. If auxotrophs had developed colonies on the CM, these were transferred onto several media each containing certain growth factors of those present in the CM. In this way the growth factor requirement of the auxotrophs was determined.

By this method the frequency of obtaining auxotrophs varied between 0.05 and 0.5 per cent as calculated from the number of the isolated colonies. The biochemical mutants were

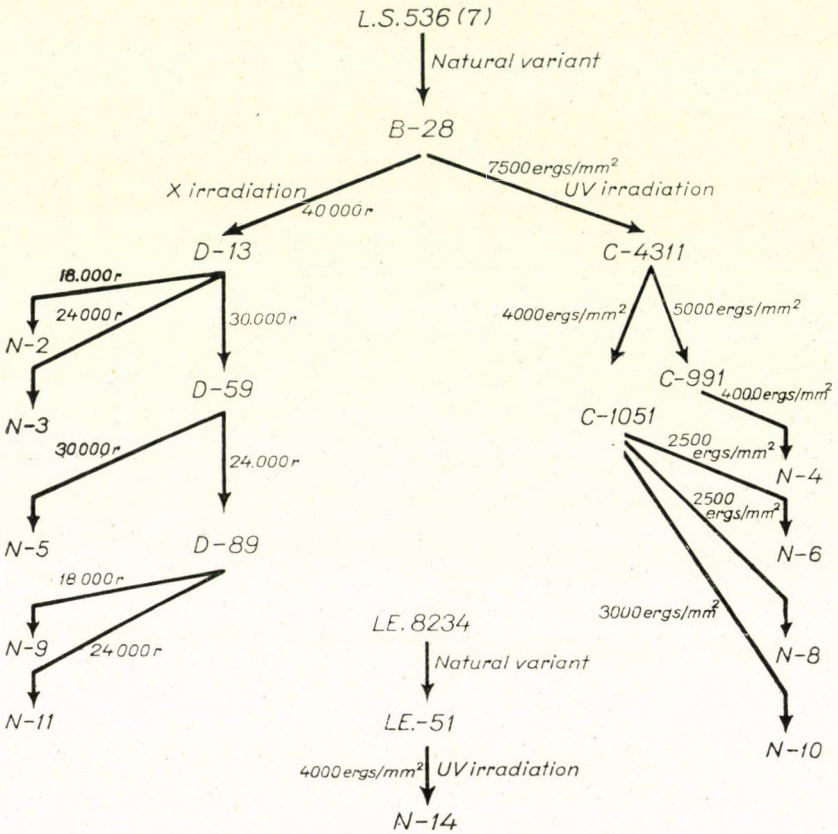

Fig. 1. Lineage of the biochemical mutants

cultivated on minimal media (MM) supplemented with the factor required for their auxotrophic growth. The concentration of the growth factor in the medium was 10^{-3} M.

Genetic recombination was demonstrated by the method of BRADLEY and LEDERBERG [7]. The conidial suspensions containing 10^5 to 10^6 conidia/ml of complementary auxotrophs were mixed in the proportion 1 : 1 and incubated on properly supplemented MM at 27° C. Thus, the method was essentially the same as that used by SERMONTI and SPADA—SERMONTI [1].

In contrast to the method of SERMONTI and SPADA—SERMONTI, in our experiments MM was not supplemented but with the factors required by the pair of biochemical mutants. The concentration of the supplementing factors varied between 5×10^{-5} and 10^{-8} *M*, depending on the biochemical mutants. Optimum concentration was established empirically.

The recombinants formed regularly developing, separate colonies on properly supplemented MM.

In addition to cultivation on properly supplemented MM, the conidial suspensions of both auxotrophs were plated singularly on MM as well. Those suspensions which had shown back mutation were not used in the recombination experiments.

Ten biochemical mutants were tested for recombination in every possible combination. The genetic markers of the auxotrophic mutants are shown in Table I.

Table I

Genetic markers of biochemical mutants

Mutant	Markers	Mutant	Markers
N—2	$P_1S^sC^gCTC^1arg^-$	N—8	$P_1S^sC^gCTC^1arg^-$
N—3	$P_1S^rC^wCTC^1arg^-$	N—9	$P_1S^rC^wCTC^1arg^-$
N—4	$P_2S^sC^gCTC^2meth^-$	N—10	$P_0S^sC^wCTC^1cys^-$
N—5	$P_0S^sC^gCTC^1arg^-am^-$	N—11	$P_1S^rC^wCTC^1meth^-am^-$
N—6	$P_1S^rC^gCTC^2arg^-cys^-$	N—14	$P_1S^rC^gCTC^1am^-$

Explanation of the symbols

P_1 = Producing water-insoluble raspberry-red pigment
P_2 = Producing water-soluble, red pigment.
P_0 = No pigment production.
$S^{s,r}$ = Streptomycin-sensitive, resistant.
$C^{g,w}$ = Conidia grey, white.
CTC^n = Chlortetracycline production in 100 U.
arg^-, cys^-, $meth^-$, am^- = arginine, cystine-cysteine, methionine, amino-nitrogen dependence

Sensitivity to streptomycin was tested on MM or supplemented MM. Strains growing well in the presence of 5 mg streptomycin sulphate per litre were regarded as resistant.

Antibiotic production was determined in shaken cultures. Cultures grown on MM or supplemented MM were inoculated in 500 ml Erlenmeyer flasks containing 100 ml of the following seed medium: 20.0 g starch; 20.0 g corn steep (dry weight); 4.0 g $CaCO_3$; distilled water to 1 litre; pH was brought to 6.8. After 48 hours' incubation at 27° C 2 per cent seed culture was brought into the fermentation medium composed of 30.0 g starch, 6.0 g corn steep liquor (dry weight), 6.0 g NH_4NO_3, 5.0 g $CaCO_3$, 2.0 g KCl, distilled water to 1 litre; pH was adjusted to 6.2. The inoculated cultures were shaken at 27° C for 72 hours, then the antibiotic activity of the fermentation liquor was determined photometrically, by the ferrichloride colour test, and microbiologically, by the agar diffusion method using the ATCC 6633 strain of *B. subtilis*.

Results

In the first part of the experiments the genetic markers of the prototrophs obtained from mixed cultures were studied. The colonies of the prototrophs were transferred to MM, and two passages were made on the same medium. The cultures thus obtained were examined for the genetic markers. The origin and the genetic markers of a few cultures are shown in Table II.

It is clearly seen that combination of the parental markers occurred in each of the cases shown in the Table.

From each of the mixed cultures 0—25 prototrophic recombinants were obtained. Three complementary auxotrophic mutants produced no recombinants under the same conditions although the experiments were repeated several times. The pairs N—2, N—10; N—3, N—4; and N—8, N—11 appeared to be incompatible.

In further experiments the antibiotic activity of the recombinants was examined. In this regard the recombinants could be classified into 3 groups, *viz.* group I with high activity (synthesizing 2000—2600 U chlortetracycline per ml); group II with medium activity (1000—1700 U per ml); group III with low activity (under 200 U per ml). The distribution of the recombinants in the 3 groups is presented in Table III.

Table II

Origin and genetic markers of some recombinants

Auxotrophic parents	Recombinants	
	Code	Markers
N—4 × N—9	J—1	$arg^+ meth^+ P_1S^rC^gCTC^{25}$
N—4 × N—8	J—6	$arg^+ meth^+ P_2CTC^1$
N—4 × N—10	J—14	$cys^+ meth^+ P_0C^gCTC^{14}$
N—4 × N—9	J—35	$arg^+ meth^+ P_1S^sC^gCTC^{26}$
N—9 × N—14	J—162	$arg^+ am^+ C^gCTC^2{}_0$
N—6 × N—11	J—179	$arg^+ cys^+ meth^+ am^+ C^wCTC^{21}$
N—2 × N—11	J—205	$arg^+ meth^+ am^+ S^sC^wCTC^2$
N—3 × N—10	J—217	$arg^+ cys^+ P_1S^sCTC^{26}$
N—4 × N—5	J—254	$arg^+ am^+ meth^+ P_0CTC^{15}$

Explanation of the symbols see Table I.

Table III

Distribution, by antibiotic activity, of the recombinants

Group	Antibiotic activity	Number of recombinants	Percentual distribution
I.	High	32	11.5
II.	Medium	88	31.7
III.	Low	158	56.8
Total		278	100.0

The antibiotic production by the initial prototrophs and by several biochemical mutants and recombinants are shown in Table IV.

Table IV

Antibiotic activity of the parental prototrophs and of some biochemical mutants and recombinants

Parental prototrophs	Antibiotic activity U/ml	Biochemical mutants	Antibiotic activity U/ml
C—991	1690	N—4	160
C—1051	1540	N—6	100
D—13	1420	N—9	80
D—59	1580	N—10	90
D—89	1460	N—11	105
Le—51	1140	N—14	85

Recombinants	Antibiotic activity U/ml	Antibiotic activity in per cent of that of the prototrophic parents	Group (see Table III)
J—1	2490	159	I
J—6	115	7	III
J—14	1385	86	II
J—35	2560	163	I
J—162	2020	155	I
J—179	2120	142	I
J—205	240	16	III
J—217	2080	140	I
J—254	1510	95	II

The group I recombinants showed a 40 to 60 per cent increase in their antibiotic activity as compared to the activity of the initial prototrophs.

It is striking that mainly parental auxotrophs deriving from two different descendant lines produced highly active recombinants (see also Fig. 1); *e. g.* a greater proportion of the recombinants deriving from the complementary auxotrophs N—4 and N—9 or N—14 and N—9 belonged to group I than of those deriving from N—4 and N—10. In Table V the recombinants have been grouped according to origin and antibiotic activity.

Eighty-one per cent of the highly active recombinants derived from parental auxotrophs belonging to two different descendant lines.

Table V

Distribution of the recombinants by lineage

Lineage of the recombinants	Number of the recombinants tested	Of these recombinants with high antibiot. activity (group I)	
		Number	Per cent
Two different descendant lines	132	26	19.7
One descendant line	146	6	4.1
Total	278	32	11.5

Discussion

Genetic recombination has been observed by other authors in 5 species of Streptomycetes, *viz. S. coelicolor*, *S. fradiae*, *S. griseoflavus*, *S. rimosus*, and *S. griseus*. Our present experiments have furnished evidence of recombination in *S. aureofaciens*. Using our own method, the transfer of the recombinants from the "tuft" may be omitted (in contrast to the method recommended by SERMONTI and SPADA—SERMONTI [1] and applied also by ALIKHANYAN [5]), since the recombinants form separate colonies in the mixed culture itself, provided the concentration of the supplementing factors in the medium is appropriate. In this case a minimum germination of the conidia and formation of colonies by the recombinants precede the conidium formation by the auxotrophs. The colonies thus obtained are directly countable. This method seems to be suited to reveal quantitative relations concerning the mechanism of recombination.

Although the present studies did not aim at the evaluation of recombinational frequencies, they provided some information in this relation. It is well known that, because of the multiplicity of the genetic interactions in Streptomycetes, the frequency of genic recombination cannot be determined exactly. Anomalous heterokaryons are often hardly distinguishable from recombinants [11] and, in addition, Streptomycetes can produce selective agents which are capable of influencing the formation of individual populations [12]. Furthermore, it is not clear, whether auxotrophic recombinants can or cannot be detected by the present technique in the case of double parental auxotrophic markers. Regarding all these circumstances, we attempted to estimate the recombinational frequencies for single parental auxotrophs on the basis of the experimental data. The recombinational frequencies for the complementary auxotrophs N—4 and N—9 were 2.2×10^{-5}, 5.8×10^{-6}, 6.5×10^{-7}, 3.4×10^{-6}, 1.2×10^{-5} and 7.1×10^{-6} in six individual experiments with the arithmetical mean 8.5×10^{-6}. Preliminary data showed no difference in the recombina-

tional frequency as to whether or not the complementary auxotrophs belonged to the same lineage.

On the basis of the present investigations a relation might be supposed to exist between the antibiotic activity of the recombinants and the mechanism of the recombination. Though the number of experiments is too small to allow final conclusions, the findings suggested that the course of recombination might be different in different groups of biochemical mutants. Recombinants with highly increased antibiotic activity may mainly be expected, if the complementary auxotrophs derive from two different descendant lines.

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TRANSFORMATION IN STREPTOMYCES

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Summary. Transformation in *Streptomyces aureofaciens* auxotrophs was induced by purified DNA preparations. Using the author's method the amount of DNA required to give rise to one transformant was about 10^{-4} μg. Interspecific transformation was demonstrated in the genus Streptomyces, namely DNA prepared from *Streptomyces griseus* gave rise to transformants in *Streptomyces aureofaciens* auxotrophs. The relation between the amount of DNA and the number of transformants was found to be polynomial; that between the dose of UV irradiation and the inactivation rate for the transforming capacity of DNA approximated an exponential relation.

Since the role of deoxyribonucleic acid (DNA) in transfer of genetic markers had been discovered [1], transformation mediated by cautiosly purified DNA preparations has been observed in numerous groups of microorganisms.

In the present work, beyond confirmation of the existence of transformation, evidence of interspecific transformation in the genus Streptomyces is provided. Some quantitative relations in transformation are also presented.

Materials and methods

Media. Minimal medium (MM). 15.0 g sucrose, 3.5 g $Ca(NO_3)_2$, 1.0 g KH_2PO_4, 0.5 g $MgSO_4 \cdot 7H_2O$, 1.0 g NaCl, 0.05 g $FeSO_4 \cdot 7H_2O$, 1 litre distilled water, 20.0 g agar; pH adjusted to 6.8.

Medium for shaken cultures of Streptomyces aureofaciens. 20.0 g starch, 20.0 g corn steep liquor (dry weight), 4.0 g $CaCO_3$, 0.22 g KH_2PO_4, 1 litre distilled water; pH adjusted to 6.8.

Medium for shaken cultures of Streptomyces griseus. 20.0 g glucose, 20.0 g soybean meal, 3 g $(NH_4)_2SO_4$, 2.5 g NaCl, 5.0 g $CaCO_3$, 0.5 g KH_2PO_4, 1 litre distilled water; pH adjusted to 6.8.

Medium for shaken cultures of Streptomyces rimosus. 5.0 g starch, 30.0 g soybean meal, 0.5 g corn steep liquor (dry weight), 4.0 g NaCl, 2.0 g $CaCO_3$, 0.33 g KH_2PO_4, 1 litre distilled water; pH adjusted to 7.0.

Microorganisms. The prototrophs and the auxotrophs used in the transformation experiments are characterized in Table I.

The strains were produced in this laboratory by UV and X-ray irradiation [2].

Preparation of conidial suspensions. Conidia obtained from cultures grown on supplemented MM were twice washed with, and suspended in, saline. The live conidia counts of the suspensions were always determined using the same medium. The growth factors required were added to MM in 10^{-3} M concentration.

Preparation of DNA. Mycelia for the preparation of DNA were obtained from shaken cultures (see above) incubated at 27° C in 500 ml Erlenmeyer flasks each containing 100 ml medium.

The method applied in the preparation of DNA was a combination of several known methods [3, 4, 5]. The shaken cultures were centrifuged, and the mycelia twice washed with saline. The sediment was frozen at —15° C and ground with quartz sand; the wet mycelium,

Table I

Genetic markers of the strains used in the experiments

Name and code of microorganism		Genetic markers
Streptomyces aureofaciens	D—242	$P_1S^sC^gCTC^{22}arg^+cys^+meth^+am^+$
	N—4	$P_2S^sC^gCTC^2arg^+cys^+meth^-am^+$
	N—6	$P_1S^rC^gCTC^2arg^-cys^-meth^+am^+$
	N—10	$P_0S^sC^wCTC^1arg^+cys^-meth^+am^+$
	N—11	$P_1S^rC^wCTC^1arg^+cys^+meth^-am^-$
Streptomyces griseus	R—574	$P_0S^rC_{\mp}CTC^0arg^+cys^+meth^+am^+$
Streptomyces rimosus	I—20	$P_0S^sC^gCTC^0arg^+cys^+meth^+am^+$

P_1 = Production of a water insoluble, raspberry coloured pigment
P_2 = Production of a water soluble, red pigment
P_0 = No pigment production
$S^{s,r}$ = Sensitive, resistant to streptomycin
$C^{g,w}$ = Grey, white conidia
CTC^n = Chlortetracycline production in 100 U
$arg^-cys^-meth^-am^-$ = arginine, cystein-cystine, methionine, aminonitrogen dependence

1 g in 8 ml, was suspended in a cold solution containing 0.1 *M* NaCl and 0.05 *M* sodium citrate and twice centrifuged. The second sediment was frozen at —15° C and resuspended in cold 2 *M* NaCl, 1 g in 5 ml. The suspension was shaken with glass beads for 10 minutes and centrifuged at 0° C; two volumes of 96 per cent ethanol were then added to the supernatant; the fibrous precipitate was filtered and kept in two volumes of 2 *M* NaCl at 0° C for 14 hours. The sample was deproteinized by five consecutive extractions with a chloroform-isoamyl-alcohol mixture. DNA was precipitated from the aqueous phase by adding 80 per cent ethanol. The dried precipitate was resuspended in sterile 2 *M* NaCl solution; the DNA preparations thus obtained were used in transformation experiments.

The DNA preparations were tested for protein contamination by the method of LOWRY *et al.* [6] and for RNA by the orcine test as modified by CERIOTTI [7]. Those showing either protein or RNA contamination were discarded. DNA was assayed with the nitrophenylhydrazine reagent recommended by WEBB and LEVY [8, 9].

Results

In the first experiments it was attempted to induce transformation by the DNA preparation obtained from the prototrophic strain *Streptomyces aureofaciens* D—242. Conidial suspensions of auxotrophs were plated on MM, about 10^4 conidia per plate. After 5 hours' incubation each plate was overlayered by 1 ml DNA solution of known concentration and the cultures were reincubated at 27° C. The transformed monoconidial colonies became visible 3—5 days later and reached their full development between the 10th and 14th days of incubation. Some of the colonies were subjected to consecutive passages on MM and their genetic markers were observed in several generations.

DNA preparations obtained from auxotrophs (*e. g. Streptomyces aureofaciens* N—11) also gave rise to transformants.

The origin and the genetic markers of several transformants are given in Fig. 1.

As shown in Fig. 1, the transformation of auxotrophs to nutritional independent prototrophs was accompanied in a few cases by transfer of other

$$|\{D\text{—}242\} \rightarrow \text{N—}4| \rightarrow \text{T—}22\ (P_2S^sC^g\ meth^+)$$
$$|\{D\text{—}242\} \rightarrow \text{N—}10| \rightarrow \text{T—}38\ (P_1S^sC^w\ cys^+)$$
$$|\{N\text{—}11\} \rightarrow \text{N—}6| \rightarrow \text{T—}13\ (P_1C^g\ arg^+\ cys^+)$$
$$|\{N\text{—}11\} \rightarrow \text{N—}10| \rightarrow \text{T—}29\ (P_0S^r\ cys^+)$$

Fig. 1. The origin and genetic markers of transformants. {} include the transforming agent showing its origin. || include the system consisting of the transforming agent and the recipient which had yielded the transformants coded with T

genetic markers; *e. g.* the ability to produce P_1 pigment was transferred to the transformant T—38 and resistance to streptomycin to T—29.

Experiments were carried out to calculate the amount of DNA required by a conidium to be transformed.

Table II

*Influence of the amount of DNA on the number of transformants**

Auxotroph	Auxotroph count per plate	Number of transformed colonies** in the presence of					DNA μg per transformant
		300	100	10	1	0	
		μg DNA per plate					
N—4	$1.5 \cdot 10^4$	197	71	8.3	2.0	0	$7.9 \cdot 10^{-5}$
N—6	$1.2 \cdot 10^4$	28	12	1.7	0.3	0	$5.9 \cdot 10^{-4}$
N—10	$1.4 \cdot 10^4$	181	92	19	1.7	0	$6.9 \cdot 10^{-5}$
N—11	$1.5 \cdot 10^4$	54	16	2.3	0.2	0	$3.5 \cdot 10^{-4}$

* Mean values, calculated from several experiments.
** The figures only show the transformation of auxotrophs to prototrophs.

It can be seen in Table II that single auxotrophs required about 10^{-4} μg DNA per transformant. The double auxotrophs N—6 and N—11 required more, on the average 7.1 times more, DNA. Thus, an increase in the degree of auxotrophy from one to two was followed by a much steeper rise in the amount of DNA required to give rise to a prototrophic transformant.

On the other hand, as calculated from the figures in Table II, the relation between the logarithm of the amount of DNA per conidium and the logarithm of the number of transformants proved to be rectilinear, at least within the given limits (Fig. 2).

Next, UV inactivation of the transforming activity of DNA preparations was studied. Samples containing 840 μg DNA per ml 2 *M* NaCl were irradiated with 1000, 2000, 3000 and 5000 erg per mm^2, respectively, under continuous stirring at +4° C. "Amikrob" lamps were used. In the experiments yielding the inactivation curve shown in Fig. 3, DNA prepared from the D—242 strain

of *Streptomyces aureofaciens*, and the auxotroph N—4 were used. The curve presented approximates in exponential relation. The second curve in Fig. 3 shows the killing rates for conidia of the D—242 strain exhibited by the same

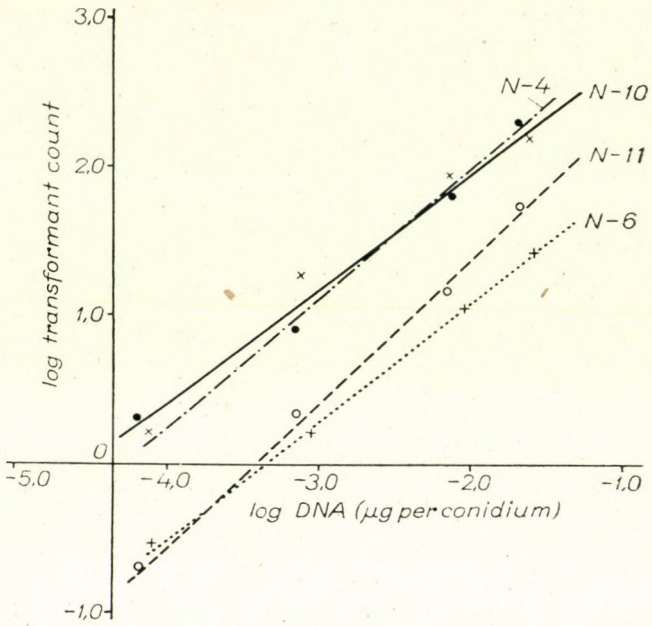

Fig. 2. Influence of the amount of DNA on the number of transformant colonies

doses of UV irradiation. (The suspension contained 8000 living conidia per ml.) The two curves run nearly parallel, the killing rates being always slightly lower than the corresponding inactivation rates.

This slight, but constant, difference suggests that the structure of DNA, which is the mediator of the genetic markers, is more labile under the given conditions than certain structures essential for life.

Finally, we attempted to induce interspecific transformation in Streptomyces. *Streptomyces griseus* and *Streptomyces rimosus* DNA preparations isolated from prototrophic strains and *Streptomyces aureofaciens* auxotrophs were used. The results are summarized in Table III.

Transformation was induced by *Streptomyces griseus* DNA in two auxotrophs of *Streptomyces aureofaciens*, but the experiments with *Streptomyces rimosus* DNA have not yielded transformants.

The existence of interspecific transformation was proven beyond doubt not only by the demonstration of the transformation of *Streptomyces aureofaciens* auxotrophs to prototrophs by DNA prepared from the *Streptomyces griseus* prototrophic strain, but also by the appearance in transformants of such genetic markers as streptomycin-resistance, which had been exclusively

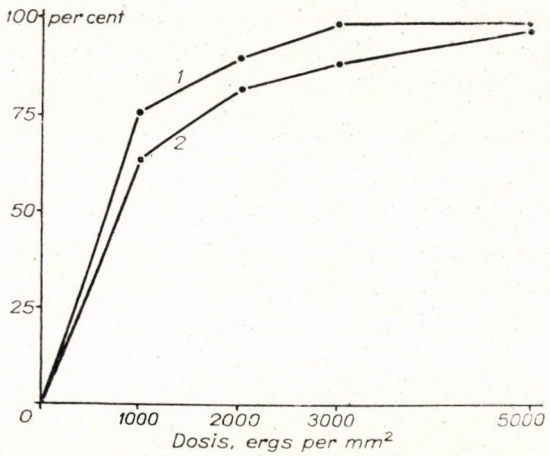

Fig. 3. Inactivation of the transforming capacity of DNA preparations, and killing Streptomyces by UV rays
1. Decrease in transforming activity.
2. Death rate.

Table III

Interspecific transformation

Prototrophic donor strains (300 μg DNA/plate)	Number of transformed colonies in the auxotrophs*				
	N—4	N—6	N—10	N—11	0
Streptomyces griseus R—574	22	6	0	0	0
Streptomyces rimosus I—20	0	0	0	0	0
Streptomyces aureofaciens D—242	197	28	181	54	0
Nil	0	0	0	0	x

* The numbers of auxotrophs agree with the figures in Table II.

characteristic of *Streptomyces griseus*. Fig. 4 shows the genetic markers of three interspecific transformants.

$$|\{S.\ griseus\} \rightarrow \text{N—4}\ | \rightarrow \text{T—58}\ (P_2S^rC^gCTC^2\ meth^+)$$
$$|\{S.\ griseus\} \rightarrow \text{N—4}\ | \rightarrow \text{T—64}\ (P_2S^sC^gCTC^2\ meth^+)$$
$$|\{S.\ griseus\} \rightarrow \text{N—6}\ | \rightarrow \text{T—69}\ (P_1S^sC^gCTC^1\ arg^+\ cys^+)$$

Fig. 4. Genetic markers of interspecific transformants. {} include the transforming agent showing its origin, || include the system consisting of the transforming agent and the recipient which had yielded the transformants coded with T

Interspecific transformation was never accompanied by any change in antibiotic activity.

As regards quantitative relations, a remarkable difference was observed between the interspecific and intraspecific transformations. While of the homologous DNA as little as 1.0×10^{-4} μg and 8.9×10^{-4} μg were sufficient to give rise to a transformant in the auxotrophs N—4 and N—6, respectively,

a single transformation induced by *Streptomyces griseus* DNA in the same auxotrophs required 9.1×10^{-4} and 4.2×10^{-3} μg DNA, respectively. We failed to give rise to transformants in two other biochemical mutants.

Discussion

The results of the present study provide further evidence that transformation can be induced by DNA preparations in the genus Streptomyces. This has to be emphasized, since in 1958 SZYBALSKI [10] still regarded the existence of transformation in this group of microorganisms as unproven. Since then HORVÁTH and BUDAY [11] have demonstrated the phenomenon in several species of Streptomyces. The present experiments revealed further details of this phenomenon as observed in the same genus.

The use of auxotrophs, first introduced by SPIZIZEN [5] for studying transformation in *B. subtilis*, was found useful in demonstrating some quantitative relations of transformation in Streptomyces.

We demonstrated by a simple method that transformation of a single individual of Streptomyces required about 10^{-4} μg DNA, *i. e.* nearly the same amount as required by bacteria according to HOTCHKISS [12], SPIZIZEN [5], and FOX and HOTCHKISS [4].

As demonstrated, the transformation in double auxotrophs requires considerably more than twice (7.1 times) as much DNA as that in single auxotrophs. This is in accordance with our finding that only few of the transformants showed accessory markers (*e. g.* streptomycin-resistance, pigment production) as results of transformation.

These findings might be explained by a disproportionate arrangement of the corresponding genetic loci in the DNA segment, or by the assumption that the DNA segments are different in size and larger segments are less frequently incorporated than smaller ones. Since, according to FOX and HOTCHKISS [4], the outcome of a transformation is determined by the formation of a "complex" by the individual to be transformed and the donor's DNA and this formation is thought to be reversible, the latter alternative seems to be acceptable.

The relation between the amount of DNA and the number of transformants is a polynomial one (Fig. 2). This agrees with HOTHKISS' results [4, 12] obtained in experiments with bacteria. According to this author, however, the number of transformants cannot increase beyond the limit, where the system has a saturation effect and the limit concentration highly depends on the quality of the DNA preparation [12]. Using comparatively low concentrations of DNA, we did not observe such a saturation effect.

As regards the inactivation of the Streptomyces DNA by UV irradiation, the shape of our dose-effect curve agreed with the "exponential" curves

obtained by ZAMENHOF [13] in experiments carried out on other microorganisms, but in our experiments the doses necessary to complete inactivation were higher (3000—4000 erg per mm^2 instead of 800—1000 erg per mm^2). However, this remarkable difference does not indicate that Streptomyces DNA is more resistant than the preparations examined by ZAMENHOF, since the conditions of irradiation were different; *e. g.* ZAMENHOF's DNA suspension contained 227 μg per ml, whereas ours as much as 840 μg per ml.

The existence of interspecific transformation in Streptomyces was repeatedly demonstrated in the present study, but not in every relation. We failed to obtain transformants with DNA prepared from *Streptomyces rimosus* in *Streptomyces aureofaciens*, and attempts to transform certain auxotrophs of *Streptomyces aureofaciens* by *Streptomyces griseus* DNA were also unsuccessful. Furthermore, the number of transformants was always less in the case of interspecific transformation (Table III). These data suggest that in the case of interspecific transformation the free course of certain unknown processes may partly or completely be inhibited. Another explanation is the assumption of genetic homology [14] between the individuals capable of giving rise to transformation. The greater the homology, the easier the incorporation of the DNA. The latter assumption might be extended to the explanation of the lower recipient property of double auxotrophs.

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INCIDENCE OF YEASTS IN HUMAN MATERIAL*

By

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Summary. From material derived from 1200 patients suspected of mycosis 433 strains of yeasts have been cultivated. Identified after the method of LODDER and KREGER—VAN RIJ, the strains could be ranged into 9 genera and 32 species. The majority of the isolated strains, derived chiefly from throat and bronchial secretion, were pathogenic *Candida albicans*, *Candida pseudotropicalis*, *Candida tropicalis*, and *Candida krusei*. A great number of yeasts known so far as saprophytes have been isolated from secretion obtained from the respiratory tract and other sites.

The respiratory tract has been found to contain great numbers of Candida species, Geotrichum strains, *Rhodotorula mucilaginosa*, and *Torulopsis inconspicua*. *Candida albicans*, *Cryptococcus diffluens*. and *Rhodotorula mucilaginosa* were isolated from blood, and mainly Rhodotorula species, in addition to *Candida albicans*, from bile. In gastric juice, Candida and Geotrichum species were common. In necropsy material *Candida krusei* was the most frequent.

The strains isolated were tested for pathogenicity by intraperitoneal inoculation of albino mice. Candida species known as pathogenic, Geotrichum strains, and *Cryptococcus neoformans* were found to be pathogenic to mice. *Candida utilis*, *Rhodotorula mucilaginosa*, *Hansenula subpelliculosa*, *Saccharomyces marxianus*, and *Saccharomyces fragilis* species also caused lesions and the death of mice.

Owing to the widespread use of broad spectrum antibiotics, secondary mycoses caused by yeasts have been noted with increasing frequency and the occurrence of endogenous mycoses has been often recorded [27]. Studies on the aetiology of these conditions and regular descriptions of the human yeast flora appear to be somewhat scarce. In the earlier case reports attention was focussed mainly on the pathological process; only in recent literature have the yeasts that occur in the human organism come to be discussed, and for no lesser reason than their occasional pathogenicity. In LODDER and KREGER—VAN RIJ's monograph on yeasts numerous species are supposed to be of human origin, but for lack of specific tests without conclusive proofs. In all probability the extensive application of antibiotics in the last decade has brought about a considerable change in the human microflora, especially in the qualitative and quantitative composition of the yeast flora.

Most of the yeasts of human origin have been isolated from the gastro-intestinal tract. In the oral cavity and the throat the most common species are Candida, Torulopsis and some ascosporogenous yeasts, such as *Saccharomyces cerevisiae*, Debaryomyces species [2, 4, 6, 10, 15, 18, 23]. The biliary

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ducts and duodenal juice harboured mostly Rhodotorula species [24, 25]. *Candida albicans* is common in the faeces; this species is a member of not only the normal intestinal flora, but also of the oral flora. *Candida pseudotropicalis*, *Candida tropicalis*, *Candida krusei*, and other Candida species are also common in faeces, from where Geotrichum species, *Torulopsis inconspicua*, *Trichosporon cutaneum*, and *Saccharomyces cerevisiae* have also been isolated [5, 10, 18, 21, 23, 26]. In addition to *Candida albicans*, *Candida krusei*, *Candida tropicalis*, *Candida pseudotropicalis*, *Candida parapsilosis*, *Candida guilliermondii*, Geotrichum species, and Cryptococcus species have been isolated from the respiratory tract [4, 5, 8, 10, 15, 23].

From the genitals *Candida albicans*, *Candida krusei*, *Candida tropicalis*, *Candida stellatoidea*, *Candida parapsilosis*, *Torulopsis glabrata* and *Rhodotorula species* have been isolated [5, 10, 14, 17, 22, 23], and from urine Candida species, even Rhodotorulae and ascosporogenous yeasts [5, 10, 23].

As stated by FRAGNER [11], 56 per cent of the fungal flora in the auditory canal is composed of Candida species.

Almost every species of yeast can be isolated from the skin surface [5, 7, 10, 13. 18, 23].

Material and methods

The following is a report on the yeasts isolated from material derived from 1200 patients suspect for systemic mycosis. In view of the origin of the samples, our results throw light on the mycoflora of the diseased organism. Of the cultivated species some have been known as pathogenic to man, others as saprophytes or facultative pathogens. The latter are apt to appear as secondary pathogens in an organism weakened by some primary disease.

As culture media, SABOURAUD's glucose agar, CSILLAG's molasses agar, and peptone agar containing penicillin and streptomycin were used. When pure cultures had failed to produce chlamydospores upon transfer to corn meal agar [20] — and were thus disproved to be *Candida albicans* — the species was identified according to LODDER and KREGER—VAN RIJ [16]. Pathogenicity tests were performed in every instance by intraperitoneal inoculation of two male albino mice. Following death of the mice resp. their sacrifice on the seventh to tenth day, the liver, kidneys and spleen were subjected to histological examination after staining with SCHIFF's periodic acid leucofuchsin.

Results

From the 1200 samples, 433 yeast strains were isolated. The distribution of the strains in the various materials is presented in Table I. The 433 strains could be classified into 9 genera; these included approximately 32 species. In agreement with the data in the literature, the Candida species recognized as pathogenic were the most common, viz. *Candida albicans*, *Candida krusei*, *Candida tropicalis*, *Candida pseudotropicalis*, *Candida parapsilosis*, *Candida guilliermondii*. *Cryptococcus neoformans* was isolated in two cases.

As shown in Table I, various yeasts known to occur in the human organism as saprophytes were also isolated. The presence of *Hansenula subpelliculosa* in human secretion has been described by ourselves [19]; however, to the best of our knowledge, no report has so far been published on the occurrence

of *Rhodotorula flava* in man. Ascosporogenous yeasts were isolated on several occasions, *e. g. Saccharomyces fragilis*, a perfect form of *Candida pseudotropicalis* [16].

Throat secretion yielded the greatest number of yeast species. As seen in Table I, here nearly every species of Candida occurred with the highest incidence. Numerous yeasts were isolated from laryngoscopic and bronchoscopic secretion, in addition to the common Candida species, *Saccharomyces fragilis*, Geotrichum strains, *Rhodotorula mucilaginosa* and *Torulopsis inconspicua.*

From the blood, yeasts were isolated in three instances, viz. *Candida albicans*, *Cryptococcus diffluens* and *Rhodotorula mucilaginosa.*

From bile, besides *Candida albicans*, Rhodotorula species were grown.

Gastric juice yielded Candida and Geotrichum species in most cases.

In *post mortem* material the high incidence of *Candida krusei* deserves special notice; this was isolated on four occasions, while *Candida albicans* in nine.

Pathogenicity tests in albino mice were carried out with every isolated strain but those of *Candida albicans*, the mouse pathogenicity of which is well-known [20, 28] (see Table II).

The scale of pathogenicity was set up arbitrarily on the basis of the mortality and the *post mortem* findings.

In agreement with the literature [12, 28], the species known as pathogen Candida, Geotrichum strains and *Cryptococcus neoformans* proved to be pathogenic to mice. Moreover, lesions and death were caused by *Candida utilis*, *Rhodotorula mucilaginosa*, *Hansenula subpelliculosa*, *Saccharomyces marxianus*, *Saccharomyces fragilis* strains isolated from man. These species have been marked in Table II with three crosses.

The species marked with two crosses did not kill the mice but it was possible to demonstrate the pathogen histologically seven to ten days following infection.

With the species marked with one cross, intraperitoneally inoculated mice survived. Cells of the inoculated fungus were discovered sporadically in the tissues and on the surface of organs. In the section only the polysaccharides of the decomposed fungal cells were stained red by Schiff's dye. Histological investigations have failed to confirm the mouse pathogenicity of 7 species. *Hansenula anomala* and *Torulopsis famata* were found to have a varying pathogenicity; *Torulopsis famata* strains isolated from cerebrospinal fluid were not pathogenic in mice.

In contrast with the pertaining literary data [3, 12, 28], numerous species known as saprophytes proved to be pathogenic to albino mice upon intraperitoneal inoculation. This may presumably be explained by all our strains having been grown from material derived from patients suspect for systemic mycosis and having thus been virulent yeasts.

Table I

The origin of cultivated yeast strains

Species	Throat secr.	Laryngeal secr.	Bronchial secr.	Blood	Urine	Cerebrospinal fluid	Bile	Gastric juice	Fistulous secr.	Other material	
										vaginal secr.	1
										culture	6
										post mortem	9
Candida albicans	142	27	49	1	11	1	6	9	2	faeces	3
										post mortem	4
krusei	9	—	2	—	—	—	—	1	—	culture	1
tropicalis	6	1	5	—	—	—	—	—	—		
pseudotropicalis	9	2	1	—	—	—	—	—	—		
stellatoidea	—	—	—	—	1	—	—	—	—		
parapsilosis	7	—	1	—	—	—	—	—	1	post mortem	1
guilliermondii	2	—	3	—	—	—	1	—	—		
utilis	4	2	—	—	—	—	—	1	—	surgical	1
mellibiosi	—	—	2	—	—	—	—	—	—	nasal secr.	1
pulcherrima	1	—	—	—	—	—	—	—	—		
rugosa	1	—	—	—	—	—	—	—	—		
mycoderma	1	—	—	—	—	—	—	—	—		
zeylanoides	4	2	—	—	—	—	—	—	—		
Cryptococcus neoformans	—	—	1	—	—	1	—	—	—		
diffluens	1	—	—	1	—	—	—	—	—		
laurentii	—	—	1	—	—	—	—	—	—		
albidus	1	—	—	—	—	—	—	—	1		
Rhodotorula mucilaginosa	2	4	3	1	—	—	2	—	—		
flava	—	—	—	—	—	—	1	—	—		
rubra	—	1	—	—	—	—	—	—	—		
glutinis	—	1	—	—	—	—	—	—	—		
sp.	1	1	1	—	—	—	—	—	—		
Torulopsis famata	2	—	2	—	1	1	—	—	1		
candida	—	—	—	—	—	—	—	—	1		
inconspicua	6	1	2	—	—	—	1	—	—		
glabrata	—	2	—	—	1	—	—	—	—		
gropengiesseri	—	—	1	—	—	—	—	—	—		
aeria	—	—	—	—	—	—	—	—	—	surgical	1
sp.	3	—	1	—	—	—	—	—	—		
Hansenula anomala	2	—	—	—	—	—	—	—	—	post mortem	1
subpelliculosa	—	—	1	—	—	—	—	—	—		
Saccharomyces cerevisiae	1	—	—	—	—	—	—	—	—	post mortem	1
marxianus	—	—	—	—	—	—	—	—	—	thoracic punct.	1
fragilis	3	1	3	—	—	—	—	—	1		
sp.	—	—	—	—	—	1	—	—	—		
Geotrichum sp.	6	2	6	—	—	—	—	3	—		
Sporobolomyces sp.	—	—	—	—	—	—	—	—	—	post mortem	1
Trichosporon cutaneum	—	—	1	—	—	—	—	—	—	post mortem	1
Total	214	47	86	3	14	4	11	14	7		33

Table II

Pathogenicity of yeasts of human origin

Species	Pathogenicity for mice	Species	Pathogenicity for mice
Candida albicans	++++	*Rhodotorula glutinis*	++
krusei	+++	*sp.*	++
tropicalis	+++	*Sporobolomyces sp.*	++
pseudotropicalis	+++	*Torulopsis inconspicua*	++
stellatoidea	+++	*glabrata*	++
parapsilosis	+++	*sp.*	++
utilis	+++	*Candida rugosa*	+
Cryptococcus neoformans	+++	*zeylanoides*	+
Rhodotorula mucilaginosa	+++	*Rhodotorula flava*	+
Hansenula subpelliculosa	+++	*Saccharomyces sp.*	+
Saccharomyces fragilis	+++	*Torulopsis famata*	changing
marxianus	+++	*Hansenula anomala*	changing
Geotrichum sp.	+++	*Candida pulcherrima*	—
Candida guilliermondii	++	*Rhodotorula rubra*	—
mellibiosi	++	*Torulopsis aeria*	—
mycoderma	++	*candida*	—
Cryptococcus diffluens	++	*gropengiesseri*	—
laurentii	++	*Saccharomyces cerevisiae*	—
albidus	++	*Trichosporon cutaneum*	—

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STUDIES ON ARTHROSPOROUS YEASTS

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Summary. Twenty-six arthrosporous strains of fungi have been identified after LODDER and KREGER—VAN RIJ's method complemented by tests for bismuth reduction, gelatin liquefaction, and sensitivity to actidion. The 26 strains have been found to show relationship in certain characteristics. Of the strains, 14 have been identified as *Geotrichum candidum*, 4 as *Trichosporon cutaneum*, 2 as *Geotrichum matalense*, 6 as *Geotrichum linkii n. sp.*

With reference to the critical evaluation of the literature on arthrosporous fungi and the results obtained by the author's investigations it is suggested that the Geotrichum genus, formerly classified in the family Mucedinaceae of the ordo Hyphomycetes, should be transferred to the sub-family Trichosporoideae of the family Cryptococcaceae.

A new species has been described under the name of *Geotrichum linkii n. sp.*

In material from patients suspected of systemic mycosis, fungi have repeatedly been found which multiply through arthrospores produced by the breaking up of true mycelium. Some of them have been found to produce budding cells, too. In the pertaining literature arthrosporous fungi isolated from the human organism are registered under the name of *Geotrichum candidum*, and are known either as members of the normal oral and enteral flora [4], or as occasional pathogens held responsible for geotrichoses [5, 5—9, 11, 12].

Yeast-like fungi forming arthrospores are known in the dairy industry under the name of *Oidium lactis* or *Oospora lactis*. On the surface of milk they sometimes form a wrinkled, mould-like membrane, and utilizing lactic acid, they neutralize sour milk, permitting the proliferation of bacteria. As shown by GARRISON's investigations, these fungi are transferred to milk from cow-manure [4]. According to PRESCOTT and DUNN [10], they occur most frequently in Cammembert cheese and some varieties of butter and have a remarkable role in lipogenesis. This species may be used as fodder yeast. Strains capable of breaking down cellulose may impair textiles.

Various strains multiplying by arthrospores and isolated from throat or bronchial secretion, gastric juice, faeces, etc. have been found to form different colonies. This has induced us to study these strains more closely, with especial regard to the question whether all of them may be classified as *Geotrichum candidum*. The findings obtained have raised the second question, namely the determination of the systemic place of the genus Geotrichum.

Table I

Assimilation of sugar by arthrosporous strains

Designation of Strain	Budding	Assimilation of Sugar					
		Gl.	G.	S.	M.	L.	R.
7/132	—	+	—	—	—	—	—
8/111	—	+	—	—	—	—	—
9/32	—	+	—	—	—	—	—
9/150	—	+	—	—	—	—	—
9/434	—	+	—	—	—	—	—
60/22	—	+	—	—	—	—	—
8/92	—	+	+	+	+	+	+
D/1	—	+	+	+	+	+	+
T.c.	+	+	+	+	+	+	+
8/25	+	+	+	+	+	+	+
8/199	+	+	+	+	+	+	+
9/436	+	+	+	+	+	+	+
G. c. (Pr.)	—	+	+	—	—	—	—
8/59	—	+	+	—	—	—	—
8/95	—	+	+	—	—	—	—
8/186	—	+	+	—	—	—	—
8/296	—	+	+	—	—	—	—
8/352	—	+	+	—	—	—	—
9/213	—	+	+	—	—	—	—
9/311	—	+	+	—	—	—	—
9/426	—	+	+	—	—	—	—
G_2	—	+	+	—	—	—	—
G_8	—	+	+	—	—	—	—
V_2	—	+	+	—	—	—	—
V_8	—	+	+	—	—	—	—
$Csat_1$	—	+	+	—	—	—	—

Code: Gl = glucose, G = galactose, S = sucrose, M = maltose, L = glucose
R = raffinose

Materials and methods

The morphological and physiological properties of the strains were determined with the methods of LODDER and KREGER—VAN RIJ [8].

Each strain was tested for pathogenicity by intraperitoneal inoculation of two male albino mice; the spleen, liver, and renal tissues of the animals were examined in sections after staining with SCHIFF's periodic acid leucofuchsin.

The bismuth reduction test was also studied, since similarly to some other yeasts, arthrosporous strains show much better growth on the bismuth sulphite agar used in medical

Table II

Investigation of arthrosporous strains

Designation of strain	Litmus milk	Gelatin liquefaction	Actidion		Bismuth reaction	Pathogenicity for mice
			100 mg/l	50 mg/l		
7/132	L.	—	+	+	+	+
8/111	P.	—	+	+	+	
9/32	—	—	+	—	+	+
9/150	—r.	—	—	—	+	+
9/434	—r.	—	—	—	+	+
60/22	—	—	—	—	+	+
8/92	L.	—	—	—	+	—
D/1	Co.r.	—	—	—	+	
T.c.	Co.r.	—	+	+	+	
8/25	—r.	—	+	+	+	+
8/199	Co.r.	—	+	—	+	+
9/436	Co.	—	+	+	+—	+
G.c.(Pr.)	L.	—	—	—	+	
8/59	Co.r.	—	—	—	+	+
8/95	—	—	+	—	+	+
8/186	L.	—	+	+	+	—
8/296	—	—	+	—	+	+
8/352	L.	—	+	—	+	
9/311	L.	—	+	—	+	+
9/426	—r.	—	—	—	+	—
$G._2$	L.	—	—	—	+—	—
$G._8$	L.	—	+	+	+	
$V._2$	—r.	—	—	—	+	
$V._8$	L.	—	+	+	+	
Csat/1	L.	—	+	+	+	

Signs

Litmus milk
L = alkalization; r = litmus reduction
Co = coagulation; P = peptonization

Actidion
+ = positive inhibition
— = no inhibition

bacteriology than on the media common in mycological work (SABOURAUD's agar, molasses-agar), and have the property of reducing bismuth, as evidenced by the dark gray or black colour of the colonies.

In addition, we studied the actidion sensitivity of our strains. The basal dilution was 1.5 to 100 mg/l actidion, and the tests were made with dilution series 1 : 2 on bismuth sulphite media.

The investigations included gelatin liquefaction tests and cultivation in litmus milk.

Results

Twenty-six arthrosporous strains were studied. Of these, 19 were isolated from patients suspect for systemic mycosis, one from sewage. Six strains have been obtained in culture, 1 strain from Prague, 1 from the State Institute of Hygiene (Dysentery Laboratory), 4 from Dr. I. Vitéz (State Institute of Hygiene).

As to micro-morphological and certain physiological characteristics, the strains were uniform. They did not form ascospores, ferment sugars, utilize nitrate, or produce starch. Among the strains forming arthrospores 2.6 to 5.2 by 4.6 to 27 microns in size, budding cells were discovered only in four.

Significant differences were recorded in sugar assimilation. In this respect our strains were divided into three groups. The strains classified with the first group assimilated only glucose; the members of the second group utilized glucose and galactose; those of the third group assimilated glucose, galactose, sucrose, maltose, and lactose. This third group consisted of two parts ; one multiplied solely with arthrospores and the other also with budding cells (Table I).

All the strains under review were found to reduce bismuth. Growth in litmus milk did not result in acidification but some strains brought about coagulation or alkalization. The strains varied in their pathogenicity for mice. Liquefaction of gelatin did not occur even on cultivation for four weeks. The use of 50 to 100 mg/l solutions of actidion caused inhibition of varying degree (Table II).

Discussion

In possession of these data we have undertaken the taxonomic classification of our strains, with due consideration of the pertaining literature.

In 1928, GILLIERMOND classified the fungi multiplying solely by breaking up of a true mycelium, *i.e.* by arthrospore formation, into group A; the yeast multiplying by both arthrospores and budding were ranged into group B. Group A included the genus Geotrichum described by LINK in 1809 [cit. 8].

The ascus-forming fungi multiplying through arthrospores, classified by GILLIERMOND into group A, were ranged by STELLING—DEKKER in 1931 into the genus Endomyces described by REESS in 1870 [cit. 8].

While studying *Oospora lactis (Syn. Oidium lactis)* in 1951 WINDISCH observed ascus formation and therefore classified the species into the genus Endomyces, under the name of *Endomyces lactis* [14]. In 1958, FRAGNER accepted the classification of WINDISCH with reservations only [3].

As stated by CARMICHAEL in 1957, *Geotrichum candidum* is an imperfect form of the yeast called *Endomyces lactis* which produces ascospores and multiplies by arthrospores [1].

According to further reports and CARMICHAEL's study, the majority of the Geotrichum species described under various names are identical with

Geotrichum candidum or may be considered to correspond to a synonym of *Trichosporon cutaneum* [1].

The physiological characteristics of the genus Geotrichum have been neglected or studied superficially by old authors. In 1957, SAEZ claimed that glucose, galactose, sucrose, maltose, and lactose were assimilated, though not fermented, by *Geotrichum candidum*, which also proved to induce coagulation and acid formation in litmus milk, but no liquefaction of gelatin [11].

In 1959 VIEU and SEGRETAIN differentiated *Geotrichum candidum* and *Trichosporon cutaneum* on the basis of the following properties [13];

a) *Geotrichum candidum* forms arthrospores, assimilates glucose and galactose, and does not split arbutin.

b) *Trichosporon cutaneum* produces arthrospores and budding cells, assimilates the above-mentioned five different sugars, and splits arbutin.

Thus of the 26 strains studied by us 14, those that formed arthrospores and assimilated glucose and galactose, may be classified as *Geotrichum candidum* according to VIEU and SEGRETAIN's criteria. Four strains, found to produce both arthrospores and budding cells, and to assimilate five different sugars, may be identified as *Trichosporon cutaneum*.

VIEU and SEGRETAIN have investigated the biological characteristics of *Geotrichum matalense* described inadequately by CASTELLANI in 1915. With due consideration of the data published by these authors, the two Geotrichum strains we had isolated and found to form no blastospores but to assimilate five different sugars, may be identified as *Geotrichum matalense* Cast.

Our remaining six strains could not be classified as any of the Geotrichum species on the basis of old, incomplete descriptions: we have therefore been compelled to define a new species, and name it *Geotrichum linkii* n. sp.

*Geotrichum linkii n. sp.** A ramifying, septated, true mycelium which breaks up into arthrospores. The arthrospores are barrel-shaped, assuming a spherical form when they become older. On malt agar and in malt extract the size of the cells varies between 4.6 to 5.2 μ by 5.2 to 15.6 μ. *Geotrichum linkii* forms rings and a pellicula; it does not develop ascospores or ferment sugars; it assimilates only glucose. It does not use up nitrate, produce starch, liquefy gelatin, acidify or coagulate litmus milk. It has been isolated in three cases from laryngeal secretion, in one case from pharyngeal secretion, in two cases from bronchial secretion.

Hence the fungi that multiply exclusively by arthrospores, as the members of the genera Endomyces and Geotrichum, owing to the absence of budding

* *Geotrichum linkii n.sp.* Mycelium verum cum arthrosporis. Cellulae elongatae et cuboideae, 4.6—5.2, 5.2—15.6 μ. Sedimentum annulusque formantur. Ascosporae non formantur. Fermentatio nulla. Assimilatur solum glucosum. Nitras kalicus non assimilatur. Amylum non formatur. Gelatinum non est liquefactum. Lac non coagulatur, acidum non formatur. Ex secretione bronchopulmonale, laryngeale et pharyngeale.

cells, do not belong to the group of yeasts. In the yeast system of LODDER and KREGER—VAN RIJ, the genus Endomyces is nevertheless classified as a member of the family Endomycetaceae on the basis of ascus formation. These fungi therefore represent a transition from the yeasts in the direction of Hyphomycetes.

As evidenced by reports in the literature and our own findings, the origin and the morphological and physiological properties of these fungi show a resemblance to those of the true yeasts. For their identification we used in agreement with SAEZ [11] the methods applied for the determination of yeasts. Our own investigations have revealed a relationship between the species Trichosporon and Geotrichum. *Geotrichum candidum*, being an imperfect form of *Endomyces lactis*, belongs into the family Cryptococcaceae, the group of imperfect yeasts.

Consequently, we suggest the transfer of the genus Geotrichum LINK from the family Mucedinaceae of ordo Hyphomycetes of the class Fungi imperfecti, to the ordo Endomycetales, in the sub-family Trichosporoideae of the family Cryptococcaceae in accordance with Fig. 1.

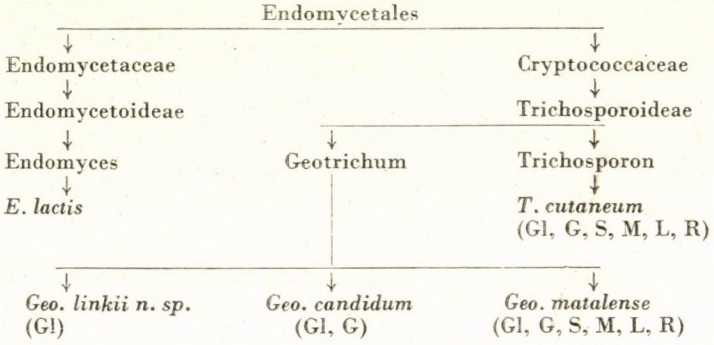

Fig. 1. Classification of the genus *Geotrichum* in the yeast system
The signs in brackets refer to assimilation of sugar by the species:
Gl = glucose G = galactose L = lactose M = maltose R = raffinose S = sucrose

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THE RESULTS OF SALK VACCINATION IN HUNGARY AS MEASURED ON THE 1959 POLIOMYELITIS EPIDEMIC

By

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Summary. The vaccination history of 1769 of the 1830 patients afflicted by the 1959 poliomyelitis epidemic in Hungary has been ascertained. The following conclusions were drawn.

1. Only 35.4 per cent of the patients had received 3 or 4 doses of vaccine, although 80 per cent of the sensitive population were at least triply vaccinated.

2. Among the triply vaccinated patients the proportion of those who had received the last dose one year, or more, earlier was considerable.

3. Among the three groups differing in the vaccination schedule the highest protection was shown by that which had received the third stimulus eight months before the epidemic. Strikingly, almost the same protection was achieved by two stimuli given 3 and 4 months before. The weakest effect was shown by the group having received the third dose 15—16 months prior to the outbreak. Thus, the immunity induced by the vaccination must have been remarkably waning in the last group.

4. The average protection rate attributable to the two years' Salk vaccination is estimated to exceed 60 per cent.

5. Case fatality was significantly lower in the vaccinated group than in the unvaccinated one.

In Hungary, like all over the world, the incidence of poliomyelitis was significantly increasing during the last decade. Epidemics occurred more and more frequently, so that in four of the six years from 1954 to 1959 the incidence rate exceeded 10 per 100 000 population [1].

It has been obvious for long that poliomyelitis cannot be suppressed but by vaccination. Since Salk's favourable results had been published [2], mass immunizations with the inactivated polio vaccine have been carried out in numerous countries.

In Hungary Salk vaccine was first used on a large scale at the peak of the 1957 epidemic. In that campaign the intracutaneous technique was applied. As demonstrated by PETRILLA [3], the incidence rate was at least 50—60 per cent less in the vaccinated than in the unvaccinated group; within the age groups involved in the campaign the incidence rate for the unvaccinated children was five times that for the vaccinated ones. According to BAKÁCS [4], about 2000 persons had escaped poliomyelitis in 1957, as a result of vaccination.

Immunization with the Salk vaccine was continued in 1958 and 1959 (Fig. 1 and Table I). The children immunized with two doses in 1957 were

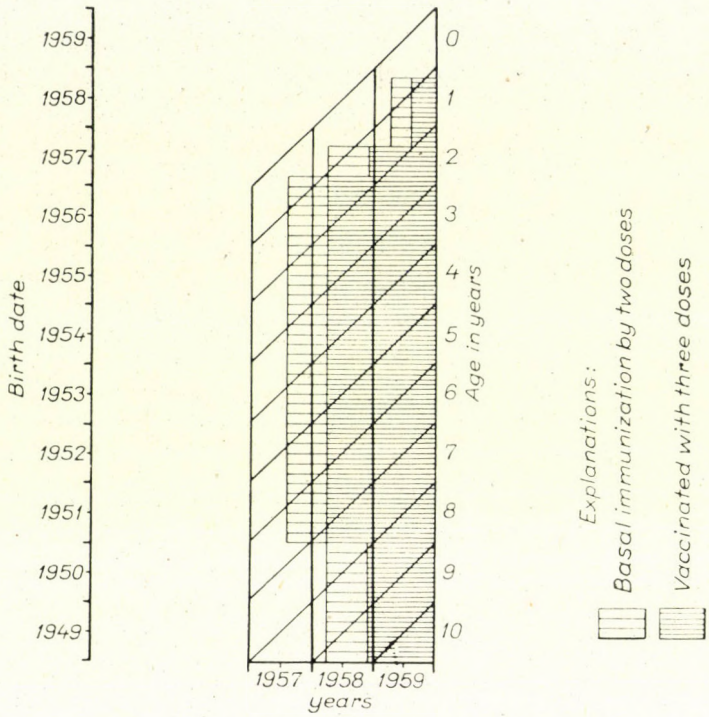

Fig. 1. Salk vaccination in Hungary, 1957—1959

Table I

Vaccination groups, 1957—1959

Group No.	Birth date	Age at the time of the epidemic, years	1st and 2nd doses	3rd dose	Time interval in months, between the last dose and the epidemic
1	1 Jan., 1944[1]—31 Dec., 1950	9—14	Feb. and Mar., 1958	Nov., 1958	8
2	1 Jan., 1951—28 Feb., 1957	2—8	July and Aug., 1957	Apr., 1958	15
3	1 Mar., 1957—31 Aug., 1957	1—2	Mar. and Apr., 1958	Nov., 1958	8
4	1 Sep., 1957—30 Sep., 1958	0—1	Mar. and Apr., 1959	July to Sep., 1959*	3*

* In most of these cases no third dose had been given, or too late to be effective.

[1] The data of those born between Jan. 1, 1940, and Dec. 31, 1943 are not included in the Table, since morbidity in this age group was very low.

given a third dose in the spring of 1958. Furthermore, those born between January 1, 1940, and August 31, 1957, but not involved in the 1957 campaign received two doses in the spring, and one in the autumn, of 1958. The children

born between September 1, 1957, and September 30, 1958, received the first two doses in March and April, 1959, and the third one in July or August of the same year. At the same time those who had escaped vaccination were also immunized, and nearly 305 000 persons were given a fourth dose of vaccine. During the three years' period over 80 per cent of the population under 15 years of age were immunized with three stimuli. The vaccine was administered in 1958 chiefly, in 1959 exclusively, intramuscularly.

As regards the incidence of poliomyelitis, the year 1958 was particularly favourable; 165 cases had occurred, less than in any of the preceding 20 years. The low incidence may be attributed to the high infection rate in 1957, to the interfering effect of the simultaneous widespread Bornholm epidemic [5, 6] and, beyond doubt, to the large-scale vaccination.

In such a favourable situation the 1959 epidemic came unexpectedly. In that year 1830 cases were registered, more than in any year except 1957. It was therefore of interest to see whether a high incidence like this indicated that all the efforts of the three years' vaccination had been in vain or without vaccination the epidemic would have been more extensive. The aim of the present report is to answer this question on the basis of the epidemiological data of the year 1959. The detailed description of the epidemic and the discussion of the vaccination's influence on the clinical course of the disease will be omitted, since these have already been published [1, 7].

In order to obtain reliable data on the immunization status of the individuals in whom poliomyelitis was diagnosed in 1959, questionnaires were sent to the Public Health and Epidemiological Station competent to the domicile of each of the patients. The returned questionnaires were controlled, and those with incomplete, or uncertain, or obviously erroneous data were sent back for completion or correction.

After another careful control the data of 1769 questionnaires referring to 90 per cent of the cases reported in 1959 were worked up.

Based on the immunization data the patients were divided into 3 categories, viz. (i) „*vaccinated*" : the number and times of vaccinations were confirmed by the vaccination registers and/or vaccination cards; (ii) vaccination condition unknown: no records were obtainable, the information supplied by the patient or the relatives were uncertain; (iii) "*unvaccinated*" : according to the records the patient had not been vaccinated, as confirmed also by the patient or relatives.

Table II shows the immunization condition of the patients. Of the 1769 patients under study 583 (33 per cent) had received no vaccine. For the sake of comparison the patients with unknown vaccination history (9 per cent) were considered unvaccinated. The number of "vaccinated" patients was 1026.

Of the "vaccinated" patients 168 had received one, 231 two doses of vaccine. Usually triply vaccinated individuals only are regarded as completely

vaccinated. In our material 511 patients were triply vaccinated, while 116 had received four doses. Thus, as few as 35.4 per cent had been completely immunized, the others (64.6 per cent) were unvaccinated or incompletely immunized. As over 80 per cent of the susceptible age groups had received three doses between 1957 and the outbreak of the epidemic, it can be stated that the incidence rate for the completely immunized population was definitely lower than that for the unvaccinated group.

Table II also presents information on the immunization data of the patients by age. The percentage of unvaccinated patients was highest under one year (71.0 per cent) and over 15 years of age (78.4 per cent); it was higher

Table II

Age incidence of poliomyelitis by vaccination status, 1959

Age in years	Unvaccinated	Vaccination status unknown	Vaccinated with 1 dose as confirmed by record	Vaccinated with 2 doses as confirmed by record	Vaccinated with 3 doses as confirmed by record	Vaccinated with 4 doses as confirmed by record	Total vaccinated	Total examined
			a) Cases					
0	259	10	65	27	4	—	96	365
1	76	22	51	94	44	—	189	287
2	44	21	27	48	87	27	189	254
3—8	86	74	24	51	319	84	478	638
9—14	9	15	—	5	5	52	62	86
15—	109	18	1	6	5	—	12	139
Total	583	160	168	231	511	116	1026	1769
			b) Per cent					
0	71.0	2.7	17.8	7.4	1.1	—	26.3	100.0
1	26.5	7.7	17.8	32.7	15.3	—	65.8	100.0
2	17.4	8.2	10.6	18.9	34.3	10.6	74.4	100.0
3—8	13.5	11.6	3.7	8.0	50.0	13,2	74.9	100.0
9—14	10.5	17.4	—	5.8	60.5	5.8	72.1	100.0
15	78.4	13.0	0.7	4.3	3.6	—	8.6	100.0
Total	33.0	9.0	9.5	13.1	28.9	6.5	58.0	100.0

for the one-year-old children (26.5 per cent) than for those between 2 and 14 years age (10.5—17.4 per cent). The divergencies should be ascribed to the low percentage of vaccinated persons under one year and over 15 years of age.

The role of the time interval between the last vaccination and the onset of the illness was also studied. As shown in Table III, nearly 6 per cent of the triply

vaccinated patients had fallen ill within two weeks following the last vaccination, that is, at a time when the booster effect had hardly developed.

Table III

Cases of poliomyelitis having three or four doses of vaccine, by age and time interval between the last vaccination and the attack*

Time interval	Age in years						Total
	0	1	2	3—8	9—14	15 and above	
Triply vaccinated							
a) Cases							
0—2 weeks	2	13	5	9	—	—	29
3 weeks—5 months	2	29	24	21	3	—	79
6—11 months.....	—	1	38	43	29	3	114
1 year and more...	—	1	20	246	20	2	289
Total	4	44	87	319	52	5	511
b) Per cent							
0—2 weeks	50.0	29.5	5.8	2.8	—	—	5.7
3 weeks—5 months	50.0	65.9	27.6	6.6	5.8	—	15.4
6—11 months.....	—	2.3	43.6	13.5	55.7	60. 0	22.3
1 year and more...	—	2.3	23.0	77.1	38.5	40.0	56.6
Total	100.0	100.0	100.0	100.0	100.0	100.0	100.0
Having four doses							
a) Cases							
0—2 weeks	—	—	14	47	3	—	64
3 weeks—5 months	—	—	13	31	2	—	46
6—11 months.....	—	—	—	3	—	—	3
1 year and more...	—	—	—	3	—	—	3
Total	—	—	27	84	5	—	116
b) Per cent							
0—2 weeks	—	—	51.9	55.9	60.0	—	55.2
3 weeks—5 months	—	—	48.1	36.9	40.0	—	39.6
6—11 months.....	—	—	—	3.6	—	—	2.6
1 year and more...	—	—	—	3.6	—	—	2.6
Total	—	—	100.0	100.0	100.0	—	100.0

* Confirmed by record

For 193 patients (37.7 per cent of the triply vaccinated patients) this interval varied between 3 weeks and 11 months; in these cases a good protection might have been expected. A markedly high proportion, nearly 57 per cent, of the triply vaccinated patients had received the third dose more than a year before the attack.

Among the 116 patients vaccinated with four doses 49 had received the last dose from 3 weeks to 11 months earlier, whereas for 64 (55.2 per cent) the interval was shorter than two weeks. In the latter cases the fourth dose was not, but the third was, expected to protect. In 48 of the 64 cases (75 per cent), however, the lack of protection might be explained by the long interval (more than a year) between the third vaccination and the attack.

It seemed interesting to study the incidence rates in the 0, 1, 2, 3—8 and 9—14-year age groups separately. Persons from 15 to 19 years of age were omitted from the analysis because of the low incidence in that age group. In Table IV the average age specific morbidity rates for the period 1952—1957 are compared with the corresponding rates for 1959.

Table IV

Age specific incidence rates for the population under 15 years of age, 1952—57 and 1959

Age in years	Incidence per 100 000		Indices taking 0 year = 100	
	1952/57	1959	1952/57	1959
0	88.5	253.2	100.0	100.0
1	155.4	187.3	175.6	74.0
2	93.7	147.8	105.8	58.4
3—8	23.4	59.6	26.5	23.5
9—14	6.4	9.7	7.2	3.8

Taking into account that only a low proportion of the infants had been vaccinated, it may be stated beyond doubt, that the shift of the peak of the age curve from 1 year to 0 year had resulted from the vaccination. To make the comparison clearer, morbidity indices were computed for the above age groups by taking the age specific morbidity rates for the 0-year age group equal to 100. The indices clearly show that the relative morbidity for each of the 1, 2 and 9—14-year age groups was considerably lower in 1959 than in the six-year period 1952—1957. The decline in the index for the 3—8-year age group was less definite.

The information obtained from this comparison is inaccurate for two reasons, *viz.* (i) the relative morbidity indices had never been constant in Hungary, especially not in the period immediately preceding Salk vaccination; (ii) not all of the 0-year-old infants can be considered unvaccinated; those born

before September 30, 1958, had received a basal immunization with two doses. Nevertheless, the difference in the degree of protection of the age groups under study is clear.

To obtain more information on the protection provided by the vaccination the morbidity rates for the four immunization groups were calculated. The total number of individuals in each of the groups was estimated on the basis of statistical data. These were, however, less exact than the figures yielded by a most recent census. For they were based on the data published by the Central Bureau of Statistics of Hungary, on the ground of the census held on January 1, 1949, and corrected according to the birth and death statistics published since then. It should be added that (i) the vaccination statistics did not contain the detailed data of the immunization condition of the individual cohorses; (ii) private vaccinations were being performed both before and after the vaccination campaigns, and (iii) a considerable number of persons who were not eligible were vaccinated during each campaign. Thus, the number of vaccinated persons could not be estimated with high accuracy, except for the youngest age groups.

It should be pointed out that in the cohorses born between January 1, 1944, and August 31, 1957, the number of the triply vaccinated subjects was only known; for this reason in the following only those vaccinated with three or more doses are included in the vaccinated population; all the others are considered unvaccinated. The poliomyelitis cases are classified by the same principle. In the youngest age groups, on the other hand, all those who received two doses in March and April, 1959, are regarded as vaccinated.

The incidence rates for the vaccinated and unvaccinated population, and the resulting protection ratios, are presented in Table V. The figures are in accordance with the relative morbidity indices shown in Table IV.

As a conclusion the protection ratio was the highest in the two groups which had received the third dose 6 to 8 months before the epidemic (69.3 and 71.1 per cent, respectively); it was hardly lower (66.3 per cent) in the youngest group immunized by two doses administered shortly before. The protection was definitely weaker (53.2 per cent) in the age group born between January 1, 1951, and February 28, 1957, *i. e.*, in the group which had received the last dose more than a year earlier.

The number of prevented cases in the population under study was calculated on the basis of the incidence among the "unvaccinated" persons, and was found to be 1147. Since the "unvaccinated" group included a considerable number of incompletely vaccinated individuals and persons who had been vaccinated out of campaign, this number should be regarded as a minimum.

The general protection ratio resulting from the three years' Salk vaccination, *i. e.* the average of the protection ratios of the four groups under study, was esetimated to at least 60—65 per cent. The real protection ratio was cer-

Table V

Incidence of poliomyelitis by vaccination groups, 1959

Designation	Vaccination groups			
	No. 1	No. 2	No. 3	No. 4
Birth date	Sep. 1, 1957-Sep. 30, 1958	Mar. 1, 1957-Aug. 31, 1957	Jan. 1, 1951-Febr. 28, 1957	Jan. 1, 1944-Dec. 31, 1950
Time of immunization				
1st dose	Mar., 1959	Mar., 1958	July, Aug., 1957	Feb., 1958
2nd dose	Apr., 1959	Apr., 1958	Aug., Sep. 1957	Mar., 1958
3rd dose	July.-Sep.,1959	Nov., 1958	Apr., 1958	Nov., 1958
Administration of vaccine				
1st dose	1.0 ml i.m.*	0.2 ml i.c.**	0.2 ml i.c.	0.2 ml i.c.
2nd dose	0.5 ml i.m.	0.5 ml i.m.	0.2 ml i.c.	0.2 ml i.c.
3rd dose	0.5 ml i.m.	0.5 ml i.m.	0.5 ml i.m.	0.5 ml m.
Vaccinated population	120 000	59 000	900 000	950 000
Unvaccinated population+	40 000	21 000	235 000	164 000
Total population	160 000	80 000	1 135 000	1 114 000
Incidence				
Vaccinated cases	1/179	2/70	2/441	2/55
Unvaccinated cases+	177	81	246	33
Total cases	356	151	687	88
Incidence per 100 000 population				
Vaccinated cases	149.2	118.6	49.0	5.8
Unvaccinated cases+	442.5	385.7	104.7	20.1
Total cases	222.5	188.8	60.5	7.9
Incidence rate in the vaccinated population in per cent of that in the unvaccinated+ population	33.7	30.7	46.8	28.9
Protection ratio per cent	66.3	69.3	53.2	71.1
Expected cases in the vaccinated population as calculated from the incidence rate in the unvaccinated population	531	228	942	191
Prevented cases	352	158	501	136

+ Unvaccinated and incompletely immunized.
1/ of these 49 had received three, 130 two, doses
2/ Three or more doses had been given
* Intramuscularly
** Intracutaneously

tainly higher, for the unvaccinated groups had included partially immunized individuals.

As regards the duration of the immunity following Salk vaccination equivocal data have been published. SALK [8] found the antibody level satisfactory as late as one or two years after the booster dose, provided the vaccine was of adequate potency. Similar is the conclusion drawn by MACLEOD [9]. In contrast to these data, other authors [10, 11, 12] found a considerable fall in the antibody titre, especially to type 1 poliovirus, one year after the last dose. The present results suggest that the immunity was remarkably waning after the first year following the third dose. The observation that two doses given 3 and 4 months earlier proved to be as potent as the third (booster) dose given 6 months earlier merits special attention.

Table V also presents the route of administration and the volume of the vaccine injected. Unfortunately, the relative potency of the intracutaneous vaccination could not be estimated in this way because intracutaneous vaccination was always followed by intramuscular one or ones, timing was variable, and the vaccination groups were different in age. Nevertheless, the two age groups each of which had received two intracutaneous stimuli followed by an intramuscular booster dose showed the best and worst protection ratio, respectively. The only difference in the immunization schedule of the two groups was the time interval between the booster dose and the epidemic. This observation confirms the importance of the time factor.

Finally, the influence of vaccination on the case fatality was studied. The data in Table VI suggest that the favourable case fatality of poliomyelitis in the year 1959 may first of all be attributed to the vaccination. Among the

Table VI

Case fatality by vaccination status, 1959

Vaccination status	Number of cases	Fatal cases	
		Number	Per cent
Vaccinated with*			
1 dose	168	5	3.0
2 doses	231	4	1.7
3 doses	511	8	1.6
4 doses	116	1	0.9
Total vaccinated	1026	18	1.8
Unvaccinated or unknown	743	31	4.2
Total	1769	49	2.8

* Confirmed by record.

1830 patients with poliomyelitis 53 (2.9 per cent) died; of the 1769 cases under study 49 ended fatally (2.8 per cent). Case fatality for the unvaccinated patients and those with uncertain immunization history was 4.2 per cent; for those who had received one, two, three and four doses, it was 3.0, 1.7, 1.6 and 0.9 per cent, respectively. Thus, the more doses had been given, the lower the rate.

Case fatality being highly dependent on age, its evaluation needs special caution. In Hungary case fatality of poliomyelitis has always been highest over 15 years of age; *e. g.* in the average of the years 1952—1958 case fatality for the groups above and below 15 was 10.3 and 5.3 per cent, respectively. Since, however, only few persons over 15 years of age had been vaccinated while a high proportion of the unvaccinated fatal cases had completed 15 years, the above conclusion might have been false at expense of the unvaccinated patients. It seemed thus more correct to restrict the comparison to the age groups under 15 years (Table VII). Doing so, case fatality in the unvaccinated

Table VII

Case fatality of poliomyelitis, by vaccination status for children of 0—14 years of age, 1959

Vaccination status	Number of		Case fatality per cent
	cases	deaths	
Unvaccinated or reported to be vaccinated	616	19	3.1
Vaccinated with 1—4 doses, confirmed by record	1014	18	1.8

group was found to be 1.7 times higher than in the vaccinated group (3.1 and 1.8 per cent respectively). This ratio is remarkably lower than the 10 : 1 ratio calculated by GEFFEN [13] by analyzing the poliomyelitis cases that occurred in England and Wales in 1958. Nevertheless, the difference in our material is also remarkable.

The advantages and disadvantages of the Salk vaccine as well as the superiority of the live attenuated vaccine to the Salk vaccine have been clearly explained by BAKÁCS [4]. After his paper had been published, vaccination with Sabin's live vaccine has produced unequivocally favourable results wherever it was applied extensively; its systematic use in the future may lead to the eradication of poliomyelitis. Nevertheless, the present results confirm the view that vaccination with SALK's formolinized vaccine was the first important success in the struggle against poliomyelitis.

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Budapest, 3 to 5 October, 1961

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С 3. по 5. октября 1961

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SOME OBSERVATIONS ON THE MECHANISM OF THE MIDDLEBROOK–DUBOS HAEMAGGLUTINATION TEST

By

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(Received April 6, 1960)

Summary. The serological behaviour of polysaccharides and proteins isolated from tubercle bacilli has been studied by the Middlebrook – Dubos test.

Polysaccharide P I was capable to adsorb onto the surface of sheep red blood cells, while under the same experimental conditions polysaccharide P II failed to do so. Previous treatment of the red blood cells with trypsin, pepsin, papain and tannin did not influence the adsorption of polysaccharides at 37° C. When using tannin-treated red blood cells, no adsorption of extract P I could be observed at room temperature.

As to adsorption, the extracts of protein character exhibited a fairly uniform behaviour. Sensitization was the most pronounced with tannin-treated red blood cells; the degree of adsorption onto papain-treated red blood cells was somewhat less, though still remarkable.

In experiments performed with tannin-treated and protein-sensitized red blood cells the grade of haemagglutination inhibition by extracts of protein character was proportional to their protein-bound polysaccharide content.

The Schmidt—Thannhauser reaction destroyed the sensitizing capacity of extract P I. A quantitative decomposition of the ribonucleic acid content of material P I was observed, while the bound lipids could still be demonstrated in the extract.

On the basis of the results an attempt has been made to explain the mechanism of the Middlebrook—Dubos test.

In the course of studies concerning the nature and specificity of antibodies it was observed that beyond agglutinating the bacterial cells, antibodies were able to precipitate and fix complement with extracts of the same organism. Accordingly, agglutination might be regarded as a precipitation that occurs on the surface of particles [1]. This hypothesis was supported by the model experiment of JONES [2], who succeeded in demonstrating the agglutination of bacterial-extract-coated collodion particles by a homologous antibody. A practical application of this observation was first accomplished by KEOGH *et al.* [3, 4], who agglutinated bacterial polysaccharide-coated red blood cells by homologous antibodies in the absence, and lysed in the presence, of complement. The above method was applied by MIDDLEBROOK and DUBOS [5] for the serodiagnosis and immunological investigation of tuberculosis. Shortly afterwards the basic principle of this type of haemagglutination was adopted for the serodiagnosis of a wide variety of diseases caused by different microorganisms (bacteria, Rickettsiae, viruses, leptospirae, *etc.*)

According to the general view [6], the specificity of bacterial-extract-coated red blood cell is determined by the polysaccharide or the protein adsorbed. There is no agreement, however, as to the nature of the mechanisms possibly involved in the adsorption. A great number of experimental data

point to the important role of lipids in the adsorption [7, 8, 9]; this hypothesis, however, cannot be accepted as of a general validity even for the Gram-negative bacteria [10]. In our own experiments performed with cell components of tubercle bacilli isolated by fractionation [11, 12, 13], only one component (polysaccharide P I) adsorbed readily onto the surface of washed normal red blood cells. The results obtained on examining the factors involved in this particular case of adsorption suggested the importance of the ribonucleic acid (RNA) present in component P I. The present paper supplies some further data on behalf of this hypothesis and gives an account of our recent studies on the adsorption of protein and polysaccharide components of tubercle bacilli onto the surface of red blood cells pretreated in different ways.

Materials and methods

Isolation of polysaccharides and proteins of tubercle bacilli was carried out as described in our earlier papers [11, 12].

The hydrolytic enzymes used were partly commercial products: crystalline desoxyribonuclease (Chinoin, Budapest), pancreatic lipase (Schuchardt), trypsin (Difco), pepsin (Grübler), papain (Merck), and part of them were prepared in our laboratory: ribonuclease as described by KUNITZ [14] and MACDONALD [15] and desoxyribonuclease according to KUNITZ [16]. Out of these substrates used as standards in control experiments desoxyribonucleic acid (DNA) was prepared by us as described by EMANUEL [17]; the RNA used was a product of Merck or B.D.H.

Chemical analysis. The quantity of polysaccharides was determined by the anthrone reagent [18], and the pentose-content by the orcinol-method of MEYBAUM [19]. DNA was determined by the diphenylamine-test of DISCHE [20], the phosphorus content according to HARWEY [21] and the nitrogen content according to CLEGHORN and JENDRASSIK [22]. Lipids were estimated by FEIGL's spot-test [23].

Serological examinations. In the Middlebrook—Dubos test [5] rabbit immune serum [12] and sheep red blood cells preserved in Alsever-solution were used. The haemagglutination and haemagglutination-inhibition tests were performed by the micro-method described earlier [11, 12]. For the sensitization of washed red blood cells 10 mg per cent solutions or still higher dilutions of our extracts were used. Sensitization of cells pre-treated in different ways was also attempted.

Tannin-treated red blood cells. According to the method of BOYDEN [24], 1 ml of a 20 per cent washed sheep red blood cell suspension was diluted to 10 ml by a mixture of equal volumes of M/15 pH 7.2 phosphate buffer and saline. Ten ml-s of tannin diluted 1 : 40.000 by saline were added to the suspension and incubated for 10 minutes at room temperature. Centrifuged cells were washed in saline and the sediment was made up to the original volume of 1 ml. For sensitization 0.5 ml of tannin-treated red blood cell suspension was added to a mixture of saline-diluted extracts and 1 ml of M/15 pH 6.5 phosphate buffer and incubated at 37° C for 2 hours. After centrifugation the sensitized cells were washed with saline containing 1 per cent normal rabbit serum, and a 0.5 per cent final suspension was prepared.

Formolized red blood cells were prepared as described by MOSKOVITZ [25], and GOLD [26]. Formalin was diluted to 1 : 30 in saline and mixed to an equal volume of washed sheep red blood cell suspension. The mixture was incubated at 37° C for 12 to 14 hours. The cells were then washed 3 times and after resuspending in distilled water or saline they were stored at 4° C until used. Trypsin, pepsin or papain treatment was carried out as described by SCHIFF [27]. To 9 ml of a 4 per cent red blood cell suspension 1 ml of a 1 per cent enzyme-solution was added and the mixture incubated at 37° C for 10 minutes. After incubation the cells were washed in saline and suspended in saline containing 1 per cent normal rabbit serum.

Desoxyribonuclease treatment of the P I extract used for sensitization was made according to the method of ALLFREY and MIRSKY [28]. Two ml of P I extract (200 mg per 100 ml in 0.05 per cent $MgSO_4$ solution) was diluted by an equal volume of 0.2 *M* pH 7.0 veronal acetate buffer and 2 ml of the appropriate enzyme (0.1 mg/ml) was added. The mixture was incubated at 37° C for 30 minutes. After incubation 3 ml of the mixture was transferred into 1 ml 3 *M*

trichloracetic acid and incubated in an ice-bath for 15 minutes. After centrifugation the amount of the acid-soluble desoxypentose compounds released into the supernatant was determined colorimetrically (Dische-test or phosphorus-determination). The rest of the enzyme-treated material was used for serological examinations.

Ribonuclease treatment was performed as described by KUNITZ [29]. Two ml of P I extract (200 mg per 100 ml in distilled water) were diluted by an equal volume of 0.1 *M* pH 7.0 veronal-acetate buffer and after adding 2 ml of ribonuclease solution (0.1 mg/ml) the mixture was incubated at 35° C for 30 minutes. After hydrolysis 3 ml of MCFADYEN reagent [30] was added to 3 ml of the reaction mixture. After 30 minutes the material was centrifuged and the quantity of the uranylacetate-soluble phosphorus compounds released on hydrolysis was determined by colorimetry (phosphorus determination). The residual 3 ml of the enzyme-treated material was used for serological examinations.

Lipase treatment of the P I extract was carried out as described by GOMORI [31] and ARCHIBALD [32]. A solution of 400 mg per 100 ml P I extract in saline was diluted by an equal volume of 0.2 *M* pH 7.2 acetate buffer. To each 5 ml sample of this solution 1 ml enzyme (1 mg/ml) solution was added. The activity of the enzyme preparate was controlled by means of a standard water soluble substrate; Tween 80 diluted in 0.2 *M* pH 7.2 acetate buffer. After incubation at 20° C for 10 minutes the quantity of the released fatty acids was determined by titration against 0.2 *N* alkali with phenol red as the indicator. Serological activity was controlled by the Middlebrook—Dubos test.

Alkaline hydrolysis of the RNA content of the P I extract was made as described by CHARGAFF [34]. This was followed by the Schmidt—Thannhauser test [33]. Solutions containing 100 mg per 100 ml or less P I extract were alkalined to 0.1 *N* by the addition of sodium hydroxide and incubated at 37° C. The samples taken at different intervals were neutralized with hydrochloric acid and the serological activity of the solution was examined by the Middlebrook—Dubos test. Before chemical analysis the neutralized solutions were dialyzed in order to remove the by-products of hydrolysis. Dialysis was performed against cold distilled water for 48 hours with frequent water changes. The dialyzed material was diluted to the calculated volume (40 mg per 100 ml) and this dilution was used for the chemical analyses.

Results

A number of experimental results supports the view that the adsorption of the different bacterial extracts onto the surface of the red blood cells follows different mechanisms. This may be ascribed partly to the different chemical structure of the extracts, partly to the mosaic-like structure of the red blood cell surface [35]. Changes of this surface or the appearance of some more deeply localised groups by hydrolytic decomposition of one or another surface component may result in increased or diminished adsorption of the bacterial extracts. This is the reason for the wide application BOYDEN's technique [24] for the adsorption of proteins onto the surface of tannin-treated red blood cells and this justifies the efforts to use red blood cells treated with different hydrolytic enzymes [36, 37, 38].

Our protein and polysaccharide preparations [13] exhibited different adsorption onto the surface of red blood cells pretreated in different ways. The pertaining experimental data are presented in Table I. In this experiment two different concentration of the extracts were used for the sensitization of normal and differently pretreated cells. The grade of sensitization was expressed in the terms of the agglutination titre obtained with appropriate rabbit immune serum.

The data of Table I permit the following conclusions.

Table I

Sensitization of differently pretreated red blood cells with extracts of M. tuberculosis H_{37} Rv as measured by haemagglutination titrations

RBC sensitized / RBC treated with	Polisaccharides				Proteins						RBC control
	P I		P II		III		IV		V		
	mg 10	per cent 1	mg 10	per cent 1	mg 10	per cent 1	mg 10	per cent 1	mg 10	per cent 1	
Untreated	512	512	4	0	4	0	8	0	0	0	0
Trypsin	512	256	4	0	8	0	32	4	4	0	0
Pepsin	512	256	4	0	8	0	8	0	4	0	0
Papain	2158	2158	16	8	64	16	256	64	128	32	8
Tannin	512	128	16	0	64	16	256	64	256	64	0
Formol	64	32	4	0	4	0	8	0	4	0	0

RBC = red blood cells.

(1) In the control experiments only papain-treated red blood cells were agglutinated by normal rabbit serum up to a 1 : 8 dilution. Red blood cells pretreated in any other way did not become agglutinable by normal rabbit serum. Thus in evaluating the experimental data, the degree of non-specific agglutination (2 degrees of dilution) caused by treatment with papain was taken into account.

(2) The sensitizing effect of P I extracts diluted to 10 resp. 1 mg per 100 ml was not influenced by the various kinds of pretreatment. The agglutination titre was in every case the same as with untreated red blood cells.

(3) Red blood cells sensitized with a 10 mg per 100 ml dilution of P II extract exhibited a different behaviour. The high titre (1 : 512) rabbit serum agglutinated red blood cells pretreated with tannin, resp. papain at a 1 : 16 dilution; other kinds of pretreatment resulted in a very low (1 : 4) agglutination titre, similarly to the titre of normal red blood cells.

(4) The 10 mg per 100 ml solution of materials of protein character exhibited uniform behaviour as to their sensitizing capacity. Sensitization was the most intensive after pretreatment with tannin, a somewhat lower though still high degree of sensitization was observed after papain treatment. Red blood cells pretreated with other enzymes exhibited moderate adsorption.

(5) Formol-treated red blood cells could not be used in the Middlebrook—Dubos test.

In our earlier experiments [39, 40] the haemagglutination-inhibiting effect of the extracts was estimated by the use of red blood cells sensitized with P I extract. The results of these experiments suggested that the haemagglutination-inhibiting effect is proportional to the polysaccharide content. The

application of the Boyden technique, however, enabled us to perform the same experiment with red blood cells sensitized by our complex extracts of protein character. The results of these experiments are presented in Table II.

Table II

The inhibitory effect of the different fractions of M. tuberculosis H_{37} Rv on the haemagglutination of tannin-treated RBC sensitized by Protein IV

	Dilutions $\times 10^3$								
	4	8	16	32	64	128	256	512	1024
P I Bovine	—	—	++	+++	++++	++++	++++	++++	++++
P I Human	—	—	—	+	+++	++++	++++	++++	++++
P II Human	—	—	—	+	+++	++++	++++	++++	++++
Protein III	—	—	—	++	+++	++++	++++	++++	++++
Protein IV	—	—	—	—	—	+++	++++	++++	++++
Protein V	—	—	—	—	—	—	—	++	++++

When making use of sera containing 16 haemagglutinating units.

The agglutination of red blood cells sensitized with protein IV could be inhibited also by the polysaccharides. The haemagglutination-inhibiting capacity of our protein materials was proportional to their polysaccharide and not to their protein content [40]. A detailed investigation of this phenomenon by cross reactions will be discussed later.

The phenomenon of adsorption has been investigated by numerous authors. The results achieved up to now suggest that it may take place by various mechanisms. Bacterial extracts *e. g.* those prepared from certain Gram-negative microorganisms will be capable of adsorption only when partially hydrolyzed by alkali, while lipo-polysaccharides originating from certain other Gram-negative microorganisms are ready for adsorption without such a treatment [41] or may even lose their sensitizing effect after hydrolysis [4, 12]. Substances active in the Middlebrook—Dubos test may display differences in the individual experiments. For the adsorption SORKIN [9] makes the lipid component of the extracts responsible, while TAKAHASHI supposes the phosphatides of the tubercle bacillus to be the active principles [42]. Up to now our results have suggested that the polysaccharide was bound to the surface by the RNA component of the P I extract. The sensitizing principle appeared to be bound to the lanthanum acetate or uranyl acetate precipitable nucleic acid-polysaccharide component of the extract [12]. The supposition was supported by the observation that the sensitizing effect was very sensitive to alkali, while the agglutination-inhibiting effect decreased only slightly after alkali

treatment. On the other hand partial acid hydrolysis suspended the haem-agglutination inhibiting effect first; the sensitizing effect disappeared only later [40].

Table III

Chemical analysis of extract P I (bovine) before and after the Schmidt—Thannhauser test

P I	Per cent of					
	Nucleic acid		Pentose	Nitrogen	Phosphorus	Anthrone (reducing materials)
	260 mμ	DNA				
Before hydrolysis	45.00	35.00	15.0	7.4	4.0	35.0
After hydrolysis	32.5	35.00	10.5	4.5	2.65	33.25

The possible role of the RNA component of the extract in the mechanism of adsorption was examined by means of alkaline hydrolysis (Schmidt—Thannhauser test) and enzyme treatment. In the Schmidt—Thannhauser test a selective hydrolysis of the RNA component of the extracts was achieved with dilute alkali at 37° C. There is, however, a possibility that, on alkaline hydrolysis a decomposition of the acetyl groups possibly present or saponification of lipids might take place. The results of the Schmidt—Thannhauser reaction are demonstrated in Figs. 1 and 2.

Fig. 1 demonstrates that in the course of the experiment a stepwise decrease ensued in the sensitizing effect of a 50 mg per 100 ml solution of P I extract. The effect came to an end in the 6th hour. If dilutions of 25 or 1 mg per 100 ml of the extract were used, the effect ceased in the 5th and 4th hour of the experiment, respectively. Under the same experimental conditions the agglutination-inhibiting capacity exhibited a moderate decrease in the early phase of the experiment, to remain essentially unchanged subsequently (Fig. 2). The changes which took place in the course of the Schmidt—Thannhauser test were controlled by chemical analysis. The results are presented in Table III.

The data presented in Table III show that alkaline hydrolysis resulted in a remarkable decrease in the quantity of materials having an adsorption maximum at 260 mμ. The DNA content was unchanged after hydrolysis. Thus the difference of the two values measured before and after hydrolysis gave directly the RNA content, which was found to be 10 per cent by chemical methods and 12.5 per cent by UV adsorption. These data were corroborated by further chemical analyses. The decrease in the phosphorus and nitrogen content was proportional to the decrease in the total nucleic acid content; the quantity of pentose decreased from 15 per cent to 10.5 per cent. In the control experiments thymus DNA and yeast RNA were hydrolyzed under identical experimental conditions. To check the procedure of hydrolysis the hydrolysates

were fractionated by lanthanum acetate and uranyl acetate, respectively, as described in an earlier paper [12]. The phosphorus and nitrogen determinations in the fractions showed the DNA was to be undamaged by hydrolysis with 0.1 *N* alkali; the total quantity of alkali-treated DNA could be precipitated from the hydrolyzates by uranyl or lanthanum acetate, compounds known to precipitate poly- and oligonucleotides, or these and also mononucleotides, respectively. On the contrary, neither uranyl acetate nor lanthanum acetate

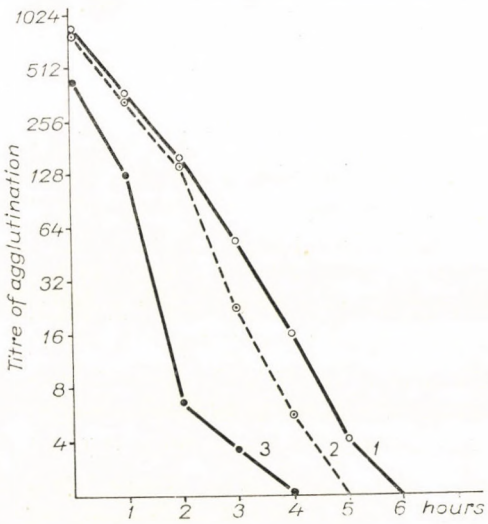

Fig. 1. Decrease of the sensitizing capacity of extract P I in the Schmidt—Thannhauser reaction
1 : 50 mg per cent solution
2 : 25 mg per cent solution
3 : 1 mg per cent solution

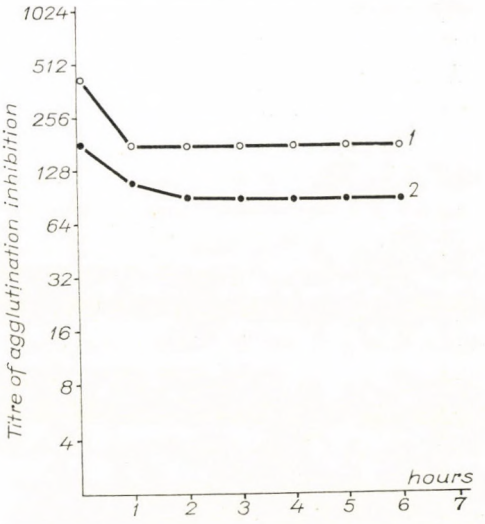

Fig. 2. The haemagglutination-inhibiting capacity of extract P I as related to the Schmidt—Thannhauser reaction
1 : with a serum containing 16 haemagglutination-inhibiting units
2 : with a serum containing 32 haemagglutination-inhibiting units

precipitated alkali-treated RNA, thus its total phosphorus and nitrogen content was present in the supernatant. That means that the total amount of RNA was quantitatively depolymerized.

Nuclease-treatment of the sensitizing P I extract may principally be regarded as a method more specific than alkaline hydrolysis. Nevertheless, as to the enzymatic decomposition of nucleic acids one has to remember that even under optimal circumstances no complete hydrolysis can be achieved and there will always be a remnant core [43, 44]. In spite of this we made attempts at enzymatic decomposition by hydrolyzing yeast RNA first. After treatment with enzyme preparations of different origin 46.7 to 51.4 per cent of the RNA was still insoluble in acid, and practically no decrease was observed in the serological activity of the ribonuclease-treated P I extract.

It was also attempted to hydrolise the P I extract with desoxyribonuclease. In this experiment the effect of the enzyme was controlled on thymus DNA. Our enzyme preparation split 76.5 per cent of the thymus DNA to acid-soluble nucleotides in 30 minutes. Under the same experimental conditions 38 per cent of the DNA content of extract P I became dialyzable without any measurable decrease in the sensitizing effect of the preparation.

As already mentioned, SORKIN and others regard the lipids as the factor responsible for the adsorption of polysaccharides onto the red blood cells. We have pointed to the possibility of a hydrolysis of lipids on the treating with 0.1 *N* alkali the RNA component of our extracts. To obtain exact data, the following experiments were performed.

The P I extract was treated with lipase. Enzymatic activity was controlled by the decomposition of Tween 80, a water-soluble substrate. From extract P I the active lipase preparation did not release any titrable material and the adsorption capacity of the extract remained unchanged.

The effect of partial alkaline hydrolysis on the lipid components of the P I extract was also examined. The preparation was hydrolyzed in 0.1 *N* alkali for 6 hours at 37° C, then neutralized and dialyzed. After dialysis, repeated hydrolysis was performed with more concentrated alkali [13]. Lipids in the hydrolysate were demonstrated by the spot-test of FEIGL. The results suggested that alkaline hydrolysis (Schmidt—Thannhauser test) of the extract P I did not split the lipids from the polysaccharide and consequently the spot-test remained positive. We may accordingly conclude that as to the adsorption of our P I material the polysaccharide-bound lipids seem to have no role.

Discussion

Many details of the haemosensitization mechanism are still unclear. It is nevertheless known that it is by different mechanisms that the different bacterial extracts are adsorbed onto the surface of red blood cells or other particles. The difference is obvious among others from the different sensitivity to heat and alkali treatment of the sensitizing substances of different origin. Bacterial extracts may be divided into 2 groups according to the above characteristics, *viz.* (1) those influenced favourably by heat or alkali treatment and (2) those that are destroyed by these factors. In some of the lipopolysaccharides from enterobacteria the adsorption capacity of the extract is greatly enhanced by heat treatment or partial alkaline hydrolysis [6, 8]. In the experiments of DAVIES [10] the sensitizing capacity of *A. aerogenes* polysaccharides was also remarkably enhanced by alkaline treatment. The sensitizing capacity of the lipopolysaccharide of *P. pseudotuberculosis*, however, could only be increased by alkaline treatment, while no change whatever could be effected by heat

treatment. Alpha haemosensitine [46], isolated from the broth culture of *M. tuberculosis* and polysaccharide P I isolated from the bacterial cell [12] equally lose their sensitizing capacity on partial hydrolysis with alkali. The mechanism activating the sensitizing capacity of bacterial extracts by heat treatment and/or partial alkaline hydrolysis is still unclear. The experiments of LÜDERITZ *et al.* suggested that on treatment with heat and alkali the lipopolysaccharide aggregates of 10 to 20 million molecular weight (*E. coli*, Salmonella) decompose to smaller particles of 200 000 molecular weight which are already capable of sensitizing [8], but their antibody-binding capacity remains unchanged [50]. It was supposed that the components liberated on depolymerization of the lipopolysaccharides were responsible for the adsorption to the lipid or protein surface groups of the red blood cells. This has been supported by a number of experimental data. Alkali-treated bacterial extracts are readily forming complexes with proteins or lipids (100 volumes of cholesterol are bound by 1 volume of lipopolysaccharide [8]). The adsorption of lipopolysaccharides onto the red blood cells is inhibited by different lipids [8, 15]. Lipids isolated from the red blood cell membrane inhibit both the formation of lipopolysaccharide complexes and their adsorption onto the red blood cell surface. On the basis of these experiments it was stated that the receptor substances on the cell surface were either lipids or proteins and the component responsible for the adsorption of lipopolysaccharides was the lipid part of the particle.

However, the above data do not yet permit to make any definite conclusions. The examinations of DAVIES [10] mentioned above support the view that the lipids play no role in the adsorption of bacterial extracts, as the polysaccharide component of *A. aerogenes* does not contain any lipids though its sensitizing effect can be activated by alkali treatment. Chemical analysis revealed that activation requires the destroying of the O-acetyl groups of the molecule.

As to the mechanisms of adsorption, the bacterial extracts of KEOGH [4], the alpha haemosensitizin of SORKIN [46], our extract P I [12], isolated by fractionating the bacterial cell and some other bacterial extracts seem to form a special group of substances [45]. All these extracts lose their sensitizing capacity on mild alkaline hydrolysis. SORKIN succeeded in isolating about 1 per cent of ether-soluble lipids from the hydrolysate of the alpha-haemosensitizin. In his supposition this lipid component is responsible for the adsorption [9]. When exposed to alkaline or acid hydrolysis, our polysaccharide P I exhibited a similar behaviour as SORKIN's haemosensitizin [12, 39]. Chemical analysis, however, suggested that the partial alkaline hydrolysis decomposed the RNA content of the P I material, while the lipids could still be demonstrated in the dialysed hydrolysate. The active role of RNA is also supported by the results obtained by fractionating the nucleic acid [12].

Sensitization, however, does not only depend on the characteristics of the individual bacterial extract but is also influenced by the surface conditions

of the red blood cell. The wide variety of possible adsorption mechanisms is suggested further by the observation that a single bacterial extract does not saturate all the red blood cell receptors, as either simultaneously or in succession several bacterial extracts of different origin can be adsorbed onto the same red blood cell [47, 48]. The different individual receptors contributing to the mosaic-like structure of the surface make the adsorption of different substances possible.

As revealed by the experimental results, adsorption of the specific substances of the Middlebrook—Dubos test (alpha haemosensitizin and polysaccharide P I) is apparently connected with the presence of lipids or of RNA. In spite of their discrepancy, these two hypotheses do not exclude each other. On the basis of our recent knowledge it might be supposed that both hypothetical mechanisms may take place and the mosaic-like structure of the red blood cell surface might supply spots of different specificity for the adsorption of one or the other substance.

The above explanation of the sensitization mechanisms is also supported by model experiments. The role of lipids as supposed by LÜDERITZ *et al.* [8] has already been discussed. The experiments of ELEY and HEDGE [52, 53] supply a physico-chemical proof and hypothetical basis for this supposition.

A number of data are available concerning the *in vitro* reactions and complex formation between nucleic acids and proteins. Such model experiments performed with isolated proteins and nucleic acids [54, 55], cytologic investigations and last but not least investigations on the protein synthesis yielded results suggesting the possible role of RNA in the adsorption of extract P I to the protein receptors of the red blood cell surface and also supply an acceptable explanation of adsorption.

The results obtained by sensitizing red blood cells subjected to various pretreatment have remarkably contributed to our recent knowledge. It must be mentioned that the data included in this paper are the results of experiments performed at 37° C, while according to the technique of BOYDEN, sensitization ought to have been made at room temperature for 10 minutes. Differences in temperature usually bring about essential qualitative differences in sensitive methods *e. g.* of the components of tubercle bacilli isolated by fractionating in 10 minutes at room temperature, extract P I is not adsorbed onto the surface of tannin-treated red blood cells. This observation is in agreement with the data of MIDDLEBROOK [58]. At 37° C, however, both the variously pretreated and the normal red blood cells may equally be sensitized by P I.

When sensitizing was performed with extracts of protein character, significant differences were observed according to the methods of pretreatment. Proteins III, IV, and V were most effective in sensitizing tannin-treated red blood cells. It was surprising that the degree of haemagglutination inhibition was proportional to the quantity of the protein-bound polysaccharide and

not to that of the protein itself. Thus it might be supposed that the coupling to the red blood cell surface is effected by the protein, while the serological specificity is determined by the free polysaccharide component of the molecule. Nevertheless, for the explanation of the phenomena observed further investigations are needed. Some interesting results might be expected especially from cross reactions and adsorption reactions.

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STUDIES ON THE CLASSIFICATION, PATHOGENICITY AND ANTIGENIC STRUCTURE OF STAPHYLOCOCCI

I. THE OCCURRENCE AND PATHOGENICITY OF MICROCOCCACEAE

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Summary. (i) In routine examinations 311 members of the family Micrococcaceae were isolated. Of the strains 233 were Staphylococcus, 75 Micrococcus. The systematic position of 3 strains could not be determined.

(ii) Because of the resemblance of the relatively frequently-occurring *Micrococcus ureae*, *M. luteus* and *M. flavus* to *Staphylococcus aureus*, these organisms cannot be distinguished by simple morphological examination.

(iii) Hyaluronidase has been shown in various species of Micrococcus.

(iv) Micrococcus and *Staphylococcus epidermidis* failed to exhibit coagulase activity. Therefore coagulase production is regarded as an exclusive property of pathogenic strains.

(v) All of the 13 coagulase-negative *Staph. aureus* strains isolated from cases of septicaemia. The explanation of this finding is discussed.

(vi) Whenever coagulase-negative strains are isolated from blood (septicaemia), other pathogenicity tests, as hyaluronidase and phosphatase production, should also be carried out.

The problem of staphylococcal infections is of an increasing clinical and hygienic interest. As to the occurrence and properties of micrococci, a group of bacteria related to staphylococci, we have only a limited number of data [1, 2]. Since both micrococci and staphylococci are ubiquitous organisms, it may be doubtful whether or not an actual strain is pathogenic. The various pathogenicity tests described are not uniform in value. Although a number of authors tried to evaluate the reactions, generally only staphylococci were examined by a small number of tests [3—5] and only the coagulase and phosphatase reactions were extended to micrococci [1].

The purposes of the present work were as follows. (1) Determination of the occurrence of various Micrococcus species in unselected material. (2) The result of pathogenicity tests with micrococci. (3) Comparison of Staphylococcus and Micrococcus by means of the pathogenicity tests.

These questions have been regarded important, as the similar morphological and cultural properties of micrococci and staphylococci are often responsible for diagnostic errors. On the other hand, as the value of various methods in determining the pathogenicity of staphylococci varies with the test employed, it may be assumed that the reactions of choice will be those that are constantly negative with saprophytic micrococci.

In the differentiation of our strains BERGEY's classification has been followed, distinguishing within the family Micrococcaceae six genera (Peptococcus, Sarcina, Gaffkya, Methanococcus, Micrococcus and Staphylococcus).

These genera are easy to differentiate on the basis of pigment production and biochemical tests.

Organisms belonging to Peptococcus, Sarcina and Tetragenus (Gaffkya) occur in the gastrointestinal tract, appendix and throat. These bacteria are known as saprophytes. Methanococcus was found only in soil or in the faeces of herbivora [6.] Micrococci are mostly isolated from human discharges, skin, milk and dairy products [1, 6]. The pathogenicity of staphylococci for human beings is well known. According to BERGEY's manual within the genus Staphylococcus two species, *Staph. aureus* and *Staph. epidermidis* are distinguished. It should be noted that *Staph. aureus* is often designated by other names. In the earlier classification of BERGEY [7], Staphylococcus had been regarded as a species of Micrococcus. Recently, however, BERGEY has abandoned this conception and established a new genus, Staphylococcus [6]. Thus the term '*Micrococcus pyogenes* var. *aureus*' should not be used any more. The *Eighth International Congress of Microbiologists* in Stockholm also accepted the term *Staphylococcus aureus*.

The genus Micrococcus can be divided into two large groups; members of group I produce yellow or white pigment, those of group II produce red pigment. Within the two groups the various species can be identified by biochemical tests (nitrate reduction, utilization of ammonia, decomposition of urea, liquefaction of gelatin and changes in pH, clot formation or peptonization in litmus milk).

Of the so-called pathogenicity tests our strains were examined for the mannitol reaction, haemolysis and urease, gelatinase, lipase, phosphatase, hyaluronidase and coagulase activity.

Materials and methods

Nitrate reduction. Tested in KNO_3 containing medium according to HALLMANN [8].

Utilization of ammonia and urea. Tested in $(NH_4)H_2PO_4$ and in urea-containing media, according to HUCKER [9].

Gelatinase activity. Tested according to CLARKE [10].

Pigment production. Agar plate, 24 hours at 37° C, then 24 hours at room temperature.

Litmus milk. Milk free from preservatives containing 5 per cent of litmus, according to HALLMANN [8].

Splitting of mannitol. Tested in 1 per cent peptone water containing 1 per cent mannitol and brom thymol blue indicator.

Coagulase activity. Tested in human plasma, according to DARÁNYI [11].

Phosphatase activity. Tested in sodium phenolphthaleinphosphate-containing broth, according to HALLMANN [12].

Hyaluronidase activity. Tested according to UJVÁRY [13] using the supernatant of 48 hour Linggood broth cultures.

Lipase activity. Tested in broth containing 5 per cent egg yolk and 1 per cent glucose.

Haemolytic activity. Tested on agar plates containing 5 per cent sheep erythrocytes.

Cultures. The strains were collected between January and April, 1958, from materials sent for routine examination by various departments of the University Medical School, Pécs. Isolation was performed on agar plates containing 5 per cent sheep blood, on the basis of the characteristic cultural and morphological behaviour of the organisms. Altogether 311 strains were collected.

Results

Organisms belonging to Micrococcus and Staphylococcus were chiefly found, therefore only members of these groups will be dealt with in the following. Of other Micrococcaceae, Sarcina was isolated in 2 cases (*Sarcina lutea*?) and Gaffkya in one case (*Gaffkya tetragena*?). The results of biochemical tests are presented in Table I.

Table I

Distribution of staphylococci and micrococci isolated from pathological materials
Total number of strains, 311

Nomenclature (Bergey's 7th ed.)	Mannitol	Pigment	Gelatin	$(NH_4)_2PO_4$	Nitrate	Urea	Litmus milk	No. of strains	Per cent of strains
Staph. aureus	+	golden yellow	+	—	+	—	acid + clot	207	65.9
Staph. epidermidis	—	white	+	—	+	—	acid + clot	26	8.6
M. ureae	d	white or none	—	+	—	+	alkali, no clot	49	16.0
M. varians	+	variable	—	+	+	—	acid + soft clot	7	2.2
M. caseolyticus	+	variable	+	+	—	—	clot, peptonization	5	1.7
M. flavus	d	yellow	+	+	—	—	acid + clot	5	1.7
M. candidus	d	white	—	—	—	—	no clot	5	1.7
M. freudenreichii	d	white	+	+	—	—	acid	2	0.6
M. conglomeratus	d	light yellow	+	+	+	—	no clot	1	0.3
M. luteus	—	yellow	—	+	—	—	soft clot	1	0.3
Undetermined								3	1.0

According to biochemical behaviour, of the 311 strains 233 belonged to the genus Staphylococcus. Of the latter 207 strains corresponded to *Staph. aureus*, 26 to *Staph. epidermidis*. The distribution of the 75 strains belonging to Micrococcus was as follows. *M. ureae*, 49; *M. varians*, 7; *M. caseolyticus*, 5; *M. flavus*, 5; *M. candidans*, 5; *M. freudenreichii*, 2; *M. luteus*, 1; *M. conglomeratus*, 1. The systematic position of 3 strains could not be determined.

The distribution according to origin of the Micrococcus strains is shown in Table II. Most micrococci were isolated from urine (*M. ureae*, 42; *M. flavus*, 4; *M. varians*, 4; *M. caseolyticus*, 2; *M. conglomeratus*, 1; *M. freudenreichii*, 1). From sputum, 3 *M. candidus*, 2 *M. ureae*, 1 *M. luteus* and 1 *M. varians* were cultured. From nose 2 *M. ureae*, 1 *M. caseolyticus*, from throat 1 *M. flavus* were isolated. In pus samples in addition to *Staph. aureus*, 1 *M. caseolyticus*, 1 *M. varians* and 1 *M. freudenreichii* occurred.

Table II

Distribution of strains according to origin

Origin of strains	*Staph. aureus*	*Staph. epidermidis*	*M. ureae*	*M. conglomeratus*	*M. caseolyticus*	*M. luteus*	*M. flavus*	*M. candidus*	*M. varians*	*M. freudenreichii*	Total
Nose	13	1	2	—	1	—	—	—	—	—	17
Throat	24	4	—	—	—	—	1	1	—	—	30
Sputum	17	3	2	—	—	1	—	3	1	—	27
Pus	73	1	—	—	1	—	—	—	1	1	77
Blood	14	—	—	—	—	—	—	—	—	—	14
Urine	26	15	42	1	2	—	4	—	4	1	95
Faeces	25	—	—	—	—	—	—	—	—	—	25
Other material	15	2	3	—	1	—	—	1	1	—	23
Total	207	26	49	1	5	1	5	5	7	2	308

Table III

Distribution of strains according to origin
Total number of strains, 75

Organism	Urine		Other material		Total
	mixed culture	pure culture	mixed culture	pure culture	
M. ureae	5	37	7	—	49
M. flavus	2	2	—	1	5
M. luteus	—	—	1	—	1
M. varians	2	2	3	—	7
M. freudenreichii	—	1	—	1	2
M. candidus	—	—	5	—	5
M. caseolyticus	—	2	3	—	5
M. conglomeratus	—	1	—	—	1

The frequent isolations of *M. ureae* from urine should be noted (Table III). The 95 samples of urine examined yielded 37 *M. ureae* in pure culture and 5 *M. ureae* in addition to other organisms. From 15 nasal, 30 throat and 27 sputum specimens only 1 *M. flavus* and 1 *M. freudenreichii* were isolated in pure culture, the remaining strains occurred with *Staph. aureus*, alpha and beta haemolytic streptococci or non-pathogenic neisseriae.

Although *M. ureae*, *M. flavus* and *M. luteus* are probably non-pathogenic, owing to their frequent occurrence they are of differential diagnostic importance. According to morphological and cultural properties, *M. ureae* is difficult

to distinguish from *Staph. epidermidis (albus)*. Differentiation should be based upon nitrate reduction and utilization of ammonia. Some *M. luteus* and *flavus* strains can be mistaken for *Staph. aureus*, as they produce haemolysis on sheep blood agar. Differentiation is, however, easy by the nitrate reduction and coagulase tests.

The results of the most important pathogenicity tests performed with our identified strains were as follows.

Decomposition of urea. Urease activity as a pathogenicity test was introduced by PÜSCHEL (cit. GRÜN [14]). This author showed that urea-decomposing strains are saprophytes. WOOHLFEIL and WOLLENBERG [15] found urease activity in 14.2 per cent of staphylococci. The presence of the enzyme was shown by RÖHMER and SCHMITZ [16] both in pathogenic and non-pathogenic strains. VOGEL [17] claimed that only coagulase negative saprophytic strains produce urease. In contrast to this opinion GRÜN [14] found 11 urea-decomposing cultures among 100 coagulase-positive strains.

Table IV

Urease activity of 308 strains

Organism	Urease-positive		Urease-negative		Total
	No.	Per cent	No.	Per cent	
Staph. aureus	6	3.0	201	97.0	207
Staph. epidermidis	3	11.4	23	88.6	26
M. ureae	49	100.0	—	0.0	49
M. varians	—	0.0	7	100.0	7
M. caseolyticus	2	40.0	3	60.0	5
M. flavus	—	0.0	5	100.0	5
M. candidus	—	0.0	5	100.0	5
M. luteus	—	0.0	1	100.0	1
M. freudenreichii	—	0.0	2	100.0	2
M. conglomeratus	1	100.0	—	0.0	1
Total	61	20.0	247	80.0	308

In the present investigations urease activity was infrequently observed with staphylococci and with most species of Micrococcus (Table IV). Urea was, however, decomposed by all *M. ureae* strains. Of 207 *Staph. aureus* strains only 6 (3.0 per cent), of 26 *Staph. epidermidis* only 3 exerted urease activity.

A positive urease reaction was given also by 2 *M. caseolyticus* and 1 *M. conglomeratus*. All the above-listed 12 urease-producing strains but two were isolated from urine. The urea-decomposing *Staph. aureus* strains gave positive coagulase, hyaluronidase and phosphatase reactions. It is evident

that these *Staph. aureus* strains cannot be regarded as non-pathogenic on the single basis of urease-positivity.

Lipase activity. GILLESPIE and ALDER [18] observed 63 per cent of their coagulase positive strains to show lipase activity. ALDER, GILLESPIE and HERDAN [19] demonstrated lipase positivity in 87 per cent of staphylococci. The value of the test as an indicator of pathogenicity has not been established. As the reaction is given only by part of the pathogenic staphylococci, lipase-positive and -negative strains may be distinguished.

In contrast to data obtained by the mentioned authors only 52 per cent of our *Staph. aureus* strains were lipase-positive. As to their origin, no connection was found between positive and negative cultures. Both types occurred with equal frequency among staphylococci isolated from boils, and among staphylococci isolated from faeces as commensal organisms. No association was revealed between lipase and other pathogenicity tests (Table V).

All members of *Staph. epidermidis* and of the genus Micrococcus were lipase-negative.

Table V

Lipase activity of 308 strains

Organism	Lipase-positive		Lipase-negative		Total
	No.	Per cent	No.	Per cent	
Staph. aureus	107	52.0	100	48.0	207
Staph. epidermidis	—	0.0	26	100.0	26
M. ureae	—	0.0	49	100.0	49
M. varians	—	0.0	7	100.0	7
M. caseolyticus	—	0.0	5	100.0	5
M. flavus	—	0.0	5	100.0	5
M. candidus	—	0.0	5	100.0	5
M. freudenreichii	—	0.0	2	100.0	2
M. luteus	—	0.0	1	100.0	1
M. conglomeratus	—	0.0	1	100.0	1

As coagulase-positive strains could be divided into two groups by means of the lipase test, the reaction was applied in epidemiological investigations. For example, the faecal flora of O. T., an infant 9 months of age was repeatedly examined at short intervals during his stay in hospital. From the infant's faeces *Staph. aureus* belonging to type "bc" (serological group 2) was cultured. As the characteristic nosocomial strain of the wards also belonged to that serotype, the problem arose how the appearance of the latter strain could be shown in the patient's faeces. The "bc" type strains isolated were lipase-

positive at the beginning; one week later, however, the patient harboured only lipase-negative Staphylococci. As the nosocomial strain had no lipase activity, it could be concluded that as in other cases it had appeared about one week after admission of the patient.

Splitting of mannitol. The question was investigated by JULIANELLE (cit, PÖHN [4]). Opinions differ as to the value of the reaction as a pathogenicity test. A differential medium devised by NYIREDY [20] for the characterization of pathogenic strains included an indicator of mannitol fermentation. LYONS [21] showed that penicillin-resistant strains exhibited a decreased mannitol-splitting capacity. Other authors [16, 22, 5] found a number of mannitol-positive strains among the coagulase-negative ones. These results have been confirmed by the present examinations.

The mannitol reaction of 308 strains was examined (Table VI). Of *Staph.*

Table VI

Comparison of the mannitol reaction with coagulase and hyaluronidase activity of 308 strains

Organism	No. of strains	Per cent of strains	Mannitol	Coagulase	Hyaluronidase	Total
Staph. aureus	167	80.9	+	+	+	207
	17	8.0	—	+	+	
	13	6.3	+	—	+	
	10	4.8	+	+	—	
Staph. epidermidis	17	65.0	—	—	—	26
	5	19.0	+	—	—	
	4	16.0	—	—	+	
M. ureae	47	96.0	—	—	—	49
	2	4.0	—	—	+	
M. varians	4	58.0	+	—	—	7
	1	14.0	+	—	+	
	2	28.0	—	—	—	
M. caseolyticus	1	20.0	+	—	—	5
	1	20.0	—	—	+	
	3	60.0	—	—	—	
M. flavus	1	20.0	+	—	+	5
	4	80.0	—	—	—	
M. candidus	5	100.0	—	—	—	5
M. freudenreichii	1	50.0	—	—	—	2
	1	50.0	—	—	+	
M. luteus	1	100	—	—	+	1
M. conglomeratus	1	100	—	—	—	1

aureus 80.9 per cent attacked mannitol. These strains were also coagulase- and hyaluronidase-positive. Of *Staph. epidermidis* cultures 19.0 per cent was mannitol-positive. These strains gave negative coagulase and hyaluronidase tests. Among micrococci 1 *M. caseolyticus*, 1 *M. flavus* and 2 *M. varians* strains attacked mannitol.

As seen in Table VI, a considerable percentage of *Staph. aureus* did not act on mannitol, while *Staph. epidermidis*, apathogenic organism, split this substance in 19.0 per cent. Taking into account that micrococci were rarely isolated, there was a considerable number of mannitol-positive strains.

Gelatinase activity. The proteolytic activity of staphylococci was first shown by ROSENBACH [23]. Some authors regarded this property as an indicator of pathogenicity [24—26]. SCHAACK [5] found no connection between the severity of the disease and the intensity of gelatin hydrolysis exhibited by the causative strain.

The results of our experiments are shown in Table VII.

Table VII

Gelatinase activity of 308 strains

Organism	Gelatinase-positive		Gelatinase-negative		Total
	No.	Per cent	No.	Per cent	
Staph. aureus	179	87.0	28	13.0	207
Staph. epidermidis	6	23.0	20	77.0	26
M. ureae	13	27.0	36	73.0	49
M. varians	—	0.0	7	100.0	7
M. caseolyticus	5	100.0	—	0.0	5
M. flavus	2	40.0	3	60.0	5
M. candidus	3	60.0	2	40.0	5
M. freudenreichii	1	50.0	1	50.0	2
M. luteus	—	0.0	1	100.0	1
M. conglomeratus	1	100.0	—	0.0	1

Of 207 *Staph. aureus* strains 28 produced no gelatinase. Of 26 *Staph. epidermidis* 6 were able to hydrolyse this substance. Among micrococci a considerable number of strains formed gelatinase, for example 27 per cent of the *M. ureae* strains. The most intensive liquefaction was caused by *M. caseolyticus*. Gelatin was hydrolyzed also by *M. candidus*, *M. conglomeratus*, *M. flavus* and *M. freudenreichii*. Accordingly, no difference in gelatin liquefaction was revealed between pathogenic and saprophytic strains. This finding is in accordance with the opinion of SCHAACK that the gelatinase reaction cannot be used for the characterization of the pathogenic property.

Haemolysis. Of the 207 *Staph. aureus* strains 2 were non-haemolytic, 7 produced a slight degree of haemolysis (Table VIII). Of the 26 *Staph. epider-*

Table VIII

Haemolytic activity of 308 strains on sheep blood agar plates

Organism	Haemolysis			Total
	+	±	−	
Staph. aureus	198	7	2	207
Staph. epidermidis	3	6	17	26
M. ureae	2	6	41	49
M. varians	1	—	6	7
M. caseolyticus	—	—	5	5
M. flavus	4	—	1	5
M. candidus	—	—	5	5
M. freudenreichii	1	—	1	2
M. luteus	1	—	—	1
M. conglomeratus	—	—	1	1
Total	210	19	79	308

midis strains 3 showed a strong, 6 a slight haemolytic activity. As to the behaviour of Micrococcus, no data can be found in BERGEY's mannual. NYIREDY [20] reported on the occurrence of haemolytic micrococci. The present experiments revealed that on sheep blood agar 4 out of 49 *M. ureae* strains produced haemolysis. In addition to these, 1 *M. luteus*, 1 *M. freudenreichii* and 1 *M. varians* were haemolytic. This finding is remarkable, since if this property is associated with the production of yellow pigment (*M. luteus*), the strain may be diagnosed as *Staph. aureus*. The data obtained are presented in Table VIII.

Coagulase, phosphatase and hyaluronidase. The coagulating capacity of staphylococci was first observed by MUCH in 1897. The connection between this property and pathogenicity was demonstrated by DARÁNYI [25]. To-day coagulase production is known as a phenomenon closely associated with pathogenicity [27—30].

The phosphatase reaction is one of the more recent tests. The phosphatase activity of staphylococci was recognized by BARBER *et al.* in 1951 [31], who showed its close parallelism to coagulase production. This was confirmed by a number of authors, while others obtained no uniform results [32, 33]. Among the strains of FÖLDES and JÁNOSIK [34] the number of phosphatase-producing strains exceeded that of the coagulase-positive strains.

According to SCHWABACHER *et al.* [35], hyaluronidase is produced in 90 per cent by pathogenic, while never by saprophytic strains. Among coagulase-negative cultures KAFFKA [3] found hyaluronidase-positives in 2.4 per cent.

In Table IX a comparison of the phosphatase, coagulase and hyaluron-

Table IX

Coagulase, phosphatase and hyaluronidase activity of 308 strains

Organism	No. of strains	Per cent of strains	Coagulase	Hyaluronidase	Phosphatase	Total
Staph. aureus	179	87.0	+	+	+	207
	13	6.3	—	+	+	
	10	4.8	+	—	+	
	5	1.9	+	+	—	
Staph. epidermidis	20	76.9	—	—	—	26
	2	7.7	—	—	+	
	2	7.7	—	+	—	
	2	7.7	—	+	+	
M. ureae	47	95.7	—	—	—	49
	2	4.3	—	+	—	
M. varians	1	14.0	—	+	—	7
	6	86.0	—	—	—	
M. caseolyticus	1	20.0	—	+	—	5
	4	80.0	—	—	—	
M. flavus	1	20.0	—	+	+	5
	2	40.0	—	—	+	
	2	40.0	—	—	—	
M. candidus	5	100.0	—	—	—	5
M. freudenreichii	1	50.0	—	—	+	2
	1	50.0	—	—	—	
M. luteus	1	100.0	—	+	+	1
M. conglomeratus	1	100.0	—	—	—	1

idase tests is presented. Of 207 *Staph. aureus* strains coagulase was produced by 194 phosphatase by 202. Of the coagulase- and phosphatase-positive strains 10 formed no hyaluronidase. Strains producing hyaluronidase but no coagulase or phosphatase were not found.

The presence of these enzymes was shown in 6 out of the 26 *Staph. epidermidis* strains. Two strains produced only hyaluronidase, 2 only phosphatase and 2 produced both.

Of 49 *M. ureae* 2 formed hyaluronidase. In addition to these, hyaluronidase was demonstrated in certain strains of *M. luteus*, *M. caseolyticus*, *M. flavus* and *M. varians* and phosphatase in *M. luteus*, *M. freudenreichii*, and *M. flavus*.

From the above data it is seen that coagulase is produced exclusively by *Staph. aureus*. This enzyme was not present in *Staph. epidermidis* and in Micrococcus, while hyaluronidase and phosphatase were formed by some strains of these organisms. The present examinations have thus confirmed the findings of THATCHER and SIMON. In addition to the known phosphatase activity of some micrococci, the production of hyaluronidase by these organisms has also been shown.

Discussion

Bacteria belonging to the genus Micrococcus have been isolated by us from all materials in which staphylococci are likely to occur. Most of the micrococcus strains were isolated from urine; among them, *M. ureae* was the commonest. This finding is notable, as the organism may be confused with *Staph. epidermidis (albus)*. *M. flavus* was isolated in pure culture from 1 throat and 4 urine specimens. In one sputum sample *M. luteus* occurred together with other bacteria. As 5 of the above-mentioned strains produced haemolysis, a simple morphological examination would have led to a diagnostic error. Thus for the proper identification of staphylococci the morphological examination by itself is not sufficient. Comparative investigations concerning the value of pathogenicity tests have so far been performed only with staphylococcal strains isolated from pathological and normal human materials. As shown by epidemiological studies, staphylococci are ubiquitous bacteria and pathogenic strains widely occur in healthy individuals. Therefore, instead of the comparison of strains of various origin, the examination of systematically defined Micrococcus, *Staph. epidermidis* and *Staph. aureus* strains was considered the best method for making a progress in elucidating the value of pathogenicity tests.

Pigment production and haemolytic activity are generally regarded as signs of pathogenicity (*Staph. aureus haemolyticus*). Pigment production, however, is dependent on a number of factors and haemolysis is a phenomenon produced by different enzymes. Apart from the variation in pigment production among staphylococci [6], several Micrococcus strains are able to form yellow pigment and give rise to haemolysis in sheep blood agar. Our examinations showed that the liquefaction of gelatin and the splitting of mannitol are not reliable pathogenicity tests, as both of them may be given by non-pathogenic strains as well as by *Staph. aureus*. Staphylococci decomposing ure cannot be regarded as saprophytic organisms, since with such strains the most important pathogenicity tests were positive. Most of the urease-producing

strains occurred in urine. The significance as a pathogenicity test of the lipase reaction cannot be clearly interpreted as yet. Among coagulase positive cultures isolated from pathological materials both lipase-producing and lipase-non-producing strains occurred. This enzyme was lacking in the coagulase-negative staphylococci and micrococci. The lipase reaction might be useful in epidemiological examinations. According to THATCHER and SIMON [1], there are phosphatase-producing strains also among micrococci. We have shown hyaluronidase production to occur in this group but coagulase production was observed only in *Staph. aureus*.

In analysing the origin of the 13 coagulase-negative *Staph. aureus* strains, it was found that all of these but 2 were isolated from blood cultures or from the organs of individuals who had died with septicaemia. The lack of coagulase activity in these strains may be attributed to an anti-coagulase produced in the patient's organism [36]. The importance of this finding lies in the fact that owing to the negative outcome of that most important pathogenicity tests, these strains may be falsely regarded as contaminating organisms. On the other hand, if this observation is not taken into account at evaluation of pathogenicity tests, the coagulase reaction would seem a less reliable diagnostic procedure. Accordingly, coagulase-negative *Staph. aureus* must be distinguished from saprophytic cocci originating from the patient's skin. If a coagulase-negative strain isolated from blood proves positive in the other tests presumably associated with pathogenicity (hyaluronidase and phosphatase), it should be regarded as probably pathogenic.

In our cases the pathogenicity of coagulase-negative *Staph. aureus* strains from blood cultures or organs from patients with septicaemia has been clearly established, as they were hyaluronidase- and phosphatase-positive, produced haemolysis and split mannitol.

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STUDIES ON THE CLASSIFICATION, PATHOGENICITY AND ANTIGENIC STRUCTURE OF STAPHYLOCOCCI

II. SEROLOGICAL TYPING OF STAPHYLOCOCCUS AUREUS*

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Summary. (i) Serological typing of 192 *Staphylococcus aureus* strains isolated from pyogenic infections has been performed by means of OEDING's method.

(ii) Strains belonging to serological group 4 were most frequently encountered.

(iii) Within the serological groups 42 types, including 22 new serotypes were distinguished.

(iv) Group 4 strains were isolated mostly from boils and phlegmons.

(v) No association was found between serotypes and the results of pathogenicity tests or antibiotic sensitivity.

(vi) The importance in epidemiological investigations of serological typing, in addition to phage typing and antibiotic sensitivity testing, is discussed.

The serological behaviour of staphylococci was first investigated in 1904 by KOLLE and OTTO [1], who divided the strains into two groups and concluded that pathogenic and saprophytic strains could be differentiated. For a similar purpose, JULIANELLE and WIEGHARD [2] elaborated a precipitation technique.

Type determination was first carried out by JULIANELLE [3], who distinguished three types by agglutination. By means of absorbed sera HINE [4] was able to divide pathogenic strains into 3, non-pathogenic strains into 2 groups. In 1936 YONEMURA [5] examined 324 strains and established 9 serological groups. Unfortunately, this author gave no description of the method employed. HEGEMAN [6] divided staphylococci on the basis of different agglutination titres into 5 groups. COWAN [7] demonstrated 3 groups (COWAN's groups I, II and III) by the use of absorbed sera. Taking into consideration these results, OEDING [8] finally succeeded in the elucidation of the antigenic structure of staphylococci and the serological typing of these organisms.

According to OEDING, staphylococci contain 9 different antigens. Antigen "d" is common in every staphylococcus, so it plays no part in type determination. This antigen is a polysaccharide-like, probably thermolabile substance. Antigens "a", "b" and "c" contain both thermostable and thermolabile components. The first two are polysaccharide-like substances, the third one is

* A preliminary report of these studies was presented at the Meeting of the Hungarian Association of Microbiologists, October 31, 1957, Budapest.

protein-like. Antigens "e" and "f" are thermolabile proteins, "h" is a thermostable protein, "i" and "k" are thermostable polysaccharides. The thermolabile antigens are destroyed at 100° C within 2½ hours.

Staphylococcal types are characterized by combinations of the various antigens. OEDING regarded antigens "abce" and their combinations as group, antigens "fhik" as type determinants. By the examination of 459 strains 13 serotypes were distinguished. The most frequently occurring "ab", "abc", "abe" and "abce" combinations constituted 80 per cent of the examined strains. Strains isolated from mastitis always contained factor "e" and belonged to group "abe" or "ae". GRÜN [9] divided 1034 strains into 11 groups. The most frequently occurring group was "abce" (20.6 per cent). The percentage distribution of groups "abc", "ab", and "b" was 16.0, 16.0 and 15.4, respectively. Other groups occurred in 8.6 to 0.6 per cent.

Differentiation of staphylococci may be performed by phage typing, serological typing and by the determination of antibiograms. Comparative trials between the first two important methods revealed no connection between phage and serological groups and types [10, 11]. This calls for a revision of the grouping of serological types, in other words, factors "abce" should not to be regarded as group antigens.

On the basis of phage and serological typing of 223 strains, OEDING and WILLIAMS [12] established 4 serological groups, which were characterized by the presence or absence of antigens "e", "i" and "k":

Group 1 : Antigen "e" is present.

Group 2 : Antigens "e", "i" and "k" are not present.

Group 3 : Antigen "i" is present.

Group 4 : Antigen "k" is present.

In the earlier classification factors "i" and "k" were type antigens. As in the new schema these factors correspond to group-determinants, the two typing methods are difficult to compare.

With factors "a", "b", "c" and "h" the new groups are divided into types. Groups 1, 2, 3 and 4 contain 9, 8, 8 and 14 serotypes, respectively. It is interesting that factor "f" is not included. In the following it will be seen that this antigen makes it possible to recognize new serotypes.

The grouping of OEDING and WILLIAMS assures a fairly good correlation between phage and serological types. Its practical value, however, will have to be established by epidemiological investigations.

In view of the increasing importance of staphylococcal infections, after performing the pathogenicity tests [13] we continued the investigations with the serological typing of *Staph. aureus*. The purpose of this work was to assess the association between various staphylococcal diseases and the antigenic structure of the causative agent, as well as to establish the value of serological typing in epidemiological examinations.

Materials and methods

Strains. A total of 192 *Staph. aureus* strains was isolated from patients treated at the Department of Paediatrics in 1958. The material included samples from suppurative processes as boils, abscesses, phlegmons, otitis, osteomyelitis, etc. and blood from septicaemia.

Pathogenicity of the strains was estimated by testing mannitol fermentation, haemolytic activity, and phosphatase, coagulase and gelatinase production. The methods have been described in the previous paper [13].

Antibiotic sensitivity was tested on agar plates by the paper disc method against penicillin, streptomycin, chloromycetin, terramycin, aureomycin, neomycin and erythromycin. The paper discs were impregnated with aqueous solutions containing 1 mg/ml of the antibiotics.

Agglutination. The test strains were kindly supplied by DR. P. OEDING (Institute "Gade", Department of Microbiology, Bergen). From the 18 hour cultures formolized suspensions containing 5×10^9 cells per ml were prepared. Before use the formalin was removed by two subsequent washings. Rabbits were injected in 3 series intravenously on three consecutive days with 0.1, 0.2, 0.4 ml, then after a 5 days interval also on three consecutive days with 0.4, 0.5, 0.8 ml and finally after another interval of 5 days with daily doses of 0.8, 1.0 and 1.0 ml of suspension. Blood samples were taken 3 to 5 days after the last injection and, provided a good titre had been obtained, the animals were bled. The end titre of the sera was determined by tube agglutination.

Absorption. The sera were diluted 1 to 160. To 8 ml of diluted serum bacteria harvested by centrifugation from 5 l of a 24 hour broth culture were added. Before centrifugation the bacteria were killed with 0.3 per cent formalin. Absorption was performed at 37° C for 3 hours then at refrigerator temperature overnight. Before use the absorbed serum was controlled with each of the standard strains. Agglutination was carried out by the slide method. The result was read by means of a hand lens after the slide had been tilted back and forth for some minutes.

Results

The agglutination titres are presented in Table I. The high titre given by living suspensions suggested that a considerable part of the antigens was thermolabile. Formalin exhibited no deleterious effect on the antigens.

Table II presents the result of slide agglutination with the test strains. It is seen that the cultures agglutinated well in the corresponding absorbed

Table I

Titres of unabsorbed Staph. aureus sera

Sera	Agglutination titre	
	living culture	culture autoclaved for $2^1/_2$ hours
3647	1 : 5120	1 : 1280
2095	1 : 10240	1 : 640
2253	1 : 160	1 : 80
B 28	1 : 10240	1 : 2560
17 A	1 : 5120	1 : 1280
F 21	1 : 10240	1 : 640
3189	1 : 5120	1 : 1280
S 365	1 : 10240	1 : 1280

sera. Serological determination of freshly isolated strains was performed similarly.

Table II

Agglutination of Staph. aureus test strains in absorbed sera

Absorbed sera	Designation and antigenic structure of strains							
	3647 abc	2095 abc	3189 bf	2253 ace	F 21 abci	17 A ah	S 365 abk	1503 ae
ac	+++	++	—	++	++	+	+++	+
a	+++	++	—	++	+	±	++	+
b	++	++	+++	—	+++	—	++	—
c	+++	+++	—	+	+	—	—	—
e	—	—	—	+++	—	—	—	++
f	—	—	+++	—	—	—	—	—
h	—	—	—	—	—	+++	—	—
i	—	—	—	—	+++	—	—	—
k	—	—	—	—	—	—	+++	—

The antigenic structure of 6 (3.0 per cent) of the 192 freshly isolated *Staph. aureus* strains could not be determined. The identified 186 strains were divided by group antigens "e", "i" and "k" in OEDING's four serological groups (Table III).

Group 1, 2 and 3 strains occurred in 15.4, 18.2 and 22.8 per cent, respectively. The highest number of strains (40.6 per cent) fell into group 4. Within the four groups altogether 42 serotypes were distinguished.

The more frequently occurring serotypes were "abhik" (19.2 per cent), "abhi" (14.0 per cent) and "abh" (8.3 per cent). Others were isolated in 1 to 7 cases and comprised 53.5 per cent of the total. It should be noted that not all of OEDING and WILLIAMS' serotypes were encountered. Among the freshly isolated strains 22 new serotypes were recognized as follows.

Group 1 : abefhi, aehi, aei, aef, bcehi
Group 2 : af, f
Group 3 : afi, ahi, abfi, fi
Group 4 : ahik, abhik, abfik, abcfhik, abchik, abefik, aeik, aefik, bhik, bceik, cfk.

Most of these serotypes were characterized by the use of antigen "f".

The occurrence of antigen "f" and its significance in type determination has so far not been described. In the present material 25 strains (13.0 per cent) belonging to 12 new serotypes contained this factor. New types with this antigen fell chiefly into group 4 (5 serotypes). In groups 2, 1 and 3 the number of "f" containing serotypes was 3, 2 and 2, respectively.

Table III

Incidence of serological groups and types

Serological group								In-agglutinable
1		2		3		4		
Type	No. of strains	Type	No. of strains	Type	No. of strains	Type	No. of strains	
abcehi	3	af	1	afi	3	ahk	1	6
abefhi	9	a	6	ai	6	aik	6	
abe	5	ah	2	ahi	2	ahik	4	
aehi	2			abhi	27	ak	1	
aei	2	ab	7	abfi	1	abhik	36	
ae	6	abh	16	abchi	1	abhk	4	
aef	1	b	2	bi	1	abik	7	
bcehi	1	f	1	fi	3	abfik	2	
						abcfhik	1	
						abchik	1	
						abefik	1	
						abehik	5	
						abehk	1	
						abeik	1	
						aeik	2	
						aefik	1	
						bhik	2	
						bceik	1	
						cfk	1	
	29 (15.4%)		35 (18.2%)		44 (22.8%)		78 (40.6%)	6 (3%)

Strains containing antigen "f" alone or antigens "fi" were encountered in 4 cases.

Table IV presents the distribution of serotypes according to origin.

It is to be noted that more than half of the cultures (53.7 per cent) isolated from phlegmons and boils fell into group 4. Other strains representing groups 1, 2 and 3 originated approximately evenly from the various pathological processes.

In OEDING's material [8], strains containing antigen "e" were most frequently recovered from mastitis. In the present investigations 11 out of 55 boils, 10 out of 56 abscesses and 5 out of 9 cases of cystitis yielded strains with antigen "e". Accordingly, these strains are common not only in mastitis, but also in other pyogenic infections. Considering certain observations

Table IV

Distribution of serological groups according to pathological conditions

Process	Serological group				Total
	1	2	3	4	
Phlegmons	1	1	2	10	14
Boils	9	9	10	27	55
Abscesses	8	12	11	14	45
Septicaemia	2	3	3	4	12
Otitis	1	2	3	4	10
Impetigo	1	2	1	3	7
Osteomyelitis	2	1	4	1	8
Clinical influenza	1	1	1	2	5
Cystitis	4	2	1	2	9
Other	—	5	5	11	21
Total	29	38	41	78	186

in our opinion the reported frequency of strains containing antigen "e" in mastitis may be attributed to an epidemic at the given hospital and not to the specific role of such serotypes in that disease.

No connection was found between antigenic structure and the coagulase, phosphatase, gelatinase and mannitol reactions. Three strains produced no haemolysis on sheep blood agar. These strains belonged to serotypes containing only antigens "fi" and "f".

Antibiotic sensitivity testing revealed that the strains were resistant to penicillin in 68.0, to chloromycetin in 9.2, to streptomycin in 8.0, to aureomycin and terramycin in 0.5 per cent. No strains resistant to erythromycin or neomycin occurred. Among the penicillin-resistant cultures the incidence of group 4 was the highest (82.8 per cent). The probable explanation of this finding is that the most frequently occurring staphylococci belong to this group.

Discussion

The increasing clinical problem of staphylococcal infections makes it imperative to extend investigations into the nature of the causative agent. In addition to problems regarding the factors influencing pathogenicity, the mechanism of infection and antibiotic resistance, the epidemiological questions with special respect to nosocomial infections are of particular importance. Epidemiological studies have been carried out so far only by means of phage typing and antibiotic-sensitivity patterns. Now OEDING's method of serological group and type determination can also be made use of in epidemiological

studies. In such work grouping of the strains into 4 groups offers little information. Now, however, following up the spread of infections has been made possible by the fact that within the four groups as many as 42 serotypes can be distinguished.

The present studies revealed the strains belonging to group 4 to predominate in phlegmons and boils, while in other conditions the serological types were distributed approximately evenly. There is little probability of a given serotype being responsible for a given disease. The high incidence of group 4 and of OEDING's serotype characterized by antigen "e" is probably due to epidemiological circumstances and not to the specific affinity of these strains to certain diseases. Our results have been compared with those of GRÜN [9] obtained on 97 strains and with the data of OEDING and WILLIAMS [12]. It is interesting that, similarly to our results, among GRÜN's strains, too, group 4 was predominating and the members of group 1 were found in the smallest number. In contrast to this, of OEDING and WILLIAMS' strains 74 belonged to group 1 and only 31 to group 4.

Five pathogenicity tests (coagulase, phosphatase, gelatinase production, haemolytic activity, and splitting of mannitol) showed no connection whatever with the serological behaviour of staphylococci.

Similarly, no association was found between antibiotic sensitivity and serotypes. The high number of penicillin-resistant strains in group 4 may be attributed to the frequent occurrence of this group. In epidemiological observations serological typing may be completed by antibiotic-sensitivity testing, as strains belonging to the same serotype may be distinguished by antibiotic sensitivity patterns.

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STUDIES ON THE CLASSIFICATION, PATHOGENICITY AND ANTIGENIC STRUCTURE OF STAPHYLOCOCCI

III. EXAMINATION OF STAPHYLOCOCCI ISOLATED FROM FAECES*

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Summary. (i) From 539 faecal samples 174 *Staphylococcus aureus*, 104 *Staphylococcus epidermidis* and 12 *Micrococcus* strains were isolated.

(ii) According to pathogenicity tests, faecal *Staph. aureus* strains corresponded to strains isolated from pyogenic infections.

(iii) Antibiotic-resistant staphylococci occurred considerably more frequently among faecal strains than among strains isolated from pyogenic infections.

(iv) Within *Staph. aureus* and *Staph. epidermidis* 51 and 25 serotypes were distinguished, respectively.

(v) It has been concluded that in cases of enteritis the simple isolation of *Staph. aureus* from faeces cannot be regarded as a proof of the aetiological role of this organism.

It is known that human faecal samples often contain staphylococci. From the faeces of children under 3 years of age these organisms were isolated in 26 per cent, from older children in 4 to 5 per cent [1, 2], while BUTTIAUX and PIERRET [3] found to occur in faeces in 9 per cent. According to MARTYN [4], there is no difference in this respect between normal infants and infants suffering from enteritis, as in the former group he obtained positive results in 46 per cent, in the latter in 45.5 per cent.

GRÜN [5] isolated staphylococci from 31 per cent of the faeces of adults without hospital connections. On examining 1173 children under and over two years of age, PRISSICK [6] isolated staphylococci in 45.8 and 28.2 per cent, respectively.

Serological typing of faecal staphylococci was first performed by OEDING and AUSTARHEIM [7]; in their material strains characterized by antigens "abc" were the most frequent. GRÜN [5] could divide 100 strains into 9 serological groups by OEDING's antigens "abce". He found no characteristic faecal serotypes; most strains contained antigens "abc" and "b". The high number (23 per cent) of inagglutinable strains was remarkable.

The present work was undertaken to study the role of staphylococci in the aetiology of infantile enteritis. So far faecal strains have been examined only for pigment production, haemolysis and coagulase activity. Therefore,

*A preliminary report of these studies was presented at the 2nd Congress of the Hungarian Association of Microbiologists, September 24, 1959, Budapest.

in addition to the systematic determination of morphologically characteristic cocci, the analysis of the results of pathogenicity tests, serological typing and antibiotic sensitivity testing was considered necessary.

Materials and methods

Strains. In February and March, 1958 and 1959, 539 faecal samples were examined from 431 infants admitted with enteritis to the Department of Paediatrics. The samples were streaked on plates of Endo and desoxycholate citrate media and on phenolphthalein phosphate agar containing 8 per cent of sodium chloride. Altogether 291 strains were isolated from the latter medium.

Biochemical tests (nitrate reduction, utilization of ammonia and urea, fermentation of mannitol, reaction in litmus milk, pigment, phosphatase, hyaluronidase and coagulase production) were carried out as indicated previously [8].

Antibiotic sensitivity was tested by the paper disc method. The antibiotic content of the individual discs was penicillin, 30 units; of streptomycin, chloromycetin, aureomycin, terramycin, erythromycin and kanamycin, 30 μg; of neomycin, 100 μg.

Serological typing was performed by the use of absorbed sera prepared with OEDING's test strains. Both living and autoclaved bacteria were examined by slide agglutination. The serological groups and types were determined as described previously [9], according to OEDING and WILLIAMS' antigenic schema [10].

Results

The systematic classification of strains was made according to BERGEY [11]. Morphologically and culturally characteristic strains (Gram-positive cocci arranged in clusters) were identified with biochemical reactions (nitrate reduction, utilization of ammonia and urea, mannitol fermentation, gelatin liquefaction and reaction in litmus milk). Of the 291 strains isolated 278 belonged to the genus Staphylococcus, 12 to Micrococcus. The systematic position of 1 strain could not be determined (Table I).

Table I

Distribution of Staphylococcus and Micrococcus strains isolated from faeces

Nomenclature (Bergey)	No. of strains	Per cent of strains
Staph. aureus	174	58.4
Staph. epidermidis	104	36.0
M. varians	5	1.7
M. conglomeratus	5	1.7
M. ureae	4	1.4
M. candidus	1	0.4
Undetermined	1	0.4
Total	291	100.0

Staph. aureus was isolated in 174 cases (58.4 per cent), *Staph. epidermidis* in 104 cases (36.0 per cent). The distribution of the 12 Micrococcus strains was *M. varians* 5, *M. ureae* 4, *M. conglomeratus* 2, *M. candidus* 1. In the faecal samples staphylococci and micrococci occurred in addition to other bacteria, as *E. coli*, Klebsiella, Proteus, etc.

The potential pathogenicity of the strains was estimated by the mannitol, hyaluronidase, coagulase and phosphatase tests. As shown in our previous paper [8], the coagulase reaction is the most reliable indicator of potential pathogenicity. Of the 174 *Staph. aureus* strains only 1 was coagulase-negative. This organism was not pathogenic, as in the other pathogenicity tests it yielded equally negative results. Of the 173 coagulase-positive strains 141 (81.0 per cent) were also mannitol-, hyaluronidase- and phosphatase-positive. Of the four pathogenicity tests only 3 was given by 18 strains (10.4 per cent) and only 2 by 14 strains (8.1 per cent). Among coagulase-positive cultures 11 mannitol-negative and 7 phosphatase-negative strains occurred (Table II).

Table II

Pathogenicity tests with staphylococci isolated from faeces

Species	No. of strains	Per cent of strains	Mannitol	Hyaluron-idase	Phosphatase	Coagulase
Staph. aureus	141	81.0	+	+	+	+
	11	6.3	—	+	+	+
	7	4.1	+	+	—	+
	1	0.5	—	—	—	—
	14	8.1	—	—	+	+
Total	174	100.0				
Staph. epidermidis	74	71.1	—	—	—	—
	7	6.7	+	+	+	—
	8	7.7	+	—	—	—
	8	7.7	+	—	+	—
	7	6.8	—	—	+	—
Total	104	100.0				

Of 104 white pigment-producing strains 7 were mannitol-, hyaluronidase- and phosphatase-positive. Eight strains were mannitol- and phosphatase-positive, 8 were only mannitol- and 7 only phosphatase-positive.

Some Micrococcus strains also gave positive pathogenicity tests. Of 5 *M. varians* 2 exercised hyaluronidase and 3 phosphatase activity. Both *M. conglomeratus* strains were phosphatase-positive. *M. ureae* and *M. candidus* gave

negative results in all these tests. In accordance with our previous observation [8], no coagulase-positive strains were found in the genus Micrococcus (Table III).

Table III

Pathogenicity tests with micrococci isolated from faeces

Species	No. of strains	Mannitol	Hyaluron-idase	Phosphatase	Coagulase
M. varians	2	+	+	+	—
	2	+	—	—	—
	1	—	—	—	—
M. conglomeratus	2	—	—	+	—
M. ureae	4	—	—	—	—
M. candidus	1	—	—	—	—

The antibiotic sensitivity of *Staph. aureus* strains was as follows. The strains were resistant to penicillin in 80.8, to streptomycin in 74.3, to chloromycetin in 53.4, to aureomycin in 61.9, to terramycin in 68.9, to erythromycin in 33.8, to neomycin in 1.1 per cent. Accordingly, the number of non-susceptible strains was very high. The frequent occurrence of erythromycin-resistant strains is of special interest. Strains resistant to kanamycin were not met with. *Staph. epidermidis* was resistant to penicillin in 76.9, to streptomycin in 50.1, to chloromycetin in 31.7, to aureomycin in 41.3, to terramycin in 47.1, to erythromycin in 17.3, to neomycin in 1.0 per cent. No kanamycin-resistant *Staph. epidermidis* was found (Table IV).

Table IV

Antibiotic sensitivity of staphyloccocci isolated from faeces

Antibiotics	Resistant *Staph. aureus* strains		Resistant *Staph. epidermidis* strains	
	no.	per cent	no.	per cent
Penicillin	156	88.8	80	76.9
Streptomycin	131	74.3	52	50.1
Chloromycetin	93	53.4	33	31.7
Aureomycin	108	61.9	43	41.3
Terramycin	120	68.9	49	47.1
Erythromycin	59	33.8	18	17.3
Neomycin	2	1.1	1	1.0
Kanamycin	0	0	0	0

The antigenic structure of 73.0 per cent of *Staph. aureus* strains could be determined. Within the groups based on OEDING and WILLIAMS' group antigens ("e", "i" and "k"), altogether 51 serotypes occurred (Table V). Of the strains 27.5 per cent belonged to group 2, 20.6 per cent to group 3 16.0 per cent to group 4 and 8.9 per cent to group 1. Predomination of a certain serotype was

Table V

Serological group and type distribution of Staph. aureus isolated from faeces

Serological group								In-agglu-tinable
1		2		3		4		
Type	No.	Type	No.	Type	No.	Type	No.	
abcefi	3	abc	3	abchi	7	abcfhik	1	47
abceh	2	abch	3	abcfhi	1	abch	1	
abce	1	b	2	bchi	3	abchk	1	
aehi	2	bf	2	bci	1	abcehik	3	
aef	1	bh	1	abi	3	abcefhk	1	
eh	2	abfh	2	abhi	3	k	2	
ace	2	ab	1	chi	4	fk	1	
abefh	1	abh	3	ci	3	ik	3	
abei	1	bc	1	ai	10	acehik	1	
		bch	1	aci	1	bchik	1	
		c	1			ahik	2	
		cfh	1			abehi(k)	4	
		af	2			abehk	4	
		a	8			abefhik	1	
		ah	7			acfik	2	
		ac	5					
		ach	5					
Total	15 (8.9%)		48 (27.5%)		36 (20.6%)		28 (16.0%)	47 (27.0%)

not observed, the various serotypes occurred with an incidence of between 0.5 and 4.6 per cent. Of the strains 27.0 per cent could not be grouped. As to group and type distribution, there was a difference between faecal strains and staphylococci isolated from pyogenic infections; namely, among faecal strains group 2, among the others group 4, were the most frequent. Among pyogenic strains types "abhik" and "abhi" were especially common (27.2 per cent). None among the faecal strains predominated.

The determination of the antigenic structure of faecal strains involved some difficulty, as agglutination was usually slow and typing was often possible

only with autoclaved bacteria. Living strains induced weak or no agglutination. Some examples are presented in Table VI. The phenomenon is characteristic of faecal strains, as with staphylococci of pyogenic origin it was observed but exceptionally.

The serological typing of *Staph. epidermidis* was also attempted. As regards the antigenic structure of this organism, few data are found in the literature. The typing of *Staph. epidermidis* was successful in 56.5 per cent (Table VII).

Table VII shows that part of the *Staph. epidermidis* strains could be included in the four *Staph. aureus* serological groups. Of the strains 33.6 per cent belonged to group 4, 15.3 to group 2, 6.7 to group 3 and 0.9 to group 1. The

Table VI

Agglutinability of strains

Designation of strains	Determinable antigens	
	Before autoclaving	After autoclaving
985	a	a b c h
991	inagglutinable	a c h
1002	inagglutinable	a
1005	inagglutinable	a h
1006	(a)	a h i k
1032	(i)	a b h i
1101	inagglutinable	a h
1207/b	inagglutinable	a b c
1103	inagglutinable	k
1119	inagglutinable	a

high incidence of strains containing only antigen "k" was remarkable (27.0 per cent). Inagglutinable strains occurred in 43.4 per cent.

GRÜN and KÜHN [13] examined 150 white pigment-producing strains which had been isolated from pyogenic infections and from throat and nasal samples. Of the strains from suppurative conditions 13 per cent, from throat and nasal samples 21.8 per cent were inagglutinable. The most frequently occurring serotypes were „abce", "abc", "ab" and "b". These examinations were not extended to the entire antigenic structure, thus to antigens "i" and "k". Accordingly, no comparison corresponding to OEDING and WILLIAMS' grouping can be made between the strains of GRÜN and KÜHN and the faecal staphylococci in our material. The occurrence of inagglutinable strains among faecal *Staph. epidermidis*, as well as among faecal *Staph. aureus* cultures, seems to be common.

Table VII

Serological group and type distribution of Staph. epidermidis isolated from faeces

Serological group								In-agglu-tinable
1		2		3		4		
Type	No.	Type	No.	Type	No.	Type	No.	
aef	1	bc	3	ai	2	abcefik	3	45
		bchi	1	abchi	1	abcfhik	1	
		ah	3	abhi	1	abik	1	
		a	2	abfi	1	afhik	1	
		abch	1	ci	1	ahk	1	
		c	2	hi	1	ak	2	
		cfh	1			cfik	1	
		abfh	1			k	25	
		ach	1					
		c	1					
Total	1 (0.9%)		16 (15.3%)		7 (6.7%)		35 (33.6%)	45 (43.4%)

Discussion

The points of the present study to be answered were

(1) the occurrence of micrococci in faecal samples and the properties of these organisms;

(2) the pathogenic role of staphylococci and other strains of Micrococci as estimated by the pathogenicity tests;

(3) antibiotic sensitivity;

(4) serological behaviour of faecal staphylococci; comparison of the distribution of serotypes among faecal strains and among strains isolated from pyogenic infections;

(5) the incidence of staphylococci in faeces as compared to that of known enteric pathogens.

ad (1) On the basis of morphological and biochemical behaviour, of the isolated strains 4.4 per cent belonged to the genus Micrococcus. These strains were members of four different species (*M. varians*, *M. conglomeratus*, *M. ureae* and *M. candidus*); the systematic position of one strain was not determined. It has been shown [8] that micrococci occur in sputum; accordingly, they may easily gain access to the intestinal tract. Some of the strains were hyaluronidase- and phosphatase-positive but neither of them produced coagulase. This finding is in accordance with our earlier observations [8]. In antibiotic sensitivity these strains did not differ from staphylococci.

ad (2) Of the pathogenicity tests, the phosphatase, hyaluronidase and mannitol reactions gave sometimes divergent results, while the coagulase test always yielded uniform ones, confirming the reliability of this reaction. According to this test, faecal staphylococci possess a potential pathogenicity, similarly to staphylococci isolated from pyogenic infections. Among white staphylococci certain strains gave positive pathogenicity tests; the coagulase test with these strains, however, was always negative.

ad (3) A considerable number of the faecal staphylococci were resistant to antibiotics, especially to the broad spectrum ones usually administered by mouth. At the same time, of 100 strains isolated from boils 32.0 per cent were resistant to penicillin, 15.0 to streptomycin, 19.0 to chloromycetin, and 8.0 per cent to aureomycin and terramycin. A comparison of the results clearly shows that the faecal strains were more frequently resistant to the examined antibiotics.

ad (4) Part of the faecal staphylococci could be included in OEDING and WILLIAMS' serological groups. Altogether 51 serotypes were distinguished. Unlike among strains from pyogenic infections, among faecal strains no predominating serogroups or serotypes occurred. The majority of the strains isolated from pyogenic conditions belonged to groups 4 [9]. The large number of serotypes among faecal strains confirms that none of the serotypes can be regarded as characteristic of the faecal flora. From the large variety of serotypes it may be concluded that the appearance of staphylococci in the faeces depends on the environmental occurrence of these organisms.

Partial antigens characteristic of *Staph. aureus* were also found in *Staph. epidermidis*. This finding shows the existence of a close relationship between the two species. *Staph. epidermidis* cultures were more frequently inagglutinable than *Staph. aureus*, and often contained only one or two of the known components. *Staph. epidermidis* strains containing only antigen "k" occurred in a considerable number (24.0 per cent).

ad (5) *Staph. aureus* was recovered from 174 (32.3 per cent) out of the 539 faecal samples examined. Known enteric pathogens were isolated from 79 samples (14.0 per cent). Of the latter organisms 78 corresponded to *E. coli* O111 : B4 serotypes associated with infantile enteritis; the remaining 1 strain was *Sh. flexneri*. In 44 out of the latter 79 specimens *Staph. aureus* occurred simultaneously with the above enteric pathogens (55.6 per cent) (43 *E. coli* O111 : B4 and 1 *Sh. flexneri*). From the remaining 35 samples (44.4 per cent) yielding enteric pathogens no staphylococci were cultured.

From these findings it may be concluded that the simple isolation of *Staph. aureus* from faeces in cases of enteritis cannot be regarded as a proof of its aetiological role in that disease.

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VARIANTS OF SH. SONNEI PHASE I

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Summary. By studying colony morphology in oblique light under gradually changing angles of incidence, six colony forms of *Sh. sonnei* cultures have been differentiated. Each of them has been found to contain phase I antigen. Investigation of these variants revealed essential differences in virulence, thus making it possible to select cultures of adequate virulence. One variant containing exclusively, or almost exclusively, phase I antigen proved to be avirulent, while the most virulent was a variant forming colonies with both phase I and phase II antigens. Accordingly, for the virulence of *Sh. sonnei* cultures not alone the phase I antigen should be made responsible.

Our methods for determining the fine structure of bacterial colonies [27, 28, 29] have been applied for studying some uncommon *Sh. sonnei* colony forms. The present report deals with the special features of colonies containing phase I antigen.

Materials and methods

Isolation of variants. The majority of the variants had been obtained from *Sh. sonnei* cultures stored on DORSET's egg medium at 3° to 10° C, or on DC agar at 37° C, by transfer on agar plates containing yeast extract. Incubation of dense suspensions of star-shaped colonies on brilliant green agar for 24 hours at 37° C was also used for the selection of variants. Upon discovery of a new colony pattern, appearing as a pin-point structure, a series of subcultures was made on C agar plates containing yeast extract [28a] until a pure or almost pure culture had resulted. Some variants failed to produce even approximately pure cultures owing to extreme dissociation; these will not be discussed in the present paper.

The purified variants were investigated for morphological, biochemical, and serological properties, toxicity and virulence. Virulence was estimated by intraabdominal infection of mice and conjunctival infection of guinea pigs.

Cell morphology. Gram-stained preparations derived from various types of colony showed some deviations which, however, have not been found characteristic.

Culture media. In addition to media prepared with yeast extract (C agar) and desoxycholic acid (D agar) [28, 28a], some usual media such as desoxycholic acid-sodium citrate agar, eosin methylene blue agar, were used.

Colony morphology was studied in oblique light under varying angles of incidence [27, 28]; C agar was found to be the medium of choice for this procedure. On horse broth or beef extract media the fine structure was less discernible.

While imprints of the star-shaped and moderately refractive colonies were characteristic [28 a], the formations left behind after removal of colonies composed of other variants could not be classified into well-defined groups.

No noteworthy differences were discovered in the *biochemical activity* of the various colony variants.

Serological investigations. Antigen structure was determined by slide agglutination with exhausted sera, Widal's test, and cross exhaustion. With some variants (2a, 3, 4, 5 and 6) phase II antigen could not be demonstrated beside phase I antigen, either by slide, or tube,

agglutination, whereas immunization of rabbits with the same variants yielded sera containing both phase I and phase II agglutinins. In such cases examination of the cultures in oblique light revealed in apparently pure cultures the sporadic presence of colonies corresponding to pure phase II variants, in structure and antigen content alike.

Tests for virulence and toxicity. For this purpose regularly-increasing quantities of bacteria, dried in a vacuum exsiccator in a refrigerator have been employed. Before performing animal experiments, the viable organism count in the dried preparation was determined. When the original variant was but slightly "contaminated" by phase II colonies, the circumstance was considered as negligible. Thus ID_{50} and LD_{50} were determined with regard to the number of phase I organisms. Animal tests were taken into account only if they proved to be reproducible.

For mouse tests albino mice weighing 17 to 19 g each from our Institute's own breed were used. Twenty animals were inoculated intraabdominally with each dose, in a volume of 0.5 ml. The mice were kept under observation for three days. In tests for virulence, bacterial suspensions were mixed at the ratio of 1: 5 in 5 per cent mucin; in tests for toxicity, suspensions exposed for an hour to streaming vapour were used. Virulence was tested also by conjunctival infection of guinea pigs with a loopful of a one-day culture, examining the capacity of the colony under investigation to cause keratoconjunctivitis shigellosa. In case of a positive finding, groups of six guinea pigs were infected, each animal in both eyes, with rising doses of the dried preparation suspended in 0.01 ml of broth.

Designation of variants on the basis of colony morphology was made by designating with figures each of the characteristic forms. In the interest of a uniform procedure, the designations applied in earlier studies [27, 28] were used. Thus No 3 has been retained for the network pattern, although the network variant described in the present report was not completely identical with the colony type previously marked 3.

Results

Variants of Sh. sonnei phase I. The present paper deals with the following variants from the phase I colony types of *Sh. sonnei* cultures.

1) Star-shaped.

2*a*) Highly refractive, homogeneous.

3) Network pattern.

4) Concentric.

5) Radial structure.

6) Colonies grown on agar plates covered by cellophane.

In some cases 2*b* variants (moderately refractive, homogeneous colonies with pure phase II antigen content) were studied as controls.

The results of these investigations are presented in Tables I to XI. The following is a brief account of the observations which are thought to deserve special mention.

1) *Star-shaped colonies.* This variant has been discussed in detail in the previous reports.

Inside the colony, the star-shaped formation contains phase I antigen, the peripheral parts phase II antigen. Thus both antigens are present, separated from each other. From the serological aspect this type may be denoted as a mixed one (Table VII); it is accordingly slightly agglutinated by trypaflavine. The variant may be regarded as the dominant pathogenic form of *Sh. sonnei.* It usually does not grow on bismuth sulphite or brilliant green agar (Table VI); however, if the inoculum is large, minute colonies develop on both

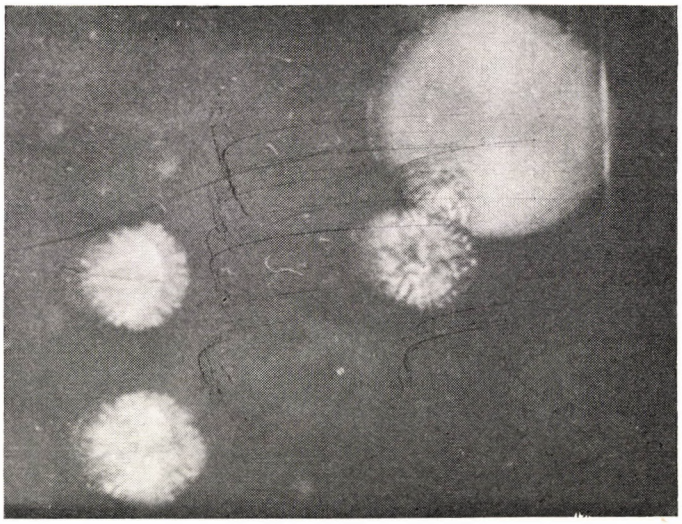

Fig. 1. A highly refractive, homogeneous colony (upper right corner) and colonies with radial structure. Magnification about ×10

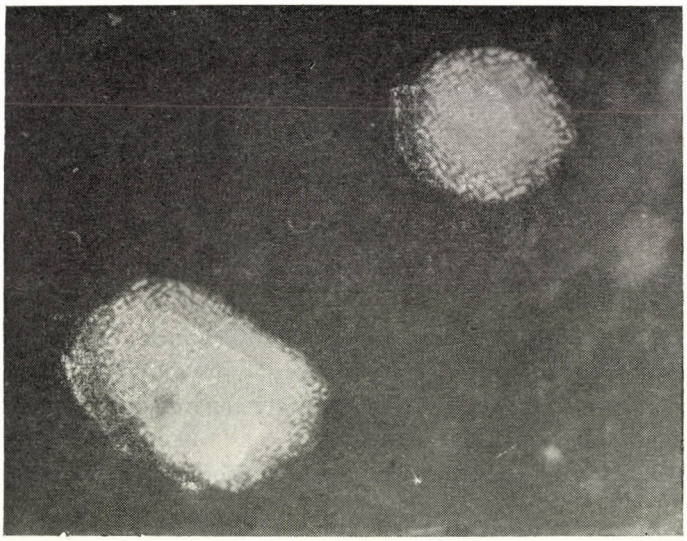

Fig. 2. Colonies with network pattern. Magnification about ×7

Table I

Structural characteristics of Sh. sonnei colony variants
C agar, incubation for 24 hours

Designation of variant	Morphological characteristics	
	in direct light	in oblique light
1. With star	Flat, but otherwise the usual S form	In less refractive colony material, more refractive shapes of varying size around the centre, usually formed by radial bundles
2a. Strongly refractive homogeneous	The usual S form	Refractive material corresponding to the star of type 1, distributed evenly over the whole colony
2b. Moderately refractive homogeneous	Slightly flatter than type 1, occasionally with somewhat irregular contours	Entirely homogeneous colony of less refractive material than that of type 2a
3. Network pattern	Porcelain-white, finely granular S form, usually smaller than type 1	In homogeneous material a network of whitish, rough lines, becoming blurred towards the centre
4. Concentric structure	The usual S form	Circles or squares formed by fine parallel lines changing in colour according to the plates position and diminishing in diameter towards the centre
5. Radial structure	Like type 3	In homogeneous material, rough, whitish lines, proceeding in radial direction from the centre
6. Grown on cellophane-covered agar	Flat, round forms of 0.5 to 2 mm diameter, with partly irregular, slightly indented or rounded margin, uneven surface and a slight protuberance in the centre.	Faintly refractive homogeneous material

culture media (Table V). Of all the investigated variants this one possesses the highest virulence and toxicity (Tables IX to XI).

2a) *Highly refractive, homogeneous colonies* (Fig. 1).

In addition to what has been reported in our earlier papers [27, 28, 28a], a remarkable refraction capacity and homogeneous distribution were observed with several of the variants. Of these, type 2*a* refers only to forms which in subcultures constantly produce patterns identical in structure with that of the mother colony. The most copious of this variant are one-day bismuth-sulphite agar cultures of a large inoculum of variant No. 1. The type under discussion is trypaflavine-negative, agglutinated only by phase I serum. By immunization of rabbits its cultures nevertheless reveal a slight amount of phase II agglutinogen (Table VII). Its virulence to mice is moderate, to guinea pigs it is second

Table II

Isolation of Sh. sonnei colony variants

Designation	Colony variant first appearance	Technique of isolation
1	Primary faecal culture on DC agar. After conjunctival infection with type 2a or 4, culture of ocular discharge on C agar	Subculture on C agar
2a	Primary faecal culture on DC agar	Subculture on C agar, and serial subcultures from highly refractive homogeneous colony
	After conjunctival infection with type 5, culture of ocular discharge on C agar	
	Culture of type 1 colony on DC agar with prolonged incubation	Repeated subculture on C agar
	One-day culture on brillant green agar of large inoculum of type 1 variant	Subcultures of pin-head sized granular colonies on C agar
2b	Primary faecal culture on DC agar	Subculture on C agar, and new subcultures of marginal part from type 1 colony on C agar
	Stored culture of type 1	Cultures grown on C agar always contain 2b variants
	Subculture of type 1 colony of C agar	Subculture of occasional 2b type colony, or of marginal parts from type 1 colony on C agar
3	Subculture of several type 1 and type 4 cultures stored for prolonged periods	Subcultures of pin-prick sized network pattern colonies on C agar
	Culture of type 4 colony on DC agar after prolonged incubation	Repeated subculture on C agar
4	Culture of type 1 colony on DC after prolonged incubation	Repeated subculture on C agar
	Culture of type 1 colony grown on Dorset culture medium, stored for prolonged periods at room temperature	Repeated subculture on C agar
5	Culture of type 4 or 2a colony on DC agar after prolonged incubation	Repeated subculture on C agar
6	One-day culture of type 1 variant on cellophane-covered C agar	Serial subculture on cellophane-covered C agar

to variant No. 1. In toxicity it does not differ from the other variants classified as phase I (Tables IX to XI).

3) *Colonies with network pattern* (Fig. 2).

This is perhaps the most interesting variant.

Inspection in direct light already reveals a whitish granular structure in the colony. In oblique light (plate position 4—8) a network is seen, formed by the crossing of less delicate lines at the margin, while a homogeneous, yellowish brownish material may be observed around the centre of the colony. A similar,

Table III

Dissociation of Sh. sonnei variants on C agar

Colony variant	Serial number of variants formed in the first subcultures
1	1, 2b
2a	2a (1, 2b)
2b	2b
3	3 (2b)
4	4 (2b)
5	5 2a
6	1 2b

Uncommon variants in parentheses

Table V

Cultures of Sh. sonnei variant colonies

Culture media	Colony		
	1	2a	2b
Horse agar	2a or 1	2a	Flat, with slightly uneven margin
Beef agar	2a or 1	2a	As on horse agar
D agar	R form with brown star	S form, homogeneous or with brown star	R shape, homogenous
DC agar	S form	S form	No growth
Eosine-methylene blue agar	S form or bomb form	S form	As on horse agar
Bismuth sulphite agar	Only in case of large inoculum, pin-prick sized colonies	In case of large inoculum, colonies of pin's head size and granular structure	No growth
Brillant green agar	In case of large inoculum, pin's head size colonies	As on bismuth sulphite agar	After 1 or 2 days colonies of pin-prick size
Broth after 24 hours	Pronounced turbidity, pronounced sedimentation	Moderate turbidity minimum sedimentation	Marked turbidity, pronounced sedimentation
After 48 hours	As after 24 hours	Faint turbidity, slight sedimentation	As after 24 hours

Table IV

Sh. sonnei variants in living organisms

Variants isolated after intraabdominal infection of albino mice or conjunctival infection of guinea pigs

Variant employed for infection	Variants grown in material derived from the organism
1	1 (2b, 2a)
2a	2a (1, 2b, 3, 4)
2b	2b
3	3
4	4, 1 (2a, 2b, 3)
5	5, 2a (1, 3, 4)
6	1 (2b)

Uncommon variants in parentheses

on different culture media

variant			
3	4	5	6
3	4	5	2a, 1, or 2b
3	4	5	As on horse agar
In plate position 2—3, network pattern	Uneven margin, peripheral network	Similar to variant 4	R form, with or without brown star
Minute network colonies	Brownish centre, network margin	No growth	S form
3	4	Network structure	2a, 1, or 2b
No growth	In case of large inoculum, few, minute colonies	No growth	No growth
No growth	No growth	No growth	As variant 1
Faint turbidity without sedimentation	Pronounced turbidity, minimum sedimentation	Pronounced turbidity, pronounced sedimentation	Pronounced turbidity, no sedimentation
Minimum turbidity, minimum sedimentation	Slight turbidity, pronounced sedimentation	Faint turbidity pronounced sedimentation	Pronounced turbidity, wreath-like sedimentation

Table VI

Number of colonies from one colony of Sh. sonnei variant on different culture media
Colony count, dilution method

Variant used for subculture	Colony variants and their number grown on				
	C agar	DC	Bismuth sulphite agar	Brillant green agar	Eosine methylen blue agar
1	1 : 6.7×10^{7} 2b : 1.2×10^{7}	1 : 3×10^{7}	0	0	1 : 6.9×10^{7} 2b : 9×10^{6}
2a	2a : 4.3×10^{7} 2b : 1.5×10^{6}	2a : 7.5×10^{6}	0	0	2a : 5×10^{7} 2b : 2×10^{5}
2b	2b : 3.2×10^{8}	2b : 1.5×10^{3}	0	2b : 2.4×10^{4}	2b : 2×10^{8}
3	3 : 4.6×10^{6}	0	0	0	3 : 4.1×10^{6}
4	4 : 2.3×10^{8}	4 : 2.7×10^{5}	0	0	4 : 1.7×10^{8}
5	5 : 3.2×10^{7}	0	0	0	5 : 2.3×10^{7}
6	1 : 4.5×10^{5} 2b : 1.1×10^{7}	1 : 6.5×10^{5}	0	0	1 : 1.5×10^{6} 2b : 1.6×10^{7}
Proportion of organisms capable of forming colonies among the organisms inoculated into culture media					
1	1 : 1 2b : 1	1.04×10^{-1}	0	0	1 : 1 2b : 1
2a	2a : 1 2b : 1	2a : 8×10^{-1}	0	0	2a : 1 2b : 1.3×10^{-1}
2b	2b : 1	2b : 5×10^{-6}	0	2b : 8×10^{-5}	2b : 1
3	3 : 1	0	0	0	3 : 1
4	4 : 1	4 : 1.2×10^{-3}	0	0	4 : 1
5	5 : 1	0	0	0	5 : 1
6	1 : 1 2b : 1	1 : 1	0	0	1 : 1 2b : 1

0 stands for values under 10^{-6}—10^{-5} in the ratio of colony-forming organisms

but perhaps somewhat less refractive structure develops in colonies containing only phase II antigen.

Variant No. 3 was isolated only under artificial conditions, from subcultures of stored No. 1 or No. 4 variants. It is trypaflavine-positive, with serological characteristics similar to those of variant No. 2a (Table VIII).

This variant is reluctant in growth (Table VI) and shows little resistance. Both on culture media and in a dried condition, its cultures soon lose viability.

In animal experiments it has proved to be the least active variant of phase I. On conjunctival infection it had no virulence in guinea pigs (Table X and XI.)

Table VII

Serological investigation of Sh. sonnei colony variants I

Tube agglutination*

Serum		Culture of variants						
		1	2a	2b	3	4	5	6
Variant 1 before exhaustion		1600	800	400	400	800	200	800
After exhaustion with cultures of type	1	0	0	0	0	0	0	0
	2a	0	0	50	0	0	0	50
	2b	200	200	0	400	200	100	50
	3	0	0	200	0	0	0	20
	4	20	0	50	0	0	0	20
	5	0	0	50	0	0	0	20
	6	20	0	20	20	0	0	0
Variant 2a before exhaustion		800	1600	50	1600	1600	400	800
After exhaustion with cultures of type	1	0	0	0	0	0	0	0
	2a	0	0	0	0	0	0	0
	2b	400	1600	0	800	1600	400	800
	3	100	0	0	0	0	0	0
	4	0	0	0	0	0	0	0
	5	0	0	20	0	0	0	0
	6	0	0	0	0	0	0	0
Variant 2b before exhaustion		100	0	800	0	0	0	20
After exhaustion with cultures of	1	0	.	0	.	.	.	0
	4	.	.	800	.	0	.	.

* Reciprocal values of agglutination titre.
0 = Before exhaustion not agglutinated by 1 : 10 titre.
. = After exhaustion not agglutinated by 1 : 20 titre.

4) *Concentric colonies.* This variant, as well as type 2*a*, corresponds to the S form. In oblique light (plate positions 4 to 7) it displays a typical structure, with lines producing roughly circular or quadrangular formations which towards the centre of the colony become smaller; on the whole, the lines present a concentric pattern.

Variant No. 4 was isolated mostly from stored, star-shaped colonies; it shows inhibited growth on DC agar (Table VI).

Table VIII

Serological investigation of Sh. sonnei colony variants II
Tube agglutination*

Serum		Culture of variants						
		1	2a	2b	3	4	5	6
Variant 3 before exhaustion		1600	1600	100	800	1600	400	3200
After exhaustion with cultures of type	1	0	0	0	0	0	0	0
	2a	20	0	0	0	0	0	0
	2b	1600	400	0	800	800	200	400
	3	0	0	20	0	0	0	0
	4	0	0	100	0	0	0	0
	5	0	0	0	20	0	0	0
	6	0	0	0	0	50	0	0
Variant 4 before exhaustion		800	1600	400	1600	1600	800	1600
After exhaustion with cultures of type	1	0	0	0	50	0	0	0
	2a	0	0	0	20	0	0	0
	2b	800	1600	0	800	800	800	1600
	3	20	0	0	0	0	0	0
	4	0	0	0	0	0	0	0
	5	0	0	0	0	0	0	0
	6	0	0	0	0	0	0	0

* Reciprocal values of agglutination titres.
0 = After exhaustion not agglutinated by 1 : 20 titre.

This variant is negative to trypaflavine. On the basis of the immunogenic action exerted by cultures containing apparently pure phase I agglutinogen, it holds a considerable amount of phase II antigen (Table VIII).

Virulence in mice and guinea pigs was found to be different (Tables IX and X); the toxicity is moderate (Table XI).

5) *Colonies with radial structure* (Fig. 1). These show some likeness to colonies of variant No. 3.

In direct light white granules are visible in the colonies. In oblique light, bundles of radial lines crossing the centre become discernible. Most of the colonies measure not more than 1 mm in diameter.

Variant No. 5 was isolated chiefly from subcultures of variant No. 4 or variant No. 2 stored on DC agar at 37° C, but it did not grow on DC agar (Tables V and VI).

Table IX

Virulence on guinea pigs of Sh. sonnei colony variants

Conjunctival infection

Number of germs in infective dose	Number of affected among 12 eyes				
	1	2a	4	5	6
	Dried preparation of colony variants				
6×10^5	4	.	.	.	.
3×10^6	10	3	.	4	4
1.5×10^7	11	8	2	7	7
7.5×10^7	.	11	5	9	9
3.75×10^8	.	.	8	.	.
ID_{50}	1.2×10^6	8.2×10^6	1.2×10^8	1.1×10^7	1.1×10^7

Relative infectivity, per cent

Colony variant	Relative efficiency	Fiducial limits
1	100	—
2a	13.0	3.7—38.5
4	0.7	0.1— 2.9

This variant is positive to trypaflavine. Serologically it is identical with variant No. 3.

Its activity in animal tests was moderate (Tables IX to XI).

6) *Colonies grown on cellophane-covered agar plates.* Star-shaped colonies transferred onto cellophane-covered C agar plates yield in one day R-shaped colonies; from a pin's head size to 2 or more mm in diameter, these appear with a webbed or indented margin, as dry, greyish formations of uneven surface showing a small protuberance in the centre. This pattern of growth is not typical of *Shigella*; under similar conditions *Proteus*, *E. coli*, etc. form colonies of the same type.

On cellophane-covered agar plates, variant No. 6 develops consistently in serial subcultures. Upon transfer onto agar plates it nevertheless reverts to variant No. 1 or 2*b* even after ten consecutive subcultures.

Serologically, variant No. 6 corresponds to a mixture of phases I and II. It is trypaflavine-positive. Cross exhaustion is similar as for variant No. 1.

The virulence for mice and guinea pigs, as related to the content in star-shaped colonies, was weaker than that of variant No. 1 (Tables IX to XI).

Table X

Virulence on mice of Sh. sonnei colony variants

Intraabdominal infection

Number of germs in infective dose	Mortality in groups of 20 mice						
	1	2a	2b	3	4	5	6
	Dried preparation of colony variants						
5^0	3	.	.	.	2	.	.
5^1	13	6	.	.	12	.	3
5^2	16	10	.	.	15	8	11
5^3	.	11	.	.	.	14	13
5^4	.	.	.	.	.	17	.
5^5	.	.	.	3	.	.	.
5^6	.	.	.	9	.	.	.
5^7	.	.	.	11	.	.	.
5^8	.	.	.	.	.	.	.
5^9	.	.	1	.	.	.	.
5^{10}	.	.	11	.	.	.	.
5^{11}	.	.	13	.	.	.	.
LD_{50} number of germs	4.8	25	1.3×10^7	4×10^4	5	60	36

Relative virulence

Colony variants	Efficiency	Limits of error
1	1	—
2a	0.1	0.02—0.4
2b	2.7×10^{-7}	0.97×10^{-7}—6.6×10^{-7}
3	1.1×10^{-4}	0.2×10^{-4}—3.8×10^{-4}

Discussion

The morphological and serological peculiarities of *Sh. sonnei* cultures have been repeatedly subjected to thorough study [1—23, 25, 26, 30, 32—35]; our investigations had the aim to differentiate further variants within the same phase on the basis of the fine structure.

In this work we had to face certain difficulties.

(*i*) Colony morphology in itself supplies insufficient information for the identification of a variant. For instance, star-shaped colonies occur also in cultures of *E. coli*; highly refractive, homogeneous colonies may appear in various forms; of the network pattern colonies, one group contains phase I

Table XI

Toxicity in mice of Sh. sonnei colony variants

Intraabdominal inoculation

Number of germs in inoculated dose	Mortality in groups of 20 mice						
	1	2a	2b	3	4	5	6
	Dried preparation of colony variants						
1.6×10^{7}	0	.	.	.	0	0	0
8×10^{7}	2	1	0	2	2	2	1
4×10^{8}	6	5	2	6	6	4	7
2×10^{9}	19	14	5	12	17	16	18
1×10^{10}	.	19	14	18	.	.	.
LD_{50}	5.9×10^{8}	9.7×10^{8}	5.5×10^{9}	1.1×10^{9}	8.9×10^{8}	8.2×10^{8}	6.2×10^{8}

Relative toxicity per cent

Colony variant	Efficiency	Limits of error
2b	1	—
1	8.6	3.9—19.2

antigen, the other phase II antigen, etc. Naturally, morphology is not characteristic of every single trait of a micro-organism; however, together with serological tests it may provide useful data, *e. g.* concerning virulence.

(*ii*) Differences in colony structure are not always so definite as to permit infallible classification into one of the types described by us. Moreover, the described forms do not embrace all the variants; intermediary forms infrequently occur. The difficulties thus involved can be minimized by the use of special culture media (Tables V and VI). For example, colonies of the variants Nos. 1, 2 and 3 can be easily differentiated on D agar (Table V). Variant 5 shows no growth on DC agar, while variant 3 does, upon applying a large inoculum (Tables V and VI).

The use of bismuth sulphite and brillant green culture media may contribute to the collection of further data.

According to the above findings, variants containing phase I antigen may differ widely in degree of virulence. Variant 1 has been found to possess the highest virulence, though phase I and phase II have been demonstrated regularly side by side within its colonies, whereas variant 3, composed almost exclusively of phase I, has proved to be avirulent; even in massive doses it has failed to cause conjunctivitis in guinea pigs.

It is therefore possible to isolate from *Sh. sonnei* cultures variants representing different degrees of virulence. Some authors have observed that phase I

of *Sh. sonnei* is not always virulent [24, 31]. This has been fully explained by our present data. An identical conclusion may be drawn from some of our previous accidental observations, namely, that the virulence of *Sh. sonnei* cultures cannot be ascribed solely and in the first place to phase I antigen.

By means of our method, the conversion of *Sh. sonnei* strains into avirulent forms can be prevented. The virulence of a strain can be maintained by isolating in oblique light the desired variant and passaging it in fresh cultures at suitable intervals.

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EFFECT OF ANTIFUNGAL AGENTS ON THE FAECAL YEAST FLORA OF MICE

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Summary. The effect of various antifungal agents on the normal faecal yeast cell flora of mice has been studied during continuous oral administration of oxytetracycline. Oral doses of nystatin, trichomycin, trypaflavine, trichothecin, flavofungin, vitamin K_5, crotocin and pentachlorphenol have been found to reduce the number of yeast cells in the faeces by more than 90 per cent. The effective doses of the agents increased in the above-mentioned order. Gentian-violet, oxyben B, and 8-oxy-quinoline proved ineffective in the doses employed. The inhibitory action of substances effective *in vivo* has been compared *in vitro* against *Candida albicans* and *Torulopsis glabrata* the latter being isolated from the faeces of mice. The relation between the data obtained and the possible therapeutic action of the agents used on *Candida albicans* in the human intestinal tract has been discussed.

Since the introduction of wide spectrum antibiotics, the incidence of fungal diseases increased, chiefly those affecting the mucous membranes and their dermal margins. The most frequent among the pathogenic fungi is *Candida albicans* which may be present in the organism, especially in the intestinal tract, also as a saprophyte [1, 2, 3]. Other yeast-like fungi also occur in the intestinal tract, though less frequently. During antibiotic treatment, *Candida albicans*, by dissemination in the organism, may sometimes bring about serious, occasionally lethal, changes in the internal organs, particularly in debilitated patients [4, 5]. Recently enteral moniliasis has also been differentiated as a separate pathologic entity [1]. Hence there exists the need of finding agents which when used concurrently with wide spectrum antibiotics are capable of preventing the proliferation of *Candida albicans* in the intestinal tract. Destruction of yeast-like fungi which present a potential danger is necessary also in preoperative intestinal sterilization. The use of fungicidal agents may be taken into consideration on such occasions.

Various fungicidal agents have been applied for the above purpose with more or less satisfactory results, in animals nystatin [6, 7], pimaricin [8] and p-oxy-benzoic acid benzylic ester [9], while in man nystatin [10, 11], amphotericin B [2, 12], undecylenic acid [13], gentian-violet [1] and the parabens [14]. However, no comparison of the effects of these agents by means of an uniform method has been performed. We have therefore studied the action of various antifungal substances on the yeast cell flora of the intestinal tract in mice concurrently with the oral administration of oxytetracycline. Apart from those

in common use, several new antibiotics and antifungal agents formerly not applied for this purpose have been examined.

Materials and methods

Experiments *in vivo* were carried out as follows. For approximately ten days before and during the experiments mice from the stock of the State Institute of Hygiene (Budapest) were kept on Sós's rat diet [15] with the difference that ground meat was substituted with casein. Per mouse 3 to 4 g of dry substance was given, together with 1 mg/g of oxytetracycline mixed in the food. The daily dose was kneaded with hot water. Water was not restricted.

For counting the yeast cells, one faecal pellet was homogenized with 1 ml water in Potter's apparatus. 0.1 ml of the required dilution was spread over the surface of broth agar plates of pH 6 containing 0.5 per cent glucose and 0.1 per cent chloramphenicol. When the surface had dried, the plates were incubated for one day at 37° C, then for another day at room temperature, whereafter the colonies were counted. Bacterial growth having been prevented by chloramphenicol, only yeast colonies could develop, as verified in each case by microscopic control. The substances used were suspended in 5 per cent gum arabic and administered through a stomach tube in volumes of 0.2 ml. (Under such conditions trypaflavine was dissolved, gentian violet only partly). With a few exceptions, the substances were administered in doses of 2, 10, 50 and 250 mg/kg, and the lowest effective dose was determined. From the materials which decompose easily, fresh suspensions were prepared and tested for efficiency *in vitro* on each occasion.

Examinations *in vitro* of antifungal substances were carried out on agar plates containing broth agar with 0.5 per cent glucose.

Results

In the faeces of the mice a yeast species was constantly present which could be identified as *Torulopsis glabrata*. Sometimes other yeast cells also appeared in the droppings. We were unable to follow their incidence, but when the cell count of *Torulopsis glabrata* diminished, the other yeasts vanished. The values recorded refer to *Torulopsis glabrata*.

In the first, informative experiments, the substances were studied in groups of three animals each; beside these, in every series of experiments three mice were given 10 mg/kg of nystatin, while three animals did not receive any drug. From three of four days prior to the onset of medication yeast cell counts were made daily and continued until, after treatment had been terminated, the yeast cell count returned to approximately its initial level. Fungicidal substances were administered once daily over a period of four days. An experiment is presented in Fig. 1.

As revealed by Fig. 1, in these experiments excessive variations were registered, since the number of excreted yeast cells showed spontaneous and daily changes, differing from animal to animal. In the further course of the experiments we therefore used groups of seven to ten animals.

As in the case of nystatin the maximum effect developed in every instance as early as the day following the third dose, in the next stage the mice were given only three day courses of treatment, and the yeast cell counts were made on the fourth day, furthermore two or three days before starting the drug, and four days after its termination. The course of these studies can be followed in Fig. 2.

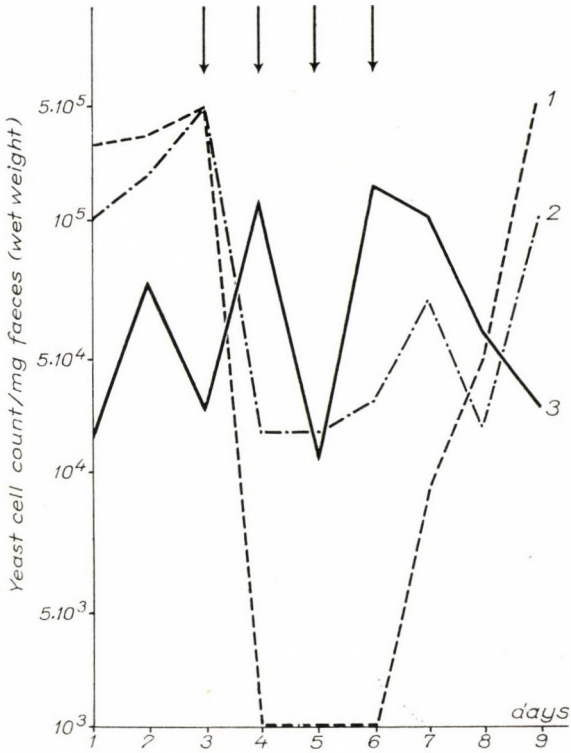

Fig. 1

1 : Nystatin 10 mg/kg
2 : Trichothecin 50 mg/kg
3 : Control
↓ Drug administration

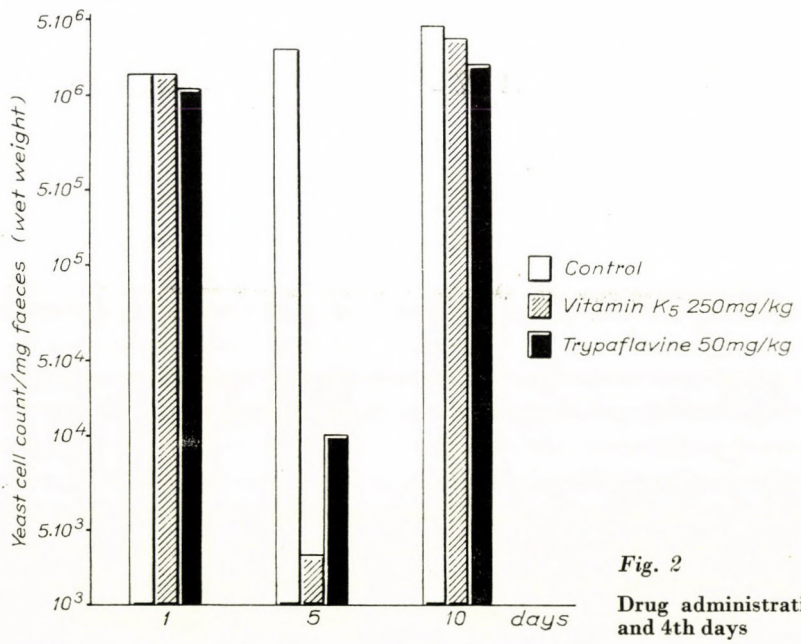

Fig. 2

Drug administration on 2nd, 3rd, and 4th days

Fig. 2 clearly demonstrates that due to the wide scatterings even larger groups of animals allowed only to differentiate by order of magnitude. Therefore in agreement with the method of SHIDLOVSKY and PRIGOT [16], who in the case of bacteria regarded 90 per cent reduction as a criterion of successful intestinal sterilization, and with reference to LINDH [7], in our experiments no result was denoted as efficient unless the cell count showed a minimum 90 per cent decrease.

Table I

Effect of antifungal agents on yeast cells in the faeces of mice

Substance	No. of mice	Reduction of yeast cells in faeces, %	Dose, mg/kg	Oral toxicity mg/kg mice
Trichomycin	8	99	3.3	>160[1]
Trypaflavine	8	99	50	400[2]
Trichothecin	9	99	100	500[2]
Vitamin K_5	6	99	250	1000[3]
Nystatin	8	94	2	>3600[1]
Flavofungin	7	90	100	>250[1]
Crotocin	7	95	250	>1000[5]
Penta-chlorphenol	8	90	250	125—200[4]

1. Literary data.
2. Minimum lethal dose, own results.
3. LD_{100}, suspension in oil, literary data.
4. LD_{50} in rat; literary data.
5. See [21].

Table I presents the substances which proved effective, with special reference to those that produced 99 resp. 90 per cent reduction.

Of the investigated substances 50 mg/kg of gentian violet, 250 mg/kg oxyben B, and 50 mg/kg 8-oxy-quinoline were ineffective, while higher doses of the latter were toxic. Even 10 mg/kg doses of nystatin failed to produce a 99 per cent effect. Trichomycin and nystatin were the most potent. Taking into consideration the effective doses of the investigated substances and their oral toxicity (see Table I), all of them but pentachlorphenol and vitamin K_5 had a satisfactory therapeutic index.

To establish the comparative effectiveness of the investigated substances against *Torulopsis glabrata* and *Candida albicans*, their action *in vitro* was studied. In Table II the substances were divided into four groups; the first was 99 per cent effective *in vivo*, the second 90 per cent effective *in vivo*, the third group ineffective *in vivo*, the fourth group ineffective both *in vivo* and *in vitro*.

Table II

In vitro effect of antifungal substances

Substance	Inhibitory concentration, mg/ml	Diameter of inhibition zone	
		Torul. glabr.	*Cand. alb.*
Trichomycin	100	23	23.3
Trypaflavine	100	18	0
Trichothecin	33	29	36
Vitamin K_5[1]	330	18	16.5
Nystatin	100	23	21
Flavofungin	33	15	18
Crotocin	100	21	26
Pentachlorphenol	1000	24	24
Gentian-violet	1000	22.5	30
Oxyben B[2]	1000	17	14
8-oxy-quinoline	100	13.5	15
Nipagin M[3]	1000	∅	∅
Yatren[4]	1000	∅	∅

1. 2-methyl-1,4-napthoquinone.
2. p-oxybenzoic acid butylester.
3. p-oxybenzoic acid methylester.
4. 7-Cl-8-oxyquinoline-5-sulphonic acid.

Most of the substances acted equaly against the two kinds of yeast cells; however, trichothecin, crotocin and gentian-violet inhibited *Candida albicans* more intensely than *Torulopsis glabrata*, while trypaflavine completely failed to inhibit Candida.

Discussion

Among the substances studied there were several which thus far have not been employed for the suppression of intestinal yeasts. Of these, pentachlorphenol was applied externally for the treatment of dermal mycoses [17], while vitamin K_5 [18] was found beneficial in systemic mycoses; 8-oxy-quinoline [19] and flavofungin [20] were also found effective in mycotic conditions by Uri *et al.* Trypaflavine was found effective *in vitro* against some yeasts in our own previous studies. Crotocin (formerly named antibiotic T) was described by the authors [21]; trichothecin was studied in view of its structural relationship to crotocin. Trichomycin was found effective upon local application [23].

In most of our experiments *in vivo*, antifungal agents were tested against the yeast species occurring normally in the intestinal tract of mice. This

method has been chosen by STERNBERG [6] as well as by ourselves because of the absence of *Candida albicans* from the intestinal flora of normal mice [6, 24]. Thus there is no way for reproducing in animal experiments conditions fully corresponding to those prevailing in the human organism. By feeding them with *Candida albicans*, mice can be made to carry Candida for a certain time, this, however, produces an abnormal microbial balance in the intestines. Changing the normal yeast flora in animals by means of drugs is a method more similar to the therapeutic action in humans since in man *Candida albicans* may belong to the normal intestinal flora. On the other hand, the findings *in vitro* allow some conclusions as to the possible inhibitory effect of these drugs on *Candida albicans* in man.

An attempt was nevertheless made to render mice *Candida albicans* carriers and to study the effect of the drugs also by this method. In agreement with STERNBERG [6], one million *Candida albicans* cells were administered daily through a stomach tube over a period of three days, and the faeces suspension was spread over the surface of PAGANO's medium [25] of which TTC was omitted because of our previous finding that latter inhibits *Candida albicans*. Colonies of *Torulopsis glabrata* and *Candida albicans* could be differentiated both on basis of the colony type and under the microscope by the chlamydospore formation on inoculation into potato agar [26]. In these experiments most drugs proved to be equally, or more effective against *Candida albicans* than against *Torulopsis glabrata*, with the exception of trypaflavine which failed to inhibit *Candida albicans* even *in vitro*. However, excretion of *Candida albicans* was not consistent in the mice; hence our findings in vivo concerning *Candida albicans* are not considered reliable.

The importance of using identical mouse strains has been confirmed by the experience that no *Torulopsis glabrata* could be detected in the mouse strain of the State Serum Institute Phylaxia, Budapest.

Although our findings cannot be applied directly to man, we suppose that they nevertheless afford some information concerning the comparative potency of the investigated substances.

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STUDIES ON THE MECHANISM OF PROPERDIN — ZYMOSAN REACTION

THE ROLE OF POLYSACCHARIDE STRUCTURE IN COMBINATION WITH PROPERDIN

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Summary. (i) The properdin-reacting and protein-adsorbing capacity of the cell wall polysaccharide of *Saccharomyces cerevisiae*, zymosan and that of its components, glucan, mannan and glycogen have been investigated. Examinations were performed also with other polysaccharides and inorganic adsorbents.

(ii) The combining capacity with properdin decreased in the following order: zymosan, mannan, glucan, cellulose, calcium phosphate gel and caolin.

(iii) With solid phase-forming substances the interaction with properdin was connected with a definite degree of protein adsorption.

(iv) Of the examined substances, the most specific combination with properdin was obtained with zymosan.

(v) In the presence of Ca and Mg ions mannan inhibited the reaction between properdin and zymosan. No inhibitory action was exerted by glycogen.

(vi) The reaction between properdin and glucan differed from that between properdin and zymosan in stability and ion requirement.

(vii) It has been concluded that the high specificity of zymosan in the reaction with properdin is due to the structural properties of mannan.

Investigations into the mechanism of the combination of the properdin system with zymosan, the cell wall polysaccharide of yeasts, have mainly been focussed on various factors participating in the process, and on the kinetics of various stages of the reaction [1—6]. No extensive studies have been performed as to the influence on the reaction of various components of zymosan. On the basis of the experiments of PILLEMER *et al.* [7] and RZUCIDLO *et al.* [8], zymosan was regarded as a glucose polymer that retained the yeast cell wall structure to some degree. An effect similar to that of zymosan was brought about with a water-insoluble polysaccharide composed of glucose units [9]. Recent investigations, however, revealed that even the highly purified zymosan, as well as the yeast cell wall, is composed of several different polysaccharides. Thus DI CARLO and FIORE [10] detected glucose, mannose and glucosamine in the hydrolysate of zymosan. From alkali-treated zymosan ROSENFELD *et al.* [11] isolated a water-insoluble glucose polymer (glucan) and water-soluble gluco-mannan polysaccharides.

In previous examinations it has been shown that the carbohydrates of zymosan are composed of glucan, mannan and glycogen, and, in addition to these, the substance contains less than 1 per cent chitin [12]. It has also been shown that differently prepared zymosans differed in glucan, mannan and

glycogen content, and that this difference decisively influenced the properdin-binding and complement-activating ability of the batches [13]. Thus it would appear that the properdin—zymosan reaction requires a specific polysaccharide structure. On the other hand, as the reaction proceeds in a heterogeneous system, this conclusion is made questionable by the possibility that actually a less defined adsorption of wide specificity occurs.

In the present paper experiments will be presented as to the dependence on the polysaccharide structure of the formation of the properdin—zymosan complex (PZ complex), which is the basic step of the reaction. In order to show whether the properdin—zymosan reaction requires a specific polysaccharide structure, the adsorption of proteins on water-insoluble polysaccharides and also the influence of water-soluble polysaccharides on the properdin—zymosan reaction have been investigated.

Materials and methods

Zymosan. This substance was prepared from autolysed cells of *Saccharomyces cerevisiae* by elastase treatment as described in reference [14]. Total carbohydrate content was 85.7 per cent. The carbohydrates were composed of 70.8 per cent mannan, 10.9 per cent glucan and 17.5 per cent glycogen. Total nitrogen content amounted to 1.6 per cent.

Polysaccharides. Isolation of glucan from the soluble polysaccharides was performed by the method of CHUNG and NICKERSON [15]. Mannan and glycogen were separated by the method of BARKER *et al.* [16]. The substances were purified by repeated precipitation with ethanol. Acetylation of zymosan was carried out according to HAWORTH *et al.* [17]. This product contained 20.6 per cent of acetyl groups. In addition to these substances the following were employed: soluble starch, alpha cellulose, dextran (molecular weight 50 000—100 000) and inulin (molecular weight 500—2000). The size of zymosan and glucan particles corresponded to approximately 100 mesh.

Proteins. Swine serum containing 10—12 units of properdin per ml; lyophilized bovine serum albumin produced by COHN's fractionation; trypsin (Novo) containing 25 Anson units per gram. For removal of Ca and Mg ions from the sera Amberlite IRC-50 (sodium cycle) was used.

Measurement of the combination with properdin. To 1 ml samples of swine serum pH 7.4 barbiturate buffer containing Ca and Mg ions [7] and different quantities of various polysaccharides or inorganic adsorbents (calcium phosphate gel, activated charcoal, caolin) were added. Final volumes were 2.0 ml. The mixtures were shaken continuously for 60 minutes at 20° C. After centrifugation the supernatants were titrated for properdin. To the blank tubes no polysaccharides or adsorbents were added [18]. The minimum quantity of polysaccharides or adsorbents removing the total properdin content of 1 ml serum was determined. The combining capacity with properdin was computed by extrapolation; the result was expressed as properdin units combined with 10 mg of substance.

Measurement of protein adsorption. In the presence or in the absence of 4×10^{-4} *M* $MgCl_2$ and 1.5×10^{-4} *M* $CaCl_2$, at an ionic strength of 0.15, various proteins were added to the examined polysaccharides and adsorbents. The mixtures were shaken continuously for 15 minutes at 20° C until adsorption equilibrium had been reached. The protein content of the centrifuged mixtures was determined by the method of LOWRY *et al.* [19], both in the supernatant and in the precipitate, which had been dissolved in 0.002 *M* NaOH. The adsorption capacity was given as mg protein adsorbed by 10 mg of adsorbent in a medium containing both substances at concentrations of 5 mg per ml.

Results

The properdin and protein binding capacities of various polysaccharides and adsorbents were compared. For the experiments the following kinds of

polysaccharides were chosen. (*a*) Polysaccharides that are composed of the same units, but, as indicated by differences in their solubility, are different in structure (1, 2, 3 and 4 in Table I). (*b*) Polysaccharides different in composition, but similar as to solubility (1, 2, 5 and 6 in Table I). (*c*) Polysaccharide constituents of zymosan (2, 4 and 6 in Table I). Of the adsorbents those known as capable of adsorbing proteins were used [20] (10 and 11 in Table I). The role of polarity in the reaction with properdin and proteins was estimated by means of activated charcoal and acetylzymosan. Calculation based on the acetyl content showed that in the latter substance 65 per cent of the hydroxy groups was acetylated.

As the direct aim of the experiments, the properdin—zymosan reaction, requires various components of complement [7], the measurements were carried out with serum and not with purified properdin. In order to show the degree of specificity of the reaction between polysaccharides and properdin, experiments were also included with properdin-free proteins such as serum albumin and trypsin. The results are summarized in Table I. Taking into consideration the sensitivity of the method and the manner of calculation (extrapolation), extremely low values were expressed as < 5 units of properdin per 10 mg and < 10 μg protein per 10 mg of polysaccharide or adsorbent.

From Table I it is seen that with the exception of mannan, water-soluble polysaccharides cannot combine with properdin, and, as all the components are in the dissolved state, no protein adsorption is possible. The insoluble

Table I

Properdin-binding and protein-adsorbing capacity of various polysaccharides and adsorbents

		Properdin-binding capacity units/10 mg	Adsorbing capacity μg protein/10 mg		
		serum	serum	albumin	trypsin
Starch	(1)	<5	<10	<10	
Glycogen	(2)	<5	<10		
Cellulose	(3)	21	460		
Glucan	(4)	26	375	740	
Inulin	(5)	<5	<10	<10	
Mannan	(6)	45	<10	<10	<10
Zymosan	(7)	80	168	300	112
Acetylzymosan	(8)	<5	210		
Charcoal	(9)	<5	<10		27
Calcium phosphat gel	(10)	13		180	20
Caolin	(11)	8	124	96	90

(solid phase-forming) polysaccharides, however, similarly to calcium phosphate gel and caolin, reacted with both properdin and the other proteins. The combining capacity with properdin decreased in the following order: zymosan > mannan > glucan > cellulose > calcium phosphate gel > caolin. Although acetylzymosan and activated charcoal formed solid phase, these substances did not react with properdin. In contrast to properdin, protein was adsorbed by all water-insoluble substances; the degree of protein adsorption was not parallel with the degree of combination with properdin. This finding is well expressed in a comparison of the specific properdin-combining capacities (ratio of properdin and serum protein bound by the same quantity of given substances). These values were 0.476 for zymosan, 0.069 for glucan, 0.045 for

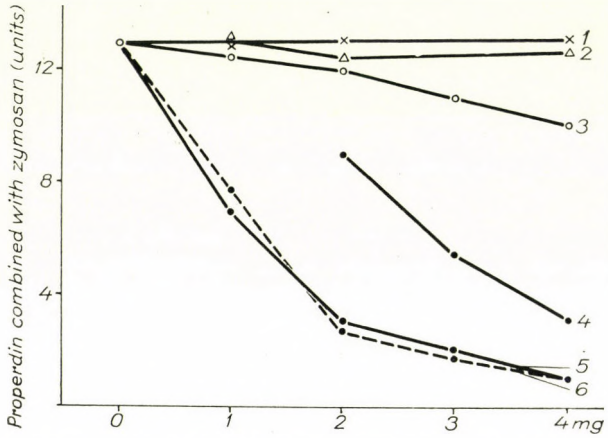

Fig. 1. The effect of soluble polysaccharides on the combination of properdin with zymosan. Reaction mixture: zymosan 2 mg, swine serum 1 ml in pH 7.4 barbiturate buffer, in the presence of 4×10^{-4} *M* $MgCl_2$ and 1.5×10^{-4} *M* $CaCl_2$. The added substances were 1, glycogen; 2, inulin; 3, dextran. Mannan was tested in the presence of 4 mg zymosan (4), of 2 mg zymosan (5) and of 2 mg zymosan + 8×10^{-4} *M* $MgCl_2$ + 3×10^{-4} *M* $CaCl_2$ (6)

cellulose and 0.064 for caolin. In other words, simultaneously with the combination with 1 unit of properdin, the other substances adsorbed 7 to 10 times more protein as compared to the amount adsorbed by zymosan.

From these findings it is evident that the reaction between zymosan and properdin was considerably more specific than that between zymosan and other proteins. As to glucan, cellulose, acetylzymosan and inorganic adsorbents there was no such difference.

Of the water-soluble polysaccharides only mannan was able to decrease the properdin content of the serum. This was striking, as on incubation with mannan no precipitation occurred, that is, a surface similar to that of zymosan, was not present. The only explanation of this finding can therefore be that mannan reacted with properdin, or, alternatively, with Ca and Mg ions, which

are needed for the formation of the properdin—zymosan complex. This consideration may be proved as follows. With the addition of increasing amounts of mannan to a system containing given quantities of zymosan and serum, the formation of the properdin—zymosan complex should decrease proportionally to the concentration of mannan. Experimental demonstration of this is presented in Fig. 1. It was also revealed that of the examined water-soluble polysaccharides mannan is the agent most likely capable of changing the course of the properdin—zymosan reaction. The inhibitory action of mannan on the formation of the properdin—zymosan complex is a function of the amounts of zymosan and mannan present in the system. When the concentration of zymosan was increased twofold, a fourfold increase of mannan was required to bring about the same degree of inhibition observed at the original concentrations. From Fig. 1 it is also clear that the degree of inhibition is not considerably altered by an increase in the concentration of Ca and Mg. Accordingly, the effect of mannan is not due to a mere ion depletion.

For the reaction with mannan, as well as for that with zymosan [7], the presence of divalent cations is essential. In a serum specimen with its Ca and Mg ions removed the quantity of adsorbed protein was not changed by the separate addition either of these ions or of mannan. Simultaneous addition of Ca and Mg ions and of 20 mg mannan, however, decreased the amount of protein adsorbed on 10 mg zymosan by approximately 30 Mg (Fig. 2). Similar experiments showed that, even in the presence of divalent cations, mannan exerted no influence on the protein adsorption by glucan. The experiments presented in Table II also demonstrate the difference between the behaviour of glucan and zymosan. Suspensions prepared in barbiturate buffer from 10 mg zymosan and 10 mg glucan were incubated with 1 ml of swine serum. After incubation the suspensions were centrifuged and washed with 5 ml barbiturate buffer. With glucan the entire amounts of adsorbed protein and properdin were shown in the eluate. In contrast, from zymosan liberation of protein occurred only partly; that of properdin was not at all observed.

Table II

Elution of serum protein and properdin bound by zymosan or glucan at 0.15 ionic strength

		Zymosan		Glucan	
		Protein, μg	Properdin units	Protein, μg	Properdin units
Bound quantity		1140	12.0	2200	7.0
Eluted quantity	I.	675	1.0	1950	6.0
	II.	119	0	288	1.5
	III.	<10	0	<10	0

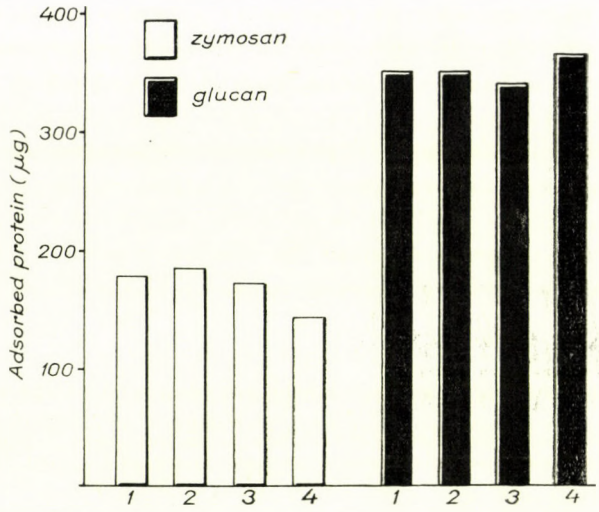

Fig. 2. The effect of Ca and Mg ions and of mannan on the protein adsorption by zymosan and glucan. Reaction mixture: 10 mg zymosan or glucan, 10 mg serum protein in pH 7.4 barbiturate buffer to give final volumes of 2.0 ml (1). The added substances were 4×10^{-4} *M* $MgCl_2$ + 1.5×10^{-4} *M* $CaCl_2$ (2), 20 mg mannan (3) and 4×10^{-4} *M* $MgCl_2$ + + 1.5×10^{-4} *M* $CaCl_2$ + 20 mg mannan (4)

Discussion

The present results show that the polysaccharide structure plays an important part in the properdin—zymosan reaction.

It is known that the formation of the properdin—zymosan complex, due to the insolubility of zymosan, is a surface reaction [2, 4, 5]. According to our investigations, the reaction requires some polarity of the surface. The lack of properdin adsorption by activated charcoal and acetylzymosan, as well as the adsorption of properdin by calcium phosphate gel, caolin, cellulose and glucan suggest the possible role of hydrophile groups, thus in polysaccharides, of hydroxy groups. It is also clear that, as compared to other serum proteins or trypsin, properdin requires the presence of more polar groups on the surface of the adsorbent.

For the formation of a specific properdin—zymosan complex, in addition to polarity, other surface properties are also needed. The hapten-inhibition-like action [21] of mannan on the properdin—zymosan reaction and the need for divalent cations suggest that the specificity of zymosan is due to the structural properties of mannan. This is experimentally confirmed by the ineffectiveness of glycogen and also by the observation that for the protein—glucan reaction no Ca and Mg ions are required; moreover, proteins including properdin, which have been adsorbed by glucan at 0.15 ionic strength are liberated when the solution is diluted. The groups in mannan that react with properdin

are at present unknown. This question awaits further experiments, which are to be carried out by the gradual breaking down of the mannan molecule.

From the basic importance of mannan in the mechanism of the properdin—zymosan reaction, some conclusions may be drawn as to the structure of the yeast cell wall. According to NORTHCOTE and HORNE [22], mannan is a component of the inner layer of the yeast cell wall. It is, however, known that the suspension of yeast cells also react with the properdin system [2, 23]. Accordingly, our experiments support the statement of EDDY and RUDIN [24], who on the basis of enzymic digestion have concluded that both glucan and mannan are present on the surface of the yeast cell wall in a mosaic-like arrangement.

The results of the present examinations suggest that investigations into the specificity of the properdin reaction should be extended in addition to zymosan also to other properdin-reacting polysaccharides, including especially bacterial cell wall components. These investigations may result in a considerable progress as to the elucidation of the immunobiological role of properdin.

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ENTEROVIRUS STUDIES IN CONNECTION WITH THE 1959 POLIOMYELITIS EPIDEMIC IN HUNGARY

By

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Summary. In 1959, when 1830 cases of poliomyelitis (18.3 per 100 000 population) were reported in Hungary, 2329 specimens derived from 1822 subjects were tested for enterovirus. Eighthundred and eighteen virus strains were isolated from 800 specimens derived from 746 persons. Of the single isolates 630 proved to be poliovirus type 1. The distribution of the remainder was poliovirus type 2, 3; poliovirus type 3, 31; Coxsackie virus, 39; ECHO virus, 53; 27 strains have not yet been identified. Seventeen patients excreted more than one strain at the same time.

Of the subjects tested 625 were suffering from poliomyelitis according to the final clinical diagnosis, *i.e.* 34.1 per cent of the reported cases were tested. Type 1 poliovirus was isolated from 73.6 per cent of these patients.

The results were analyzed by clinical diagnosis, age, seasonal incidence and geographical distribution.

Paired sera of 16 virologically negative patients whose disease was diagnosed clinically as poliomyelitis, were tested for neutralizing antibodies to each of the three types of poliovirus. Two of these patients were triple negative. Among 89 patients with aseptic meningitis 8 showed appreciable antibody response to poliovirus type 1. Attempts to isolate virus from these patients have failed.

In a Budapest nursery a single case of poliomyelitis occurred; it was proved to be caused by type 1 poliovirus. The contacts of the patient were tested for carrier state and for changes in the serum antibodies during the subsequent two weeks' period. Eighty-one per cent of the children were found to be infected with poliovirus type 1. At the same time the Coxsackie B2 virus, which was also present in the nursery, failed to disseminate, probably because of interference by the poliovirus.

Hungary experienced her severest poliomyelitis epidemic in the year 1957, with 2334 cases (23.8 per 100 000 population) [1]. The epidemic was caused by poliovirus type 1 [2, 3, 4]. Mass immunization with the Salk vaccine had been started during the epidemic; its epidemiological effectiveness was demonstrated [5]. Regular mass vaccination was continued in 1958 and 1959. In spite of this a further poliomyelitis epidemic with 1830 reported cases (18.3 per 100 000 population) occurred in 1959. From epidemiological aspects this epidemic has been analyzed in detail by RUDNAI [6].

The present report deals with the enterovirus studies conducted by us throughout the year 1959, and the virological analysis of the unexpected epidemic.

Materials and methods

The materials were prepared, and the experiments were conducted, as described elsewhere [9]. All the specimens collected for virus isolation were tested in monkey kidney cell cultures; 108 faecal specimens, 50 CSF and 25 miscellaneous specimens were examined in sucking mice as well.

The isolates were identified by the neutralization test, as already described [9].

Human sera were tested for neutralizing antibodies to poliovirus types 1, 2 and 3, and Coxsackie type B2 in monkey kidney cell cultures, based on the cytopathic effect. Fourfold dilution series of serum was tested against 100 CPD_{50} of virus. Serum—virus mixtures were kept at + 4°C for 12 hours, and subsequently inoculated into 3 cell cultures each. The inoculated cultures were observed for 7 days.

The patients' data were obtained from the physicians in special questionaries. Additional data for patients with poliomyelitis were supplied by the Epidemiological Department of our Institute.

Results

Virus isolation experiments. In the year 1959, 2329 specimens originating from 1822 subjects were tested for enterovirus in this laboratory. Eight-hundred and eighteen strains were isolated from 800 specimens derived from 746 persons.

Table Ia

Individuals tested for enteroviruses and isolations obtained from them

	Grouping by clinical diagnosis									
	A Poliomyelitis		*B* Suspect poliomyelitis		*C* Aseptic meningitis or encephalitis		*D* Non-neurological diseases		*E* Poliomyelitis contacts	
	number of cases	per cent	number of cases	per cent	number of cases	per cent	number of cases	per cent	number of cases	per cent
Number of subjects tested	625	100	260	100	325	100	455	100	157	100
Number of virus excretors	489	78	86	33	39	12	74	16	58	37
Of these polio 1	460	73.6	55	21.2	17	5.2	15	3.3	54	34.3
polio 2	2	0.3	—	—	—	—	—	—	—	—
polio 3	8	1.3	9	3.4	2	0.6	12	2.6	—	—
ECHO	11	1.8	12	4.6	5	1.5	22	4.8	3	1.9
Coxsackie	6	1.0	5	1.9	5	1.5	16	3.5	1	0.6
Unidentified virus excretors	2	0.3	5	1.9	10	3.1	9	2.0	—	—

In Table Ia and Ib the cases are grouped according to clinical diagnosis. Group A includes the patients whose name remained in the final, purified official list of patients with poliomyelitis. The cases belonging to group B were initially diagnosed as poliomyelitis, some of them were even notified officially, but revoked thereafter. In these cases differences in reflexes, paresis of extremities or of the facial nerve were observed. In 239 cases belonging to group C the diagnosis was aseptic meningitis, the remainder showed meningoencephalitis or encephalitis. In group D non-neurological cases were included with a diagno-

sis of "febrile state" in 38 cases, "febrile illness with atypical rash" in 37 cases, "Bornholm disease" in 43 cases, "herpangina" in 37 cases; the illness was not properly characterized in 300 cases. Group E consisted of healthy subjects belonging to 4 communities in each of which poliomyelitis had occurred.

The high incidence of type 1 poliovirus suggests that for the epidemic that type of virus was responsible. It was isolated from 73.6 per cent, whereas the two other types altogether only from 1.6 per cent, of the patients with poliomyelitis. It was widely disseminated in the other groups as well. Type 2 poliovirus was yielded only by 2 persons of those tested in 1959; both were suffering from poliomyelitis. Excretion of type 3 poliovirus was demonstrated in each group of patients, except group E (most frequently in group B).

Excretion of polioviruses was commoner in group B (24.6 per cent) than in group C (5.8 per cent) or in group D (5.9 per cent), suggesting that part of the revoked cases was nevertheless caused by poliovirus. The frequency of poliovirus type 3 was the highest in group B (even higher than in group A).

The incidence of non-polio enteroviruses was lowest in groups A and E, *i.e.* among patients with poliomyelitis and their contacts, and highest in group D. Within this group virus was isolated from 28 per cent of the patients with Bornholm disease, and from 24 per cent of those with herpangina. The isolation rates for patients with febrile state, atypical rash and non-characterized illness were 13, 2.7 and 6.7 per cent, respectively. Among the Coxsackie viruses type B2 (18 cases), among the ECHO viruses type 1 (20 cases), were isolated in the greatest number.

In 17 cases two or more types of virus were isolated from the same specimen. Most frequently poliovirus type 1 and a non-polio enterovirus (in 5 cases ECHO 1) were excreted simultaneously.

It should be noted that mass vaccinations with SABIN's attenuated poliovirus strains were started in Hungary late in 1959. In *Győr-Sopron* county two doses of the trivalent vaccine were administered in November and December of that year, whereas the monovalent type 1 vaccine was fed everywhere else in December. Thirty-two strains of poliovirus (Table Ib, in parentheses) were excreted by patients with poliomyelitis, after feedings.

The bulk of the virus strains was isolated from faecal specimens. Only 4 strains were recovered from CSF, 10 from throat washings, and 3 (2 poliovirus type 1 and 1 poliovirus type 3) from *post mortem* CNS specimens. Fifty-two per cent of the specimens from poliomyelitis patients were taken during the first week, 31 per cent in the second, whereas 10 per cent in the third week, of illness. Five per cent were taken later. The respective poliovirus isolation rates were 81, 69, 67 and 43 per cent. In 2 per cent of the cases the time of sample taking was unknown. The failure of isolating virus from certain cases of poliomyelitis (group A) was obviously due to late sampling.

Table Ib

Specimens tested for enteroviruses and isolations obtained from them

Disease groups	Specimens tested			Type of virus isolated																						Mixed strains
	type	number	positive	Polio					Coxsackie					ECHO												?	
				1	(1)	2	3	(3)	A	B2	B3	B4	B5	1	2	4	6	7	8	9	11	12	14	19		
(A) Poliomyelitis	Faeces	648	498	446	16	3	6	2	2	2	—	2	—	6	—	—	—	1	2	—	—	—	—	1	2	3P1 + E1; 1P1 + E8; 1P1 + E14; 1P1+?; 1E2+CA9
	CSF	23	0	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
	Miscell.	24	4	2	1	—	—	—	—	—	—	—	—	—	—	1	—	—	—	—	—	—	—	—	—	—
(B) Suspect poliomyelitis	Faeces	263	87	46	5	—	9	—	—	4	—	—	1	2	1	—	1	1	1	1	—	1	4	—	5	1P1+E7; 1P1+ +E17; 1P1+ CB4; 1P1+?; 1(P1+2+3)
	CSF	21	0	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
	Miscell.	19	1	1	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
(C) Aseptic meningitis or encephalitis	Faeces	299	37	13	5	—	2	—	—	3	—	1	1	—	—	1	—	—	—	2	—	—	—	—	9	—
	CSF	180	4	—	—	—	—	—	—	1	—	—	—	—	—	1	—	—	—	—	—	—	1	—	1	—
	Miscell.	25	1	1	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
(D) Non-neurological diseases	Faeces	420	74	12	2	—	10	—	2	7	2	4	2	4	1	2	1	6	—	—	4	1	3	—	9	1P1+E1; 1P3+CB4
	CSF	40	0	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
	Miscell.	148	6	—	—	—	2	—	—	2	—	1	—	—	—	—	—	—	—	—	—	—	—	—	1	—
(E) Poliomyelitis contacts	Faeces	218	87	79	—	—	—	—	—	2	—	—	—	3	—	—	—	—	—	—	—	—	—	—	—	1P1+E1; 1P1+CB2; 1P1+?
	Miscell.	1	1	1	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Total		2329	800	601	29	3	29	2	4	21	2	8	4	15	2	5	2	8	3	3	4	2	8	1	27	17

Note. ? = unidentified E = ECHO CB = Coxsackie B
P = Polio CA = Coxsackie A ( ) = Strains isolated after mass vaccinations with live attenuated polioviruses

Of the 1830 poliomyelitis cases which had occurred in 1959, 34.1 per cent were tested for virus (Table II). The monthly incidence of poliomyelitis, the monthly number of the cases tested, and the results are shown in Fig. 1. During the seven months between the onset of the epidemic (June) and the

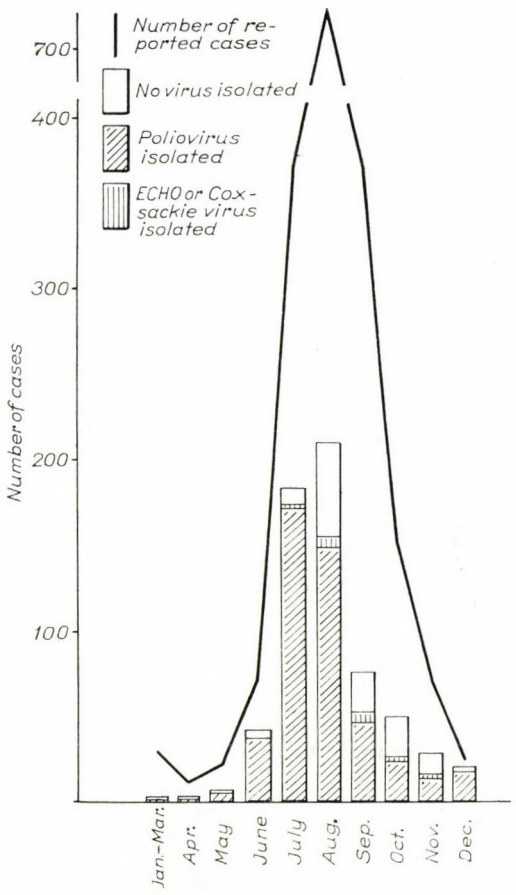

Fig. 1. Monthly distribution of the poliomyelitis cases tested, as compared with the monthly incidence of poliomyelitis

end of the year, 61, 50, 29, 21, 34, 42 and 80 per cent, respectively, of the cases reported as poliomyelitis were tested for virus. The poliovirus isolation rate, which was rather high during the first 5 months of the year and persisted at a high level during the first two epidemic months, remarkably decreased after the peak of the epidemic. The respective monthly rates from August to November were 71, 61, 46 and 44 per cent. In December the poliovirus isolation rate rose again, but the results were not comparable with the earlier ones because

of the mass immunizations with the live poliovirus vaccine in December. The drop in the isolation rate after the peak of the epidemic will be analyzed later.

In Fig. 2 the percentage occurrence of the different enteroviruses in groups A—D is illustrated, by months. It is clearly seen that the trend of the isolation rate for group B was the opposite of that for group A, *i.e.* simulta-

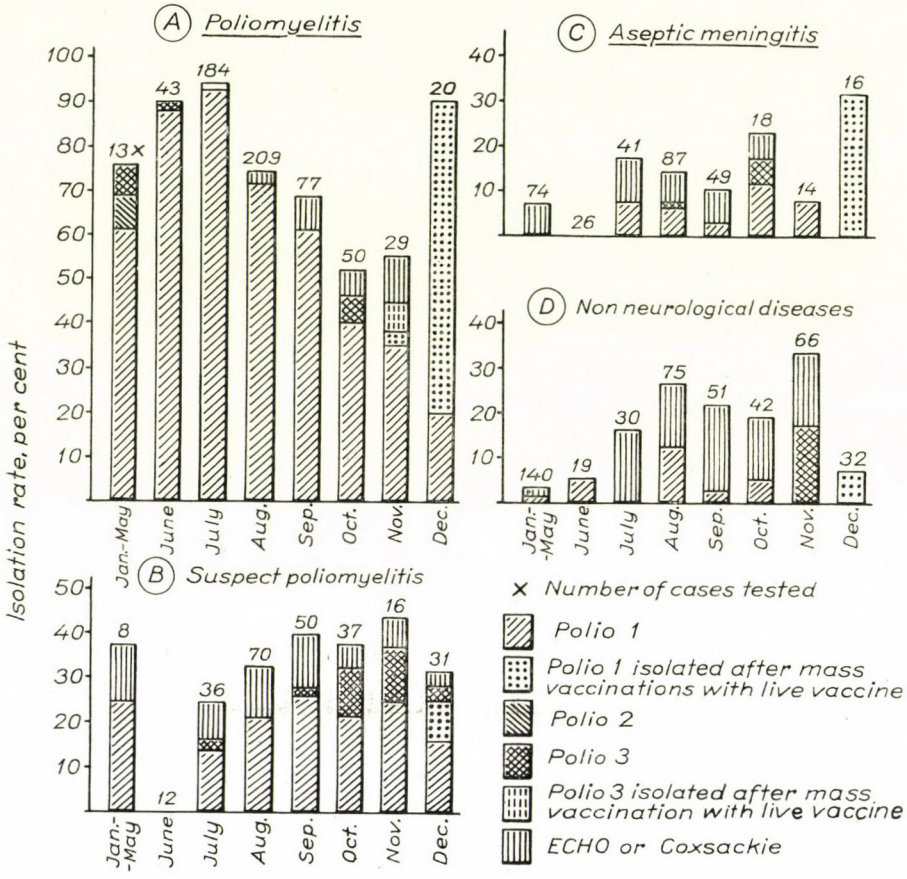

Fig. 2. Monthly isolation rates

neously with the decrease in the isolation frequency for patients with poliomyelitis there was an increase in the percentage of the suspect cases excreting virus. It is noteworthy that in June only poliovirus was isolated from each group of patients, whereas both earlier and later other enteroviruses also occurred. The frequency of the Coxsackie and ECHO viruses showed a gradual rise even in the group of officially registered cases of poliomyelitis. The data suggest that in the early stage of the epidemic the type 1 poliovirus suppressed dissemination of other enteroviruses. Infections by type 3 poliovirus were demon-

strable almost throughout the year. The incidence of such infections among patients with various diseases showed a remarkable rise after the peak of the epidemic.

The figures in Table II show that the age distribution of the cases examined by us agreed with that of the total cases of poliomyelitis reported in Hungary in 1959. Fewer than one third of the patients were examined in the group of the 6—9 years' old patients only.

Table II

Distribution, by age, of the reported, and of the tested cases of poliomyelitis

Age group in year	Reported cases Number	Tested cases	
		Number	Per cent
0—2	941	317	33.7
3—5	529	181	34.2
6—9	154	41	26.6
10—14	66	33	50.0
>15	139	53	38.1
Unknown	1	—	—
Total	1830	625	34.1

Fig. 3 illustrates the isolation rates analyzed by age and clinical diagnosis. The frequency of virus isolation showed gradual decrease with increasing age in each group, including group A. Regarding the cumulative data of groups A—D the decrease was most prominent in the frequency of type 1 poliovirus; somewhat less definite, but also gradual for type 3 virus. The rate of Coxsackie B virus isolation did not show a similar relation to age. In the 5 age groups illustrated in Fig. 3 the respective percentages of Coxsackie B viruses were 1.5, 1.5, 2.3, 1.7 and 2.8 per cent. Of the ECHO viruses the types 6, 8, 11, 12, 14 and 19 were not isolated but from subjects under 5 years of age, whereas the types 1, 2, 4, 7, 8 and 9 were isolated from older persons as well.

In group C the number of persons over 15 years of age was the highest, whereas that of children under 2 years was the lowest. The age distribution was thus different from that of the patients with poliomyelitis or the suspect cases under study. This difference suggests that the physicians were tended to report cases of aseptic meningitis occurring in childhood as poliomyelitis or suspect cases.

The number of the tested poliomyelitis cases for geographical area are illustrated in Fig. 4. The rates, tested cases per reported cases are presented in

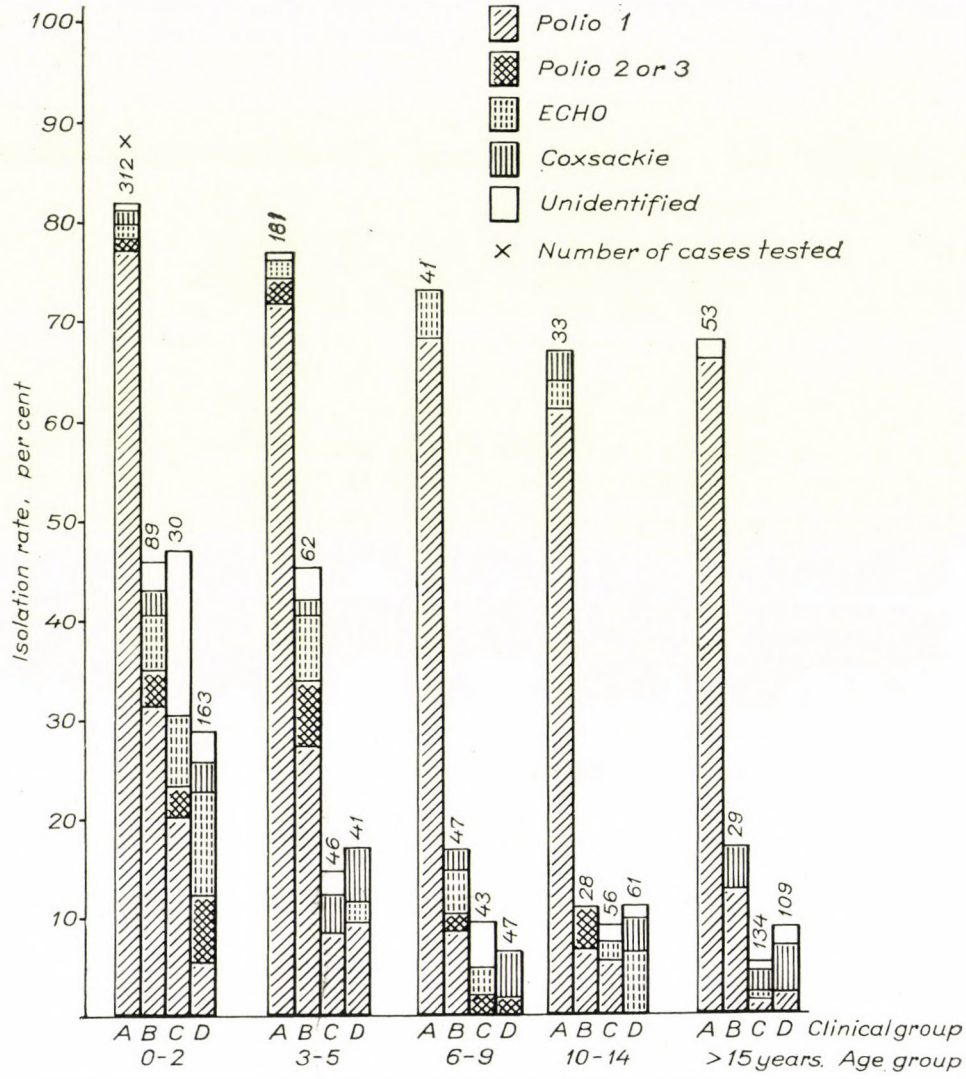

Fig. 3. Isolation rates for five age groups

parentheses. Approximately half (319) of the tested patients lived in Budapest or in Pest county. It should be added that 30.9 per cent of the patients reported as poliomyelitis were residents of these two areas. Budapest had reported 324, Pest county 242 cases.

Poliovirus was isolated from 90 and 81 per cent of the poliomyelitis cases reported from Budapest and Pest county, respectively. The percentage for Fejér county was similar (87 per cent). Elsewhere the isolation rates were lower, provided more than 20 cases were examined. The percentage of positive patients

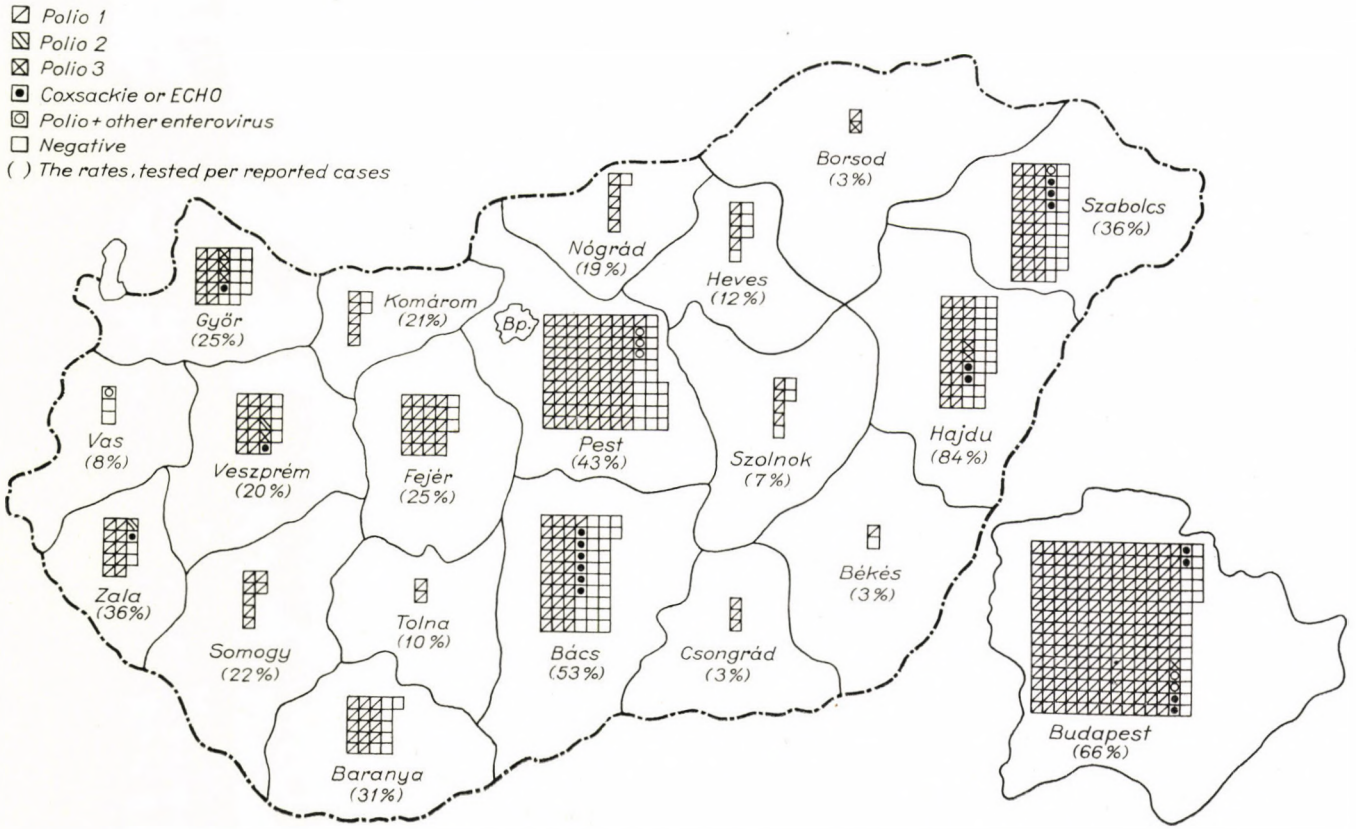

Fig. 4. Geographical distribution of the poliomyelitis cases tested

Table III

Neutralization tests with paired sera of patients reported as poliomyelitis, but yielding no virus

No.	Patient initials, sex, age	Symptoms reported	Serum**	Antibody titre*		
				polio 1	polio 2	polio 3
1.	Gy. P. ♂ 2 years	Disturbance of walk, absence of reflexes, meningeal symptoms	I	<4	<4	<4
			II	<4	<4	<4
2.	É. S. ♀ 5 years	Paresis of left lower extremity	I	>1024	16	<4
			II	>1024	n. t.	<4
3.	L. Sz. ♂ 9 years	Mild paresis of left lower extremity	I	>1024	16	>1024
			II	>1024	16	>1024
4.	R. I. ♀ 2 years	Partial quadriplegia, respiratory failure	I	<4	1024	<4
			II	<4	>1024	<4
5.	I. Sz. ♀ 6 years	Meningeal symptoms, slight disturbance in walk	I	>1024	>1024	64
			II	>1024	>1024	64
6.	É. K. ♀ 3 years	Meningeal symptoms, absence of patellar reflex	I	>1024	16	1024
			II	>1024	16	1024
7.	L. D. ♂ 21 yeras	Quadriplegia, respiratory paralysis	I	>1024	64	>1024
			II	>1024	>1024	>1024
8.	L. H. ♂ 3 years	Paralysis of both lower extremities	I	64	>1024	<4
			II	1024	n. t.	<4
9.	Gy. M. ♂ 10 years	Meningeal symptoms	I	1024	>1024	>1024
			II	1024	n. t.	>1024
10.	R. T. ♀ 3 years	Paralysis of limbs	I	256	16	<4
			II	256	16	<4
11.	E. B. ♀ 7 months	Paresis of right lower extremity	I	<4	<4	<4
			II	<4	<4	<4
12.	S. K. ♂ 7 years	Paresis of limbs	I	>1024	>1024	>1024
			II	>1024	1024	>1024
13.	I. M. ♀ 1 year	Paralysis of left lower extremity	I	>1024	<4	<4
			II	>1024	<4	<4
14.	Gy. P. ♂ 17 years	Limb pain, paralysis of bladder	I	256	1024	>1024
			II	256	>1024	>1024
15.	B. G. ♂ 4 years	Meningeal symptoms, paralysis of both lower extremities	I	<4	n. t.	n. t.
			II	>256	n. t.	n. t.
16.	J. K. ♂ 9 months	Mild paresis of left arm, meningeal symptoms	I	<4	n. t.	n. t.
			II	<4	n. t.	n. t.

* = Reciprocal of serum dilution.
**I = Acute sample. II = Convalescent sample. n. t. = Not tested.

was the lowest in Bács county (50 per cent). In the same county 9.7 per cent of the poliomyelitis patients tested were proved to excrete some other enterovirus.

The two patients excreting poliovirus type 2 lived not far from each other (the one in Veszprém county, the other in Zala county). Unlike this, type 3 poliovirus infections had occurred in many distant parts of Hungary, such as in the counties Borsod, Fejér, Hajdu, Pest, Szabolcs and Veszprém, and in the town Budapest. (The strains isolated from non-poliomyelitis cases are not illustrated in Fig. 4.)

Neutralization tests. Nineteen per cent of the poliomyelitis patients tested during the first week of illness did not yield virus. Serum pairs were available from 16 virologically negative patients. Fourteen serum pairs were tested for antibodies to all three types of poliovirus, whereas two pairs only for type 1 antibodies.

The results are shown in Table III. Two triple negative patients gave no antibody response. These cannot be considered to have suffered from poliomyelitis from aetiological aspect. Two patients showed antibody response to type 1, and one to type 2 poliovirus. The neutralization tests gave no appreciable information as to whether the other cases had been caused by poliovirus.

Considering the low incidence of virus excretion in group C, it can be stated that only few of the cases with the clinical diagnosis of aseptic meningitis or encephalitis had been caused by type 1 poliovirus (Table I). To check these results, neutralization tests against type 1 poliovirus were carried out in the paired sera of 89 virologically negative patients (Table IV).

Table IV

Antibody content to poliovirus type 1 of paried sera of patients with aseptic meningitis yielding no virus

		Titre of the 1st sample					Total
		<4	4	16	64	≧256	
Titre of the 2nd sample	<4	7	—	—	—	—	7
	4	1	4	—	—	—	5
	16	1	—	7	1	—	9
	64	—	2	1	1	1	5
	≧256	—	1	2	1	59	63
Total		9	7	10	3	60	89

Rise in titre was found in 8 cases suggesting that infection by poliovirus type 1 had occurred in 8.9 per cent of the cases tested between the first and the second sampling. In some of these cases poliovirus type 1 might have been the causative agent, but the possibility that the antibodies had been induced by accidental infection during the long period of hospitalization cannot be excluded.

Studies on contacts. These studies were performed in 4 children communities in each of which poliomyelitis had occurred. In a Budapest nursery the studies were performed with especial care. In this nursery a typical case of poliomyelitis was observed on June 16, 1959. The child was hospitalized, and poliovirus type 1 was isolated from her faeces. Two days after the onset faecal and blood samples were taken from the children (2—4 years of age) and the staff of the nursery. Sampling was repeated two weeks later. The results of the virus isolation experiments are summarized in Table V.

Table V

Virus excretion in a Budapest nursery following the observation of a single case of poliomyelitis

Virus excretion		Children		Adults
2 days after onset	17 days after onset	Number	Per cent	Number
Pl	Pl	28	81	0
Pl	—	7		0
—	Pl	2		1
n. t.	Pl	1		0
Pl	Pl + CB2	1		0
CB2	CB2	1	19	0
—	—	6		10
—	n. t.	2		3
n. t.	—	0		1

Explanation : Pl = Poliovirus type 1
CB2 = Coxsackie B2
— = Negative
n. t. = Not tested

According to the cumulative results of the tests with the two-day samples and the 17 day ones, poliovirus type 1 was isolated from 81 per cent of the children, none of which had been ill between the two samplings. Among the adult contacts only one proved to excrete virus. One of the children was excreting Coxsackie B2 virus at the time of the first sampling. This virus was not disseminated during the two weeks' period in spite of the conditions which were

rather favourable to the spreading of enteroviruses as shown by the rapid dissemination of the type 1 poliovirus.

The antibody content of the paired sera collected from the same nursery was titrated by the neutralization test. According to the data thus obtained 75, 69, 50 and 25 per cent of the children had antibodies to poliovirus 1, 2, and 3, and Coxsackie B2 virus, respectively, at the time of the first sampling. Titres 1 : 4 or higher were considered positive. An appreciable rise in titre was demonstrated only against poliovirus type 1; 97 per cent of the children had become positive by the end of the observation period and antibody response was demonstrated in the paired sera of a number of children who had had initial antibodies to poliovirus type 1. The serological state of the adults under study had remained unchanged.

Discussion

In Hungary the 1959 poliomyelitis epidemic was the first one subjected to an appropriate aetiological analysis. In addition to our studies MÉCS *et al.* reported on virological examination of the poliomyelitis cases observed in the town Szeged and its environs [4]. During past epidemics only limited numbers of cases were examined [2, 3, 4, 10, 11, 12], thus the type or the types of virus responsible for the epidemics could not be established reliably. The present study involved one third of the cases occurring in the year 1959, and the distribution by age and domicile of the cases tested was closely similar to that of the total number of cases. The data thus obtained allowed to draw some conclusions in addition to stating that the type 1 poliovirus had been responsible for the 1959 epidemic in Hungary.

Our failure to isolate poliovirus from 25 per cent of the clinically diagnosed poliomyelitis cases was attributable partly to methodological causes as suggested by the fact that three of 16 virologically negative patients gave appreciable antibody response to poliovirus. The role of other factors is suggested by the following data. (a) Of the 16 patients with poliomyelitis 2 had no antibodies to either of the three types, of poliovirus even in the convalescent serum samples. (b) The isolation rate considerably decreased after the peak of the epidemic. (c) In certain areas a cumulation of negative cases was observed.

The existence of serologically negative cases among those diagnosed as poliomyelitis suggests that a certain proportion of the diseases was not caused by poliovirus. It is thus reasonable to suppose that the cumulation of virologically negative cases in certain areas during the second half of the epidemic was due, at least partly, to false diagnoses. Considering the difficulties of the clinical diagnosis of poliomyelitis, it is not surprising that after the epidemic had developed, the physicians tended to report mild atypical cases as poliomyelitis.

Another factor should be taken into consideration in the analysis of the geographical differences. The patients living in Budapest or Pest county were hospitalized in the *László Central Hospital for Infectious Diseases*, which has been in close co-operation with this laboratory. They took samples soon after the admission of the patient, and the samples arrived in this laboratory on the day of sampling. In contrast to this, country hospitals usually took the samples at a later stage, and shipping needed at least 24 hours. Since the epidemic had arisen in Budapest, and spread in the country subsequently, the decrease in the isolation rate after the peak of the epidemic may partly be attributed to the gradually increasing percentage of county patients.

The poliovirus isolation rate for patients with poliomyelitis tended downward with increasing age. Hence one might think that false diagnoses were commoner among older subjects. In fact, however, the isolation rate was similarly related to age in the groups of non-poliomyelitis patients (groups B—D) suggesting that accidental excretion of poliovirus occurred most frequently among the youngest population. It is thus probable that a certain proportion of the patients with a false diagnosis of poliomyelitis were accidental excretors of poliovirus, and such cases occurred most frequently among the youngest patients.

The fact that in June type 1 poliovirus strains were isolated almost exclusively, suggests that the dissemination of this type of virus was most extensive at the beginning of the epidemic. This is thought to be another factor in the decrease of the isolation rate after the peak of the epidemic.

Although a certain proportion of the cases registered as poliomyelitis could not be accepted, the extent of the epidemic is properly characterized by the rough number of the registered cases, for many unreported or revoked suspect cases were due to poliovirus. This is shown by the four times higher incidence of polioviruses in group B than among patients with aseptic meningitis (group C) or non-neurological diseases (group D).

From 19 cases reported as poliomyelitis no poliovirus but ECHO or Coxsackie virus was isolated. Serum pairs were unfortunately not available from any of these cases. So the question whether the isolates were the only pathogens, or polioviruses were also involved, cannot be answered. We believe that for some of these poliomyelitis-like cases the isolated strains were responsible.

Type 2 poliovirus infection was found in three cases only. In two the infection was demonstrated by the isolation of type 2 virus, in the third one by the specific antibody response. Type 3 poliovirus infections were rare throughout the year, but widely spread over the country; their incidence began to rise after the peak of the epidemic. It should be noted that the majority of the type 3 infections did not appear as typical poliomyelitis. Type 3 strains were isolated from only 1.3 per cent of the poliomyelitis patients tested (group A); of the poliomyelitis-like cases 3.4 per cent, of the non-neurological cases

2.6 per cent yielded this type of virus. The three type 3 strains isolated by MÉCS *et al.* [4] in 1959 had also originated from non-poliomyelitis cases. The mild appearance of the type 3 infections might be attributed to the three years' Salk vaccination, but it might be supposed that the circulating strains were mild. The latter assumption is supported by the *rct/40*$^-$ character of 5 of the 11 type 3 poliovirus strains isolated from healthy children in Győr-Sopron county before the immunization campaign with the live poliovirus vaccine [13].

Our findings in the Budapest nursery were characteristic of the natural antagonism between enteroviruses. On the days following the observation of a case of poliomyelitis 75 per cent of the contact children proved to excrete the causative poliovirus. A single child had then excreted Coxsackie B virus and she did so two weeks later. Although the conditions for a dissemination of enteroviruses were favourable, as shown by the rapid spread of the type 1 poliovirus, only one new excretor of Coxsackie B2 virus was found at the time of the second sampling. Since 75 per cent of the children had no antibodies to the Coxsackie B2 virus, specific factors could not hinder the dissemination of this virus. Consequently, the interference between type 1 poliovirus and Coxsackie B2 virus seems to offer the best explanation for the findings.

The 1959 poliomyelitis epidemic in Hungary has provided evidence that type 1 poliomyelitis epidemics cannot be prevented by mass vaccinations with the Salk vaccine [7, 8]. This recognition led to the highly successful country-wide immunization campaign with the live poliovirus vaccine late in 1959 and early in 1960. Based on the excellent results of the campaign we hope that the 1959 epidemic offered the last opportunity of analyzing extensive poliomyelitis epidemics in Hungary.

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ASSOCIATION BETWEEN LIPID METABOLISM AND ANTIBIOTIC SENSITIVITY

I. THE LIPID COMPOSITION OF ANTIBIOTIC SENSITIVE AND RESISTANT STAPHYLOCOCCUS AUREUS STRAINS

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Summary. (i) The lipid composition and fatty acid constituents of two *Staphylococcus aureus* strains differing in antibiotic sensitivity have been compared. The sensitive strain contained 3.6 per cent lipids; the resistant strain, 5.3 per cent.

(ii) As to phosphatides, a considerable difference was found between the two strains. Of the total phosphatides the resistant *Staph. aureus* contained nearly 2 times, of the cephalin-type phosphatides nearly 2.5 times more than the sensitive organism.

(iii) The total lipid content and within this the phosphatide content of a highly antibiotic-sensitive *Streptococcus haemolyticus* strain was very low (2.5 and 0.7 per cent respectively).

(iv) As to fatty acids, no characteristic difference was found between the sensitive and resistant *Staph. aureus*. In the solid fraction of fatty acids 5 components were shown, namely arachic, stearic, palmitic and myristic acids and an unidentified fatty acid containing probably 19 carbon atoms.

(v) In the liquid fraction of fatty acids 5 main components were distinguished, none of which corresponded to an unsaturated acid. It was striking that unsaturated fatty acids comprised only a very small part of the lipids in both *Staph. aureus* strains.

The biological role of bacterial lipids has scarcely been studied. The high resistance to environmental effects of certain organisms, as *Mycobacterium tuberculosis* and members of the Pseudomonas group is explained by the protective action of lipids, in which these bacteria are richer than others. According to VÁCZI *et al.* [1, 2] with Gram-negative enteric bacteria there is a definite difference between the lipid content of strains sensitive to antibiotics, and of strains that have acquired resistance *in vivo* or *in vitro*; the resistant organisms namely contain more of these substances than the sensitive bacteria. These examinations revealed an association between antibiotic resistance and the lipid content of the cell. It is known that Gram-positive bacteria contain considerably less lipids than the Gram-negatives [3]. The participation of these substances in the constitution of Gram-positive cells has not been fully investigated and confirmed. In the present study the lipid content, the distribution of lipid fractions and fatty acids in an antibiotic-sensitive *Staph. aureus* (strain 100) and in a polyresistant (resistant to 6 antibiotics) *Staph. aureus* (strain 53) have been studied.

As a comparison, the lipid content and the composition of the lipid fractions of a highly antibiotic-sensitive *Str. haemolyticus* (strain T 25) was also examined.

Materials and methods

Test strains. Staph. aureus strain Duncan 100; international test strain, sensitive to antibiotics. *Staph. aureus* strain 53; resistant to penicillin, streptomycin, polymyxin, chloramphenicol, aureomycin, terramycin and erythromycin. *Str. haemolyticus* strain T 25; highly susceptible to antibiotics.

Antibiotic sensitivity was estimated by the disc and tube dilution methods as described previously [2a].

Determination of lipid content. Twenty-four hour standard broth shake cultures were used. The bacteria were harvested by supercentrifugation at 30 000 r. p. m. The wet bacteria were immediately transferred without washing to dry aceton and kept in the refrigerator until used. Before use the cells were dried under vacuum in the presence of phosphorus pentoxide. The bacteria were then finely ground and analytically measured in 4 to 8 g quantities into an extraction tube. Extraction was performed by use of a modified Soxhlet apparatus, with a 2 : 1 chloroform—methanol mixture for 24 hours. In order to remove water-soluble substances, the crude preparation was washed in water according to FOLCH *et al.* [4]. The remaining sample was dried in nitrogen under reduced pressure then treated with ethyl ether. The ether was evaporated *in vacuo* and the weight of the residue was determined. The material obtained was regarded as the total extractible lipids. The values were expressed as the lipid content of 100 g of dried bacteria.

Determination of bound lipids. After the extraction 5 to 10 g of dry bacteria were heated under a reflux in a solution containing equal parts of hydrochloric acid and water for 4 hours. Lipids were then extracted with petroleum ether. The lipids corresponded partly to unsaponifiable substances, partly to fatty acids originating from the decomposition of complex lipids.

Determination of lipid fractions. The total lipid content was dissolved in a small amount of ether, then the phosphatides were precipitated with aceton in large excess (25—30 parts). The mixture was left in the refrigerator for 6 to 8 hours then centrifuged. The deposit was dissolved in a small amount of ether and the cephalin-type phosphatides were precipitated by the addition of absolute alcohol, in large excess (10—15 parts).

Isolation of fatty acids. After the total lipid content had been dissolved in a small amount of ether, 5 per cent methyl alcoholic solution of potassium hydroxide was added in excess and the mixture was left to stand in nitrogen atmosphere for 18 to 24 hours at room temperature. During that time hydrolysis became practically complete. The mixture was then diluted with water and unsaponifiable lipids were separated with petroleum ether. Then the aqueous solution was acidified to pH 2—3 with 10 per cent hydrochloric acid and the liberated fatty acids were extracted with petroleum ether. After removal of the acid components by washing in water, the petroleum ether fraction was dried first over anhydrous sodium sulphate then in nitrogen in a vessel of known weight. In order to simplify the analysis, the obtained fatty acid mixture was separated into *solid* and liquid fractions by TWITCHELL's lead-salt alcohol method [5]. The solid fraction consists primarily of saturated acids with a melting point over 25° C. The members of the liquid fraction are usually unsaturated fatty acids with a melting point lower than 25° C. The liquid fraction may, of course, contain saturated acids with a melting point under 25° C, and *vice versa*, in the solid fraction there may be higher, poorly saturated acids which melt over 25° C. The separation — in addition that it makes the analysis more easy to perform — allows the enrichment of components occurring in very slight quantities.

Determination of fatty acids. The chromatographic procedure described by KAUFMANN and NIETSCH [6] was used as follows.

Schleicher-Schuell No. 2043/B paper was impregnated with a fraction of petroleum (boiling point 190—220° C). A 90 per cent watery solution of acetic acid saturated with petroleum served as the solvent. To obtain good separation of fatty acids, the solvent front had to run as far as 30 to 35 cm. Depending on the temperature, this was reached within 36 to 50 hours. After the removal of petroleum and solvent, the chromatogram was dipped in aqueous copper acetate solution in order to form copper salts of fatty acids. The excess copper acetate was washed out with tap water and then the spots were developed with dilute rubeanic acid. The various fatty acids were indicated by dark-green spots on white background. Unsaturated fatty acids were detected with osmium tetroxide vapours, which blackened their spots. In order to identify the spots, parallel chromatograms were run with mixtures of known fatty acid composition. Quantitative estimation was performed by densitometric measurement.

Results

The results concerning the total lipid content of the two *Staph. aureus* strains differing in antibiotic sensitivity are presented in Table I. The total extractible and strongly bound lipid content of *Staph. aureus* strain 100 (sensitive), of *Staph. aureus* strain 53 (polyresistant) and of *Str. haemolyticus* strain T/25 (highly sensitive) are shown in columns 1, 2 and 3, respectively.

Table I

The lipid content of various organisms in dry weight percentage
Average data of 5 parallel cultivations. Limits of error: ±10%

	Staph. aureus 100	*Staph. aureus* 53	*Str. haemolyticus* T/25
	per cents		
Extractible lipid	3.1	4.4	2.4
Strongly bound lipid	0.5	0.9	0.1
Total lipid	3.6	5.3	2.5

The results were obtained by averaging the analysis of 5 parallel cultivations. The differences were not more than ± 10 per cent.

From Table I it is seen that the extractible lipid and the bound lipid content of the resistant *Staph. aureus* is considerably higher than that of the sensitive strain. The percentage difference is 42, or, as regards the total lipid content, 47. The extractible and bound lipid contents of the highly sensitive *Str. haemolyticus* strain T/25 were considerably less than those of the sensitive *Staph. aureus* strain (2.4 and 2.5 per cent, respectively).

In subsequent experiments the lipid composition of sensitive and resistant staphylococci was compared. The neutral lipids were separated from the biologically active lipids (phosphatides). The latter were further fractionated into lecithin and cephalin-type phosphatides. The results are presented in Table II.

Table II

Composition of extractible lipids of antibiotic sensitive and polyresistant Staph. aureus strains in dry weight percentage
Average data of 5 parallel cultivations. Limits of error: ±10%

		Staph. aureus		Difference
		strain 100	strain 53	
		per cent		
Neutral lipids		1.3	1.0	−23
Phosphatides	Lecithin	1.3	2.2	+69
	Cephalin	0.5	1.2	+140
Extractible lipids		3.1	4.4	+42

The extractible lipid fractions of the sensitive and resistant staphylococci are shown in column 1 and 2 of Table II. Column 3 shows the percentage difference between the lipids of the two strains, where the data obtained with the sensitive organism are taken as 100. It is seen that the lipid content of the resistant strain was 42 per cent more than that of the sensitive one. The sensitive *Staph. aureus* was richer in neutral lipids, while the resistant strain contained more of the phosphatides. The phosphatide content in the latter was nearly twice that in the former (3.4 as compared to 1.8 per cent). The difference was remarkably high as regards the cephalin-type phosphatides, from which the resistant organism contained nearly 2.5 times more than the sensitive strains.

Taking into consideration the high difference in the lipid composition of the antibiotic-sensitive and -resistant staphylococci, a comparison was made between two Gram-positive cocci, namely *Staph. aureus* strain Duncan 100 and *Str. haemolyticus* strain T/25, which, although both sensitive to antibiotics, differed in the degree of sensitivity. The results are shown in Table III.

Table III

Composition of extractible lipids of Staph. aureus and Str. haemolyticus in dry weight percentage
Average data of 5 parallel cultivations. Limits of error: ±10%

		Staph. aureus strain 100	*Str. haemolyticus* strain T/25	Difference
		per cent		
Neutral lipids		1.3	1.7	+31
Phosphatides	Lecithin	1.3	0.5	—62
	Cephalin	0.5	0.2	—60
Extractible lipids		3.1	2.4	—23

In column 1 of Table III the lipid fractions of the sensitive staphylococcus, in column 2 those of the highly sensitive streptococcus are presented. As it is clear from the differences set out in column 3, *Str. haemolyticus* contained less of the lipids, and within these, much less of the phosphatides, than the sensitive *Staph. aureus* did. The former was richer only in neutral lipids. Thus the difference existing between these strains was of the type observed at the comparison of sensitive and resistant staphylococci.

In order to gain further information of the bacterial lipids, the fatty acid constituents were analysed. As very small amounts (10 to 80 mg) of fatty acids were only available, the analysis had to be performed with paper chromatography. The results are presented in the following tables. The data refer to mol%; the results could not be expressed in exact weight%, because the molecular weight of some components was not known.

Table IV

Distribution of solid fraction fatty acids obtained from Staph. aureus strains

Designation of spot	*Staph. aureus* strain 53	*Staph. aureus* strain 100
	per cents	
5	27.3	25.0
4	20.3	17.8
2—3	30.2	29.8
1	22.2	27.4

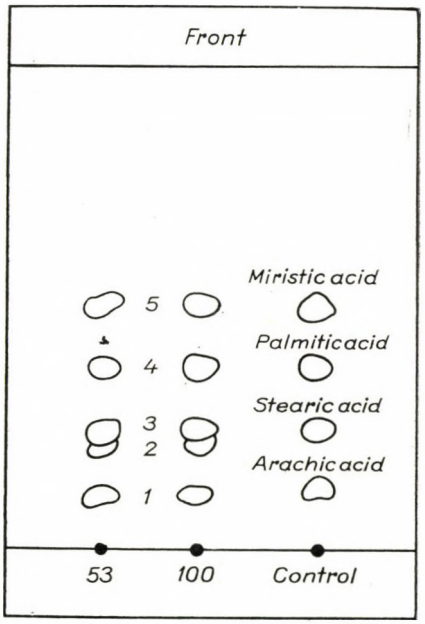

Fig. 1. Characteristic chromatogram of solid fraction fatty acids obtained from *Staph. aureus* strains

Fig. 1 and Table IV show the characteristic chromatogram of the solid fatty acid fraction isolated from *Staph. aureus*, and the percentage distribution of the components. Spots denoted with 1, 3, 4 and 5 were undoubtedly identical with those of the control fatty acids. Thus they corresponded to arachic, stearic, palmitic and myristic acids, in this succession. Spot 2 was a fatty acid presumably containing 19 carbon atoms. Spots 2 and 3 could be evaluated only together. In the data the second decimal was rounded up. From Table IV it can be concluded that no characteristic difference exists between the solid fraction fatty acids of the two strains.

Table V

Percentage distribution of liquid fraction fatty acids obtained from Staph. aureus strains

Designation of spot	*Staph. aureus* strain 53	*Staph. aureus* strain 100
	per cents	
5	74.8	71.9
4	21.3	23.5
2—3	2.2	3.4
1	1.7	1.2

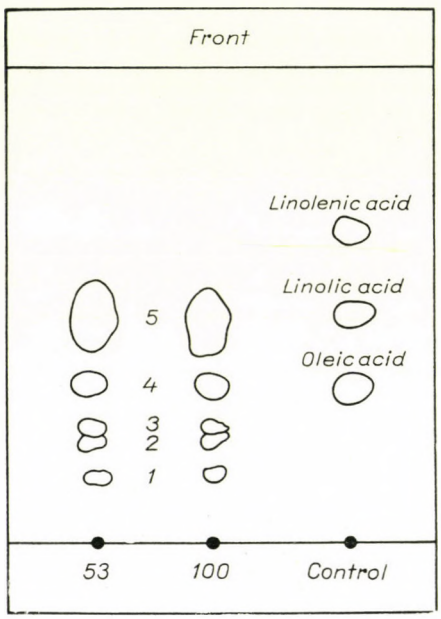

Fig. 2. Characteristic chromatogram of liquid fraction fatty acids obtained from *Staph. aureus* strains

Fig. 2 and Table V show the characteristic chromatogram and the percentage distribution of the liquid fraction fatty acids in the two staphylococcal strains. By the usual technique 5 spots appeared; osmium tetroxide vapours produced 3 smaller spots with the resistant strain and 1 smaller spot with the sensitive organism. The major part of the fatty acids showed up in spots 4 and 5. These two spots contained about 90 per cent of the liquid fraction acids.

Indicating a low content of unsaturated compounds, the spots showed slight blackening on exposition to osmium tetroxide vapours. Despite the fact that these were arranged as spots of oleic and linolic acids, they corresponded to unidentified saturated fatty acids. This was unexpected, as unsaturated fatty acids are important constituents of the cell. The presence of these sub-

stances makes it possible that under normal conditions, at body temperature, the lipids are maintained in the fluid state, which is required for the normal functioning of the cells.

In order to prove the lack, or the presence of only small quantities, of unsaturated acids, the liquid fractions of the two bacteria were examined as follows. In the first step the sample was treated with alkaline potassium hypermanganate at room temperature as described by CROMBIE *et al.* [7]. Under these circumstances potassium hypermanganate breaks up the unsaturated bonds. On the chromatogram the subsequently isolated fatty acids showed no blackening with osmium tetroxide. Spots 1 to 5 appeared in about the same size as obtained at chromatography of the untreated liquid fraction.

The fatty acid spots were examined in hydrogenation experiments in the presence of a highly active palladium catalyst, under a pressure of 2 atm. The chromatogram of the fatty acids treated this way showed no important difference from that of the untreated samples. Only one or two smaller spots indicated the very low unsaturated acid content of the fraction.

Discussion

Lipids constitute only a small part of the bacterial cell, and so, because of the number of their components and the imperfect analytical methods, little progress has been made in their examination. Owing to the basic investigations of ANDERSON [8], the lipid constitution of *M. tuberculosis* is the one best understood. Yet, in spite of numerous studies on this subject, the whole lipid spectrum of this organism has not been elucidated. The constitution of its phosphatides, which, according to DE SÜTŐ NAGY and ANDERSON [9] comprise 6 per cent of the total lipids, is unknown.

Of Gram-positive bacteria, the 7 per cent lipid-containing *C. diphtheriae* has been investigated more thoroughly in this respect [10].

Of the investigations into the lipids of Gram-negative enteric bacteria those of ECKSTEIN [11], WESTPHAL *et al.* [12], BOIVIN *et al.* [13] and the more recent examinations of CMELIK [14] should be mentioned. The analytical methods applied by these authors are not uniform, thus the results can be compared with difficulty or not at all.

VÁCZI *et al.* [1, 2] showed differences between the lipid and especially the phosphatide constitution of chloramphenicol-sensitive and -resistant *E. coli* and *S. paratyphi B.* From these investigations it has been concluded that an association exists between the lipid content, lipid metabolism and antibiotic sensitivity of Gram-negative enteric bacteria.

The purpose of the present studies was the determination under constant circumstances of the lipid content, the lipid fractions and fatty acids of two *Staph. aureus* strains with very different antibiotic sensitivity. The experiments were completed with the lipid analysis of a *Str. haemolyticus* strain.

It was found that staphylococci contained relatively high amounts of lipids (3.6 per cent on the average). The lipid content of the resistant *Staph. aureus* (5.3 per cent) was 47 per cent more than that of the sensitive strain. The lipid content of the highly antibiotic-sensitive *Str. haemolyticus* was the lowest (2.5 per cent).

Beyond the differences in total lipid content found in repeated experiments, the differences in lipid fractions may be regarded as significant. The high quantitative difference between the two staphylococcal cells as to the biologically active lipid fraction shows that these materials may be important factors in the determination of antibiotic susceptibility. This consideration is supported by the finding that the difference between the lipid content of the highly sensitive *Str. haemolyticus* and of the sensitive *Staph. aureus* was the most considerable as regards the phosphatides.

Of our data concerning the fatty acid components of staphylo-lipids, two points should be emphasized. One of these is that in this respect *no significant difference was found between the two cells differing in antibiotic sensitivity*, the other is that *unsaturated fatty acids occurred only in very small quantites.* According to CMELIK [14], in Salmonellae saturated and unsaturated fatty acids are present in approximately equal amounts. Our recent examinations also showed that a considerable part of the fatty acids of *E. coli* corresponded to unsaturated acids. The fact that in the liquid fraction of staphylococcal fatty acids no larger amounts of unsaturated acids could be demonstrated by different methods, although the melting point of these lipids was very low, points to the extraordinary structure of these materials.

The question thus arises, how the nature of fatty acids of the liquid fraction that do not give the characteristic reactions of unsaturated acids can be explained. If they are saturated, a linear-chain structure seems unlikely, since they are fluid at room temperature. It may be assumed that these substances correspond to forked-chain fatty acids. It is known that the melting point of palmitic acid is 62.2° C, while 9-methyl palmitic acid melts at 22° C and 11 methyl palmitic acid at 19° C. The melting point of 11-methyl stearic acid is only 8° C. Thus in the examined bacteria the fluidity of fatty acids is probably due to a forked-chain structure. Investigations into the finer structure and composition of these fatty acids are in progress.

The boundary surface consisting of complex lipids and lipoproteins plays an important part in the entrance to the cell and in the intracellular movement of materials acting on the bacterial organism. The influence of lipids on the biological properties of the cell is unquestionably important. The present investigations have revealed differences between the lipid constitution of Gram-positive cocci with different antibiotic sensitivity. The difference has been found the highest with the biologically active phosphatides.

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ASSOCIATION BETWEEN LIPID METABOLISM AND ANTIBIOTIC SENSITIVITY

II. THE INFLUENCE OF ESTERASE INHIBITORS ON THE ANTIBIOTIC SENSITIVITY OF STAPHYLOCOCCUS AUREUS STRAINS

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Summary. (i) The effect of esterase inhibitors on the antibiotic sensitivity of *Staphylococcus aureus* has been investigated. It was shown that staphylococcal strains sensitive to antibiotics or resistant only to penicillin exerted a higher lipolytic activity than polyresistant strains.

(ii) Of the esterase inhibitors paraoxone, methyl parathiooxone and sodium oleate at concentrations of 0.5 mg per 100 ml, considerably increased the penicillin sensitivity of resistant staphylococci; a slight increase was observed in terramycin sensitivity.

(iii) While the endopenicillinase activity of staphylococci was decreased only slightly by high concentrations of methyl parathiooxone, formation of the enzyme was strongly inhibited.

(iv) The phosphatide content of polyresistant staphylococci grown in the presence of methyl parathiooxone decreased considerably. The lipid composition of strains cultivated under this condition became similar to that of the sensitive organisms.

(v) The aspects of aimed drug research based on the association between lipid metabolism and antibiotic sensitivity are discussed.

In the preceding paper a comparison between polyresistant and antibiotic-sensitive *Staph. aureus* strains has revealed a higher lipid content in resistant bacterial organism than in sensitive ones. The distribution of lipid fractions also differed in the two kinds of bacteria [1]. A similar difference in lipid constitution was found between antibiotic-sensitive and -resistant Gram-negative enteric bacteria (*E. coli*, Salmonella) [2]. From these findings it has been concluded to an association between lipid metabolism and antibiotic susceptibility. In the present paper we shall present experiments aimed at establishing whether the lipolytic activity of *Staph. aureus* varies with antibiotic sensitivity, and, on the other hand, whether esterase inhibitors acting on the enzymes of lipid metabolism exercise any influence on the antibiotic-sensitivity of this organism.

Materials and methods

Test strains. The antibiotic sensitivity of the *Staph. aureus* strains used was as follows. Strain 100 and 102: sensitive to antibiotics: strain 1: resistant only to penicillin; strains 50, 52 and 53: resistant to penicillin, streptomycin, aureomycin, terramycin, chloramphenicol and erythromycin. In the lipase activity experiments 77 *Staph. aureus* strains freshly isolated from pathological processes were also included. All the strains were coagulase-positive, produced haemolysin and split mannitol.

Assay of lipolytic activity. Lipase activity was estimated as described by Sierra [3]. As substrates, Tween 20, 40, 60 or 80 at 1 per cent concentrations together with 0.01 per cent calcium chloride were incorporated in melted agar medium. The agar was then poured into

Petri dishes and the strains to be examined were streaked on the surface of the plates. Readings were made after an incubation for 48 hours at 37° C. Lipase activity was indicated by zones of calcium soap around the colonies.

Antibiotic sensitivity was estimated by the disc and tube dilution method as described in reference [4].

Assay of penicillinase activity. Penicillinase activity was measured in a Warburg apparatus by the manometric method of HENRY and HOUSEWRIGHT [5]. Into the centre vessel 2.5 ml 0.047 *M* sodium hydrocarbonate and 1 ml of bacterial suspension, into the side arm 50 000 units of Penicillin-G-Na dissolved in 0.5 ml 0.047 *M* sodium hydrocarbonate were measured. In the tubes a gas mixture containing 5 per cent CO_2 and 95 per cent N_2 was circulated for 30 minutes. Measurements were carried out at 37° C.

Results

Table I shows the lipolytic activity of 77 pathogenic *Staph. aureus* strains with varying antibiotic sensitivity.

While more than 80 per cent of the strains sensitive to antibiotics or resistant only to penicillin exhibited lipolytic activity, the 31 polyresistant strains were lipase-positive only in 45.6 per cent. With the qualitative method the lipase activity of the first group of strains seemed considerably stronger than that of the polyresistant organisms. Thus it may be concluded that sensitive and polyresistant staphylococci differ in enzymes participating in the lipid metabolism. Therefore it was assumed that the two groups of organisms might differ in sensitivity to the blocking action of substances inhibiting these enzymes.

Table I

Lipolytic activity of Staph. aureus strains with varying antibiotic sensitivity

No. of strains	Antibiotic sensitivity	Decomposition of Tween	
		percentage of strains	
		positive	negative
17	sensitive	83	17
29	penicillin-resistant	83	17
31	polyresistant	45	55

In subsequent experiments the effect of various esterase inhibitors was examined on the penicillin- and terramycin-sensitivity of an antibiotic-sensitive (strain 100), a penicillin-resistant (strain 1) and a polyresistant (strain 53) *Staph. aureus*. Estimation of sensitivity was performed by means of antibiotic-containing discs. The esterase inhibitors were incorporated into agar medium at concentrations of 0.5 to 0.1 mg per 100 ml. These concentrations did not influence the growth of the cultures.

As shown in Table II, of the esterase inhibitors mainly the paraoxone-type compounds and sodium oleate had a definite effect on the antibiotic sensitivity. These substances highly increased the penicillin sensitivity of the penicillin-resistant and polyresistant strains. The susceptibility of the penicillin-

Table II

Effect of esterase inhibitors on the penicillin- and terramycin-sensitivity of Staph. aureus
Average of 3 parallel experiments. Limits of error: ±5%

Esterase inhibitors	Concentration, mg%	Diameter of inhibition zone, mm					
		Sensitive strain		Penicillin-resistant strain		Polyresistant strain	
		penicillin	terramycin	penicillin	terramycin	penicillin	terramycin
Sodium fluoride	0.5	32	29	0	8	0	0
Tetraethyl pyrophosphate	0.5	31	32	13	13	0	0
Parathiooxone (Ekatox)	0.1	34	34	27	16	31	17
Methyl parathiooxone (Wofatox)	0.01	27	30	20	12	30	16
Sodium oleate	0.06	31	29	19	8	20	0
Control		29		8		0	

sensitive strain was only slightly increased. A less well-defined increase in sensitivity appeared against terramycin. The enzyme inhibitors did not increase the sensitivity against other antibiotics; on the contrary, in some strains tetraethyl pyrophosphate caused a marked decrease in streptomycin sensitivity.

The penicillin-sensitivity-increasing effect of paraoxone-type compounds and sodium oleate was uniform not only with the above strains, but with 20 antibiotic resistant *Staph. aureus* strains isolated from pathological materials. It was therefore thought necessary to examine the mechanism of the phenomenon. In subsequent experiments methyl parathiooxone was used as an esterase inhibitor.

Table III shows the effect of 0.5 mg per 100 ml methyl parathiooxone on the penicillin sensitivity of 2 sensitive, 1 penicillin resistant and 3 polyresistant *Staph. aureus* strains. The experiments were performed in broth with the tube dilution method [4]. It is clear that while the penicillin susceptibility of antibiotic-sensitive strains (100 and 102) changed only slightly or not at all, with the penicillin-resistant (1) and polyresistant (50, 52 and 53) cultures a 64 to 128-fold increase was found. Densitometric measurements showed that methyl parathiooxone at a concentration of 0.5 mg per 100 ml did not influence the growth of the cultures. Thus the increase in penicillin sensitivity could not be due to an inhibitory effect on the viability or multiplication of the bacteria.

Table III

Effect of methyl parathiooxone on the penicillin sensitivity of Staph. aureus strains

Strain	Antibiotic sensitivity	Penicillin sensitivity units/ml		Increase in sensitivity
		0.5 mg per 100 ml M. P. T.	control	
100	sensitive	0.03	0.03	0
102	sensitive	0.03	0.06	2×
1	penicillin-resistant	1.56	100.0	64×
50	polyresistant	1.56	200.0	128×
52	polyresistant	3.12	200.0	64×
53	polyresistant	1.56	100.0	64×

As esterase inhibitors caused an increase in sensitivity especially to penicillin, the effect of methyl parathiooxone on intracellular penicillinase activity was examined. The experiments were carried out by the use of a Warburg apparatus as described by HENRY and HOUSEWRIGHT [5]. A 24 hour broth culture of strain 53 was washed and part of the bacteria was dried by treatment with acetone and ether. The results obtained with living and dried bacteria are presented in Table IV.

Table IV shows the penicillinase activity of strains 53 in the presence of various concentrations of methyl parathiooxone (1.0—25 mg per 100 ml). It is seen that by this material the endopenicillinase activity was only slightly decreased. The high increase in sensitivity when the strains were grown in the presence of this substance thus could not be explained by a direct effect on the enzyme. It should be noted that the penicillin sensitivity of the exopenicillinase-producing *B. subtilis* was not altered in the presence of methyl parathiooxone.

Table IV

Penicillinase activity of a polyresistant Staph. aureus strain at different concentrations of methyl parathiooxone

Average of 3 parallel experiments

Preparation	Concentration of M. P. T. mg per 100 ml	μl CO_2/mg bacterium/hour
Living suspension	0	117
	1	106
	5	95
Acetone dried bacteria	0	43.5
	1	42.6
	5	42.5
	25	34.6

In the following experiments the influence of methyl parathiooxone on the endopenicillinase production by *Staph. aureus* was investigated. The data as to whether a difference exists in the endopenicillinase formation by the organism cultured with or without methyl parathiooxone are presented in Table V.

Table V

Penicillinase activity of polyresistant Staph. aureus at different times of growth in broth shake culture containing methyl parathiooxone 0.5 mg per 100 ml

Average of 3 parallel experiments. Limits of error: ±10%

Age of culture hours	μl CO_2/mg bacterium/hour		Decrease of activity, %
	Control culture	cultured with M. P. T.	
4	91	103	+12
6	96	30	—67
8	131	35	—76
24	224	20	—91

It is clear that the penicillinase activity of the culture grown in the presence of 0.5 mg per 100 ml methyl parathiooxone was one tenth of that of the control culture. Thus the esterase inhibitor caused a metabolic change in the cells which resulted in a considerably decreased production of the enzyme. That mainly penicillin sensitivity was affected was obviously due to this blocking action. The inhibitory effect appeared after the start of logarithmic growth, that is, after the 4th to 6th hour of cultivation. After the 6th hour the intracellular penicillinase activity of the cells decreased and remained at a low level until the end of cultivation. At the 24th hour it was only 10 per cent of the activity exhibited by the control culture. In the lag phase the activity of the treated culture was even somewhat higher than that of the control. Accordingly, methyl parathiooxone acts in the logarithmic phase of growth and exerts no blocking effect in the lag phase. It was questionable whether the lipid composition of cells which gained an increased penicillin sensitivity this way, was not altered during the cultivation with methyl parathiooxone.

Table VI shows the results of the lipid analysis of *Staph. aureus* strain 100 (sensitive) and strain 53 (polyresistant) after cultivation in the presence or in the absence of methyl parathiooxone. From the table it can be seen that when cultured in the presence of this substance, the lipid composition of the polyresistant strain became similar to that of the sensitive organism. In the resistant strain the neutral lipid—phospholipid ratio was 1 : 3, in the sensitive strain in was nearly 1 : 1. Methyl parathiooxone decreased the total phosphatides so that the neutral lipid—phosphatide ratio became similar to the ratio observed in sensitive cultures.

Table VI

Changes in lipid constitution of Staph. aureus cultivated in the presence of methyl parathiooxone, 0.5 mg per 100 ml

Average of 2 parallel experiments. Limits of error: ±10%

Strain		Lipid content, %		Difference, %
		control culture	cultured with M. P. T.	
Staph. aureus strain 100 (sensitive)	Neutral lipids	1.3	1.2	—8
	Phosphatides			
	(a) Lecithin	1.3	1.2	—8
	(b) Cephalin	0.5	0.4	—25
	Total	3.1	2.8	—11
Staph. aureus strain 53 (polyresistant)	Neutral lipids	1.0	1.3	+30
	Phosphatides			
	(a) Lecithin	2.2	0.9	—59
	(b) Cephalin	1.2	0.3	—75
	Total	4.4	2.5	—43

Discussion

The increasing resistance to antibiotics of some microorganisms, such as staphylococci, has been observed all over the world. It is important to gain exact knowledge of the mechanism by which various bacterial cultures exhibit a differing degree of antibiotic sensitivity. The elucidation of the metabolism of resistant cells may contribute to an aimed drug research, by allowing the synthesis of specifically acting new substances.

Esterases play a basic part in lipid metabolism. From the increased lipid content it can be concluded that an extreme degree of enzymatic activity connected with the formation and decomposition of lipids is displayed by the resistant cell. The role of esterases in the metabolic processes has not been cleared sufficiently. While according to LUBERT *et al.* [6] bacterial lipases are of wide specificity, the aliesterases and aromesterases possess a much narrower substrate specificity. Although aliesterases are present in every living cell, their physiological function is not exactly known. According to MENDEL *et al.* [7], unlike in lower organisms (viruses, bacteria and fungi), in higher organisms these enzymes are not essential for the function of the cells. This opinion has been confirmed by the examinations of RUYS [8] and MYERS *et al.* [9], who showed that aliesterase inhibitors hinder the growth of *Mycobact. tuberculosis*. In contrast to the finding of CUTCHINS *et al.* [10], who stated that bacterial esterases are non-specific, SIERRA [11] and GREUELL and SIERRA [12] showed various specific esterases in Pseudomonadaceae.

The present data revealed a direct association between the lipid metabolism and antibiotic sensitivity of staphylococci. The association is especially definite in connection with penicillin resistance. According to COOPER [13], a lipid-like material in the outer layer of the cell is responsible for the binding of penicillin. The actual quality of this material may influence the endopenicillinase production, and, in connection with this the penicillin sensitivity of the organism.

The biological role of bacterial lipids is not exactly known, although their importance in the permeability of the cell has been established. These substances undoubtedly play a part in the higher resistance to environmental effects of certain bacteria *(Mycobacterium tuberculosis, Pseudomonas pyocyanea)*. In case of polyresistant staphylococci, antibiotic resistance is a problem closely connected with the permeability of the cell. Accordingly, the association between the amounts of active lipids present and the antibiotic sensitivity is obvious.

On the basis of the present results it is thought that in aimed systematic research of drugs against resistant microorganisms the above findings should be taken into consideration.

MARUZELLA and BLOCK [14] demonstrated that certain plant oils strongly increase the effect of penicillin on staphylococci. In this case the increased sensitivity was probably due to the inhibited endopenicillinase production caused by the specific esterase-inhibiting effect of oleic acid.

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A VITAMIN B_{12} ANTAGONIST ISOLATED FROM PROPIONBACTERIUM SHERMANII

I. ISOLATION, STRUCTURE AND MICROBIOLOGICAL EXAMINATIONS

By

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(Received May 26, 1960)

Summary. Together with vitamin B_{12} isolated from *Propionbacterium shermanii* a red-coloured factor (Factor X) was discovered by paper chromatography. Autobiographic examination revealed that in contrast to vitamin B_{12} this factor exerted an inhibitory (anti-vitamin) effect on the growth of *E. coli*. The acid substance was isolated in crystalline form. The inhibition index of Factor X was determined and it was demonstrated to be a competitive inhibitor of vitamin B_{12}.

Shortly after the isolation of crystalline vitamin B_{12} from the liver certain microorganisms were found to be able to produce this substance RICKES *et al.* succeeded in isolating crystalline vitamin B_{12} from *Streptomyces griseus* [1]. Recently it has been demonstrated that under adequate conditions vitamin B_{12} may be produced also by other microorganisms [1—4]. Nevertheless these microorganisms simultaneously produce also different vitamin B_{12} analogues displaying different biological and microbiological activity.

In the present paper a vitamin B_{12} derivative, isolated from *Propionbacterium shermanii* will be described. Contrary to vitamin B_{12} this derivative inhibited the growth of *Escherichia coli*, exerting an anti-vitamin effect.

Materials and methods

Microbiological determination of vitamin B_{12}. The microbiological activity of vitamin B_{12} was examined by the agar plate method using an *E. coli* mutant cultivated for 16 to 18 hours on the agar medium described by TSHAIKOVSKAYA and DRUZHININA [5].

Photometric determination of vitamin B_{12} and Factor X. Pure vitamin B_{12} was estimated by photometry by means of Pulfrich's photometer: extinction was measured at 530 mμ. From the extinction value the concentration of vitamin B_{12} was determined by the equation

$$\mu g \text{ Vitamin } B_{12}/ml = \frac{E_{530} \times 10^4}{63}$$

As according to our observations the ultraviolet absorption spectrum of Factor X corresponds to that of vitamin B_{12} — both having their maximum at 550 mμ, — the concentration of Factor X in pure solutions free of yellow and brown colours was determined similarly as vitamin B_{12}.

Fermentation was performed with a strain of *Propionbacterium shermanii*, at pH 5.2 to 5.5, under anaerobic conditions for 120 to 160 hours. The medium contained corn steep liquor, starch sugar and 5 μg/ml Co $(NO_3)_2$ [6, 7, 8]. As propionic acid, a by-product of fermentation would acidify the mixture, solid $CaCO_3$ was added to buffer the fermentation mixture.

Twenty-four hours preceding the completion of the fermentation process, 1 g/m³ 5.6 dimethyl-benzimidazol was added to the medium to convert the factor B to vitamin B_{12}.

Isolation of vitamin B_{12} from the fermentation mixture. After completion of the fermentation the mixture was boiled for one hour in order to enhance release of vitamin B_{12} from the bacterium. Purification of the vitamin was performed by repeated adsorption to Fuller's earth and activated charcoal, followed by extractions by solvents; purification was completed by crystallizing the vitamin from aqueous acetone.

Paper chromatography (ascending) was performed on Whatman's paper No. 1. A mixture of water-saturated secondary butanol containing 0.01 per cent NaCN was used as solvent. Chromatography was run for 14 to 16 hours and the chromatogram was dried at room temperature.

Paper electrophoresis was carried out on Macherey-Nagel paper No. 214. The samples to be examined were dropped onto the middle of the paper strip. A polymethylmetakrylat apparatus was used. Depending on the width of the paper strip, the intensity was 20 to 40 mA at a potential difference of 280 V (9 V/cm). For the separation of Factor X from vitamin B_{12} an 0.1 *M*, pH 6.5 phosphate buffer containing 0.1 per cent NaCl was used. The electrophoresis was run for 6 to 8 hours. For the separation of vitamin B_{12} and the so-called Factor B, 1 *N* acetic acid was used; in this case 16 hours' running was necessary.

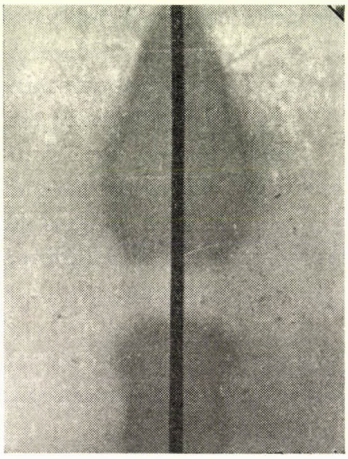

Fig. 1. Autobiographic development of paper chromatogram of crystalline vitamin B_{12} isolated from the fermentation fluid of *Propionbacterium shermanii*

Characterization of the materials separated by paper chromatography and paper electrophoresis. The quantitative determination of the materials separated by the above methods was carried out by cutting out the clearly separated spots and extracting them with 0.1 per cent NaCN solution. The extracts were analyzed by photometry and by microbiological methods.

Qualitative determination was performed by autobiography. A 2 mm strip was cut lengthwise from the chromatogram and placed on an agar plate used for the quantitative determination of vitamin B_{12} with *E. coli*. After an incubation for 16 to 18 hours, *E. coli* growth zones corresponding to the growth enhancing or inhibiting factors were well visible around the paper strip.

Ultraviolet absorption spectra of vitamin B_{12} and Factor X were determined by a Beckman DU spectrophotometer.

Re-synthesis of Factor X to vitamin B_{12}. Synthesis of the amide from ammonia and the mixed anhydride obtained from the monocarboxylic acid and chloroformic acid ethyl ester [9] was carried out as follows. 11.8 mg of crystalline Factor X (of a 85.4 per cent purity, as demonstrated by spectrophotometry) was dried for 15 minutes *in vacuo* at 120°C. The dehydrated material was dissolved in 0.3 absolute dimethyl formamide, and to this solution 0.005 ml anhydrous triethyl amine was added. As a result a small quantity of precipitate appeared in the mixture. Then the reaction mixture was cooled to — 5°C and after adding 0.03 ml of a 10-per

cent solution of chloroformic acid ethyl ester it was kept at this temperature for 10 minutes. Subsequently dry ammonia gas was bubbled through the mixture for half an hour. The mixture was then allowed to stand at room temperature for 30 minutes and at 50°C for 5 minutes. This was followed by the addition of 5 ml of ethyl ether; the red coloured sediment formed was then dissolved in water and the reaction products were separated by electrophoresis.

Results

Paper chromatography of isolated crystalline vitamin B_{12} yielded two red spots. One was identical with that of vitamin B_{12} run in parallel (Rf, 0.27). The other had an Rf value of 0.14, and might therefore be regarded as Factor I. Identification of the spots was made autobiographically (Fig. 1).

Fig. 1 demonstrates that around the spot corresponding to vitamin B_{12} an intensive growth of *E. coli* was visible, while around the margins of the strip moderate growth took place resulting from the traces of vitamin B_{12}. At the spot corresponding to Rf 0.14 there were not even traces of *E. coli* growth. This allowed the conclusion that the spot with an Rf of 0.14 was not identical with Factor I, the latter being also a growth factor for *E. coli*. The unknown substance was designated Factor X.

Isolation of Factor X. To obtain information about the acid, neutral or alkaline character of Factor X, electrophoresis of the solution containing Factor X together with vitamin B_{12} was performed. This showed a 0.3 cm migration of B_{12} towards the negative pole and 1.0 cm migration of Factor X towards the positive pole, showing the acid character of Factor X.

Vitamin B_{12} was easily extracted from an aqueous solution by phenol at pH 5 to 9. It was supposed that in alkaline environment the acid Factor X might form a salt, and thus to be less easily extractable by phenol than vitamin B_{12}. Consequently the aqueous vitamin B_{12} solution containing Factor X was repeatedly extracted by a 1:4 phenol-chloroform mixture until the pink colour of the phenol phase had faded. By adding 50 per cent aqueous acetone to the phenol phase, vitamin B_{12} was transferred to the watery phase. From the aqueous phase the organic solvents were distilled and the concentrate was examined chromatographically in secondary butanol. After neutralization the aqueous residuum of phenol extraction was de-phenolized by extraction with ether, and examined by chromatography parallel to vitamin B_{12}. The chromatogram is presented in Fig. 2.

It is obvious from Fig. 2 that the two materials may easily be separated by extraction with phenol from an alkaline aqueous solution. In this way a quantity of Factor X was isolated from fermentation liquids of *Propionbacterium shermanii*. Unlike vitamin B_{12}, on the addition of acetone no crystalline Factor X precipitated from the concentrate but it was easily crystallized from hot aqueous solution.

Examination of crystalline Factor X. Paper chromatography of crystalline Factor X in secondary butanol yielded a spot with an Rf value of 0.14.

Paper electrophoresis performed at pH 2 in acetic acid and at pH 6.5 in phosphate buffer revealed the homogeneity of the material.

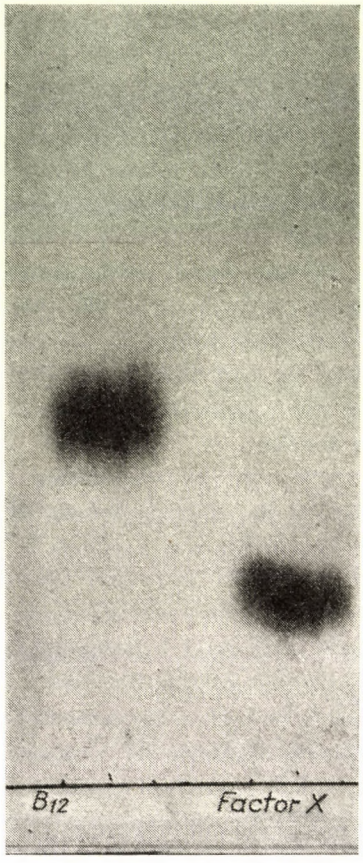

Fig. 2. Paper chromatogram of vitamin B_{12} and Factor X, prepared with secondary butanol

The *ultraviolet absorption spectrum* of vitamin B_{12} and Factor X was determined (Fig. 3).

Fig. 3 shows that the absorption curves of both materials were the same.

Microbiological examinations. As mentioned above, Factor X exerted an inhibitory effect on the growth of *E. coli*. The inhibitory effect was complete if Factor X had been applied in a concentration 40 times higher than that of vitamin B_{12}. Accordingly, the inhibition index of Factor X for the growth stimulating effect of vitamin B_{12} was 40, with *E. coli* as the test organism.

For the determination of the inhibition index, different quantities of Factor X were added to different concentrations of vitamin B_{12} (1.0, 0.2, 0.04 and 0.008 μg) and their effect on the growth of *E. coli* was examined. It was found that the growth enhancing effect of vitamin B_{12} could in every concentration be eliminated by the addition of a 40 times higher concentration of Factor X.

Examination of the structure of Factor X. This was started on the basis of the literary data [10] that the product obtained from vitamin B_{12} on hydrolysis in mildly acid medium contained free carboxyl instead of acid amide and had

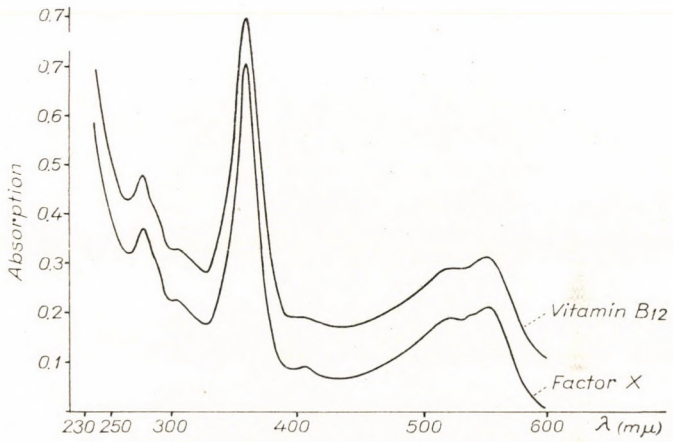

Fig. 3. Ultraviolet absorption spectra of vitamin B_{12} and Factor X

no effect on *E. coli*. As Factor X was acid and inactive in stimulating *E. coli*, it was supposed to be identical with the carboxyl containing derivative of vitamin B_{12}. This supposition was proved by the following experiments.

1. Identification with one of the products of mild acid hydrolysis of vitamin B_{12}. For this purpose crystalline vitamin B_{12} was dissolved in a pH 2.5 buffer solution and kept at 40°C. After 48 and 72 hours, respectively, samples were taken and examined by chromatography and electrophoresis. In these examinations samples of vitamin B_{12} and Factor X were always run parallel. Out of the homogeneous starting material two different red-coloured substances arose after 48 hours. One of them showed a Rf value identical to that of vitamin B_{12}, while the other, present only in minor quantities, had an Rf similar to that of Factor X (Fig. 4).

After hydrolysis for 72 hours the residual vitamin B_{12} was removed from the reaction mixture and the rest was examined in itself. It was found that the least acid product was identical with Factor X (Fig. 5).

2. A different way for identifying Factor X was the re-synthesis of the supposed monocarboxylic acid to acid amide, *i. e.* active vitamin B_{12}. This was

performed as described in the literature [9], through the mixed anhydride of Factor X, with ammonia. After completion of the reaction, the products were separated by electrophoresis in a pH 6.5 buffer. The results of quantitative evaluation are shown in Table I.

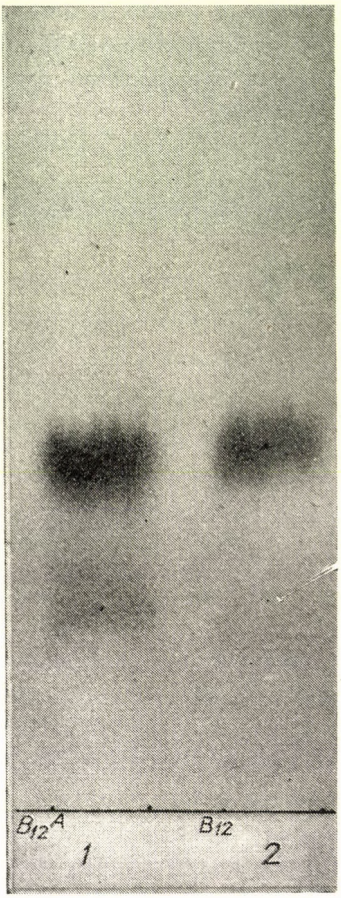

Fig. 4. Paper chromatogram prepared with secondary butanol. 1. Vitamin B_{12} after acid hydrolysis for 48 hours; 2. Vitamin B_{12}

Table I

	Photometric determination	Microbiological determination
Vitamin B_{12}	4400 μg	3950 μg
Factor X	3030 μg	inhibition

According to the photometric determination, 44 per cent, and according to the microbiological assay 39.5 per cent of the starting material was re-synthesized to vitamin B_{12}, and 30 per cent of the starting material was regained unchanged.

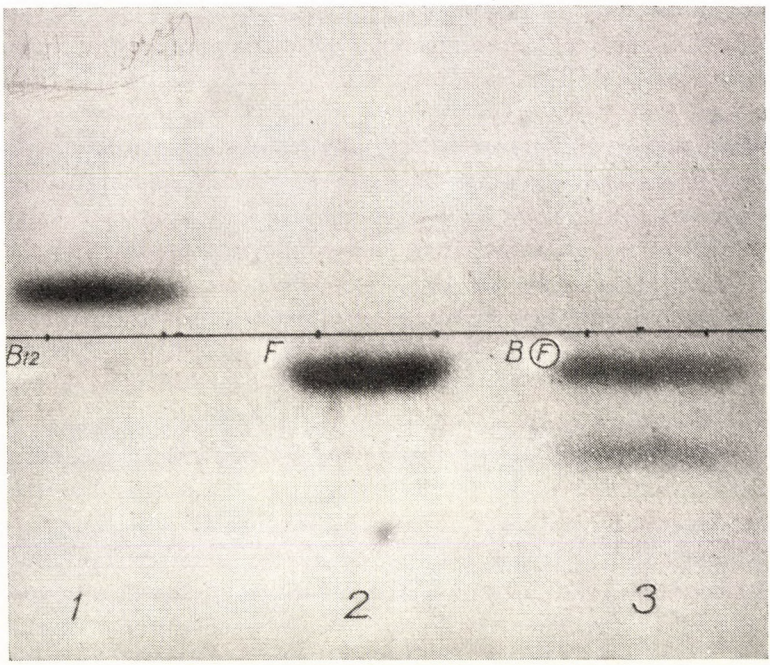

Fig. 5. Electrophoresis in pH 6.5 phosphate buffer solution. 1. Vitamin B_{12} 2. Factor X, 3. Acid hydrolysis products of vitamin B_{12} after the removal of vitamin B_{12}

Discussion

Numerous analogous compounds were isolated from vitamin B_{12} producing microorganisms. Nevertheless, up to now compounds antagonistic to vitamin B_{12} were only described as hydrolysis products or their derivatives [11]. Thus it was thought to be of a certain interest to study the naturally occurring B_{12} antagonist isolated from *Propionbacterium shermanii*.

This acid material, isolated in a crystalline form free of vitamin B_{12}, proved to be homogeneous both by paper chromatography and by electrophoresis. Microbiological investigations by the agar plate method showed the material to have a growth-inhibiting effect on *E. coli* [12].

The inhibition index was found to be 40. As by changing the concentration of the metabolite (B_{12}) no changes in the inhibition index could be elicited, the antagonism had to be regarded as competitive [13]. As according to our

observations the inhibitory effect of Factor X appeared to be practically independent of the concentration of vitamin B_{12}, the inhibition was regarded as competitive. According to SHAW [14], antimetabolites have three main characteristic properties, *viz.*, (i) a structure similar to that of the metabolite; (ii) their effect is identical with that elicited by the lack of the corresponding metabolites; (iii) their effect antagonises that of the metabolites.

As our examinations demonstrated Factor X to possess all these properties, it might be regarded as an anti-metabolite of vitamin B_{12} in the metabolism of *E. coli*.

One of the starting points of our examinations concerning the structure of the isolated substance was the identity of the UV absorption spectra of vitamin B_{12} and Factor X. On the basis of this fact it might be concluded that as to their nucleotid components there is no difference between the two compounds. Another observation concerning the structure was the demonstration of the acid character of Factor X. It was supposed that out of the six acid amide groups of vitamin B_{12}, one or more may be substituted by free carboxyl-residues. According to literary data the acid hydrolysis product of vitamin B_{12} cannot be utilized for the growth of *E. coli*. It has been demonstrated that Factor X was identical with one of the hydrolysis products of vitamin B_{12}; according to paper chromatographic and electrophoretic examinations this derivative is the least acid product of hydrolysis, *i. e.* monocarboxyl acid.

The supposition, that the structure of Factor X differs from that of vitamin B_{12} by containing free carboxyl instead of one of the acid amide groups, has been proved by the fact that the mixed anhydride of Factor X and chloroformic acid may be transformed with ammonia to acid amide, *i. e.* vitamin B_{12}.

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A VITAMIN B_{12} ANTAGONIST ISOLATED FROM PROPIONBACTERIUM SHERMANII

II. INVESTIGATIONS ON THE ORIGIN OF FACTOR X

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Summary. Experiments performed with labelled vitamin B_{12} and isolation of vitamin B_{12} at neutral pH showed that the monocarboxylic acid derivative of vitamin B_{12} isolated from *Propionbacterium shermanii* was not an artefact but was produced during the fermentation process.

On fermentation the quantity of B_{12} monocarboxylic acid exhibited a gradual decrease; thus it should be regarded as an intermediary product of the vitamin's biosynthesis rather than a decomposition product.

In a previous paper [1] we reported about the isolation of a monocarboxylic acid derivative of vitamin B_{12}, which was found to have an inhibitory effect on the growth of *E. coli*. In the following we shall report on further investigations into the origin of Factor X.

Materials and methods

Photometric and microbiologic examination of vitamin B_{12}, the fermentation procedure, the isolation of vitamin B_{12}, and the quantitative evaluation of paper chromatography and paper electrophoresis were performed as already described [1].

Preparation of Cobalt 60 *labelled vitamin* B_{12}. During the fermentation process cobalt salt is added to the agar medium to enhance vitamin B_{12} production. Radiocobalt is readily built into the vitamin B_{12} molecule [2, 3, 4, 5]; for labelling Co^{60} with a half life of 5.3 years was used, thus the decrease of activity during the experimental period was negligible.

In a glass fermentor 50 litre volumes of fermentation mixture were prepared. The fermentation mixtures contained a total of 50 μc of radioactivity each. The produced Co^{60} containing vitamin B_{12} was isolated in the same way as ordinary vitamin B_{12}. According to the paper chromatographic and paper electrophoretic examinations, the activity in the crystalline product was 0.23 μc/mg. Activity was tested in the fluid phase, in 50 ml containers where the GM tube was surrounded by the fluid.

Isolation of vitamin B_{12} *and Factor X from the bacteria.* The bacteria sedimented by centrifugation were extracted twice with 70 per cent ethanol containing 0.1 per cent NaCN, by boiling for an hour. After the ethanol was distilled away, the concentrate was purified by charcoal adsorption and then vitamin B_{12} and Factor X were extracted from the neutralized aqueous solution by a 1:4 phenol chloroform mixture. The material transferred into water by shaking with acetone was then evaporated and the total concentrate transferred to a paper strip for electrophoresis.

Results

Isolation of vitamin B_{12} *from the fermentation liquid at a neutral pH.* At the end of the fermentation process the vitamin B_{12} was inside the bacterial cell. The fermentation mixture — at a pH of from 5.2 to 5.5 — was boiled in

order to destroy the bacterial cells and free the vitamin. Factor X was isolated in exactly the same way. To eliminate boiling in slightly acid environment, bacteria were sedimented in a supercentrifuge at 18 000 r. p. m. Vitamin B_{12} was released from the cell suspension by boiling in ethanol at pH 7. After the

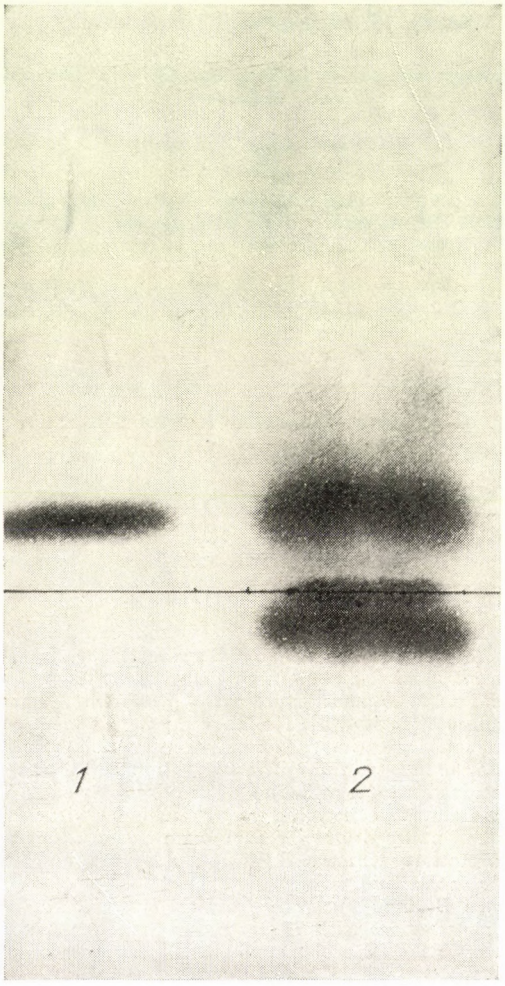

Fig. 1. Eletrophoresis in pH 6.5 phosphate buffer. 1. Factor X, 2. Fermentation liquid of *Propionbacterium shermanii* purified at pH 7

distillation of ethanol the material was adsorbed from the neutral medium onto activated charcoal and eluated by a water-ethanol mixture. After evaporation the eluate was dissolved in a phosphate buffer of pH 6.5 and examined by electrophoresis. Factor X was run in parallel (Fig. 1).

Eletrophoretic analysis demonstrated besides vitamin B_{12} also the presence of a considerable quantity of another substance, migrating as Factor X did. The identity was further supported by the microbiological examination, as the substance showed an inhibitory effect.

Examinations with labelled vitamin B_{12}. With the Co^{60} labelled vitamin B_{12} the following experiment was performed. To 50 litre fermentation mixture of *Propionbacterium shermanii* 30 mg of labelled vitamin B_{12} was added after fermentation. This quantity amounted to about 20 per cent of the total vitamin B_{12} contained in the mixture. After repeated extractions by phenol the aqueous and the phenolic phases were purified separately by adsorption to charcoal. The purified materials were concentrated and the total volume was transferred to paper strips and electrophoresis was carried out in a pH 6.5 buffer solution. After electrophoresis the spots were cut out of the paper, eluted by water, assayed for radioactivity and examined photometrically. The results are shown in Table I.

Table I

	Photometric determination	Radioactivity
Vitamin B_{12}	5.2 mg	PM
Factor X	4.55 mg	PM

Thus, Factor X isolated by electrophoresis exhibited only traces (0.9 per cent) of activity, *i. e.* within the limits of error.

Changes in the amount of Factor X in the medium during fermentation. In the above described glass apparatus 5 litre of fermentation liquid was prepared. Before starting the fermentation 2.0 mg/litre of Factor X was added to the mixture. After 96 hours of incubation half of the fluid was removed from the fermentor. The remaining half was incubated for a total of 140 hours The two parts were separately purified in a similar way. The total amount of the red-coloured concentrates was analyzed separately by paper electrophoresis in pH 6.5 phosphate buffer. The paper strips were extracted by 0.5 per cent NaCN solution and analyzed photometrically. The results are shown in Table II.

Table II

	Vitamin B_{12}/litre	Factor X/litre
96 hours	574 μg (100 per cent)	637 (100 per cent)
140 hours	1090 μg (190 ,, ,,)	287 (45 ,, ,,)

Discussion

Factor X isolated from the fermentation liquid of *Propionbacterium shermanii* is a monocarboxylic acid derivative of vitamin B_{12} [1]. By hydrolyzing in slightly acid medium the acid amide groups of vitamin B_{12} gradually decompose and derivatives containing free carboxyl groups are formed.

As the acid amide groups of vitamin B_{12} are gradually decomposed on hydrolysis in slightly acid media and form derivatives with free carboxyl groups, it might be argued that the Factor X isolated by us is not of fermentation origin [5], but an artefact produced during the purification process. This argument was contradicted by two experiments. First, vitamin B_{12} was isolated from the fermentation fluid at a neutral pH, and Factor X could still be demonstrated, though no hydrolysis of acid amides was likely to have taken place under the given conditions.

Second, the fermentation origin of Factor X was decisively proved by the examinations performed with radioactive vitamin B_{12}. As radioactive vitamin B_{12} was added to the fermentation fluid after fermentation had been finished and before preparation was started, an eventual radioactivity of Factor X would prove its non-fermentation origin. Nevertheless, Factor X isolated in this experiment did not exhibit any radioactivity, so its fermentation origin seemed to be obvious.

The fermentation origin of Factor X was also proved by the fact that by the same procedure as used with *Propionbacterium shermanii*, vitamin B_{12} was isolated from many other microorganisms as well (*e. g. Streptomyces griseus, Streptomyces rimosus, Rhizobium meliloti, etc).* Nevertheless no presence of Factor X could be demonstrated in any of the vitamin B_{12} preparations obtained from these microorganisms. Thus the production of Factor X might be regarded as characteristic of fermentation with our Propioni bacterium strain.

Vitamin B_{12} has 3 labile acid amide groups [6]. Thus, even in the first step of decomposition isomeric compounds are formed [7] which are easily distinguished by chromatography in secondary butanol. Factor X isolated in our laboratory did not contain isomers demonstrable by this method; this may be regarded as a further proof of its natural origin.

As to the origin of Factor X two suggestions might be made. An intermediary phase of B_{12} synthesis by the bacterium might be supposed, on the basis of JUILLARD's hypothesis [8] concerning the carboxylic derivative of factor B. The transformation of carboxylic acids to acid amides as a terminal phase of B_{12} vitamin biosynthesis has been supposed also by BERNHAUER [9]. On the other hand it was proved by PAWELKIEWICZ [10] for the Corynebacterium, that the carboxylic acid derivative of factor B was a decomposition product of the same factor.

To decide this problem, at the start of fermentation Factor X was added to the medium and its quantity was controlled during and after fermentation.

As a result it was found that while the quantity of vitamin B_{12} increased from 574 μg/litre, measured at the 96th hour, to 637 μg/litre at the 140th hour, the quantity of Factor X decreased from 637μg/litre to 287μg/litre within the same interval. Accordingly, either a transformation of Factor X to vitamin B_{12}, or the occurrence of an equilibrium situation of a reversible process might be supposed. It is quite possible, however, that a shift in the equilibrium state of a reversible process is the reason why we got decreasing values for Factor X and increasing values for vitamin B_{12} at two different times of fermentation. Our further experiments will have the task to decide upon this question.

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MICROBIOLOGICAL EXAMINATION OF A NATURAL VITAMIN B_{12} ANTAGONIST

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Summary. 1. Large quantities of a competitive vitamin B_{12} antagonist were isolated from cultures of a specific *Propionbacterium shermanii* strain, and a simple method for the demonstration of this competitive antagonism was developed.

2. A method was developed for the quantitative determination of the antagonistic factor by agar diffusion.

3. A theoretical explanation of the phenomena observed in agar plate experiments has been attempted.

In the fermentation fluid of *Propionbacterium shermanii* besides vitamin B_{12} a specific factor was found which proved to be antagonistic to vitamin B_{12} when tested on *E. coli* strain 113—3. A large quantity of this factor — corresponding to about 20 to 25 per cent of the amount of vitamin B_{12} produced — could be demonstrated in the cultures of one of our *P. shermanii* strains. Our other *P. shermanii* and *P. freudenreichii* strains produced the same factor in small quantities, in some cases in traces only. The factor was isolated in crystalline form, its chemical structure was elucidated, and its competitive antagonism to vitamin B_{12} was demonstrated [1].

The present paper gives a detailed account of examinations on this competitive antagonism together with the description of an adequate procedure for the demonstration of the competitive antagonism in this case, and of an agar diffusion method for the quantitative determination of the antagonistic factor.

Materials and methods

The antagonistic factor was prepared in a crystalline form [1] from a *P. shermanii* strain isolated in Hungary.

The crystalline vitamin B_{12} preparation (Chinoin, Budapest) used in the experiments contained less than 2 per cent of factors, as revealed by paper chromatography.

The antagonistic action of the factor in question was examined on *E. coli* 113—3 by the agar plate method described by TSHAIKOVSKAYA and DRUZHININA [2].

The agar plates were photographed according to the method of HUNTER [3].

Results

First, the inhibitory concentration of the antagonistic factor was determined. Solutions were prepared containing 0.1 μg/ml of vitamin B_{12} each and

different concentrations of the antagonistic factor. It was found by the agar plate method that concentrations of the antagonistic factor of 0.12 μg/ml or less did not measurably influence the growth zone obtained in the presence of vitamin B_{12}. When using higher concentrations, a proportional gradual increase of the growth zone diameter and a fading away of the zone margins were observed. At concentration levels of 4 μg/ml *(i. e.* 40 times higher than that of the vitamin B_{12} concentration present), or higher, either a disappearance or a fading of the growth zone ensued, the latter leaving but a very faint halo impossible to read. When repeating this experiment with the modification, of using a constant concentration of 0.025 μg/ml of vitamin B_{12}, similar results were achieved as a 40-fold concentration of the antagonistic factor resulted in complete inhibiton. The experimental data are presented in Table I. The results suggested the competitive nature of the antagonism.

Table I

Effect of different concentrations of the antagonistic factor on the diameter of growth zones produced by vitamin B_{12}

Factor concentration μg/ml	Diameter of growth zone in mm	
	At	
	0.100	0.025
	μg/ml concentration of vitamin B_{12}	
4	0.00	0.00
2	22.0	0.00
1	21.6	0.00
0.5	20.8	20.00
0.25	20.8	19.00
0.125	20.6	17.10
0.06	20.6	17.00
0.03	20.6	16.90
0.015	20.6	16.90
0.00	20.6	16.60

For comparison, solutions of vitamin B_{12} containing also sulphuric acid, formalin and/or oxytetracycline were prepared. When these solutions were tested on agar plates, growth zones with clear margins and inhibition areas were produced at appropriate concentrations. The diameter of the growth zones was usually greater than that observed at corresponding concentrations of vitamin B_{12} alone. This antagonism between vitamin B_{12} and the above antiseptics is obviously of quite a different character than that observed when the factor was used.

The following experiment also demonstrated the competitive nature of the antagonism between the factor in question and vitamin B_{12}.

Instead of the usual 2.5 cm distance holes were punched in the agar plates 2 cm apart, as measured between the centres of the holes. When titrating an 0.1 μg/ml concentration of vitamin B_{12} on these plates, confluent growth zones appeared. When however titrating the same vitamin concentration in the presence of the antagonistic factor (for such experiments the optimal concentration of the latter was 0.5 to 1.5 μg/ml) no confluence of the growth zones occurred, but they assumed a somewhat quadrangular shape separated by

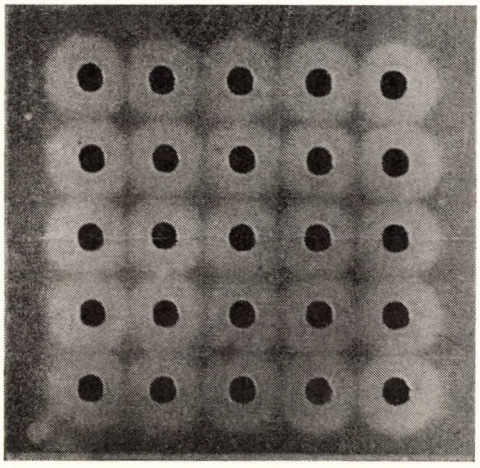

Fig. 1. Growth zones induced by a mixture of vitamin B_{12} and antagonistic factor on an agar plate inoculated with *E. coli* 113—3. Distance between hole centres: 2 cm. Concentration: 0.1 μg/ml vitamin B_{12} and 0.75 μg/ml antagonistic factor

narrow sharp lines where no growth was visible. A photograph of a typical experiment is presented in Fig. 1. An explanation of this phenomenon will be given later.

In the above experiments the antagonistic factor was demonstrated indirectly, by its influence on the growth zone induced by vitamin B_{12}. Subsequently a method for the direct demonstration of the antagonistic factor was developed.

For that purpose an agar medium was prepared as described by TSHAIKOVSKAYA and DRUZHININA for the determination of vitamin B_{12}, and 1/300 μg/ml pure vitamin B_{12} and an inoculum of *E. coli* 113—3 were added. In cultures prepared in this way the growth of the organism caused an uniform opalescence. The addition of antagonistic factor to such cultures resulted in an inhibition phenomenon similar to that produced by antibiotics. The autobiogram of the antagonistic factor on a chromatogram strip is demonstrated in Fig. 2.

The quantitative relation of the inhibition zone to the concentration of the antagonistic factor was also examined. The data obtained are summarized in Table II.

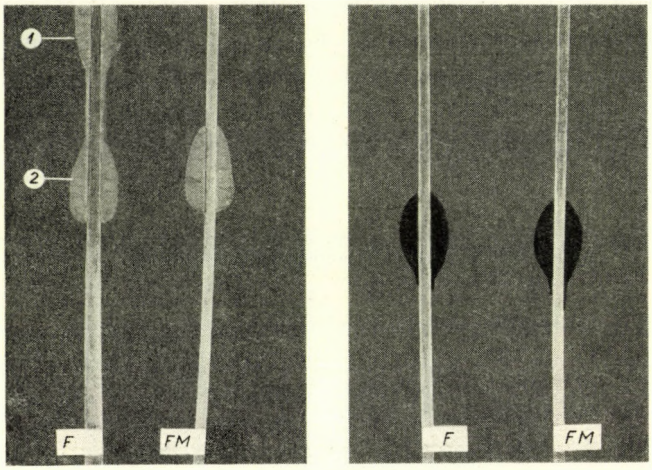

Fig. 2

Left : Autobiogram of unpurified antagonistic factor on an agar plate used for the determination of vitamin B_{12}. 1. Spot corresponding to factor B, 2. Spot corresponding to cyanocobalamine. The sharp margin at the bottom of the spot indicates the antagonistic effect

Right : Autobiogram of the same substances on a plate containing vitamin B_{12}. On this plate no vitamin B_{12} can be demonstrated but a well defined inhibition zone is visible on the site of inhibition merely suggested on the previous picture at the bottom margin of the cyanocobalamine spot

Table II

Diameter of inhibition zones produced by different concentrations of the factor

Concentration of the factor µg/ml	log. conc.	Diameter of inhibition zones (mm)	Difference in the diameters of the adjacent zones (Δd)
400	8.64	47.3	0.6
200	7.64	46.7	1.3
100	6.64	45.4	1.6
— — 50 — — —	— — 5.64 — —	— — 43.8 — —	— — — — — — — —
25	4.64	41.0	2.8
12.5	3.64	38.1	2.9
6.25	2.64	35.3	2.8
3.12	1.64	32.3	3.0

Table II demonstrates that within the range of 3 to 50 μg/ml the logarithmus of the factor concentrations were directly proportional to the diameters of the inhibition zone, *i. e.* the correlation was the same as with other agar diffusion methods for determination of antibiotics and vitamins. Accordingly, within the range of the concentrations mentioned, the antagonistic factor may be quantitatively determined in any solution not containing vitamin B_{12} (separation by paper chromatography). A standard solution prepared from crystalline factor and adjusted by photometry should always be used simultaneously as a comparative standard. The zones are very regular in form and though somewhat faded they have sufficiently sharp edges to make reading easy.

Discussion

The developmental mechanism of the growth zones induced by vitamin B_{12} on agar plates has been described recently [4, 5]. In the course of the agar diffusion process the diffusion of the vitamin continues until its total quantity has become bound to bacteria. Accordingly, the phenomenon demonstrated in Table I may be explained by the hypothesis that the avidity of the vitamin to bacteria is much greater than that of the antagonistic factor. If the concentration of the vitamin is not too high, the simultaneous addition of vitamin B_{12} and factor to the culture results in the absorption of the vitamin by the bacteria, thus the formation of a normal zone will take place. The factor diffuses beyond the zone, although this process is not visible. If a mixture of the vitamin and its antagonistic factor is, however, put into adjacent holes, the space for the diffusion is limited and the concentration of the factor may rise to the inhibition limit (1:40), resulting in an inhibition of the confluence of the adjacent zones (see Fig. 1).

On the basis of this explanation the possibility of a competitive antagonism between vitamin B_{12} and the factor in question might be supposed. As on the other hand certain other experiments are known to have shown the competitive nature of this antagonism, the above explanation seems to be justified.

A certain theoretical importance might have the fact that on agar plates with little vitamin B_{12} content the antagonistic factor exhibits an inhibition phenomenon similar to that caused by antibiotics. Thus the factor might either be regarded as an anti-vitamin or as a specific vitamin B_{12} sensitive antibiotic effective against *E. coli* 113—3.

In the case of *Staphylococcus aureus*, oxamycin plays the role of a competitive antagonist of the enzyme system building d-alanine into uridine-nucleo tides [6]. Oxamycin is generally regarded as an antibiotic. Similarly, the competitive antagonist just described might probably be a competitive inhibitor for an eventual enzyme system with vitamin B_{12} as the prosthetic group.

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ANTISTREPTOLYSIN-O TITRE OF HEALTHY ADULTS IN BUDAPEST

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Summary. The average ASO titre of 600 healthy blood donors was found to be 125.6 units, with a standard deviation from 63.8 to 247.2. In 16.66 per cent of the cases, titres were below 63.8, and in 12.84 per cent over 247.2, units, while titres varied between the two limits in 70.5 per cent of the cases.

The numerical distribution of the titres with 35 to 470 units around the average was in agreement with Gauss's normal distribution, but the number of titres with 25 to 30 and those with 555 to 800 units was significantly higher than might have been expected according to Gauss's normal distribution.

Over the age of 18 to 20 years, average titres showed a gradual decrease with advancing age.

The average titres for females with the exception of the 41 to 50 year age group were higher than those for males. The difference between the mean titres for males and females was not significant statistically.

ASO titres high in the spring underwent a slight decrease in the following months, to rise again in the autumn.

Titration was performed by our own method. This method is technically more simple, but more reliable and supplies figures more suitable for mathematical evaluation than the usual ones, the distance between the single titres being characterized throughout by the quotient $\sqrt[4]{2}$. Statistical computations can therefore be made instead of the titres with simple ordinals, and only the results must be converted into titres.

The distribution of streptococcal diseases is not uniform in the various countries; owing to latent and manifest infections, this fact is reflected also by geographical differences in the antistreptolysin-O (ASO) titres of the healthy population [1, 2]. We have studied this question as no data concerning conditions in Hungary have been published.

Materials and methods

Test material. Serum samples obtained from 600 unselected donors of the *National Blood Service* in Budapest have been tested. Prior to withdrawal of blood the donors had been subjected to medical and laboratory examination and were found to be healthy and fit to act as blood donors. Their age ranged between 18 and 71 years.

O-Streptolysin, lyophilized, was produced by ourselves. One ampoule contained 10—14 combining units of O-streptolysin (OSL), lyophilized in the reduced state. It was dissolved in 10 ml physiological saline immediately before use.

Standard Antistreptolysin-O, produced by the *Statens Seruminstitut, Copenhagen*. For routine use we prepared a substandard according to IPSEN's principles [3], of identical titre.

Method of titration. The examinations were carried out as follows.

Of the inactivated sera to be titrated a 1:100 basal dilution is prepared with physiologic saline of which 1.0, 0.7, 0.5, 0.35, 0.25 and 0.18 ml are measured into 6 tubes, and the volume is made up to 1.0 ml by physiologic saline so that the serum dilutions in the tubes correspond

Table I

Products of standard units and reciprocals of serum dilutions

Dilution of serum	ml	Standard units						
		1.00	0.84	0.70	0.60	0.50	0.42	0.35
	1.00	12.5	10.5	8.75	7.5	6.25	5.25	4.37
		15	12.5	10.5	8.75	7.5	6.25	5.25
	0.70	17.5	15	12.5	10.5	8.75	7.5	6.25
		21	17.5	15	12.5	10.5	8.75	7.5
	0.50	25	21	17.5	15	12.5	10.5	8.75
1 : 12.5		30	25	21	17.5	15	12.5	10.5
	0.35	35	30	25	21	17.5	15	12.5
		42	35	30	25	21	17.5	15
	0.25	50	42	35	30	25	21	17.5
		60	50	42	35	30	25	21
	0.18	70	60	50	42	35	30	25
		84	70	60	50	42	35	30
	1.00	100	84	70	60	50	42	35
		120	100	84	70	60	50	42
	0.70	143	120	100	85	70	60	50
		170	143	120	100	85	70	60
	0.50	200	170	140	120	100	84	70
1 : 100		240	200	170	140	120	100	84
	0.35	286	240	200	170	143	120	100
		336	286	240	200	170	143	120
	0.25	400	336	280	240	200	170	140
		470	400	336	280	240	200	170
	0.18	555	466	388	333	278	233	194
		672	560	480	400	336	280	240
	1.00	800	672	560	480	400	336	280
		960	800	672	560	480	400	336
	0.70	1142	960	800	685	570	480	400
		1344	1120	960	800	672	560	480
	0.50	1600	1344	1120	960	800	672	560
1 : 800		1920	1600	1344	1120	960	800	672
	0.35	2284	1920	1600	1370	1142	960	800
		2688	2240	1920	1600	1344	1120	990
	0.25	3200	2688	2240	1920	1600	1344	1120
		3740	3200	2688	2240	1920	1600	1344
	0.18	4444	3733	3111	2666	2222	1866	1555

to 100, 143, 200, 286, 400, 555 (566) respectively, and form a geometrical progression with the common ratio of approximately $\sqrt{2}$. In case of haemolysis throughout the series, dilution is repeated, starting at 1:12.5, while in the absence of haemolysis in all tubes, repeated dilution is started at 1:800. Of these dilutions exactly the same amounts should be measured into six tubes as of the 1:100 dilution. After making up the volume to 1.0 ml, the following dilutions are obtained: 12.5, 17.5, 25, 35, 50, 70 and 800, 1142, 1600, 2284, 3200, 4444. The 18 grades 12.5 to 4444 thus obtained constitute a geometrical progression with $\sqrt{2}$ as the common ratio.

A dilution of 1.0 unit/ml is prepared from the Copenhagen standard ASO or a substandard of equivalent value; of this dilution 1.0, 0.84, 0.70, 0.60, 0.50, 0.42 and 0.35 ml, respectively, are measured into 7 tubes. The volumes are completed to 1.0 ml by physiologic saline. Then each tube contains as many standard ASO units as the ml amount of the 1.0 unit/ml dilution it holds. These ASO units form a diminishing geometric progression with $\sqrt[4]{2}$ as the common ratio; hence the standard series is exactly twice as dense as the test serum series.

Into each tube 0.5 ml of freshly dissolved lyophilized homogeneous OSL solution is pipetted. The tubes are incubated for 15 minutes in a water-bath of 37° C.

Then 0.5 ml of 2 per cent washed sheep erythrocyte or 5 per cent washed rabbit erythrocyte suspension is added to each tube. After shaking the tubes are placed into a water-bath of 37°C for 45 minutes.

After the second incubation the degree of haemolysis is read with the naked eye in every tube. The ++ tube showing approximately 50 per cent haemolysis is considered as the titre.

The effect of the OSL solution is indicated by the ++ tube of the standard series. Since our OSL solution contained about 0.5 to 0.7 combining unit per 0.5 ml, 50 per cent haemolysis had to occur in one of the tubes of the standard series between the units 0.35 and 1.00. The unit value of this standard tube multiplied by it the dilution grade of that test tube which gives likewise 50 per cent haemolysis yield the ASO titre of the test serum in international units.

The standard series is dense enough to include with great probability a ++ tube, one with 50 per cent haemolysis. On the other hand, in the test series, which is but half as dense as the standard series a ++++ tube might immediately be followed by a ∅ tube. In such cases, the value obtained by interpolation with the quotient $\sqrt[4]{2}$ between two steps is regarded as the titre. In this way the dilutions and the interpolated values form a geometrical progression with the common ratio $\sqrt[4]{2}$, as does the standard series. Dilution grades multiplied by standard values are presented in Table I.

Mathematical evaluation of the results. The data are computed from the logarithms of the individual steps in question. The difference between two successive steps is thus $\frac{\log 2}{4} = 0.07526$. For sake of simplicity, however, we start instead with zero and assign to each consecutive step the next whole number. Computations are then made with these values. For example, if the lowest titre is 25 units, then 25 represents the 0 value, 30 corresponds to 1, 35 to 2, *etc.* Accordingly, any X_n value can be converted into titre by the formula:

$$(\sqrt[4]{2})^{X_n} \times 25$$

Results

1. The ASO titres for the 600 samples of serum, their standard deviation and the average values are presented in Table II.

Converted into titres, the data in Table II yield for the average titre, 125.6 units; for the upper and lower limits, 63.8 and 247.2 units, respectively. In our opinion, the zone of simple standard deviation for the average titre may be regarded as the "normal" for healthy adults. Of our material 70.5 per cent, *i. e.* 423 persons, had a titre between 63 and 247 units. In 100 persons, *i. e.* 16.66 per cent, the titre was lower; in 77 persons, *i. e.* 12.84 per cent, it was higher.

2. We ascertained by means of the χ^2 test whether the distribution of the various titres in our material followed the normal distribution of Gauss. The data are given in Table III and illustrated graphically in Fig. 1.

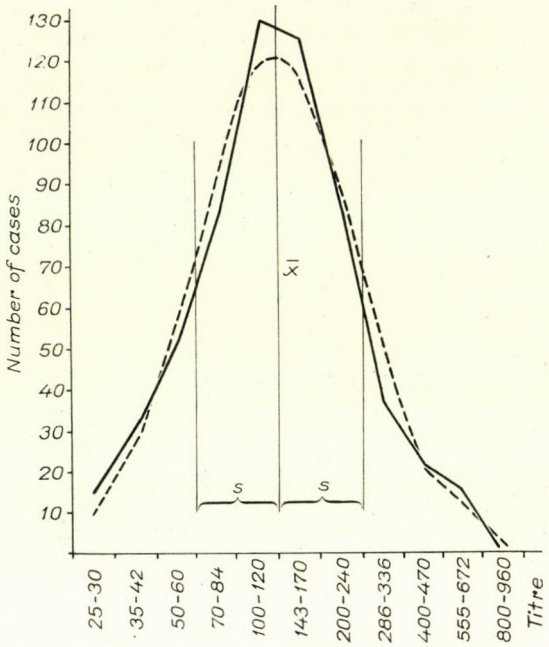

Fig. 1. Real and computed distribution of titres. Legend:
——— real distribution
— — — computed Gauss-distribution
$\bar{x}$ = average titre
S = simple dispersion

As demonstrated in Table III P was < 5 per cent for all data, the deviation of the two curves was statistically significant. Deviation from the normal was due chiefly to the first and last groups of values. In the last column of Table III, the value of χ^2 was calculated also with omission of the first and last groups when P was > 25 per cent. In other words the distribution curves in the region of the values between 2 and 17 corresponded to the normal Gauss curve. These results evidence a higher incidence of lowest and highest titres than may be expected to result from random dispersion.

3. Average values and their standard deviation according to sex and age are presented in Table IV. The average values are illustrated graphically in Fig. 2.

These data show that (a) the average titre decreased with advancing age; diminution was gradual in the case of males and total groups, while these were minor fluctuations in the case of females.

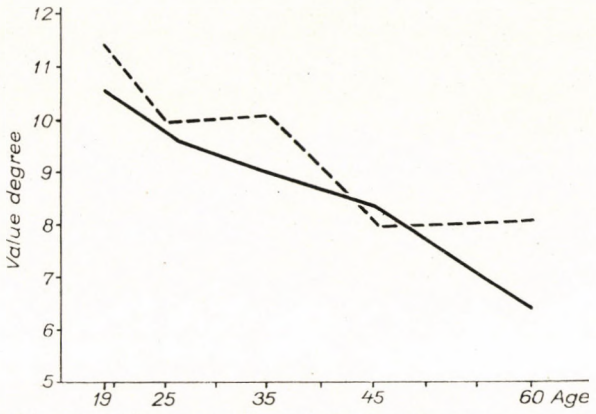

Fig. 2. Average titres according to sexes and age groups. Legend:
——— males
— — — — females

Table II

Value-degree	Titre	Number of cases		
		Male	Female	Total
0	25	2	3	5
1	30	5	5	10
2	35	7	7	14
3	42	12	6	18
4	50	9	5	14
5	60	27	12	39
6	70	21	22	43
7	84	25	15	40
8	100	35	25	60
9	120	35	35	70
10	143	30	26	56
11	170	42	28	70
12	200	31	11	42
13	240	24	18	42
14	286	10	11	21
15	336	6	11	17
16	400	9	8	17
17	470	3	2	5
18	555	4	4	8
19	672	4	4	8
20	800	—	1	1
Total		341	259	600

average: $\overline{X} = \frac{1}{n} \sum_{i=0}^{n} X_i = 9.321$ value degree

standard deviation: $s = \sqrt{\frac{S_{xx}}{n-1}} = \pm 3.908$ value degree

$9.321 \pm 3.908 = 5.413 — 13.229$

Table III

Value degree	Number of cases m	Normal distribution m'	$\frac{(m-m')^2}{m}$	$\frac{(m-m')^2}{m}$
0— 1	15	9.8	2.555	—
2— 3	32	27.4	0.770	0.770
4— 5	53	57.8	0.398	0.398
6— 7	83	95.0	1.515	1.515
8— 9	130	119.8	0.868	0.868
10—11	126	117.2	0.577	0.577
12—13	84	88.3	0.209	0.209
14—15	38	51.6	3.583	3.583
16—17	22	23.3	0.072	0.072
18—20	17	9.8	5.285	—
Total	600	600.0	$\chi^2_{(8)} = 15.832$ $P = <5\%$	$\chi^2_{(6)} = 7.992$ $P = >25\%$

Table IV

Age class	Male				Female				Total			
	Number of cases	average titre			Number of cases	average titre			Number of cases	average titre		
		in v. d.-s	standard deviation	in ASO units		in v. d.-s	standard deviation	in ASO units		in v. d.-s	standard deviation	in ASO units
18—20	23	10.56	±3.20	155.6	16	11.37	±3.66	179.0	39	10.89	±3.35	164.75
21—30	167	9.73	±3.74	134.7	96	9.95	±4.13	140.0	263	9.81	±3.90	136.50
31—40	81	9.01	±3.66	119.0	58	10.18	±4.03	145.6	139	9.51	±3.75	129.75
41—50	46	8.42	±4.09	107.3	47	8.05	±3.68	100.7	93	8.23	±3.82	104.00
51—70	24	6.50	±3.10	77.3	42	8.22	±4.16	103.8	66	7.60	±3.85	93.20
18—70	341	9.21	±3.79	123.1	259	9.46	±4.04	128.6	600	9.321	±3.908	125.60

(b) Except for the 41 to 50 years age-group the average titre was higher in females than males. Other authors [4, 5] have failed to find such a difference; neither was our difference statistically significant according to Student's *t* test.

(c) The standard deviation of the age-group averages for males was between ± 3.10 and ± 4.09; for females, between ± 3.66 and ± 4.16, thus slightly higher for females than for males, but no correlation could be discovered between the extent of variance on the one hand and the size and age of the groups, on the other.

4. It was striking that a number of persons pronounced healthy after general medical examination yielded a high ASO titre. The examinations were made in the spring, and it was thought that the increased titres might be due

Table V

Difference in value degrees	Number of repeated cases		Total
	before Sept. 15	after Sept. 15	
−7		1	1
−6			
−5	1		1
−4	1		1
−3	2	1	3
−2	2		2
−1	2	1	3
0	1		1
+1	1		1
+2		1	1
+3			
+4		3	3
+5		1	1
+6		1	1
+7		1	1
Total	10	10	20
Average diff. in v. degrees	−2.0	+2.1	+0.1

to some past transient streptococcal infection. Therefore, after the lapse of 3 to 7 months between June and November, samples were again collected from 20 persons with elevated titre. At the beginning of the summer the titres were somewhat lower; in the autumn months an increase occurred in all persons but three. When the data reflecting the changes of titres were divided arbitrarily into two columns according to whether repeated examination had taken place before or after September 15, it was found that ten persons during the summer months yielded titres that were, on the average 2 values lower than their spring titres whereas those of the other ten persons were on the average 2.1 values higher in autumn than they had been in spring. These data are presented in Table V.

In spring, the average titre for the 20 persons amounted to 466.6 units; by bringing the average deviations into correlation with the initial average, the following titre values were obtained:

$$466 : (\sqrt[4]{2})^{2.0} = 329.4$$

$$466 \cdot (\sqrt[4]{2})^{2.1} = 670.6$$

Discussion

Determination of the "normal" ASO titre presents the same theoretical problems as the definition of any biological characteristic norm, namely establishment of the criteria of normality. As concerns the case in point, we thought that by the aid of statistical methods we would define the values occurring most frequently among healthy adults and regard these as normal. The term "most frequently occurring values" denotes the calculated average values within the limits of simple variance. On this basis an ASO titre of from 63 to 247 units may be considered normal for the adults of Budapest. Of course, even normal values change under the influence of age, seasons, and naturally also social conditions affecting health. Despite all these varying factors 70.5 per cent of our cases remained within the limits of simple dispersion; moreover, the averages for the various age-groups were likewise within these limits.

Several authors [4, 6, 7] claimed the upper limit of normal titre to be 200 units. However, even these authors reported on titres over 200 units in 17 to 22 per cent of healthy individuals. Therefore, KÖHLER [8*a*], relying on the evidence of his experiments performed at Rostock, put the upper limit of the normal titre at 250 units. On the basis of our own results, we are in agreement with KÖHLER. As regards our material, this value practically agreed with those 247 units which constitute the upper limit of the simple standard deviation of the average titre.

As seen in Table III, the incidence of extremely low and high values was more common in our material than in Gauss' distribution. Though very high and very low titres were recorded also in symptomless subjects, in our opinion they are caused by pathological factors even in the absence of any other sign.

The diagnostic value of the ASO titre seems to be reduced by the pathologically high values encountered in nearly 13 per cent of the healthy population. The persons belonging to this group are most probably susceptible to streptococcal infections, manifesting a tendency to develop tonsillitis, pharyngitis, or harbouring latent foci; in such cases recurring antigenic stimuli are maintaining the ASO titre at a permanently high level. In periods free from inflammation, the traces of the sustained, more or less mild infections cannot be detected either by medical examination, ESR or by taking temperatures. The data presented in Table V show that their titres diminished during the summer months free from droplet infection, and even continued to diminish until they had received a new streptococcal antigenic stimulus in the course of the autumnal season of droplet infection. With those of KÖHLER and SCHMIDT [9], our data furnish evidence to show the seasonal fluctuations of the ASO titre.

As regards the connection between values and age-groups, our findings are in agreement with those reported by RANTZ *et al.* [10] and KÖHLER [5],

who found the highest values in persons between 8 and 20 years of age and observed a subsequent gradual decline.

The average titre for females was usually higher than that for males. However, the difference was not significant enough to warrant the consideration of sex in the evaluation of individual titres.

Considering the eventual connection between geographical latitude and the normal ASO titre [8*c*], our data agree with those of CHRIST [7] collected in Frankfurt a. M. and of SCHULTZE *et al.* [4] in Marburg, who found a normal average ASO titre of 155 units.

As the methods published in the literature and partly tested by ourselves [4, 7, 11—17], all included some impractical elements, we thought it necessary to elaborate a modified procedure. For instance, for the determination of the combining unit several authors are adding varying amounts of OSL to constant amounts of standard; this procedure yields less serviceable data than do the results of the contrary method, the one usually employed in toxin-antitoxin titration, and based on the principle of "varying amounts of antitoxin-unchanged amounts of toxin". This principle has been applied also by IPSEN [3], and KALBAK [15].

In a well-planned titration, the series of serum dilutions should in general satisfy the following criteria. (a) The distance between concentrations should be in accordance with some mathematical system, and titres should admit of mathematical evaluation; (b) the distance between the titres should be in proportion to the sensitivity of reaction; (c) preparation of the dilutions should be easy and accurate.

Mathematical considerations have been taken into account also in the methods of JOHNSON [17], LOEFGREN [18], and MASSEL and MILLER [19] who so selected the serial titre figures that their logarithms form arithmetical progressions increasing by 0.1 or 0.125. However, their methods seems to have weak points in other respects. The above-mentioned first requirement is satisfied by a simple series of twofold dilutions as determined by the quotient 2 [3, 15, 16]; yet we share the opinion of HEDBERG [20] that titre figures rising by 100 per cent are much too widely-spaced for a reaction where the limit of error is $\pm$ 20 per cent according to KALBAK [15], $\pm$ 30 per cent according to HOLLINGER [21], and about 10 per cent according to our own experiments [22]. Consequently, a series of twofold dilutions gives much rougher results than actually allowed by the sensitivity of the reaction. As regards the requirement of easy accomplishment and accuracy, errors are more likely to occur with serial twofold dilutions and are better avoided if only a single basal dilution is used from which the necessary amounts are separately pipetted into each tube, as reported also by COBURN and PAULI [12], IPSEN [3], and KALBAK [15]. In the method of the two last-named authors the transfer with a pipette of such small quantities as, for instance, 0.063 ml or 0.031 ml appears to be impractical.

The other features of our method have also been elaborated with a view to ensuring accuracy, simplicity, and expediency. In choosing the volume of every one component and the total volume of all components we have followed TODD [11]: 1.0 ml of serum dilution, 0.5 ml of OSL, 0.5 ml of erythrocyte suspension. Physiological saline has been employed for all dilutions, since — in agreement with KÖHLER [8*b*] — we deemed the use of various buffers unnecessary. We found the application of 5 per cent suspensions of rabbit erythrocytes or 2 per cent suspensions of sheep erythrocytes equally suitable; this is in agreement with the experience of SCHULTZE *et al* [4]. As to reading the results, we approve of the procedures adopted by IPSEN [3], KALBAK [15], and LIAO [23] who determine the titre on the basis of the tubes showing approximately 50 per cent haemolysis, since the haemolysis curve manifests less fluctuation at this point than at the points of zero or 100 per cent haemolysis.

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DIE WIRKUNG VERSCHIEDENER ANTIBIOTIKA AUF DAS WACHSTUM DER RHIZOBIEN

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Zusammenfassung. Das Verhalten von acht Rhizobien-Stämmen, die aus Leguminosen verschiedener Standorte Ungarns stammten, wurde gegen die Antibiotika Neomycin, Primycin, Chlortetracyclin, Tetracyclinhydrat, Erythromycin und Grubilin-Na untersucht.

Das Verhalten der Mikroorganismen bei der Prüfung gegen Antibiotika war verschieden. Am wirksamsten erwiesen sich Neomycin und Chlortetracyclin, die in bezug auf die Rhizobien zu den Antibiotika mit dem breitesten und aktivsten Wirkungsspektrum gerechnet werden können, während Tetracyclinhydrat, Erythromycin und Primycin schwächere Wirkung ausübten (d. h. sich als Substanzen mit engerem Wirkungsspektrum auf die Rhizobien erwiesen).

Angesichts der Tatsache, daß auch die Antibiotikumproduktion der im Boden lebenden Mikroorganismen eine wesentliche Rolle im Lebenskampf der dort lebenden Mikrobengruppen spielt, erscheint die Untersuchung der Frage in bodenmikrobiologischer Beziehung wichtig. Unsere Versuche in vitro lieferten gewisse Anhaltspunkte zur Erkennung der im Boden herrschenden Verhältnisse, da die bei unserer Arbeit verwendeten Antiobiotika — mit Ausnahme von Primycin — von Bodenmikroorganismen stammen.

Material und Methoden

Bakterienstämme. Die untersuchten 8 Rhizobien-Stämme stammten von drei verschiedenen Pflanzen, die in jeweils anderen Standorten Ungarns angebaut wurden. Ihre Identifizierung erfolgte auf Grund der morphologischen, kulturellen, biochemischen Merkmale und mit Hilfe von Gefäßversuchen (Knöllchenbildung und Stickstoffbindung).

Im Hinblick darauf, daß in bezug auf die Artzugehörigkeit der Rhizobien gegenwärtig noch kein einheitlicher Standpunkt vorherrscht, haben wir statt der in den verbreiteten Bestimmungsbüchern [1, 7] angegebenen Bakteriennamen die Pflanze bezeichnet, aus deren Knöllchen das fragliche Bakterium stammte.

So wurden die mit II/2 und VIII/1 bezeichneten Bakterienstämme aus Wurzelknöllchen von *Vicia villosa*, die Stämme I/6, IX/3 und XIII/1 von *Medicago sativa*, die Bakterienstämme VII/2, VII/5 und XXV/1 von *Trifolium pratense* isoliert.

Folgende Antibiotika wurden benutzt: Neomycin Byk-Sulfat *(Chemische Fabrik Byk-Gulden-Lomberg GmbH., Konstanz)*; Primycin *(Pharmakologisches Institut der Medizinischen Universität, Debrecen)*; Chlortetracyclin *(Forschungsinstitut der Arzneimittelindustrie, Budapest)* ; Tetracyclinhydrat *(Chinoin, Budapest)* ; Erythromycin lactobionate *(Abbott Laboratories, Chicago, Ill., U. S. A.)* ; Grubilin-Na *(Pharmakologisches Institut der Medizinischen Universität, Debrecen)*.

Zur Züchtung der Bakterienstämme und bei den Untersuchungen über ihr Verhalten den Antibiotika gegenüber verwendeten wir Bohnenagar-Nährböden. Bei den Untersuchungen wurde die von OTTE und KÖHLER [8] empfohlene Plattenmethode mit einer geringen Modifikation angewandt.

Von den Antibiotika stellten wir den Lösungsvorschriften entsprechende Stammlösungen her. Aus den Stammlösungen wurden unter sterilen Bedingungen im Verhältnis 1:10 noch weitere 4—4 Verdünnungstufen zubereitet. Letzten Endes enthielt der Nährboden je ml die in Tabelle I angeführten Mengen der Antibiotika.

Ergebnisse und Schlußfolgerungen

Die Untersuchungsergebnisse sind in Tabelle I zusammengefaßt. Die Wirkung der untersuchten Antibiotika kann direkt verglichen werden, weil wir in allen Fällen dieselben Antibiotikummengen verwendet haben. Am wirksamsten waren Neomycin und Chlortetracyclin, mit denen selbst bei der Nährbodenkonzentration 5 μg/ml — einen bzw. bei Chlortetracyclin zwei Bakterienstämme ausgenommen — Hemmungswirkung erzielt wurde.

Schwächeren Hemmungseffekt als Neomycin und Chlortetracyclin zeigte Tetracyclinhydrat, noch schwächere Wirkung wies Erythromycin, noch geringere als diese Primycin auf.

Die größte Resistenz zeigten die unserseits untersuchten Bakterienstämme gegenüber Grubilin-Na. Sämtliche untersuchten Bakterienstämme wuchsen noch bei der Nährbodenkonzentration 50 μg/ml. Diese Feststellungen stimmen mit den Untersuchungsergebnissen von Vályi-Nagy, Uri und Szilágyi [10] sowie Uri [9] überein, nach welchen Primycin in erster Linie auf Grampositive Bakterien wirke und Grubilin-Na ein sehr enges Spektrum besitze bzw. hauptsächlich ein antifungales Antibiotikum sei. Wir glauben, daß die bei unseren Untersuchungen angewandten Dosen geeignet waren, um die Resistenz der Rhizobien-Stämme gegen Antibiotika festzustellen bzw. um die Empfindlichkeit der Rhizobien gegenüber Antibiotika mit unterschiedlichem Spektrum auf obige Weise zu charakterisieren.

Antibiotika und andere auf Bakterien hemmend wirkende Stoffe werden bei der Bekämpfung von Krankheiten der Leguminosen immer häufiger angewandt [13]. In bezug auf die Wirkung dieser Stoffe auf Rhizobien wird über Wuchsförderung [2, 11], als auch über Hemmung [2, 12], berichtet.

Vintika [11] vermochte durch die Behandlung der Rhizobien mit Antibiotika (z. B. Penicillin) den Ernteertrag des Rotklees um 10.9%, die Menge der Wurzelknöllchen um 17.3% zu steigern und den Stickstoffgehalt des Bodens um 26.0% zu vermehren.

Wir haben bereits früher orientierende Untersuchungen über die Empfindlichkeit der Rhizobien einzelnen Antibiotika und Sulfonamiden gegenüber ausgeführt [5]. Darüber hinaus studierten wir die Resistenz der Rhizobien gegenüber den Antibiotika Penicillin, Streptomycin, Viomycin, Chloramphenicol, Oxytetracyclin und Kanamycin [6].

Tabelle I

Die Wirkung der Antibiotika auf verschiedene Rhizobien-Stämme

Antibiotika	Aintibiotikum-gehalt/ml Nährboden	Bakterienstämme							
		VIII/1	I/6	IX/3	VII/5	XXV/1	XIII/1	VII/2	II/2
Neomycin	50 µg	−	−	−	−	−	−	−	−
	5,0 „	−	−	−	−	−	−	+	−
	0,5 „	+	+	+	+	+	+	+	+
	0,05 „	+	+	+	+	+	+	+	+
	0,005 „	+	+	+	+	+	+	+	+
	Kontrolle	+	+	+	+	+	+	+	+
Primycin	50 µg	−	+	+	+	−	+	+	−
	5,0 „	−	+	+	+	+	+	+	+
	0,5 „	−	+	+	+	+	+	+	+
	0,05 „	−	+	+	+	+	+	+	+
	0,005 „	+	+	+	+	+	+	+	+
	Kontrolle	+	+	+	+	+	+	+	+
Chlortetracyclin	50 µg	−	−	−	−	−	−	−	−
	5,0 „	−	−	+	−	−	+	−	−
	0,5 „	+	−	+	+	−	+	−	+
	0,05 „	+	+	+	+	−	+	+	+
	0,005 „	+	+	+	+	+	+	+	+
	Kontrolle	+	+	+	+	+	+	+	+
Tetracyclinhydrat	50 µg	−	−	−	−	−	−	−	−
	5,0 „	+	−	+	+	−	+	−	+
	0,5 „	+	+	+	+	+	+	+	+
	0,05 „	+	+	+	+	+	+	+	+
	0,005 „	+	+	+	+	+	+	+	+
	Kontrolle	+	+	+	+	+	+	+	+
Erythromycin	50 µg	−	+	+	−	−	−	+	−
	5,0 „	−	+	+	−	+	+	+	−
	0,5 „	−	+	+	+	+	+	+	−
	0,05 „	+	+	+	+	+	+	+	+
	0,005 „	+	+	+	+	+	+	+	+
	Kontrolle	+	+	+	+	+	+	+	+
Grubilin-Na	50 µg	+	+	+	+	+	+	+	+
	5,0 „	+	+	+	+	+	+	+	+
	0,5 „	+	+	+	+	+	+	+	+
	0,05 „	+	+	+	+	+	+	+	+
	0,005 „	+	+	+	+	+	+	+	+
	Kontrolle	+	+	+	+	+	+	+	+

Zeichenerklärung: + = Wachstum
− = Kein Wachstum (Hemmungswirkung)

Erwähnt sei schließlich, daß vorliegende Arbeit zugleich unsere früheren Untersuchungen zwecks Feststellung der Empfindlichkeit von chitinabbauenden Mikroorganismen [4] und Rhizosphären-Bakterien [3] Antibiotika gegenüber ergänzt.

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VARIABILITY OF STREPTOMYCES RIMOSUS

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Summary. The natural and artificial variants of *Streptomyces rimosus* species which produce oxytetracycline have been studied and their conditions of growth as well as morphological, physiological, and biochemical properties have been compared. On the culture media used, colony form, the shape of sporangia and spores, the antibiotic yield, furthermore the colour of the vegetative mycelium appeared as conservative features. Differences were noted in the readiness to proliferate on various culture media, in the colour of spores *en masse* and the capacity for sporulation, and variations were observed as regards physiological and biochemical features.

The variants were found to display no antagonism to one another.

As a result of research into antibiotic producing Actinomycetes numerous reports have been published on the isolation of increasing numbers of Streptomyces derived from soil samples of various origin but producing a similar antibiotic. Upon taxonomic investigation of the newly isolated strains some have been classified into the group of the classical species, while others have been described as new species. The classification of Actinomyces producing the same antibiotic into different species is inconsistent with the apparently obvious assumption that each species of Actinomyces produces a given antibiotic [1].

To elucidate the question, close inquiry into characteristic traits of a few especially significant Streptomyces species would seem to be expedient. Such studies are pursued with the objective (1) of an analytical comparison to determine the variations shown within the species; (2) to determine as far as possible the relationship of a species to other species producing the same antibiotic.

In the present paper an attempt is made to elucidate the cultural, morphological, physiological, and biological variability of *Streptomyces rimosus* variants producing oxytetracycline. First we compared some typical features of natural and artificial variants derived from three species producing oxytetracycline and identified as *Streptomyces (Actinomyces) rimosus*. Relying on the findings thus obtained we intend to subject to analytical comparison several Streptomycetes producing oxytetracycline and described as new species.

In studies of similar nature to follow the changes of antibiotic production have usually been followed solely by tests on microorganisms. Thus the possibility was barred to check by a reliable method whether the antibacterial action was due to one or several antibacterial substances.

In the studies to be reported below we employed Streptomycetes producing antibiotics of clearly defined chemical structure, and paper chromatography to differentiate the antibiotics.

Materials and methods

Strains. After previous selection from numerous variants, the following oxytetracycline-producing actinomyces strains were used for the tests.

1. *Streptomyces rimosus* strains RS-21 (own culture).

(a) Natural variant I—22; good pigmentation, good sporulation, favourable yield.

(b) Natural variant Aa—4; faint pigmentation, poor sporulation, poor yield.

(c) Variant TO, obtained by ultraviolet irradiation; moderate pigmentation, moderate sporulation, abundant yield.

2. *Streptomyces (Actinomyces) rimosus* strain L SzT-118 (VNIIA, Moscow).

(a) Natural variant 3-LSzT-118; good pigmentation, no or poor sporulation, good yield.

(b) Variant S—10/2 obtained by ultraviolet irradiation; moderate pigmentation, no or very poor sporulation, good yield.

(c) Variant H—1, obtained by beta-irradiation; good pigmentation, moderate sporulation, moderate yield.

3. *Streptomyces rimosus strain* "V" (isolated by DR. K. PÓLYA and producing oxytetracycline).

(a) Originally isolated strain; moderate pigmentation, moderate sporulation, poor yield.

(b) Natural variant VA—5; good pigmentation, good sporulation, good yield.

(c) Variant FV, produced by treatment with formalin; good pigmentation, good sporulation, moderate yield.

Data concerning pigmentation and sporulation were obtained by growing the microorganisms on a solid glycerine culture medium; the yield was determined by shaking.

Culture media. The culture media prescribed by WAKSMAN for testing Actinomycetes have been employed without modification [2]. The media were as follows. (1) CZAPEK's solid agar (with glucose); (2) glucose-asparagine agar; (3) glycerine agar; (4) tyrosine agar; (5) meat-extract peptone agar; (6) glucose-peptone agar; (7) starch agar "A"; (8) starch agar "B"; (9) eggalbumin agar; (10) potato agar; (11) potato-glucose agar; (12) starch-nitrate agar; (13) yeast extract agar; (14) EMERSON's solid medium; (15) meat-extract-peptone-gelatine; (16) peptone gelatine; (17) glucose broth; (18) bouillon; (19) sucrose solution; (20) starch solution; (21) yeast extract "B" solution; (22) fluid corn-steep culture medium; (23) fluid soy-meal medium; (24) synthetic ammonlactate medium; (25) potato plugs; (26) milk; (27) cellulose medium.

In test for sugar utilization we used WAKSMAN's carbon culture medium [3], replacing the CH source of the culture medium by the corresponding sugar. The sugars employed were mannose, rhamnose, laevulose, maltose, d-xylose, raffinose, l-sorbose, d-ribose, galactose, saccharose, arabinose, glucose, fructose, lactose.

The materials were of analytical purity. Soviet agar-agar was used to consolidate media.

Technical conditions. Inoculations were evaluated after 28 days' incubation at 28° C, with the exception of milk cultures, which were read after 10 days' incubation at 28° and 37° C, and tests for CH consumption which were read after 14 days' incubation at 28° C.

Antagonism of the strains was studied on potato-glucose culture medium No. 11, by cross-streaking.

Pigments were examined by paper chromatography. The antibiotics derived from the variants were determined with the autobiographic method, by paper chromatography. *Bacillus subtilis* FDA 6633 and a strain of *Candida albicans* served as test microorganisms.

Results

The characteristic features of growth of the strains have been summarized in Tables I to VI.

Among the investigated Actinomycetes, strain H—1 displayed remarkable qualities regarding growth on various media. This variant failed to proliferate on starch solution, peptone gelatine, and synthetic ammonium lactate

media. Of the variants originating from *Actinomyces rimosus* and *Streptomyces rimosus*, disposition to growth was much stronger with the latter. In addition, there were certain differences in growth between the strains.

Table I

Growth of Streptomyces rimosus variants on different culture media

Culture medium	Streptomyces rimosus								
	I-22	Aa-4	TO	LSzT-118	S-10/2	H-1	V	VA-5	FV
1.	4	2	2	1	3	3	2	4	2
2.	4	3	3—4	3	3	3	4	4	4
3.	4	4	3	2	2—3	3	4	3	4
4.	3	4	3—4	3	3	3—4	3	3	3
5.	2	3	3	3	3	2	1	2	4
6.	4	4	5	4	4	4	4	4	4
7.	3	4	4	4	2	2	5	5—4	3
8.	2	3	3	1	2	2	3	3	2
9.	2	2	3	2	2—3	3	2	2	1
10.	4—5	4	5	5	4	5	4—5	5	4
11.	3	3	4	4	3	3	3	3	3
12.	3	4	3—4	3	2	2	5	4	3
13.	4	5	4	4	4	4	4	4	4
14.	3	3	4	1	2	2	4	3	2
15.	5	5	5	5	5	4	5	5	5
16.	3	3	2	3	4	0	4	4	4
17.	3—4	4	3	2	3	1	2	3	3
18.	2	4	2	1	3	1	2	3	1
19.	4	4	1	2	2	2	3	3	3
20.	4	4	3	2	1	0	4	5	4
21.	4	3	3	2	2	1	3	4	4
22.	4	4	3	2	1	1	3	3	3
23.	3	4	1	2	2	1	3	2—3	2
24.	3	4	1	3	3	0	3	3	3
25.	5	3	4	4	4	5	4	5	5
26.	4	4	5	4	4	3	4	4	4
27.	0	0	0	0	0	1	0	0	0

Key. 5 = excellent growth; 4 = fair growth; 3 = moderate growth; 2 = faint growth; 1 = very poor growth; 0 = no growth.

Table II shows the characteristic colony forms of the variants on various solid culture media. The so-called level-line fragmentation [4] described as

Table II

Colony forms of Streptomyces rimosus on solid culture media

Strains	Colonial morphology (Culture media 1 to 14)
I-22	Regularly circular, convex, conic; slight tendency to crease, level-line fragmentation, smooth-broad margin
Aa—4	Regularly circular, convex, conical or crateriform, crinkled colonies; level-line fragmentation, smooth-broad margin
TO	Regularly circular, convex, conical colonies; slight tendency to crease; mild tendency to flattening; level-line fragmentation, smooth-broad margin.
LSzT-118	Circular, convex, conical or crateriform colonies; creased, colonial surface; level-line fragmentation; dissected, wide margin
S-10/2	Regularly circular, slightly convex, conical or crateriform colonies with creased surface; no level-line fragmentation; dissected, broad margin
H—1	Regularly circular flat, occasionally strongly creased colonies, penetrating into the culture medium; level-line fragmentation, smooth-broad margin
V	Regularly circular, convex, conical or crateriform colonies with creased surface; level-line fragmentation; smooth, narrow margin
VA—5	Regularly circular, convex, crateriform colonies with creased surface; level-line fragmentation; smooth, narrow margin
FV	Regularly circular, convex, crateriform colonies; slight tendency to creasing; level-line fragmentation; smooth narrow margin

characteristic of *Streptomyces rimosus* was not present in the case of every variant. In order of precedence, the most consistent features were the regular, circular pattern, creased surface, level-line fragmentation, and convex form of growth.

The colony margin generally described as characteristic of *Streptomyces rimosus* was found to vary according to strain and medium. For instance, on tyrosine agar no margin developed. However, the distribution of smooth or dissected and broad or narrow formations was hereditary, reproducing the pattern characteristic of the strain. Flat colonies and colonial parts extending into the medium were displayed by the variant H—1. The colonies obtained from single spores differed in size with every medium and variant.

Table III comprises details of the colony forms that developed on fluid media. The changes were mostly qualitative. A pattern common with every variant was a growth in submerged stocks on bouillon and minute circular, flat colonies adhering to the inner wall of the tube in sugar solutions. These latter forms were not encountered with strain H—1.

Table III/a

Colony forms of Streptomyces rimosus variants on fluid culture media

Culture media	Streptomyces rimosus		
	I-22	Aa-4	TO
17.	Superficial colonies of F-3—4 colour adhering to the wall of the dish, or colourless, submerged colonies	Large, colourless stocks which disintegrate upon shaking	Thick membrane of F-3—4 colour on the surface, profuse sedimentation
18.	Submerged, colourless jellied stocks which disintegrate upon shaking (disintegration into threads)	Submerged, colourless stocks which disintegrate into threads upon shaking	Faint superficial growth, large submerged, colourless stocks that disintegrate into threads upon shaking
19.	Colonies of A-4—5 colour growing on the surface and on the walls of the vessels; colourless or greyish-white sedimentation	Colonies of F-2 colour growing on the surface	Colonies of AF-1 colour growing below the surface and adhering to the wall of the vessel
20.	Superficial colonies of F-6—7 colour	Superficial colonies of F-4 colour	Superficial colonies of F-6 colour
21.	Superficial colonies of A-3 colour adhering to the wall of the vessel	Superficial colonies of A-2—3 colour adhering to the wall of the vessel	Mostly submerged contiguous colonies of F-1–5 colour, resistant to shaking
22.	Colourless, submerged stocks; colonies of F-3—4 colour on the surface, adhering to the wall of the vessel	Colourless, submerged stocks; colonies of F-3 colour on the surface	Colonies of F-1—2 colour growing on the surface or submerged
23.	Superficial colonies of F-3 colour or colourless colonies growing submerged and disintegrated into threads upon shaking	No surface growth; submerged, colourless stocks that disintegrate upon shaking	Superficial colonies of F-3—4 colour growing along the walls of the vessel; submerged, colourless stocks
24.	Colonies of A-2 colour adherent to the vessel wall below the surface	Colonies of A-2 colour adhering to vessel wall below the surface	Slight colourless sedimentation

The colour of the vegetative mycelia was compared on several solid and fluid media (Table IV). Variants displayed only quantitative changes. In fluid media slight differences from brown to brownish-green were apparent in every case. A brownish-green basic colour was usually a standard feature of the vegetative mycelium. A characteristic smell of earth was experienced with every variant.

Sporulation data are presented in Table V. Every variant was sporogenic on CZAPEK's medium and potato plugs, whereas on glucose asparagine, tyrosine agar, ammonium lactate and cellulose no sporulation took place. Spore

formation was found to show a high variability in different culture media. Owing to this extreme variability, it was difficult to distinguish newly isolated strains on the basis of sporulation. On yeast extract and potato plugs the variant *H*-1, for instance, proliferated in concentric circles. Two sporulation zones enclosed sterile areas. In such cases profuse spore formation was seen especially at the colony margins.

Data referring to the colour of aerial mycelia are shown in Table VI. Notwithstanding the striking dominance of white, there was a significant shift of colours in the direction of butter-yellow, bluish-white, and mouse-grey. The colour of aerial mycelium was studied only where microscopic examination disclosed the presence of sporulation.

Table III/b

Colony forms of Streptomyces rimosus variants on fluid culture media

Culture media	Streptomyces rimosus		
	LSzT-118	S-10/2	H-1
17.	Slight sedimentation of F-1—2 colour	No superficial growth, moderate amount of sedimentation of F-1 colour	No superficial growth, slight sedimentation of F-1 colour
18.	Colourless submerged stocks; shaking caused turbidity	Colourless, submerged stocks; shaking caused turbidity	Colourless, submerged stocks which disintegrate into threads upon shaking
19.	Superficial colonies of F-1—2 colour adhering to the walls of vessel	Superficial colonies of A-3—4 colour adherent to the walls of the vessel, detached by shaking	Colonies of A-3—4 colour appearing as sedimentation
20.	Few superficial, mostly submerged colonies of F-8 colour	Few superficial, mostly submerged colonies of F-8 colour	No growth
21.	Submerged colonies of F-3 colour; no superficial growth	Mostly submerged colonies of F-2 colour	Submerged, colourless stocks which disintegrate upon shaking
22.	Superficial colonies of F-4 colour adherent to the vessel walls; colourless submerged stocks	Superficial colonies of F-2 colour adherent to the vessel walls	Colourless, submerged stocks, homogeneous turbidity upon shaking
23.	Colourless, submerged stocks which disintegrate into threads upon shaking	Colourless superficial colonies adhering to the vessel walls; colourless sedimentation	Colourless, submerged stocks, disintegrating into threads upon shaking
24.	Sedimentation of F-4—5 colour	Sedimenlation of tiny flakes of F-4 colour	No growth

Table III/c

Colony forms of Streptomyces rimosus variants on fluid cullture media

Culture media	Streptomyces rimosus		
	V	VA-5	FV
17.	Moderate amount of superficial growth of F-3 colour; very little sedimentation	Superficial growth of F-2—3 colour; slight sedimentation	Superficial growth of F-3 colour; moderate sedimentation
18.	Submerged, colourless stocks, disintegrating into threads upon shaking	Submerged, colourless stocks, disintegrating into threads upon shaking	Submerged, colourless stocks, disintegrating into threads upon shaking
19.	Superficial colonies of A-4 colour adhering to the wall of the vessel	Colonies of F-4 colour below the fluid surface adhering to the wall of the vessel	Superficial colonies of A-4 colour adhering to the vessel wall
20.	Submerged colonies of F-6 colour	Superficial growth of F-5 coloured colonies	Profuse sedimentation of AF-5 colour
21.	F-3—4 coloured colonies submerged or adhering to the wall of the vessel	F-3—4 coloured colonies submerged or superficially adhering to the wall of the vessel	Submerged colonies of F-4 growth; no superficial growth
22.	Submerged colourless stocks	Submerged colourless stocks	Submerged colourless stocks
23.	Superficial colonies of FA-2 colour; submerged colourless stocks	No superficial growth submerged colourless stocks which disintegrate into threads upon shaking	No superficial growth colourless stocks resistant to shaking
24.	Submerged colonies of A-H colour, adhering to the wall of the vessel	Submerged colonies of A-5 colour, adhering to the wall of the vessel	Submerged colonies of AF-4 colour adhering to the wall of the vessel

Investigation of the shape of sporophores assumed by sporiferous variants (Fig. 1, Fig. 2), revealed the consistent presence of open spiral forms [5]. Variants had short, cylindrical spores. Sporulation proceeded by fragmentation.

Allowing the native fermentation juice of soy-meal culture medium to migrate on *Macherey-Nagel*'s paper No 214 in a mixture of water, butanol and acetic acid at the ratio of 5:4:1, after various kinds of treatments the following Rf values were obtained.

A spot at Rf 0.80 appearing as orange in ultraviolet light, could not be identified,

A spot at Rf 0.62, yellowish-light brown in direct light and showing bluish fluorescence in ultraviolet light, proved to be inactive against *B. subtilis*

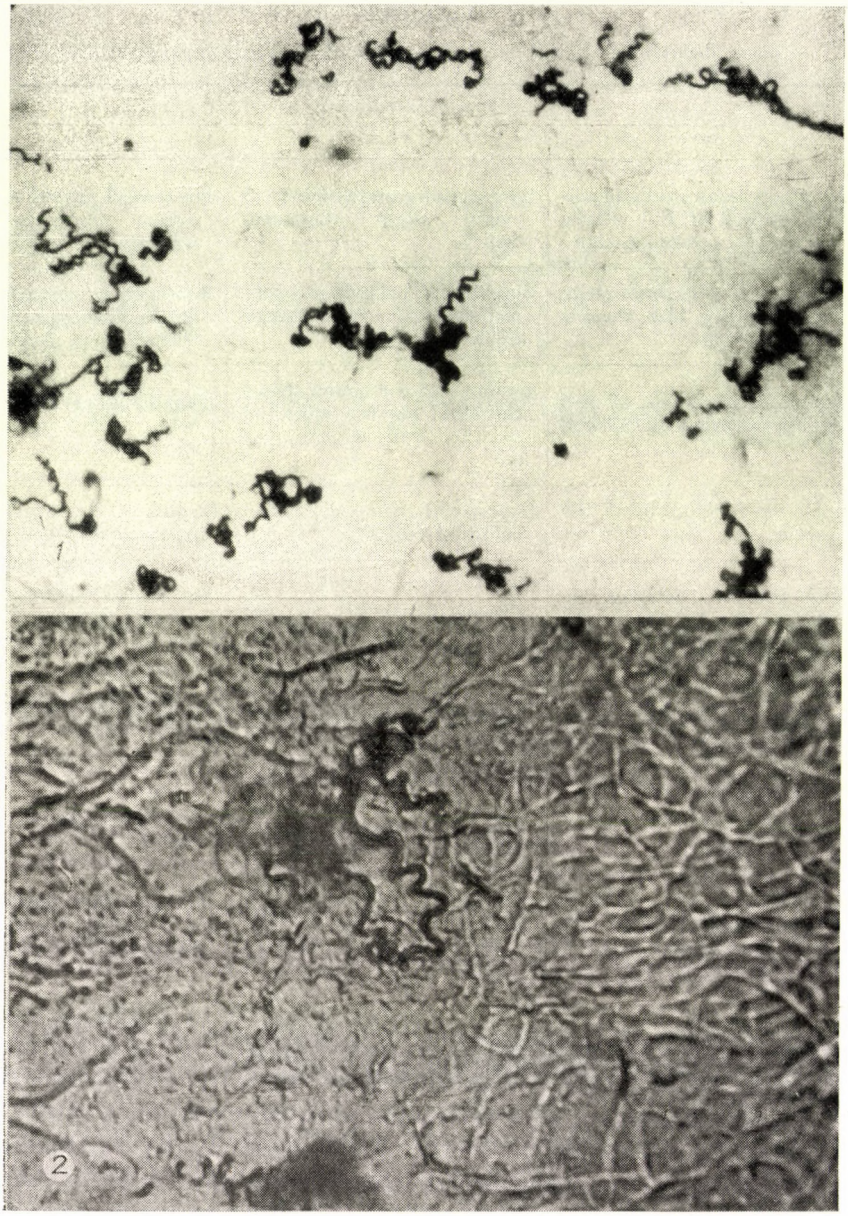

Fig. 1. Spiral sporangium of *Streptomyces rimosus* variant RS-21 (I-22) CZAPEK-DOX's culture medium (with glucose as CH-source); magnification, ×250

Fig. 2. Spiral sporangium of *Streptomyces rimosus* variant TO CZAPEK-DOX's culture medium; magnification, ×1000

Table IV

*Colour of Streptomyces rimosus variants on solid culture media**

Culture media	Streptomyces rimosus								
	I-22	Aa-4	TO	LSzT-118	S-10/2	H-1	V	VA-5	FV
1.	A-8	A-5	A-8—9	A-7	A-7—8	A-6—7	A-8	A-7—8	A-7
2.	A-8—9	A-3	A-8—9	A-4—5	A-7	A-8, F-7	A-8—9	A-8	A-7—8
4.	A-5	A-3	AF-5—6	A-6—7	A-4—6	A-2	A-6—7	A-3—4	A-4—5
6.	A-8—9	A-8	A-7—9	A-6	AF-2—3	A-8—9	A-9	A-9	A-9
7.	A-9	A-8	A-9	A-6—7	A-9	A-9	A-9	A-9	A-8
10.	A-6—7	A-5—7	A-9	AF-4	F-8	A-5—7	A-6—7	A-5	A-5—6

* Colour determination based on I. PACLT, Farbenbestimmung in der Biologie, Jena, 1958.
Key: A = aurantiacus; F = flavus.
The increasing sequence of numbers represents the deepening of the colour.

Table V

Sporulation of Streptomyces rimosus variants according to culture medium

Culture medium No	Streptomyces rimosus								
	I-22	Aa-4	TO	LSzT-118	S 10/2	H-1	V	VA-5	FV
1.	+++	++	+	±	+	++	+++	++++	++
2.	∅	∅	+	+	++	∅	+	++	∅
3.	++	+	∅	∅	∅	++	+	+	∅
4.	∅	∅	∅	∅	∅	∅	∅	∅	∅
5.	∅	∅	+	∅	∅	∅	+	++	∅
6.	++	∅	+++	∅	∅	+++	+	++	+
7.	+++	∅	+	++	∅	+	+	+	+++
8.	∅	∅	+	∅	∅	∅	+	+	+
9.	∅	∅	∅	∅	∅	+	∅	∅	∅
10.	+++	+	∅	+	∅	++++	++	++	+++
11.	+++	∅	∅	+	∅	++++	+	+	++
12.	+	∅	∅	∅	∅	+	+	+	++
13.	∅	∅	∅	∅	∅	+++	∅	++	++
14.	++	∅	+	∅	∅	∅	+	++	+++
15.	+++	++	∅	+	∅	+++	++++	+++	++
16.	++	∅	∅	∅	∅	−	+	++	+
17.	+	∅	∅	∅	∅	∅	++	+	+
18.	∅	∅	∅	∅	∅	∅	∅	∅	∅
19.	+++	++	∅	+	+	∅	++	∅	∅
20.	+	+	∅	∅	∅	−	∅	+	∅
21.	+	∅	∅	∅	∅	∅	∅	∅	∅
22.	++	+	∅	∅	∅	∅	∅	∅	∅
23.	++	∅	∅	+	∅	∅	+	+	∅
24.	∅	∅	∅	∅	∅	∅	∅	∅	∅
25.	++++	++	+++	+	+	++++	++++	++++	++++
26.	++	∅	+	∅	∅	+	+	+	+
27.	∅	∅	∅	∅	∅	∅	∅	∅	∅

Signs: ++++ = excellent sporulation; +++ = good sporulation; ++ = mediocre sporulation; + = poor sporulation; ∅ = no sporulation; − = no growth.

and *Candida albicans*. Owing to its characteristic colour it was identified as pigment material.

A spot at Rf 0.44, yellowish-brown in direct light and yellow in ultraviolet light, when tested by autobiography was found to inhibit the growth of *B. subtilis* and to react positively to ferrochloride. After elution in methanol hydrochloride, the ultraviolet spectrum permitted its identification as oxytetracycline.

Table VI

Colour of sporiferous air mycelium of Streptomyces rimosus variants on different culture media

Culture media	Streptomyces rimosus								
	I-22	Aa-4	TO	LSzT-118	S-10/2	H-1	V	VA-5	FV
1	white	white	bluish white	white	white	white	white	white	pale grey
2	—	—	white	white	white	—	light grey	light grey	—
3	yellowish white	yellowish white	—	—	—	white	white	white	—
5	—	—	white	—	—	—	white	yellowish white	—
6	yellowish white	—	bluish white	—	—	greyish white	greyish white	yellowish white	greyish white
7	white	—	bluish white	white	—	white	greyish white	greyish white	mouse grey
8	—	—	white	—	—	—	—	white	pale grey
9	—	—	—	—	—	white	—	—	—
10	white	yellowish white	—	white	—	yellowish white	greyish white	greyish white	grey
11	white	—	—	white	—	yellowish white	yellowish grey	yellowish grey	pale grey
12	white	—	—	—	—	greyish white	greyish white	greyish white	greyish white
13	white	—	—	—	—	greyish white	—	greyish	grey
15	greyish white	greyish	—	white	—	yellowish greyish white	white	greyish white	mouse grey
16	greyish white	—	—	—	—	—	greyish white	greyish white	grey
17	white	—	—	—	—	—	white	white	grey
19	white	yellowish white	—	white	white	—	white	—	—

Table VI cont'd

Culture media	Streptomyces rimosus								
	I-22	Aa-4	TO	LSzT-118	S-10/2	H-1	V	VA-5	FV
20	yellowish white	white	—	—	—	—	—	white	—
21	white	—	—	—	—	—	—	—	—
22	greyish white	white	—	—	—	—	—	—	—
23	white	—	—	white	—	—	light greyish white	greyish	—
25	white with pale greyish tint	greyish white	greyish white	white	white	greyish white	greyish bluish white	greyish white	grey
26	yellowish white	—	greyish white	—	—	yellowish white	light grey	greyish white	greyish white

A spot appearing at Rf 0.35 showing bluish fluorescence in ultraviolet light and a negative reaction to ninhydrin and ferrochloride, was found to be inactive against *B. subtilis* and to inhibit the growth of *Candida albicans*. On the basis of its ultraviolet spectrum the substance was identified as rimocidine.

A spot at Rf 0.30, showing greenish-blue fluorescence in ultraviolet light, proved to be inactive biologically. It gave a positive reaction to ninhydrin. The ninhydrin-positive spots encountered in this Rf domain displayed quantitative and qualitative differences with each variant.

At Rf 0.11 another inactive spot, appearing yellowish-brown in direct light and giving a bluish fluorescence in ultraviolet light, and positive with ninhydrin, could be observed with every strain. This provided support for demonstrating the presence of another pigment which migrates together with ninhydrin positive material.

Thus with *Str. rimosus* variants, the coloration of culture media is due to the yellow and yellowish-brown pigments accumulated during cultivation and to a coloured antibiotic of the tetracycline type. The quantity of the produced material and the ratio of the constituents depend on the culture medium. In the media, no antibiotic degradation products were demonstrated except for a few instances, as *e.g.* apoterramycin at Rf 0.40. The antibiotic designated P—85 was not present.

The general physiological properties of the variants have been summarized in Table VII. Apart from reducing methylene blue and coagulating milk at 37°C the physiological features of the variants differed from one another. Qualitative dissimilarities occurred particularly in nitrate reduction and gelatine liquefaction, furthermore in coagulation of milk at 28°C.

The data reflecting sugar utilization are presented in Table VIII. The uniform utilization of arabinose, glucose, and fructose was noteworthy. The capacity for sugar utilization is in general hereditary with natural variants. In the case of artificial variants this phenomenon is sometimes absent.

The variants showed no antagonism to one another.

Table VII

General physiological properties of Streptomyces rimosus variants

	Streptomyces rimosus								
	I-22	Aa-4	TO	LSzT-118	S-10/2	H-1	V	VA-5	FV
Gelatine liquefaction	very poor	very poor	very poor	very poor	good	absent	weak	weak	weak
Milk coagulation at 28°C	absent	absent	absent	absent	absent	present	very poor	absent	absent
Milk digestion at 28°C	weak	weak	weak	good	moderate	moderate	good	moderate	good
Milk coagulation at 37°C	present	present	present	present	present	present	present	present	present
Milk digestion at 37°C	moderate	good	good	very good	good	moderate	very good	good	good
Milk pH change	absent	absent	absent	absent	absent	faint acidification	absent	absent	absent
Starch digestion	moderate	good	moderate	slight	slight	very poor or absent	good	good	moderate
Growth on cellulose	absent	absent	absent	absent	absent	slight	absent	absent	absent
Nitrate reduction	faint	absent	absent	absent	absent	absent	moderate	faint	absent
Methylene blue reduction	positive	positive	positive	positive	positive	positive	positive	positive	positive

Table VIII

Sugar consumption of Streptomyces rimosus variants

Kind of sugar	Streptomyces rimosus								
	I-22	Aa-4	TO	LSzT-118	S-10/2	H-1	V	VA-5	FV
Mannose	+	+	+	∅	+	∅	∅	∅	+
Rhamnose	+	+	±	∅	∅	∅	±	±	±
Laevulose	∅	∅	+	+	±	+	∅	∅	∅
Maltose	+	+	+	∅	+	±	±	±	+
d-xylose	+	±	±	±	+	∅	±	+	+
Raffinose	∅	∅	±	+	±	∅	±	±	±
l-sorbose	∅	∅	∅	±	∅	∅	∅	∅	±
d-ribose	∅	∅	∅	+	∅	+	∅	∅	∅
Galactose	∅	+	∅	+	±	+	+	+	+
Saccharose	+	+	±	+	+	∅	+	+	±
Arabinose	+	+	+	+	+	+	+	+	±
Glucose	+	+	+	+	+	+	+	+	+
Fructose	±	+	+	+	+	+	±	+	+
Lactose	+	+	±	+	∅	+	+	+	+

Signs: + = growth;
± = weak growth;
∅ = no growth.

Discussion

In the selection of variants for comparative studies the properties noted in previous experiments were taken into consideration. Of the variants in our possession we endeavoured to choose first of all those that had displayed differences in physiological characteristics or growth, and to render these differences conspicuous by the use of several media.

The cultivation characteristics permitted of closer comparison by establishing the relationships between strains and culture media. Our studies were aimed mainly at the features usually taken into consideration in the classification of Streptomycetes.

Some cultural, morphological, physiological, and biochemical properties were common to all variants; others manifested more or less marked differences with the individual strains. The features common with every variant were as follows.

Growth characteristics. (a) Growth on CZAPEK—DOX's culture medium (with glucose as the CH source); excellent growth on potato media and meat extract-peptone gelatine; good growth on glucose-peptone agar "A"; moderate growth on tyrosine agar.

(b) On all solid culture media, round colonies with creased surface were formed, with smooth or dissected transparent margins formed of colourless vegetative mycelium which is not observable on tyrosine culture media.

(c) In fluid bouillon, growth in colourless submerged stocks.

(d) The ground colour of the vegetative mycelium is aurantiacus on solid media, and flavus on fluid culture media.

(e) Sporulation takes place on CZAPEK—DOX's culture medium (with glucose as source of CH) and potato plugs, but is absent in tyrosine, synthetic ammonium lactate, and cellulose media, as well as from bouillon.

Morphological characteristics. (a) Sporangia display an open spiral form. (b) The shape of spores is short, cylindrical. (c) Sporulation takes place through fragmentation.

Biological features. (a) Rapid coagulation of milk at 37°C. (b) Reduction of methylene blue.

Biochemical properties. (a) Capacity to utilize glucose, arabinose, fructose.

Antibiotics, pigmentation, antagonism. (a) Production of oxytetracycline on organic culture media. (b) Production of rimocidine on organic culture media. (c) The presence of a yellow (Rf, 0.62) and a yellowish-brown (Rf, 0.11) pigment on soybean. (d) Variants show no antagonism to one another.

From the above findings it is clear that for the taxonomic classification of *Streptomyces rimosus* there are few constant traits that would serve as unequivocally characteristic of the species.

An additional difficulty is presented by the tendency of sporiferous aerial mycelium to change in colour, assuming greyish or yellowish hues according to the strain and the medium. This may interfere with the differentiation of a species from others. — In comparative investigations it nevertheless appears expedient to determine the aerial mycelium of colonies grown on potato plugs according to its colour, with due regard to sporulation.

The level-line fragmentation described as typical of *Streptomyces rimosus* [4] has been found to lack constancy; so has the appearance of spores at sites of colonial development [6]. The lack of these important distinctive features renders determination increasingly difficult.

Owing to the extreme variability of the characteristics classification of the species according to the systems of KRAINSKY, DUCHE, GAUZE, or PRIDHAM may involve the danger of classing some variants into different series, particularly when cultivation has been undertaken on a small number of media. The situation is further aggravated by the circumstance that some variants may develop additional modifications.

In contrast to the above difficulties, the apparently stable micromorphological properties of the species, the invariable nature of the organs of reproduction, may supply a reliable key to determination. However, the exploitation of this fact in the case of oxytetracycline producing Actinomyces is contrary

to WAKSMAN's generally valid statement that Actinomycetes differ from one another rather in physiological and biochemical features than in morphological pattern [3]. Judging by the findings of KRASSILNIKOV [7], GAUZE [8], and PRIDHAM [5] this principle is valid, within one species rather than among species. Our investigations into the *Streptomyces rimosus* variants seem to confirm this view.

On the basis of the vegetative mycelium's colour, the *Streptomyces rimosus* species which produces oxytetracycline is listed as flavus [9]. In the course of our experiments the flavus ground colour of the vegetative mycelium was seen in fluid culture media; on solid culture media an aurantiacus colour dominated.

The colour of the vegetative mycelium is due to accumulated pigments. This points to the significance of a close study of pigments [10]; this could replace or furnish a basis for species differentiation according to the colour of the vegetative mycelium. For similar reasons it is expedient to perform comparative analysis by paper chromatography of the vegetative mycelium pigments found dissolved in various fluid and solid culture media. The ideal method would nevertheless be a series of experiments where the pigments derived from the mycelium and culture media are identified by their chemical properties.

The chromatographic tests have proved the identity of the pigments contained by variants of *Streptomyces rimosus* on certain media. Moreover, antibiotic production and quality have also proved to be unaltered.

Paper chromatography followed by autobiographic development and tests involving several species of microorganism, as well as subsequent identification of demonstrably antagonistic spots with standard antibiotics has been deemed to be an especially efficient procedure for assessing antibiotic action.

The quantity of the oxytetracycline produced varied according to variant and medium. Among a great number of variants, examined for another purpose, we found strain where the oxytetracycline yield decreased to unmeasurable levels. The absence of oxytetracycline synthesis, however, failed to result in the synthesis of a new antibiotic.

The stability of the capacity for antibiotic production is hereditary and highly specific [1]. The development of antagonistic features in nature proceeds very slowly under environmental influences [11].It would therefore be unreasonable to apply artificial mutagenic forces in order to develop Streptomyces variants which, in addition to the existing active principle, would synthesize some new antibiotic differing from the original in chemical structure and even superseding it.

On the strength of our experiments it may be stated that in a comparison of the *Streptomyces rimosus* species — admitting of wide variations — with other oxytetracycline producing species it is advisable to rely on the micro-

morphological pattern, growth, antibiotic properties, and pigment production, in the first place. Further physiological and biochemical characteristics will only serve to complete the analysis.

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DIE VERTEILUNG VON COLI-ENDOTOXIN IM KANINCHENORGANISMUS WÄHREND DER SHWARTZMANSCHEN REAKTION

Von

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Zusammenfassung. Nach unseren bisherigen Versuchen ist chromiertes Endotoxin zur Vorbereitung des Shwartzmanschen Phänomens ungeeignet. Bei Anwendung des mit P^{32} markierten Coli-Endotoxins zur Auslösung wurde festgestellt, daß die Verteilung des Endotoxins im tierischen Organismus durch die Endotoxinpräparation im allgemeinen nicht wesentlich beeinflußt wird; auch nach der Präparation wird die größte Menge von der Leber, sodann der Reihe nach von der Lunge, Niere und dem Herzen gebunden, ebenso wie dies im Zusammenhang mit anderen makromolekulären Antigenen von mehreren Autoren festgestellt wurde. Mit spezifischer Aktivitätsbestimmung konnte nachgewiesen werden, daß die Endotoxinaffinität der vorbereiteten Hautregion im Vergleich zum Bindungsvermögen der intakten Haut um etwa 40% zunimmt. Von der Haut wird bereits in der ersten Stunde diejenige Endotoxinmenge gebunden, die im Zeitpunkt des Erscheinens der hämorrhagischen Reaktion (4 Stunden nach der i. v. Injektion) bestimmt werden kann. Nach 24 Stunden zeigen die präparierte und nicht präparierte Haut jedoch ungefähr den gleichen Endotoxingehalt. Das bei der Auslösung eingespritzte Endotoxin gelangt wahrscheinlich mit den Leukozyten in den hämorrhagischen Fleck. Ferner wurde festgestellt, daß das zur Vorbereitung der Shwartzmanschen Reaktion angewendete Endotoxin von der Präparationsstelle vorwiegend resorbiert wird und nicht akkumuliert, so daß es bei der Entwicklung des Shwartzmanschen Phänomens seine Wirkung auf die Zellen oder Kapillaren der präparierten Region nicht direkt, sondern indirekt nach einem nicht näher bekannten Mechanismus ausübt.

Im Verlauf der in unserem Institut durchgeführten Studien über das Shwartzmansche Phänomen hat sich schon seit längerer Zeit die Notwendigkeit ergeben, das Schicksal und die Lokalisation des eingespritzten Endotoxins in den verschiedenen Teilen des Organismus zu klären, um den Mechanismus der zustande kommenden pathologischen Prozesse besser verstehen zu können. Braude, Carey und Zalesky [1] haben als erste mit radioaktivem Na_2CrO markiertes Endotoxin beim Studium der physiologischen Wirkungen von Endotoxinen benutzt. Ihre Versuche ergaben, daß die Toxizität des Coli-Endotoxins durch die Markierung mit $Na_2Cr^{51}O_4$ bzw. $Cr^{51}Cl_3$ nicht herabgesetzt wird und daß zwischen dem Ausmaß der Radioaktivität und Toxizität bei Versuchen in vivo enge Parallelität besteht.

Material und Methoden

Im Rahmen unserer Versuche wünschten wir zunächst klarzustellen, ob die Endotoxine nach Chromierung zur Auslösung des Shwartzmanschen Phänomens geeignet sind. Zur Chromierung benutzten wir erst gereinigtes Typhus-Endotoxin [2], von dem 5 mg/ml mit 0,2 ml $Cr^{51}(NO_3)_3$ bzw. $Na_2Cr^{51}O_4$ während 1—2 Stunden inkubiert, dann 24 Stunden erst fließendem Wasser, danach dest. Wasser gegenüber dialysiert wurden, wonach wir die Aktivität des Dialysates bestimmten. Es stellte sich heraus, daß vom Endotoxin unter diesen Bedingungen keine nachweisbare Isotopchrommenge gebunden wird.

Hiernach gaben wir nach dem Verfahren von BRAUDE, CAREY, SUTHERLAND und ZALESKY [3] zu 100 mg gereinigtem Coli-Endotoxin (Herstellung s. weiter unten) aus Sparsamkeitsgründen 0,56 mg nicht radioaktives $CrCl_3$ in 25 ml Phosphatpuffer pH = 7,0 (0,3530 g NaH_2PO_4 + 0,639 g Na_2HPO_4 + 0,172 g NaCl ad 1000 g H_2O). Nach 24stündiger Inkubation bei 37° C wurde das chromierte Endotoxin durch Sättigung mit 68 Gewichts Äthylalkohol gefällt und der Niederschlag zentrifugiert. Nach mehrmaligem Waschen mit absolutem Alkohol und Äther erfolgte die Trocknung an der Luft bis zur Gewichtskonstanz. Den Chromgehalt des Endotoxins bestimmten wir kolorimetrisch nach der Diphenylkarbazid-Methode von ROWLAND [4], wobei wir einen fest gebundenen Chromgehalt ermittelten, der mit den Literaturangaben übereinstimmte.

Das gereinigte Coli-Endotoxin wurde auf 1%igem Dextrose-Agar-Nährboden aus einem 24 Stunden gezüchteten *E. coli* O111 Stamm so hergestellt, daß wir die Bakterien nach Abwaschen vom Nährboden dreimal in physiol. NaCl Lösung bzw. in Wasser durchspülten und nach jeder Spülung zentrifugierten. Die gewonnene Bakterienmasse wurde 24 Stunden in 96%igem Alkohol suspendiert und anschließend dreimal mit Alkohol und einmal mit Äther gewaschen. Die getrocknete Masse pulverisierten wir und hydrolysierten sie in einem mit Rückflußkühler versehenen Kolben 1½ Stunden bei 10° C. Sodann wurde das ganze Substrat durch Filtrierpapierbrei sorgfältig filtriert, das Filtrat 48 Stunden dialysiert und das Endotoxin anschließend mit der 6fachen Menge 96%igen Alkohols im Eisschrank gefällt. Der Niederschlag wurde zentrifugiert, mit Alkohol dreimal, mit Äther einmal gewaschen und an der Luft bis zur Gewichtskonstanz getrocknet.

Mit dem auf diese Weise hergestellten und chromierten Coli-Endotoxin präparierten wir die Kaninchen vorschriftsmäßig intrakutan, während wir zur Auslösung gewöhnliches, nicht chromiertes Coli-Endotoxin benutzten. Dieses chromierte Coli-Endotoxin erwies sich indessen als ungeeignet zur Präparation, da die Reaktion bei den Kaninchen nach wiederholter i. v. Einspritzung der Endotoxindosis nicht auftrat. Diese Beobachtung scheint uns wichtig, weil sie zeigt, daß das chromierte Endotoxin, im Gegensatz zu den mit einer anderen Methode gewonnenen Ergebnissen von BRAUDE *und Mitarbeiter* [1, 3], dem gewöhnlichen, nicht chromierten Endotoxin — zumindest im Zusammenhang mit dem Shwartzmanschen Phänomen — nicht gleichwertig ist.

Nach diesen erfolglosen Versuchen gingen wir derart vor, daß wir dem 1%igen Dextrose-Agar-Nährboden je ml 5 μC P^{32} in Form von Na_2HPO_4 zusetzten, den Nährboden mit *E. coli* O111 Stämmen beimpften, 24 Stunden im Thermostaten inkubierten, dann die Bakterien abspülten, die Waschflüssigkeit zentrifugierten, die gewonnene Bakterienmasse 24 Stunden in 96%igem Alkohol suspendiert im Eisschrank aufbewahrten und nach neuerlichen Alkoholspülungen mit 0,1 *n* Essigsäure im Rückflußkühler 1½ Stunden kochten. Hiernach wurde die Flüssigkeit durch Filtrierpapierbrei abgesaugt, 48 Stunden dialysiert und bei der Endoxinfällung 24 Stunden mit der 6fachen Menge 96%igen Alkohols im Eisschrank aufbewahrt. Das ausgefällte Endotoxin wurde mehrmals mit Alkohol und Äther gewaschen, bis zur Gewichtskonstanz getrocknet und seine Aktivität bestimmt.

10 Kaninchen präparierten wir durch intrakutane Einspritzung von 0,1 ml verschiedener Verdünnungen von 1 mg/ml gewöhnlicher Ty.-Endotoxinlösung in die Rückenhaut. 24 Stunden später wurde die Shwartzmansche Reaktion durch das mit P^{32} markierte Coli-Endotoxin ausgelöst. Die Tiere erhielten zur Auslösung i. v. mit etwa 1—20 mg Isotop markiertes Endotoxin. Diese Menge entsprach einer Aktivität von 70 000—700 000 Imp/min. 24 Stunden nach der Auslösung, als sich das Phänomen entwickelt hatte, wurden die Tiere getötet, ihre Organe herausgenommen, die Organgewichte ermittelt und in einer Probe von gemessener Menge die Aktivität bestimmt. Die Messungen erfolgten unter Anwendung des mit einem 2,8 mg/cm² dicken Glimmerendfenster versehenen GM-Zählers Marke Orion Typ 1871.

Ergebnisse und Besprechung

Abb. 1 zeigt die prozentuale Verteilung des bei der Auslösung i. v. injizierten Endotoxins in den einzelnen Organen, wobei die Durchschnittswerte angegeben sind. Wie aus Abb. 1 hervorgeht, wurde die größte Endotoxinspeicherung in der Leber festgestellt, dann der Reihe nach in der Lunge, Niere, im Herzen und in der Milz. Diese Feststellungen stimmen mit den Literaturangaben sowie mit den Ergebnissen der eigenen Versuche überein, die wir im Zusammenhang mit der Speicherung der mit Isotop markierten ver-

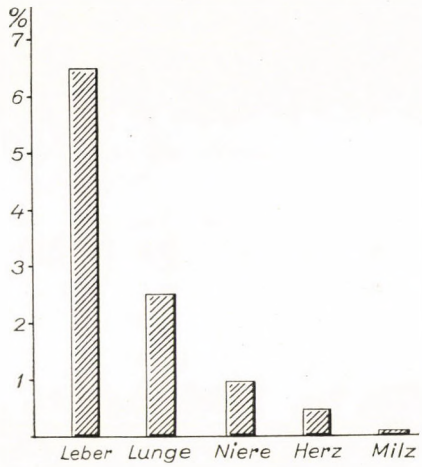

Abb. 1. **Prozentuale Verteilung des mit P^{32} markierten Coli-Endotoxins während des Shwartzmanschen Phänomens. Präparation: gereinigtes Typhus-Endotoxin, 0.1 mg i. c. Auslösung: mit P^{32} markiertes Coli-Endotoxin, 1—20 mg i. v. ≈ 70 000—700 000 Imp/min**

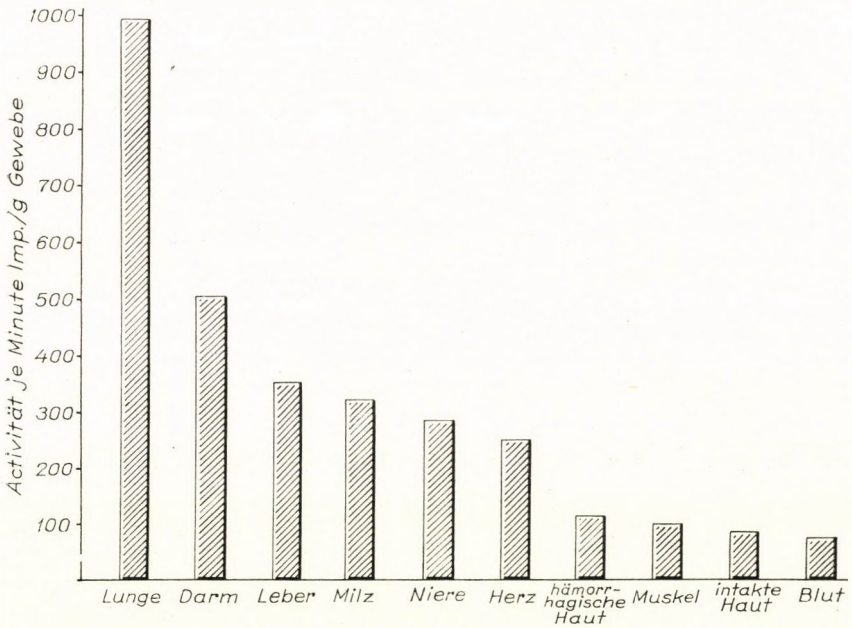

Abb. 2. **Die spezifische Aktivität im Verlauf des Schwartzmanschen Phänomens. Präparation: gereinigtes Typhus-Endotoxin 0,1 mg i. c. Auslösung: mit P^{32} markiertes Coli-Endotoxin 1—20 mg i. v. ≈ 70 000—700 000 Imp/min**

schiedenen Antigene durchgeführt haben [5]. Im Endergebnis wird somit die Verteilung der i. v. eingeführten Makromoleküle während der Shwartzmanschen Reaktion durch die Präparation nicht wesentlich beeinflußt. Die

Endotoxinspeicherung zeigt im großen und ganzen Parallelität mit dem Phagozytosevermögen der einzelnen Organe.

In Abb. 2 ist die spezifische Aktivität je Minute Imp/g Gewebe dargestellt. Wie aus dem Diagramm ersichtlich, ist die spezifische Aktivität in der Lunge am höchsten, dann folgen der Reihe nach der Darm, die Leber, Milz, Niere, das Herz, Muskeln und Blut. Auf Grund der spezifischen Aktivität betrachten wir es als das wesentlichste Ergebnis, daß die markierte Endotoxinmenge in der hämorrhagisch nekrotisierten Hautregion um etwa 40% höher ist als im intakten Hautbereich. Dies beweist, daß die Endotoxinaffinität der Haut in der präparierten Region von dem zur Präparation benutzten Endotoxin gesteigert wird. Die Affinitätssteigerung dürfte auf den pathologischen Veränderungen beruhen, — bekanntermaßen entstehen im Verlauf

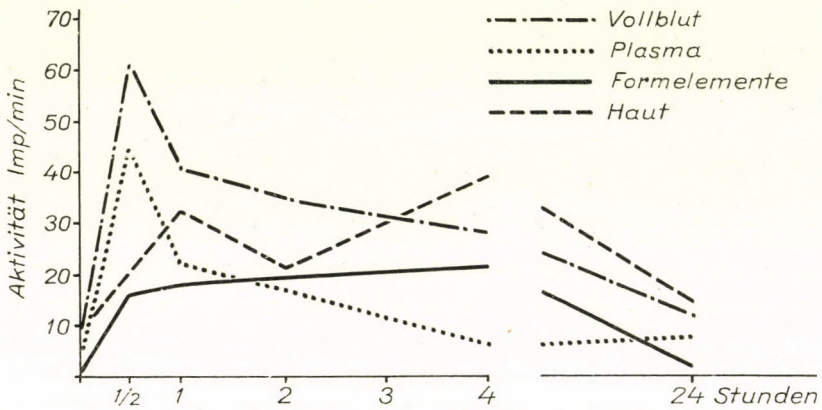

Abb. 3

der hämorrhagischen Nekrose Leukozyten-Thromben in den Hautgefäßen, und die angehäuften weißen Blutzellen, die laut Literaturangaben große Endotoxinmengen enthalten [6—7], sind zweifellos imstande, eine derartige Aktivitätssteigerung hervorzurufen. Möglich ist aber auch, daß von dem bei der Präparation eingeführten Endotoxin eine pathologische Veränderung der Hautgewebszellen zustande gebracht wird und diese zur Erhöhung der Endotoxinaffinität im fraglichen Bezirk führt.

In Fortsetzung dieser Versuche wollten wir anstelle statischer Untersuchungen die Endotoxinspeicherung der intakten Haut und im präparierten Hautbezirk auch in kontinuierlich durchgeführten Reihenuntersuchungen verfolgen. Zu diesem Zweck präparierten wir die Rückenhaut von 2—3 kg schweren Kaninchen mit gleichen Typhus-Endotoxinmengen an 4 Stellen. Zur Auslösung des Shwartzmanschen Phänomens wurde mit P^{32} markiertes Coli-Endotoxin in der Dosierung von 2—5 mg/kg benutzt. Nach Verabreichung des Endotoxins verfolgten wir die Aktivitätsveränderungen in Blut

und Plasma kontinuierlich in den ersten 4 Stunden, d. h. bis zu dem Zeitpunkt, wo sich die hämorrhagische Reaktion entwickelte. Ebenso beobachteten wir die Aktivitätsveränderungen der präparierten Hautregion. Die Resultate eines derartigen Versuchs sind in Abb. 3 wiedergegeben.

Wie Abb. 3 entnommen werden kann, ist die Aktivität im Blut und Plasma 1/2 Stunde nach der i. v. Injektion am höchsten und nach 4 Stunden bedeutend, auf etwa 1/3 des in der 30. Minute gemessenen Wertes, gesunken. Zugleich erreichte die Hautaktivität bereits in der ersten Stunde nach der Auslösung das Niveau, das in der 4. Stunde, zur Zeit des Erscheinens der hämorrhagischen Reaktion, zutage trat. Nach 24 Stunden hat die Hautaktivität stark abgenommen und war in den meisten Fällen nicht höher als die der nicht präparierten Haut.

Aus allen diesen Ergebnissen darf geschlossen werden, daß das i. v. eingespritzte Endotoxin von den RES-Elementen sehr rasch aufgenommen wird und das Endotoxin aus dem zirkulierenden Blut während der konsekutiven Leukopenie in die an RES-Elementen reichen Gewebe verschwindet. Im präparierten Hautbezirk aber kommt es offenbar unter Wirkung der vorangegangenen Präparation mit Endotoxin bereits in der ersten Stunde zur maximalen Akkumulation der im Blut kreisenden Endotoxinmenge.

Es ist anzunehmen, daß das zirkulierende Endotoxin mit den Leukozyten in den hämorrhagischen Fleck gelangt. Hierfür zeugt unsere Beobachtung, daß sich die Verteilung der Aktivität zwischen Plasma und Formelementen in den zu verschiedenen Zeitpunkten entnommenen Blutproben von Stunde zu Stunde verändert. Eine halbe Stunde nach der Auslösung zeigte das Plasma noch doppelt so große Aktivität wie die Formelemente, nach einer Stunde war die Aktivität der Formelemente und des Plasmas annähernd gleich und nach 4 Stunden die der Formelemente wesentlich höher als die des Plasmas.

In den weiteren Versuchen verfolgten wir das Schicksal des präparierenden Endotoxins im Verlauf des Shwartzmanschen Phänomens. Zu diesem Zweck nahmen wir die Präparation mit dem mit P^{32} markierten Coli-Endotoxin vor, während wir zur Auslösung unmarkiertes Typhus-Endotoxin gebrauchten. Nach Entwicklung des Phänomens, etwa 4 Stunden nach der Auslösung, wurden die nekrotisierte Hautregion, der diese direkt begrenzende ödematöse Rand sowie der intakt erscheinende Hautbezirk über den ödematösen Rand hinaus exzindiert und die spezifische Aktivität dieser Gewebe ermittelt. Auch von weiter entfernten Hautgebieten schnitten wir zur Kontrolle Hautflecke heraus und bestimmten ihre Aktivität.

Diese Versuche zeigten, daß nur etwa 5—10% des zur intrakutanen Präparation verwendeten isotopmarkierten Endotoxins 4 Stunden nach der Auslösung an der Präparationsstelle aufgefunden werden können. Das meiste Endotoxin bleibt im Bereich des hämorrhagischen Flecks zurück, während die Aktivität im ödematösen Rand und in dem darüber hinausreichenden Gebiet mit der Aktivität der Kontrollbezirke übereinstimmt.

Daraus darf geschlossen werden, daß der überwiegende Teil des zur Präparation dienenden Endotoxins von der Präparationsstelle resorbiert wird, d. h sich in der Umgebung gleichmäßig ausbreitet. Auf dieser Grundlage ergibt sich die Arbeitshypothese, daß das präparierende Endotoxin hauptsächlich im präparierten Hautbereich biochemische Veränderungen herbeiführt, von denen die Endotoxinaffinität dieses Bezirks 24 Stunden lang gesteigert wird, ohne daß es zur Akkumulation des präparierenden Endotoxins in diesem Gebiet kommt.

Wie bekannt, geht die Shwartzman-Reaktivität eine gewisse Zeit nach Einführung des präparierenden Endotoxins verloren.

Um klarzustellen, ob das zur Präparation benutzte Endotoxin zugleich mit dem Aufhören der Reaktivität ganz verschwindet, bereiteten wir die Haut der Kaninchen mit isotopmarkiertem Endotoxin vor. Nach 48 Stunden spritzten wir i. v. unmarkiertes Typhus-Endotoxin ein, wonach es zur Entwicklung des Phänomens kam. Versuchten wir aber, die Reaktion nach 72 Stunden auszulösen, so kam die hämorrhagische Nekrose nicht zustande. In beiden Fällen haben wir die präparierten Hautregionen bei der Auslösung sowie nach Entwicklung des Phänomens exzindiert und die Aktivität der Haut bestimmt. Überraschenderweise zeigte die bei den Aktivitätsmessungen in der Haut nachgewiesene Endotoxinmenge in den reaktiven und areaktiven Fällen keinen wesentlichen Unterschied. Auch dieser Befund beweist, daß die quantitative Anwesenheit des in die präparierte Hautregion eingeführten Endotoxins bei der Entwicklung des Shwartzmanschen Phänomens nicht ausschlaggebend ist. Aller Wahrscheinlichkeit nach hängt die Auslösbarkeit des Phänomens von der Persistenz der vom Endotoxin auf die Zellen oder Kapillaren der Haut ausgeübten biologischen Wirkungen ab.

Welcher Natur diese vom Endotoxin hervorgerufenen primären Effekte sind, wissen wir nicht. Es bedarf weiterer, hauptsächlich biochemischer Untersuchungen zur Klärung der Frage, welche Veränderungen das zur Präparation benutzte Endotoxin in den Zellen zustande bringt, von denen die Auslösbarkeit der Shwartzmanschen Reaktion abhängt.

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A STANDARDIZED METHOD FOR THE DETERMINATION OF STAPHYLOCOCCAL ALPHA-ANTITOXIN

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Summary. The accuracy of staphylococcal alpha-antitoxin determination may be enhanced by the use of physiologic saline with gelatine for serum and toxin dilutions as well as for the preparation of erythrocyte suspensions, owing to the stabilizing effect of gelatin on the diluted staphylococcal alpha toxin.

The sensitivity of the erythrocytes of the same rabbit to staphylococcal toxin proved to be inconstant.

Inaccuracies resulting from the lability of the diluted toxin as well as from the varying sensitivity and fragility of rabbit erythrocytes may considerably be reduced by the introduction of a five tube standard antitoxin series.

By the method recommended the antitoxin values may be determined with an average deviation of ± 6 per cent at the L/10 level, and of ± 10 per cent at the L/100 level, respectively.

The staphylococcal alpha-antitoxin (antistaphylolysin) content of sera or other biological fluids is determined by a haemolytic reaction. The titration methods evolved by different laboratories are, however, not uniform. The concentration of erythrocytes varies from 0.25 to 33 per cent, the amount of red blood cell suspension from 0.025 to 1.0 ml [23, 5, 13, 20, 15, 16]. Some authors take a partial haemolysis while others the complete one as the end point when reading the results [13, 15, 20, 16]. Owing to the discrepancies in performing the titration, the deviation of the results by re-testing may exceed even 50 per cent.

During our studies of the problem of staphylococcal alpha-antitoxin determination of human and animal sera, attempts were made to ensure enhanced accuracy. In the present paper some of our studies in that field as well as a standardized method evolved by us will be reported.

Materials and methods

Sera. Sera separated from human blood drawn aseptically were heated at 56°C for 30 minutes and stored at 4—10°C or at — 10°C when tested after several days. Animal sera preserved with 0.5 per cent phenol were kept at 4—10°C.

Rabbit erythrocyte suspension. Rabbit blood was collected into 3.8 per cent sodium citrate solution and washed three times with physiological saline. A 2 per cent erythrocyte suspension was used immediately, or after an additional washing if having been stored 24 hours at 4°C.

Staphylococcal alpha toxin. Lingood broth cultures inoculated with strain *Wood 46* were incubated for 7 days and filtered through a Seitz filtre. In other experiments, the supernatants of shaken broth cultures of 40 hours were used with 0.5 per cent phenol as a preservative.*

* We are indebted to Dr. L. ZALAY and Dr. G. DÓBIÁS for the supply of toxin samples from shaken cultures.

The d. h. m. values of the toxin were determined as follows. Decreasing amounts of toxin in 1.0 ml of physiologic saline were added to tubes of about 12 mm diameter. After adding 1.0 ml of erythrocyte suspension the tubes were gently shaken and placed in a 37°C water bath for 1 hour shaken at intervals. The degree of haemolysis was estimated after further 60 minutes at room temperature. The minimum quantity of toxin resulting in a just perceptible but distinct haemolysis was taken as the d. h. m. value.

The Lh (limes haemolysans) values of the toxin, *i. e.* the amounts neutralized by 1.0 IU staphylococcal alpha-antitoxin or by its fractions, *e.g.* by 0.1 IU antitoxin (Lh/10), were titrated in the following way. Increasing amounts of toxin in 1.0 ml of physiologic saline were added to tubes. Staphylococcal antitoxin of exactly known titre was diluted to contain double amounts of alpha-antitoxin pro ml of the IU values to be combined with the toxin. To each tube, 0.5 ml of this dilution was added. At the beginning we used the International Standard for Staphylococcus-Alpha-Antitoxin from *Statens Seruminstitut, Copenhagen*, and continued our experiments with an antitoxin preparation from the *Institute for Serobacteriological Production and Research "Human", Budapest*, corresponding to the international standard in containing exactly 20.0 IU per ml. After gently shaking, the toxin-serum mixtures were allowed to stand for 30 minutes at room temperature. Subsequently, 1.0 ml of erythrocyte suspension was added and the carefully shaken tubes were placed in a 37°C water bath for 1 hour. The further steps were identical with those of d. h. m. determination. The slightest amount of toxin resulting in distinct haemolysis affecting 8—10 per cent of the erythrocytes, thus observable with the naked eye, was taken as the Lh value.

Determination of staphylococcal alpha-antitoxin. The titrations were carried out on the 1, $1/2$, $1/10$ and $1/100$ IU levels, respectively. Decreasing amounts of sera to be investigated were added to tubes in 1.0 ml of physiologic saline. Then one test dose of toxin in 0.5 ml was added to each tube and the serum-toxin mixtures were allowed to react for 30 minutes at room temperature. After the addition of 1.0 ml of erythrocyte suspension and careful shaking the tubes were placed in a 37°C water bath. The procedure was continued in the same way as with the Lh determination.

Results

Stabilization of the diluted staphylococcal alpha-toxin. Staphylococcal alpha-toxin is very sensitive to various physical effects. It may be damaged at 45°C for 60 minutes and is rapidly destroyed at 58—60°C [5, 18, 19]. Shaking or repeated pouring of diluted or undiluted toxin as well as air-bubbling in the presence of nitrogen or 1 per cent $Na_2S_2O_4$ results in a sharp decrease of its haemolytic activity [6, 15, 19]. In case of some labile toxins one may fail in preparing two dilutions of equal strength. Retesting of most staphylococcal alpha toxins does not result in reproducible d. h. m. or Lh values.

The errors arising from the partial destruction of the toxin may be diminished by parallel testing of a standard serum of known and constant value and taking its reaction into account by reading and calculating the results [17]. It is however, more suitable to render the toxin itself stable. According to MARKS the dilution of staphylococcal alpha- and beta-toxins with broth has a favourable effect on their stability [15]. JACKSON [11] protects beta-toxin by diluting it with gelatin. For anti-alpha-staphylolysin titration TOWERS and GLADSTONE [22] recommend physiologic saline containing gelatin, but without any mention on its stabilizing effect. Gelatin may increase the stability of diluted diphtheria toxin and Schick-toxin, too [14]. Tetanus toxin, also very labile at high dilutions [3, 7, 10, 9], is stabilized by peptone.

On the basis of these data we attempted to stabilize the staphylococcal alpha-toxin. Our experiments with peptone have led to inconclusive results.

Gelatin, however, at a concentration of 0.05—1.0 per cent dissolved in saline-phosphate buffer of pH 7.2, was found to possess a suitable stabilizing effect as demonstrated in Table I.

Table I

Stabilizing effect of 0.1 per cent gelatin on the physiologic saline dilutions of staphylococcal alpha-toxin No 21/2—4

Dilution degree	Dilution fluid	Manipulations	D. h. m.	Lh/100
1/5	saline-phosphate buffer	—	0.001	—
1/100	saline-phosphate buffer	—	0.003	0.009
1/100	saline-phosphate buffer	repeated pouring*	0.006	0.012
1/100	saline-phosphate buffer + 0.1 per cent gelatin	—	0.0015	0.005
1/100	saline-phosphate buffer + 0.1 per cent gelatin	repeated pouring*	0.0015	0.006

* Ten times from one tube to the other

Sensitivity of rabbit erythrocytes to staphylococcal alpha-toxin. The sensitivity of rabbit erythrocytes to staphylococcal alpha-toxin is not uniform. The Lh values of the same toxin may vary considerably from rabbit to rabbit, especially, when complete haemolysis is taken as the end point. Having established the Lh value of the toxin with erythrocytes from one rabbit and performing the antitoxin titration with erythrocytes of another animal, the deviation in titre at re-testing may reach 77 per cent, while using the blood of the same rabbit for Lh determination and antitoxin titration, the error does not exceed 35 per cent [21]. The sensitivity of erythrocytes of the same animal may, however, also undergo a change. ALBERTSEN [1] found the blood samples drawn at different points of time from the same horse to exhibit inconstant sensitivity to the haemolysin of *Corynebacterium pyogenes*. We made some similar observations on rabbits. The Lh/2 and Lh/10 values of the same toxin were repeatedly titrated employing two alpha-antitoxin standard sera of different batches but of conforming strength. In these investigations lasting 9 months erythrocytes of the same rabbit were used throughout. As seen in Fig. 1, both values showed certain fluctuations due partly to the lability of the toxin, these experiments having been carried out with physiologic saline without gelatin. The differences were most marked in June an July, reaching 32 and 33 per cent, respectively, at the Lh/2 level and 27 and 37 per cent, respectively, at the Lh/10 level. The nearly parallel run of the four curves seemed to verify the variation in sensitivity of the erythrocytes of this individual rabbit.

Estimating the degree of haemolysis. Haemolysis is generally read after 1 hour in a 37°C water bath and subsequent 1 hour at room temperature. The accuracy of the reading may be enhanced by shortening this second incubation period. Haemolysis namely proceeds at room temperature as described in the literature [19] and demonstrated in Table II. After incubation in a water bath the reaction may be interrupted by rapid centrifugation, this being the most correct way for estimating the results. If centrifugation is

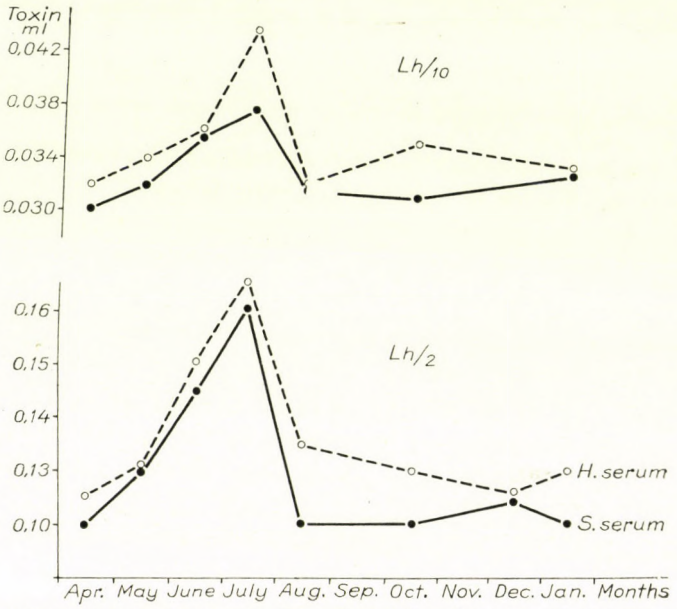

Fig. 1. Fluctuations of the Lh/2 and Lh/10 values of staphylococcal alpha-toxin No 21/2—4 tested against erythrocytes of the same rabbit

omitted, it is advisable to read the test after about 25 minutes at room temperature, the degree of haemolysis being observable with the naked eye at this point of time.

The significance of standard serum. The errors in antistaphylolysin titration may be overcome by parallel testing of a control serum of known and constant strength. The use of standard alpha-antitoxin was introduced by PACKALÉN and BERGQUIST [17], on grounds of the antistreptolysin titration method evolved by IPSEN [8]. The standard series starts with 1/25 IU, with a 20 per cent reduction in consecutive tubes. The sera of patients were investigated in twofold dilutions satisfactory for clinical purpose. Independently of the above cited authors, we introduced a control series analogous with that used by JENSEN [12] for the intracutaneous titration of diphtheria toxin in rabbits. The middle, *i. e.* the 3rd, tube of the series contained IU amounts equivalent to the titration level, the lst and 2nd tubes 20 and 10 per cent more, the 4th and 5th ones

Table II

Occurrence of haemolysis at room temperature after 37°C water bath

	Staphylococcal alpha-antitoxin			
	0.012 IU	0.011 IU	0.010 IU	0.009 IU
	Staphylococcal alpha-toxin			
	0.038 ml	0.038 ml	0.038 ml	0.038 ml
Reading				
after 15 minutes	—	—(?)	+	++
after 25 minutes	—	+.	++.	++
after 40 minutes	—	+	++.	++
After centrifuging				
immediately	—	—	+.	++
after 15 minutes	—	—	+.	++
after 25 minutes	—	—	+.	++
after 40 minutes	—	—	+.	++

Legends: —: no haemolysis
+. + ++. ++: haemolysis of increasing degree.

10 and 20 per cent less, respectively. By titration at the 1 IU level 1.2, 1.1, 1.0, 0.9 and 0.8 IU of antitoxin is added to the control tubes. If working at the 1/10 or 1/100 IU levels, the corresponding tubes contain ten or hundred times less standard serum, respectively. With the first traces of haemolysis in the 3rd tube the titre need not be corrected. Otherwise it is necessary to convert the titration results into real values by multiplicating with a factor of 1.2—0.8 according to the first appearance of haemolysis in the control tubes. The significance of the control series is demonstrated in Table III, showing the results of repeated titrations of the same serum employing physiologic saline with and without 0.1 per cent gelatin, respectively. Omitting the control series, the staphylococcal alpha-antitoxin sheep serum No 4A would have been declared to contain 130 or 110 IU per ml at the first titration and 140 or 120—125 IU per ml at re-testing. The correct values obtained by factorization proved to be 110—112 and 108—112 IU per ml, respectively. The lower the titration level, the more importance must be attributed to the control series. Higher dilutions of toxin involve more manipulations resulting in major destruction and a deviation in the results which *e. g.* at the 1/100 IU level, omitting standardization, may reach 35 per cent [21]. By introducing our control series the deviation does not exceed $\pm$ 6 per cent at the 1/10 IU level, and $\pm$ 10 per cent at the 1/100 IU level.

Table III

Titration of staphylococcal sheep antitoxin No 4A at the 1.0 IU level on two different occasions

Dilution fluid	IU supposed					IU content of control tubes					Factor	Result
	100	110	120	130	140	1.2	1.1	1.0	0.9	0.8		
Saline-phosphate buffer	−	−	−	+	++	−	−	−	−	++	0.85 or 0.8	130×0.85 = 110.5 IU or 140×0.8 = 112.0 IU
Saline-phosphate buffer containing 0.1 per cent gelatin	−	+	++.	++	++	−	−	+	++	+++	1.0	110×1.0 = 110.0 IU
Saline-phosphate buffer	−	−	−	−	+	−	−	−	−	+	0.8	140×0.8 = 112.0 IU
Saline-phosphate buffer containing 0.1 per cent gelatin	−	−	+.	++	+++	−	−	−	+	++	0.9	120×0.9 = 108.0 IU

Legends: −: no haemolysis
+., +, ++., ++, +++: haemolysis of increasing degree.

Discussion

With staphylococcal antitoxin titration, various sources of error must be taken into account. Alpha-toxin may be denatured by simple physical effects [6]. Although our observations furnished convincing proof for the stabilizing effect of gelatin, all manipulations with the toxin must be carried out very carefully. In our experience the extent of haemolysis is influenced even by the frequency and extent of shaking the tubes intended for preventing the sedimentation of erythrocytes. Four or five shakings at the 10th, 30th and 50th minutes of incubation proved to be the most suitable, as confirmed by the observations of DÓBIÁS ET AL. [4].

The errors arising from the different sensitivity of the erythrocytes of individual animals may be eliminated by the use of red blood cells from the same rabbit, preferentially of albino type with no antistaphylolysin in its serum. The possible sensitivity changes of the erythrocytes of the chosen rabbit, as well as the eventual concentration differences of red blood cell suspensions prepared at different points of time, finally the occasional variations in the strength of toxin, may be overcome by parallel testing of a standard serum allowing the conversion of the titration results into real values by factorization.

On the basis of our experience, a standardized method for staphylococcal alpha-antitoxin determination has been elaborated as follows.

Buffered physiologic saline of pH 7.2 with 0.1 per cent gelatin is used for the dilution of serum and toxin samples, for washing and suspending the erythrocytes. In 100 ml of sterile buffer (8.5 g NaCl, 0.565 g $Na_2HPO_4 \cdot 2H_2O$, 0.135 g KH_2PO_4, ad 1000 ml of distilled water) 0.1 g gelatin is dissolved by boiling. The solution remains practically sterile and keeps its pH value constant or only not significantly lowered. After having been cooled it is ready for use. Dilution of the toxin is carried out carefully. After adding the appropriate amount of toxin to the bottom of an Erlenmeyer flask and allowing the dilution fluid to run along the side of the flask, mixing is achieved by rotatory motion.

The test dosis *e. g.* the Lh/10 value of the toxin is determined as Table IV demonstrates. The figures in Table IV show that the Lh/10 value of the toxin proved to be 0.036 ml. Since this amount of toxin must be contained in 0.5 ml at the antitoxin titration, the proper dilution is prepared to contain 0.072 ml of toxin per ml.

If fully ignorant of the antitoxin content of the serum, a pilot titration is carried out. A titration at the 1/10 IV level presented in Table V shows that

Table IV

		Tube No				
		1.	2.	3.	4.	control
1 : 10 dilution of toxin	ml	0.30	0.33	0.36	0.40	—
Dilution fluid	ml	0.70	0.67	0.64	0.60	1.5
0.2 IU per ml dilution of standard serum	ml	0.5	0.5	0.5	0.5	—
After gentle shaking and incubation for 30 minutes at room temperature						
2 per cent rabbit erythrocyte suspension	ml	1.0	1.0	1.0	1.0	1.0
After gentle shaking and incubation for 60 minutes in a 37°C water bath (additional shakings at 10th, 30th and 50th minutes)						
Degree of haemolysis in the supernatants after 25 minutes at room temperature		—	—	+	++	—

Table V

		Tube No.					
		1.	2.	3.	4.	5	control
Dilution of serum		1/1	1/10	1/10	1/10	1/10	1/1
Amount of serum dilution	ml	1.0	1.0	0.2	0.1	0.05	1.0
Dilution fluid	ml	—	—	0.8	0.9	0.95	0.5
Dilution of toxin (0.72 + 9.28 = 2 Lh/10/ml)	ml	0.5	0.5	0.5	0.5	0.5	—
Incubation for 30 minutes at room temperature							
2 per cent rabbit erythrocyte suspension	ml	1.0	1.0	1.0	1.0	1.0	1.0
Incubation for 60 minutes in a 37°C water bath (shaking at 10th, 30th and 50th minutes)							
IU expected		0.1	1.0	5.0	10.0	20.0	—
Degree of haemolysis in the supernatants after 25 minutes at room temperature		—	—	++	+++	+++	—

the titre of the serum lies between 1.0 and 5.0 IU per ml. (The titre is calculated by dividing the titration level, 0.1 in this case, by the amount of serum, in the presence of which the first traces of haemolysis have appeared.)

For a correct evaluation, the titration is repeated introducing a control series as follows (see Table VI).

Table VI

	Tube No					Control series				
	1.	2.	3.	4.	5.	1.	2.	3.	4.	5.
1 : 10 dilution of serum ml	1.0	0.5	0.33	0.25	0.20	—	—	—	—	—
0.2 IU per ml dilution of standard serum ml	—	—	—	—	—	0.6	0.55	0.5	0.45	0.40
Dilution fluid ml	—	0.5	0.67	0.75	0.80	0.4	0.45	0.5	0.55	0.60
Dilution of toxin (0.72 + 9.28=2 Lh/10/ml) ml	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5

For further steps see by pilot titration (Table V)

IU per ml	1.0	2.0	3.0	4.0	5.0	IU content of tubes				
						0.12	0.11	0.10	0.09	0.08
Degree of haemolysis in the supernatants after 25 minutes at room temperature	—	—	+	++	+++	—	—	—	+	++

According to the haemolysis observed in the control tubes, the titration level is 0.09 IU instead of the 0.1 as originally supposed. Thus, the actual titre of the serum equals 0.9 times 3, *i. e.* 2.7 IU.

The accuracy of the titration may be enhanced by serum dilutions at closer steps. If examining values under 0.1 IU, the titration must be carried out at the 1/100 IU level. In this case the Lh/100 amount of toxin is used, the determination of which is identical with that of the Lh/10 value, but the standard serum is diluted to contain 0.02 IU per ml. At the antitoxin titration 0.012—0.008 IU is added to the control tubes according to the titration level. For comparable results the determinations must be carried out at identical titration levels.

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METABOLIC STUDIES WITH STREPTOMYCES RIMOSUS

By

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Summary. Storage at —10°C of spores of *Streptomyces rimosus* BS—21, or the serial subculturing of this organism, leads to a diminution of its oxytetracycline yield. This is accompanied by changes in morphologic features, and by changes in metabolic activity. In media containing glucose and various amino acids, *e. g.* glycine, DL-alanine, β-alanine, and DL-serine. the greatest observable divergence between variants with good and bad yields appears in their pyruvic acid metabolism. Loss of productivity is greatest in media which contain serine, Some of the variants which have a tendency to decrease their production would still give an unchanged yield of oxytetracycline in other media but produce on a lower level when kept in media containing serine. When the production capacity of *Streptomyces rimosus* falls, its constitutive serine-desaminase activity increases, its capacity to utilize pyruvic acid decreases.

Investigations on the metabolism and oxytetracycline production of *Streptomyces rimosus* BS—21 have been carried out in these laboratories for several years [1, 2, 3]. During storage the production capacity of this organism diminishes, a similar phenomenon can also be brought about by serial subculturing. Beside morphologic signs the fall in production capacity is accompanied by changes in metabolic activity. The metabolism of variants which differ in their production capacity has been investigated in synthetic media, and this paper presents results gathered during this work.

Materials and methods

Experiments on the metabolism of natural variants of strain BS—21/37, —72, and —76, of *Streptomyces rimosus*, have been carried out. In medium B (see below) these variants produced, respectively, 2000, 100 and 2000 μg/ml of oxytetracycline. Inoculations were made with suspensions of spores which had been kept in storage at — 10°C. The medium used for the production of spores was composed of (per cent w/v): soybean meal (defatted), 1; yeast extract, 10; glucose, 1; sodium chloride, 0.3; calcium carbonate, 0.5; pH 7 before sterilization.

Preparation of the yeast extract: 1 kg of yeast is liquified with ethyl acetate, mixed with one liter of water, autoclaved at 121°C for 20 minutes, and allowed to settle for several hours. The somewhat turbid supernatant liquid layer is used in the media.

To determine the production capacity 100 ml portions of medium B were shaken in 500 ml Erlenmeyer flasks. This medium was composed of (per cent w/v) soybean meal (defatted), 3; potato starch, 2.5; corn steep liquor (50% dry substance), 0.1; NaCl, 0.3; $CaCO_3$, 0.5; palm oil, 1; pH 7 before sterilization. For inoculation 1 ml of suspension of spores produced in shaken cultures was taken.

In experiments with synthetic media inocula were shaken in 100 ml portions of the medium, in 500 ml Erlenmeyer flasks. The medium was composed of (per cent w/v): glucose, 1; glycine, 0.5; NaCl, 0.3; KH_2PO_4, 0.013; Tween-80, 0.002; pH 7 before sterilization. The medium was made with 1 ml spore suspension prepared as mentioned before. Incubation time of the inocula was 96 hours.

The experiments concerning metabolism in synthetic media have been carried out with 40 ml portions of the medium in 100 ml Erlenmeyer flasks. This medium was composed of

(per cent w/v): glucose, 1.5; NaCl, 0.3; Tween-80, 0.002; glycine, 0.5; or some other amino acid with the same nitrogen content as 0.5% of glycine, and, in the form of KH_2PO_4, the quantities of phosphate mentioned in the experimental part of this paper. Before sterilization the pH value was 7. Media were made up with tap water, and inoculated with 0.4 ml of an inoculum grown in the synthetic medium described before.

The media were sterilized in steam at 121°C for 20 minutes. The rotary shaker made 300 revolutions per minute around a circular path with a 2 cm diameter. The temperature was kept at 28°C throughout.

Analytical methods. The concentration of oxytetracycline was determined by the agar diffusion method with *Bacillus subtilis* ATCC 6633 [4]. The following determinations were made from the supernatant of the centrifugated samples; glucose has been determined by SZÁRA's [5] method (for details see: [2]); amino nitrogen has been measured as described by BOISSONAS [6], and pyruvic acid has been analysed by FRIEDMAN and HAUGEN's method [7]. Measurement of the amino nitrogen in fermentation samples has been carried out as follows. Sheets of filter paper were inmersed in a solution of 3% (w/v) NaOH in abs. ethanol, dried at 60°C and cut into disks of 28 mm diameter. The sample was allowed to drop upon such an impregnated disk whilst hot air (about 80°C) was blown thereon to dry the liquid. The amount of amino nitrogen brought upon the paper was from 5 to 30 μg. Using this process, the ammonia content of the sample was driven off. The amount of the amino nitrogen on the paper was referred to a glycine standard, except in the case of β-alanine, where a calibration curve was used; the exactness of this procedure was verified by paper chromatography (in chromatograms no significant amounts of α-amino acid could be found). The determination of alanine was carried out by quantitative paper chromatography as described by BRAUN *et al.* [8]. The paper chromatography of the keto-acids was carried out by CAVALLINI and FRONTALI's method [9]. Dry substance was determined by centrifugal sedimentation, washing with water, and drying n an exsiccator over phosphorus pentoxide.

Results

Streptomyces rimosus BS—21 produced spores well in shaken cultures. It has been found that a suspension of these spores kept in storage at —10°C is suitable not only for inoculation but for the preservation of the species as well. In point of production of oxytetracycline and other morphologic features no substantial difference was observed between fermentations inoculated with submerged cultures or with cultures raised on a solid nutrient containing glycerol, corn steep liquor and yeast extract.

Based upon observations made in the course of experiments, the change of two features has been systematically investigated; the loss of oxytetracycline production capacity in the B-medium, and the degree of loss of spore production measured by plating on a potato-infusion-agar medium. Transplanted from shaken cultures into shaken cultures, the first generations had the same capacity of production, and on potato-infusion-agar media the number of spore-free variants was less than 0.1%. Later on, oxytetracycline production capacity fell gradually, and at the same time, on potato-infusion-agar cultures, the number of colonies unable to produce spores became greater. These simultaneous changes are dependent on the serial number of a generation, and on the duration of storage at —10°C; a longer period of storage resulted in a greater degree of loss of production capacity caused by a certain number of transplantations. Experiments on metabolism have been carried out with natural variants produced during this storage.

Experiments on metabolism in media containing glycine. Based upon experience gathered during work with laboratory fermenters [2], the experiments on metabolism began with media containing glycine. The two variants differing most from each other were used in these experiments. The variant BS—21/76 produces 2000 μg/ml oxytetracycline; its spore-free colonies, on potato-infusion-agar number less than 0.1%. The variant BS—21/72 produces 100 μg of oxytetracycline per ml, its spore-free variants amount to 99%. Experimental results referring to media containing different quantities of phosphates are summarized in Figs 1/*a*—*j*.

Consumption of the various ingredients of the medium increases with increasing phosphate contents. Variant BS—21/72 exhibits greater metabolic activity than the variant BS—21/76. Greater metabolic activity is accompanied by a more rapid succession of the phases of development; the breaking off of parts from mycelium threads and the shaping of these bacillus-like fragments into spores proceed more rapidly, the degree of autolysis is greater. Under identical circumstances the dry matter contents of the fermentation broth are lower. The most pronounced divergence is shown in the pyruvic acid concentration during fermentation. In the case of the variant BS—21/76 of *Streptomyces rimosus* a measurable pyruvic acid level is to be found only when phosphate concentrations are low, with variant BS—21/72 pyruvic acid production was evident even at the highest phosphate values, and at low phosphate concentrations the occurrence of 0.15—1.2 mg/ml of pyruvic acid has been noted.

The quantity of oxytetracycline produced by each of these variants depends on the phosphate concentration of the medium. At phosphate contents of 30 μg/ml, the variant with good production capacity produced 550 μg/ml oxytetracycline; the optimum concentration of phosphate for the variant with small production capacity is 20 μg/ml, and the quantity of oxytetracycline produced is 400 μg/ml. A better productivity of the variant BS—21/72 was evident in the synthetic medium compared with its productivity in medium B containing soybean meal, starch, and fat. The cause of this might be found in the fact that in this medium a substantial degree of basicity is evolved in consequence of rapid metabolism, the pH rises above 9 and this inhibits production. In a synthetic medium the shift towards higher pH values is smaller and does not reach pH 8. At high phosphate concentrations (90—120 μg/ml) pH values higher than 8 are sometimes observed in the second half of the fermentation.

In media containing glycine, secretion of alanine can be observed during fermentation (Table I). The degree of this secretion depends on the concentration of phosphate in the medium. The highest contents of alanine were found, as a rule, with phosphate concentrations favourable to oxytetracycline production.

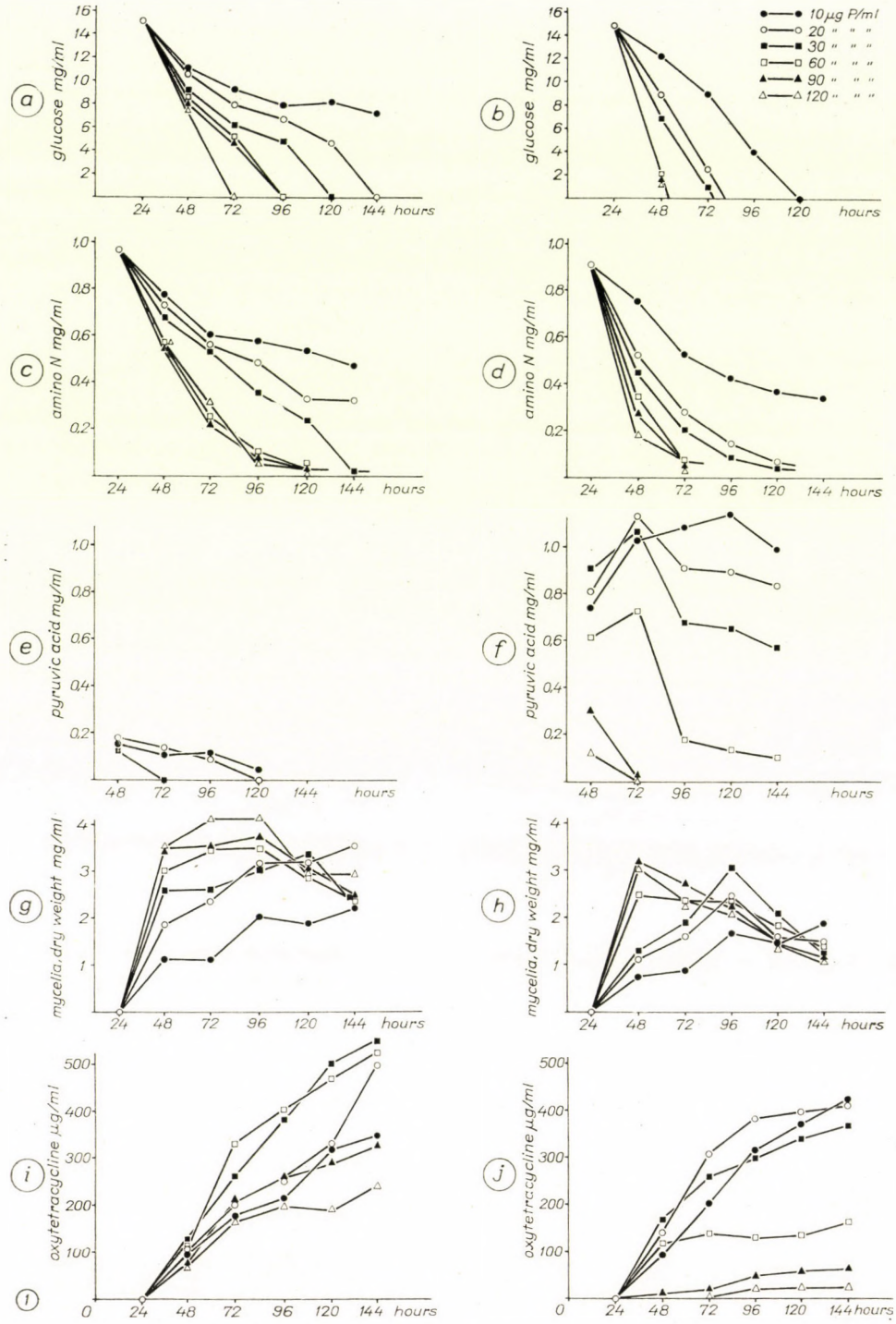

Fig. 1/a—j. Investigations of the metabolism and of the production of oxytetracycline in media containing glycine and glucose. Experiments at different phosphate concentrations. Figures 1/*a*, *c*, *e*, *g*, and *i* refer to variant BS—21/76; Figures 1/*b*, *d*, *f*, *h*, and *j* refer to variant BS—21/72

Table I

Formation of alanine in media containing glycine, with different quantities of phosphate added, in the case of variants BS—21/76, and BS—21/72

Fermentation time, hours	Variant BS—21/76						Variant BS—21/72					
	10	20	30	60	90	120	10	20	30	60	90	120
	μgP/ml alanine N mg/ml						μgP/ml alanine N mg/ml					
48	0.03	0.07	0.09	0.04	0.02	0.02	0.08	0.09	0.09	0.02	0.03	0.02
72	0.06	0.08	0.11	0	0	0	0.10	0.10	0	0	0	0
96	0.10	0.10	0.08	0	0	0	0.08	0.06	0	0	0	0
120	0.09	0.08	0.04	0	0	0	0.06	0.03	0	0	0	0
144	0.08	0.06	0	0	0	0	0.03	0	0	0	0	0

Investigations of metabolism in media containing DL-alanine, β-alanine, and DL-serine. In further experiments the metabolism of two natural variants

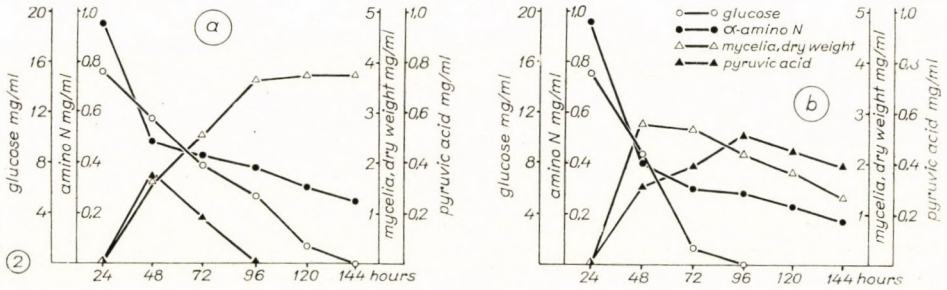

Fig. 2/a—b. Investigation of the metabolism in media containing DL-alanine and glucose. Figure 2/*a* refers to variant BS—21/76; Figure 2/*b* refers to variant BS—21/72. Phosphate concentration of the media was 30 μg/ml of P. Content of DL-alanine in the medium, 0.6% (w/v)

compared in glycine medium were compared also in media containing DL-alanine, β-alanine, and DL-serine. These experiments were carried out under identical conditions with varied concentrations of phosphate. Except with the medium containing DL-serine, the results were similar to those found with media containing glycine. For comparison, Figs. 2/*a—b*, 3/*a—b*, and 4/*a—b* show the changes in metabolism in the case of a concentration of phosphorus of 30 μg/ml.

The consumption of sugar by the variant BS—21/76 proceeds with similar velocity no matter which of the three sources of nitrogen is present in the medium, but with β-alanine sugar consumption begins one day later. The consumption of DL-alanine is more rapid than that of DL-serine. The consumption of β-alanine, beginning after some delay, is similar to the consumption of DL-alanine. Accumulation of pyruvic acid is low. The consumption of sugar

by the variant BS—21/72 is more rapid with each of the three sources of nitrogen. Similarly, its consumption of DL-alanine and β-alanine is more rapid, but its consumption of DL-serine is slower. Accumulation of pyruvic acid is considerably increased. The quantity of the mycelium produced is less, and autolysis rather pronounced.

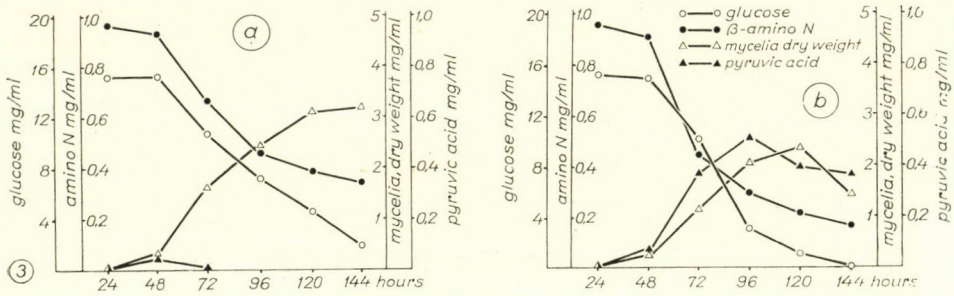

Fig. 3/a—b. Investigation of the metabolism in media containing β-alanine and glucose. Figure 3/*a* refers to variant BS—21/76; Figure 3/*b* refers to variant BS—21/72. Phosphate concentration of the media was 30 μg/ml of P. Content of β-alanine in the medium, 0.6% (w/v)

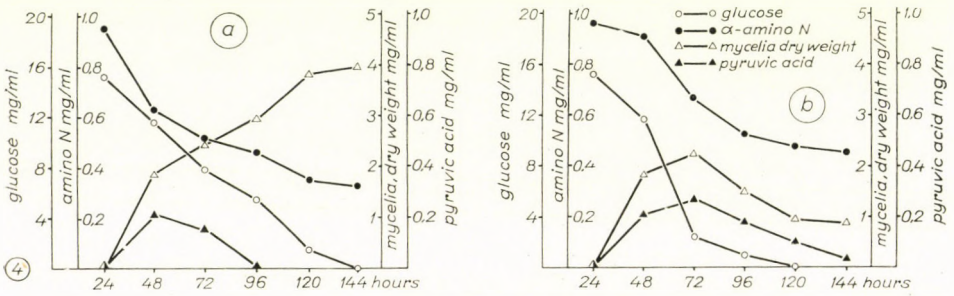

Fig. 4/a—b. Investigation of the metabolism in media containing DL-serine and glucose. Figure 4/*a* refers to variant BS—21/76. Figure 4/*b* refers to variant BS—21/72. Phosphate concentration of the media was 30 μg/ml of P. Content of DL-serine in the medium, 0.7% (w/v)

The variant BS—21/76 produces oxytetracycline in similar quantities in media containing different sources of nitrogen (Tables II, III, and IV). 30 μg P/ml is the optimum concentration of phosphates for production. The variant BS—21/72 produces 200—250 μg/ml oxytetracycline in the presence of DL-alanine and β-alanine; and 20 μg P/ml is the most favourable concentration of phosphates. In the presence of DL-serine no production could be registered.

In media containing β-alanine the consumption of the components of the media, and, together with this, the production of oxytetracycline starts with a delay of about 24 hours. If 10% of the β-alanine is substituted by DL-alanine, this delay fails to occur, and the course of metabolism is with both variants the same as with DL-alanine.

Table II

Oxytetracycline output in media containing DL-alanine, and phosphate at different concentrations

Fermentation time, hours	Variant BS—21/76						Variant BS—21/72					
	10	20	30	60	90	120	10	20	30	60	90	120
	μgP/ml oxytetracycline μg/ml						μgP/ml oxytetracycline μg/ml					
48	100	131	200	200	116	84	31	39	42	0	0	0
72	168	346	333	263	163	133	80	120	135	21	0	0
96	201	360	462	300	227	138	144	165	187	15	12	0
120	218	390	537	352	235	150	190	200	150	30	15	7
144	363	525	550	402	315	162	216	230	150	40	15	9

DL-alanine contents in medium, 0.6%.

Table III

Oxytetracycline output in media containing β-alanine, and phosphate at different concentration

Fermentation time, hours	Variant BS—21/76						Variant BS—21/72					
	10	20	30	60	90	120	10	20	30	60	90	120
	μgP/ml oxytetracycline μg/ml						μgP/ml oxytetracycline μg/ml					
48	0	0	0	0	0	0	0	0	0	0	0	0
72	69	87	115	165	121	72	52	39	31	0	0	0
96	110	187	241	362	258	155	105	195	200	21	18	15
120	210	229	380	440	249	187	176	240	210	36	17	13
144	210	363	485	460	258	163	228	264	255	27	17	14

β-alanine contents in medium, 0.6%.

Table IV

Oxytetracycline output in media containing DL-serine, and phosphate at different concentrations

Fermentation time, hours	Variant BS—21/76						Variant BS—21/72					
	10	20	30	60	90	120	10	20	30	60	90	1 20
	μgP/ml oxytetracycline μg/ml						μgP/ml oxytetracycline μg/ml					
48	13	35	43	56	25	14	0	0	0	0	0	0
72	110	218	290	234	168	120	0	0	0	0	0	0
96	160	310	310	240	233	144	0	0	0	0	0	0
120	221	345	420	221	185	128	10	7	0	0	0	0
144	216	400	430	259	167	130	14	21	18	0	0	0

DL-serine contents in medium, 0.7%.

Further experiments with media containing DL-serine. The behaviour of the two variants used, in media containing glycine, DL-alanine, and β-alanine, was the same. The changes in metabolism in media containing DL-serine show a different pattern, the variant of reduced production capacity ceases to produce oxytetracycline. Experiments carried out with further natural variants show that in nutrient broth containing DL-serine the production decreases when under the same experimental conditions a production decrease in medium B, and in synthetic media containing the other three amino acids, cannot yet be noted. The variants characterized by such a behaviour show marked tendency to stop production altogether. An instance of this is furnished by variant BS—21/37. On nutrient B it produces 2000 μg/ml oxytetracycline, and the number of its spore-free variants does not increase substantially. Table V presents metabolic changes and oxytetracycline production of this variant, in a medium containing 30 μg/ml phosphorus. It is to be seen that the behaviour of this variant in a medium containing glycine is nearly the same as that of variant BS—21/76. In a medium with DL-serine production capacity falls to 200 μg/ml, and a high production value for pyruvic acid can be noted. If 10% of the DL-serine is substituted by DL-alanine, production rises to the value noted in media containing glycine, the consumption of amino acid and glucose does not change substantially, and no considerable production of pyruvic acid is observable.

Table V

Investigation of the metabolic behaviour of variant BS—21/37, and of its oxytetracycline production

Fermentation time, hours	Designation of medium											
	1	2	3	1	2	3	1	2	3	1	2	3
	oxytetracycline μg/ml			pyruvic acid mg/ml			amino nitrogen mg/ml			glucose mg/ml		
48	165	0	110	0.23	0.16	0.18	0.54	0.82	0.83	8.15	11.45	9.00
72	300	27	235	0.18	0.35	0.06	0.35	0.70	0.65	3.25	7.20	6.50
96	470	100	450	0.05	0.15	0	0.22	0.58	0.49	1.00	3.30	2.50
120	510	170	460	0	0.07	0	0.16	0.46	0.43	0	0.70	0
144	480	200	460	0	0.04	0	0.12	0.45	0.41	0	0.50	0

The media contained 30 μg/ml of P.
Amino acid in medium 1. glycine, 0.5%,
2. DL-serine, 0.7%,
3. DL-serine, 0.63, and DL-alanine, 0.06%.

This "protective influence" of DL-alanine cannot be reproduced with glycine and, in fact, in media containing glycine the production of oxytetracycline of the variant BS—21/37 can be reduced by serine. (This impeding effect can be parried by DL-alanine.) In media containing DL-alanine no such

impeding influence of DL-serine is displayed. Pyruvic acid increases in the medium when the production falls (see Table VI).

Table VI

Oxytetracycline production with variant BS—21/37 in media containing glycine, DL-alanine, DL-serine, and their mixtures

Concentration of amino acids in the medium, %	Oxytetracycline μg/ml	Pyruvic acid mg/ml
Glycine 0.5%	480	0.18
DL-serine 0.7%	200	0.35
DL-alanine 0.6%	470	0.25
DL-serine 0.63%, glycine 0.05%	220	0.35
DL-serine 0.63%, DL-alanine 0.06%	460	0.06
Glycine 0.37%, DL-serine 0.17%	350	0.28
DL-alanine 0.45%, DL-serine 0.17%	470	0.22

The media contained 30 μg/ml of P. The pyruvic acid values were determined at the 72nd hour of fermentation. Oxytetracycline yields were determined after 144 hours.

These phenomena are not perceived, of course, with a variant of good production capacity (var. BS—21/76) because its output of oxytetracycline in serine containing medium is similar to that in other media. As we have seen, variant BS—21/72 does not produce oxytetracycline in media containing serine, this production cannot be influenced by the addition of 10% alanine, but the reduced production observed in glycine media is further reduced under the influence of serine.

Consumption of serine, and of pyruvic acid, by washed mycelia. Streptomyces rimosus BS—21/76 utilizes glucose, and amino acids, serine excepted, slower than does the variant BS—21/72. Because of this, the consumption of serine, and of pyruvic acid, by washed mycelia of these two variants were investigated. The results are shown in Table VII. The ability of the variant with smaller production capacity to decompose serine is greater, but its consumption of pyruvic acid is smaller.

Table VII

DL-serine and pyruvic acid consumed by washed mycelia

	Variant BS—21/72	Variant BS—21/76
Consumption of pyruvic acid mg/g mycelium (dry)	23 (19—25)	28 (24—41)
Consumption of DL-serine mg/g mycelium (dry)	46 (40—53)	36 (28—41)

Mycelia were grown in media containing glycine and glucose, and washed three times with distilled water in a centrifuge. The washed mycelia were suspended in a mixture of the

following composition: 0.3% NaCl, 0.013 KH_2PO_4, and 0.1% pyruvic acid resp. 0.1% DL-serine. (pH 7.0 with NaOH.) The concentration of the mycelia (on dry basis) in the experiments was 4—6 mg.

The gradual decrease of production capacity brought about by serial subculturing. The experiments described were carried out with a natural variant which was bred in the course of the maintenance of strain BS—21. The loss of production capacity of this strain can be brought about also by serial subculturing (Table VIII), as shown by the investigations of PERLMAN *et al.* [10]. Starting with a variant which already contains 1% of a variant without spores, its subculturing every fifth day from shaken culture into shaken culture causes a gradual diminution of its production capacity. In parallel with this diminution goes an increase of metabolic activity, and a lowering of the optimum phosphate value of production; with higher concentrations of phosphate a strongly pronounced decrease of production is to be observed, and the output in pyruvic acid increases gradually.

Table VIII

Production capacity loss of subsequent generations grown in serial subculturing, in different media

Generation number	Oxytetracycline μg/ml in medium B	Frequency of variants with spores, in %, in potato-infusion agar media	Optimum phosphate concentr. μg/ml	Oxytetracycline μg/ml				
				at optimum phosphate concentration	at a phosphate concentration of 120 μg/ml	glucose mg/ml	amino nitrogen mg/ml	pyruvic acid mg/ml
				in media containing glycine and glucose				
1	2000	99	30	550	230	10.5	0.62	0.07
2	1700	95	30	550	200	10.1	0.59	0.09
3	1500	90	30	450	180	9.7	0.58	0.40
4	500	64	20—30	450	100	8.5	0.51	0.44
5	100	13	20	380	65	8.3	0.44	0.72
6	30	2	10—20	350	40	7.9	0.42	0.77
7	30	1	10—20	350	15	7.5	0.40	0.82

Glucose, amino nitrogen, and pyruvic acid contents were determined in samples of culture 72 hours old, grown in media containing glycine, glucose, and 10 μg/ml of P. Oxytetracycline was determined in cultures 144 hours old.

Discussion

Strain BS—21 of *Streptomyces rimosus* loses its production capacity in the course of serial subculturing as other strains do which produce antibiotics. But unlike the investigations of PERLMAN *et al.* [10] which have shown that with *Streptomyces grieseus* subculturing does not change the metabolic activity of the organism, our results suggest that the metabolic activity of *Streptomyces rimosus* becomes accelerated. With enhanced metabolism the phases

of development of the latter organism follow each other more rapidly. The optimum value of phosphate concentration for the production of oxytetracycline falls and the output in pyruvic acid grows substantially during fermentation.

Experiments carried out in synthetic media containing glycine, DL-alanine, β-alanine, and DL-serine, show that in media containing DL-serine the loss in production capacity is the most pronounced. Loss of production capacity in media containing DL-serine is observed also with variants the production capacity of which does not change in other media.

This enhanced susceptibility in respect to the media containing serine can be explained perhaps by the fact that the ability of variants with lower production capacity to decompose serine, which ability is uncommonly strong in the case of strains of good production capacities, becomes even stronger. Serine deaminase is chiefly responsible for the destruction of serine. The activity of this enzyme leads to the liberation of ammonia and pyruvic acid, and the latter, when accumulated, can cause difficulties in the intermediary metabolic process. SZENTIRMAI and HORVÁTH [11] had shown that ultrasonic destruction of the mycelium of *Streptomyces rimosus* BS—21 yielded protein solutions which produced from 0.3 to 1.0 mg of pyruvic acid per mg protein per hour. This enzymatic activity is common to mycelia raised on different sources of nitrogen. Mycelia raised in media containing serine do not show increased activity.

When the loss of production capacity is manifest only in media containing serine, this loss can be warded off by the addition of DL alanine, or β-alanine, in quantities corresponding to 10% of serine. A similar protective influence cannot be attributed to glycine. In the case of these variants, and in media containing glycine, a reduction of output can be caused by the addition of serine. There is no similar effect of serine in media containing DL-alanine. The production experiments show that the utilization of glycine goes through the glycine-serine transformation.

The experiments with labelled carbon carried out by SNELL *et al.* [12] have shown that the molecule of oxytetracycline is built up with the intermediary formation of the active acetate. DOSKOCIL *et al.* [13], in the course of their investigations of the metabolism of *Streptomyces rimosus*, have measured the contents of pyruvic acid of fermentation samples and explained their result by ascribing some role in the synthesis of the oxytetracycline molecule to the active acetate which is formed from pyruvic acid. These experiments on metabolism support this supposition. With loss of production capacity the yield of pyruvic acid by *Streptomyces rimosus* increases, the cause of this probably being a loss in the degradation capacity of this organism. In media containing DL-serine the increase of pyruvic acid output, and similarly a loss of production capacity, is already manifest when the same effect cannot yet be observed in media containing other amino acids used as nitrogen sources. If the weaker production of oxytetracycline of the variant BS—21/37 is increased

by the addition of DL-alanine, hardly any change in the consumption of glucose and amino acid follows, but the substantial accumulation of pyruvic acid ceases.

It has been observed in the cases of *Streptomyces aureofaciens* and *Streptomyces rimosus* that at higher concentrations of phosphate in the medium the yield of the antibiotic substances is less, and the accumulation of pyruvic acid is stronger [14, 15, 16, 17]. Based upon these observations the activity of enzymes of glucose metabolism has been investigated in cell-free extracts. It was shown that the activity of several enzymes was reduced by phosphates [18, 19]. If this effect plays a role in the whole cell, then by the concentrations of phosphate the degradation of glucose is switched over from the hexose-monophosphate shunt towards EMBDEN and MEYERHOF's glycolytic pathway. Based upon these experiments it was supposed that the heptulose phosphate plays some part in the building up of the chlorotetracycline molecule. Under the experimental conditions of our investigations with *Streptomyces rimosus* BS—21, when the phosphate concentration was greater, the accumulation of pyruvic acid was smaller. Although we observed the sensitivity of the enzymes taking part in the intermediary glucose metabolism towards phosphates in cell-free extracts as described by the authors cited before, their conclusions are not supported by these experiments because with growing concentrations of phosphates the production of pyruvic acid decreases.

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SPECIES-SPECIFIC ANTIGENS OF PSEUDOMONAS TABACI

By

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Summary. Untreated cultures of *Ps. tabaci* and *Ps. angulata* cannot be differentiated from *Ps. lachrymans*, *Ps. mors-prunorum* and *Ps. mori* on the basis of agglutination titres in sera prepared with living *Ps. tabaci*. *Ps. tabaci* and *Ps. angulata* become easily distinguishable from the above organisms when prior to agglutination the cultures are heated at 100°C for 1 hour.

The aspecific reactions are due to thermolabile antigens, which "cover" the species-specific thermostable antigens of *Ps. tabaci*.

Ps. tabaci and *Ps. angulata* may be adequately distinguished from the related organism also by agar gel diffusion. As compared to agglutination, this method is more specific, much simpler to carry out and can be read after short incubation.

In 1925 BROOKS, NAIN and RHODES [3] showed that numerous phytopathogenic organisms belonging to the genus Pseudomonas are related in antigenic structure. Thus sera prepared with the causative agent of tobacco wildfire agglutinated eight other species in addition to the homologous bacterium.

It has therefore been concluded by several authors that the various species of Pseudomonas cannot be differentiated serologically. In ISRAILSKY's monograph [6] it is stated that serological identification is possible only if the plant host is known from which the organism in question has originated.

As different procedures have been applied for the serological examination of the genus Pseudomonas, it was thought advisable to select the method most suitable for the differentiation of *Ps. tabaci*.

Materials and methods

Test strains. Of the 24 strains used 12 corresponded to *Ps. tabaci*, 2 to *Ps. angulata*, 6 to *Ps. lachrymans*, 1 to *Ps. mors-prunorum* and 3 to *Ps. mori*. The cultures were obtained partly from abroad, partly from isolations carried out in this country. *Ps. angulata* strains PA3 and PA11 were supplied by *Dr.* M. P. STARR, *University of California, Davis; Ps. lachrymans* strain 277 and *Ps. mors-prunorum* strain 330 were obtained from the *National Collection of Plant Pathogenic Bacteria, England.* Our own isolations were performed during the years 1955—1960 from pathological plant material originating from various parts of Hungary. Pathogenicity of the cultures was estimated by greenhouse inoculation experiments.

Immune sera. For the preparation of sera rabbits were selected which possessed no antibodies against the organisms examined. As previous studies [7] showed that high titre sera were less specific, sera with relatively low titre were preferred. These were obtained by intravenously injecting rabbits with 1, 2, 4, 5 and 5 ml antigen on every other day. The antigens were prepared by suspending the growth of 24 hour agar slant cultures in 2 ml saline. Seven days after the last injection the animals were bled. The sera were preserved with 0.5 per cent phenol.

Antigens for the serological reactions. Onto agar plates in Kolle flasks 4 ml portions of broth were pipetted. The broth in each flask was inoculated with a loopful of culture. After incubation at 27°C for 24 hours very thick suspensions were harvested. The suspensions were diluted with saline so as to give equal densities.

Agglutination tubes were incubated at 37°C for 2 hours, then left to stand at room temperature overnight.

Agar gel diffusion was performed with the method of OUCHTERLONY [11, 12]. Into a Petri dish 10 cm in diameter 15 ml agar was poured. The gel contained 1.5 per cent washed agar, 0.85 per cent sodium chloride, 0.025 per cent thymerosal and 0.01 per cent methylorange. Into the agar plate holes 15 mm in diameter were punched, 5 mm apart.

For gel diffusion the same sera were employed as were used for agglutination. As a control, a serum prepared with living *Ps. mori* strain TO1 was applied.

Extraction of antigens was performed with LANCEFIELD's method modified by SZENT-IVÁNYI [17], as follows. A thick suspension harvested from Kolle flask was centrifuged. The cells were resuspended in about 4 ml of 0.05 *N* hydrochloric acid containing sodium chloride at molar concentration. Then the cells were heated in a water bath at 100°C for 10 minutes. After cooling in tap water, the mixtures were neutralized in the presence of 1 drop of 0.2 per cent phenol red indicator as follows. *N* sodium hydroxide containing molar sodium chloride was added until the colour of the suspension had become deep red. Then 0.1 *N* hydrochloric acid containing molar sodium chloride was added until the colour had changed into orange indicating a neutral reaction.

Results

Ps. tabaci produced two characteristic colony types. One of them was strongly mucoid, the other was non-mucoid. Sera were produced with strain H20 forming mucoid colonies and with strain H11 growing in non-mucoid colonies. It should be mentioned that the colonial variation of *Ps. tabaci* was noted in a serological study by STAPP [14].

The results of agglutionation of untreated bacteria in sera prepared with living strains H20 (mucoid) and H11 (non-mucoid) is presented in Table I. As both sera gave identical results, the data were summarized.

Table I

Agglutination of untreated bacteria in sera H20-K and H11-K
Sera H20-K and H11-K were prepared with untreated antigens

Species	No. of strains	Agglutination titre
Ps. tabaci	12	1 : 1280
Ps. angulata	2	1 : 1280
Ps. lachrymans	2	1 : 1280*
Ps. lachrymans	3	1 : 640
Ps. lachrymans	1	1 : 640*
Ps. mors-prunorum	1	1 : 640
Ps. mori	1	1 : 1280
Ps. mori	2	1 : 640

* Partial agglutination.

From Table I it is clear that *Ps. tabaci* cannot be distinguished from the related species on the basis of agglutination titres*.

If the suspensions were heated in a water bath at 100°C for 1 hour, *Ps. tabaci* and *Ps. angulata* could sharply be differentiated from other species (Table II).

Table II

Agglutination of bacteria boiled for 1 hour in sera H20-K and H11-K

Sera H20-K and H11-K were prepared with untreated antigens

Species	No. of strains	Agglutination titre
Ps. tabaci	12	1 : 1280
Ps. angulata	2	1 : 1280
Ps. lachrymans	3	1 : 40*
Ps. lachrymans	3	0
Ps. mors-prunorum	1	0
Ps. mori	3	0

* Partial agglutination.

From Table II it is clear that the 12 *Ps. tabaci* strains originating from various parts of Hungary behaved similarly.

Results obtained with the two different sera were identical. This shows that the antigenic structure of the mucoid and non-mucoid variants of *Ps. tabaci* is identical.

The uniformity of the reactions of *Ps. tabaci* and *Ps. angulata* could be expected, the identity of these organisms having been known [2, 10, 13, 14].

It is interesting that whether the tests were prepared with living or with boiled cultures, the agglutination appeared in the "H"-type, floccular form.

Accordingly, the species-specific antigens of *Ps. tabaci* are thermostable, while aspecific reactions are due to thermolabile antigens. This was proved also by absorption experiments. Sera H20 and H11 were absorbed with suspensions of the homologous organism which had been boiled for 1 hour. The absorbed sera, which contained no antibodies against thermostable antigens, agglutinated the untreated bacteria non-specifically, similarly to the unabsorbed sera.

From the results it would seem that if related organisms have to be differentiated by the use of untreated antigens, the immune sera should be prepared with boiled bacteria. Therefore O sera were prepared with *Ps. tabaci* strain H11.

* It should be noted that *Ps. mori* var. *huszi*, which causes the same changes in mulberries as *Ps. mori*, gave no reaction in *Ps. tabaci* serum, and was therefore omitted from subsequent experiments.

Strain H11 was subjected to various thermal treatments. Some decrease in the agglutination titre (from 1 : 1280 to 1 : 640) against the homologous serum was observed when the cells had been heated for 2 hours at 1 atm. In order perfectly to denature the thermolabile antigens, an O serum was prepared with bacteria heated for 2 hours at 1 atm. Rabbits were injected similarly as with untreated antigens, except that instead of 5 injections 6 were administered. At the 6th injection another 5 ml dose was given. This additional dose was necessary as with heated bacteria a weaker agglutinin response was observed.

Agglutination of untreated bacteria and bacteria boiled for 1 hour in serum H11-A prepared with autoclaved antigen is presented in Table III

Table III

Agglutination of untreated bacteria and bacteria boiled for 1 hour in serum H11-A

Serum H11-A was prepared with antigen autoclaved for 2 hours at 1 atm.

Species	No. of strains	Agglutination titre	
		untreated antigen	boiled antigen
Ps. tabaci	12	1 : 160	1 : 1280
Ps. angulata	2	1 : 160	1 : 1280
Ps. lachrymans	6	1 : 80	1 : 40
Ps. mors-prunorum	1	0	0
Ps. mori	3	0	0

From Table III it is seen that serum H11-A agglutinated untreated bacteria at a low titre only. The agglutination was not species-specific, since in addition to *Ps. tabaci* and *Ps. angulata*, *Ps. lachrymans* also reacted. When the strains had been kept in a water bath for 1 hour at 100°C, only *Ps. tabaci* and *Ps. angulata* gave high titre agglutination, and thus were sharply differentiated from the related bacteria.

From these experiments it follows that the agglutinogenic and agglutinating properties of the thermolabile antigens are not perfectly inactivated by heat and thus living strains react in sera prepared with autoclaved suspensions. The same is shown by Table II regarding the remaining trace agglutination of *Ps. lachrymans*. Consequently, in order to differentiate between *Ps. tabaci* and *Ps. angulata* and the related organisms, heat-treated antigens should be applied even when sera prepared with autoclaved bacteria are used. From this it follows that for the differentiation of these species there is no need to apply sera prepared with boiled antigens.

According to STAPP [15, 16], for the identification of phytopathogenic bacteria the precipitation test is more specific and sensitive than agglutination.

It was therefore considered advisable to examine our strains with the former method.

According to STAPP the antigen for precipitation should be prepared from a 6-week old cell autolysate. This method is impractical because of the time-consuming preparation of the antigen. We attempted to work out a more rapid method by the use of the gel diffusion technique. This method revealed not only the positivity or negativity of the reaction, but also a possible finer antigenic structure.

Agglutination experiments showed that the species-specific antigens of *Ps. tabaci* are thermostable. The extraction of these components from the cells was attempted as follows.

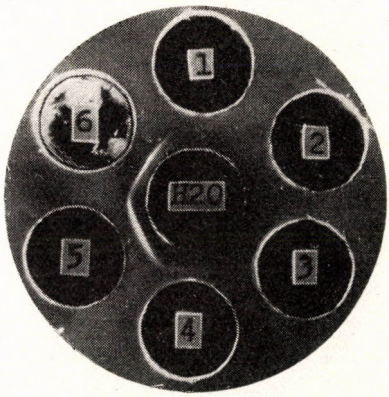

Fig. 1. Agar diffusion test with *Ps. tabaci* H20 serum.
Incubation: 48 hrs. Antigens: 1. Untreated. 2. Boiled for 10 min. 3. Boiled for 1 hr. Boiled for 2 hrs. 5. Autoclaved for 2 hrs. at 1 atm. 6. Acid-treated.

Five ml samples of a thick suspension of *Ps. tabaci* strain H20 were boiled for 10 minutes, 1 hour, and 2 hours. Another 5 ml sample was autoclaved at 1 atm. for 2 hours. An untreated suspension served as the control. The agar diffusion test was performed with the antigens prepared in this manner. After an incubation at room temperature for 12 hours, no reaction occurred either with the untreated antigen or with the suspensions boiled for 10 minutes or for 1 hour. A weak precipitation line was obtained with the suspension boiled for 2 hours. A marked line appeared with the preparation autoclaved for 2 hours at 1 atm. Accordingly, large amounts of well-diffusible antigens could be extracted from the cells only by heat-treatment under increased pressure. As this procedure was time-consuming, LANCEFIELD's method modified by SZENT-IVÁNYI [17] was applied when agar diffusion was performed with acid-extracted antigens, a precipitation line of the same intensity was obtained as with the autoclaved antigen (Fig. 1).

Table IV

Agar diffusion reactions of acid-treated antigens

Sera H20-K, H11-K and T01-K were prepared with untreated antigens; serum H11-A was prepared with antigen autoclaved for 2 hours at 1 atm.

Species	No. of strains	Serum			
		H20-K	H11-K	T01-K	H11-A
Ps. tabaci	12	+	+	—	—
Ps. angulata	2	+	+	—	—
Ps. lachrymans	6	—	—	—	—
Ps. mors-prunorum	1	—	—	—	—
Ps. mori	3	—	—	+	—

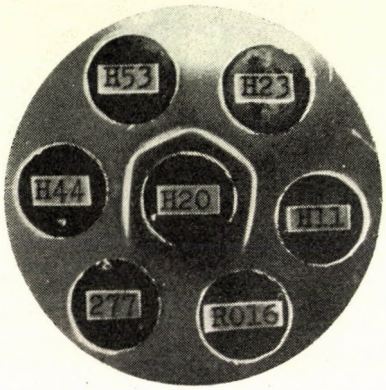

Fig. 2. Agar diffusion test of 4 *Ps. tabaci* and 2 *Ps. lachrymans* strains with *Ps. tabaci* H20 serum
Incubation: 48 hours

All strains were treated with both autoclaving and acid extraction. The preparations were tested by agar diffusion against all the sera. As the two kinds of extract behaved similarly, in Table IV only the reactions given by acid-extracted antigens are presented. Positive reactions were generally readable after an incubation at room temperature for 12 hours. With strain H42 the reaction became intense only after 48 hours.

From Table IV it is clear that reactions obtained with the diffusion method are specific. Sera prepared with *Ps. tabaci* reacted only with *Ps. tabaci* and *Ps. angulata*, and similarly, serum *Ps. mori* precipitated only antigens obtained from the homologous strains. The serum prepared with heat-treated antigen gave no reaction with either of the extracts. The explanation of this finding may be that heat treatment had so denatured the antigens that they were unable to give rise to appreciable amounts of antibodies against the diffusible antigens.

From the results it is also clear that the antigenic structure of *Ps. tabaci* is identical with that of *Ps. angulata*. Similarly, there were no antigenic differences among *Ps. mori* strains (Fig. 2).

Discussion

STAPP [14] claimed that *Ps. tabaci* was antigenically homogeneous and in this respect there was no difference between this organism and *Ps. angulata* and *Ps. mellea*. This finding was confirmed by other authors (OBERMEISTER [10], BRAUN [2] and REID *et al.* [13]). In contrast, NOVIKOVA [9] dinstinguished two serological types among *Ps. tabaci* strains, and showed that *Ps. tabaci* and *Ps. mellea* differed in antigenic structure. According to REID *et al.* [13], *Ps. tabaci* is antigenically identical with *Ps. angulata*, *Ps. fluorescens* and *Ps. syringae*. ISRAILSKY and CHISTOSERDOVA [5] in a study of fluorescent pigment producing bacteria showed that sera prepared with *Bacterium citriputeale (Ps. syringae)* gave a high titre agglutination with a *Ps. tabaci* strain isolated in Kiev. Serum *Ps. mori* showed a strong reaction with various *Ps. tabaci* strains isolated in different parts of the U. S. S. R. ARTEMIEVA [1] observed that the serological behaviour of *Ps. phaseolicola* is closely related to that of *Ps. mori*. According to FRIEDMAN [4] *Ps. lapsa* cannot be differentiated from *Ps. syringae* on the basis of agglutination titres obtained in *Ps. syringae* serum. KLEMENT *et al.* [7] showed that serum *Ps. mori* agglutinated 10 other Pseudomonas species. Serum *Ps. mori* var. *huszi* agglutinated 11 species in addition to the homologous bacterium.

It should be noted that the above investigations were carried out with different methods.

Some authors used sera prepared with living bacteria and untreated antigens for the agglutination. According to STAPP [16], for the differentiation of phytopathogenic organisms the precipitation test is more sensitive and specific than agglutination. REID *et al.* [13] distinguished DAWSON's M and S phases in *Ps. tabaci* and examined both phases as to their being suitable for identification of the strains. Phase S was produced by subculturing the strain in anti-M serum. Some strains formed no stable S phase. Recently MUSHIN *et al.* [8] compared by agglutination 9 different species belonging to the genus Pseudomonas. With O sera and O antigens 5 species could be distinguished, while strains belonging to the remaining 4 species gave no reaction. Since these authors used no H sera, it cannot be assumed whether O sera were really needed for the differentiation of these bacteria. KLEMENT *et al.* [7] used both O and H sera for the identification of *Ps. mori* and *P. mori* var. *huszi*. They showed that these bacteria can be distinguished with both O and H sera. As expected, the lower titre H sera were more specific than high titre sera.

As seen, no uniform methods have so far been elaborated for the serological determination of organisms belonging to the genus Pseudomonas, and it may be assumed that due to the inadequacy of the methods certain species have not yet been recognized.

From the present investigations it has been concluded that *Ps. tabaci* can adequately be distinguished from other species. For this purpose both the agglutination and the gel diffusion techniques can be applied. As compared to agglutination, the latter method offers a more specific result and is easier to perform. Differentiation being so simple is due to the fact that Pseudomonas bacteria possess a common group-specific, thermolabile antigen, while the species-specific antigen is thermostable.

The methods described are supposed to be useful not only for the recognition of *Ps. tabaci* but also for the differentiation of other Pseudomonas organisms.

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PHAGE-TYPING OF SALMONELLA PARATYPHI B

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Summary. A survey during the years 1956—1960 has shown that FELIX and CALLOW's phage-typing of *S. paratyphi B* is not sufficient for epidemiological purposes. For the improvement of the results SCHOLTENS' "natural grouping" based on the lysogenic state of strains has been introduced.

The methods described by FELIX and CALLOW and by SCHOLTENS were compared in parallel examinations of 360 strains. On repeated examinations of carriers constant results were obtained with the natural grouping method. The completion of the internationally used method of FELIX and CALLOW with the natural grouping has been considered useful and necessary.

With natural grouping 7 out of SCHOLTENS' 12 new types were encountered (Leeuwarden, Beccles 22, Taunton-Kampen, Taunton-the Hague, 87, Sittard and J7).

Phage-typing of *S. typhi*, elaborated by CRAIGIE and YEN [1] and successfully applied in epidemiological investigations, yielded a basis for the classification of *S. paratyphi B*. By the use of adapted phages FELIX and CALLOW [2] in 1943 classified *S. paratyphi B* first into 4 types, then by further phages into 10 types and several variants. The phage reaction of the types and variants were demonstrated in Tables 1 and 6 of FELIX and CALLOW's paper [3]. While all phages used for the typing of *S. typhi* had been derived from phage ViII by adaptation, of *S. paratyphi B* phages 2, 3a, 3aI, 3b, Jersey, Taunton and Dundee had been adapted from phage 1; phages Beccles and B. A. O. R. had originated from other sources. The site of action of *S. paratyphi B* phages is not entirely known. The role of somatic antigen 5 does not seem decisive, as variants lacking this factor can also be classified by the typing phages.

Materials and methods

Phage-typing of *S. paratyphi B* by FELIX and CALLOW's method has been performed in this laboratory since 1956. Until December 31, 1960, 715 strains isolated from chronic carriers, temporary excretors and patients were examined.

The used phages were: (i) The international typing phages of FELIX and CALLOW 1, 2, 3a, 3aI, 3b, Jersey, Beccles, Taunton, B. A. O. R., Dundee, 1010 (later phage Worksop was also applied). (ii) Phages e, d, c, f, h, b, liberated by SCHOLTENS from mixed cultures; spontaneously liberated natural phages I, IVa, IVb, VI and finally phage Beccles-Meppel, which had been obtained by adaptation.

The phage-typing technique was the same as that employed in the typing of *S. typhi* [4].

Results

The percentage distribution of 715 *S. paratyphi B* strains according to phage types is presented in Table I.

CRAIGIE and FELIX [4] and ANDERSON [5] often stressed that the occurrence of various types in a given country had to be calculated according to the number of foci, as otherwise in the course of outbreaks the high occurrence of the causative types might alter the real distribution. Thus in Table I the percentage of types is presented according to epidemic sources.

Table I

Percentage distribution of phage-types of S. paratyphi B counted by foci

Examinations performed by FELIX and CALOW's method May, 1956 — December, 1960
Total number of foci, 274. Total number of persons, 393. Total number of strains, 715

Phage-type		Average percentage 1956—1960	Phage-type	Average percentage 1956—1960
1	Most common	5.0	Jersey	1.5
	Variation 1	0.4	Beccles	2.5
	Variation 2	0.4	Taunton	59.5
	Variation 3, 4	0.4	B. A. O. R.	5.8
2		—	Dundee	5.5
3a	Most common	1.5	Untypable	4.0
	Variation 1	0.7	Degraded	0.4
	Variation 2	—		
3aI	Most common	2.5		
	Variation 1	2.2		
	Variation 2	6.6		
3b	Variation 3	0.4		
	Variation ?*	0.7		

* Reaction different from that given by any known type.

Within 5 years the average incidence of type Taunton was 59.5 per cent. This type particularly emerged in 1960 (77.6 per cent), when it caused several outbreaks originating from various sources. During the indicated period the order of type frequency was as follows: Taunton, 3aI, B. A. O. R., Dundee, 1. Untypable strains occurred in 4 per cent on the average.

A comparison of our results with those obtained in other countries showed that with the exception of England, Norway and France, in Europe generally type Taunton predominated. In England and Norway type 1, in France Dundee were the most frequent types. In Canada and Japan type 3a was the one most often encountered (Table II).

Table II

*Percentage distribution of phage-types of S. paratyphi B in various countries counted by foci**

	1	2	3a	3aI	3b	Jersey	Beccles	Taunton	B. A. O. R.	Dundee	Untypable
Austria	12.6	—	1.6	11.8	0.8	0.5	2.1	55.5	8.4	3.9	2.8
England	29.9	5.2	13.0	3.4	3.7	2.2	3.1	17.7	1.5	10.1	10.2
France	4.4	—	0.7	1.9	1.4	18.9	6.1	24.3	0.5	39.4	2.4
Netherlands ...	12.8	—	6.2	12.9	2.1	10.4	9.4	28.4	7.0	5.4	5.4
Germany											
Frankfurt/M.	12.5	0.2	5.5	14.4	3.6	2.2	5.1	38.3	10.0	5.2	4.1
Wernigerode	11.4	—	4.4	14.5	1.9	0.9	0.9	47.5	8.4	3.1	7.0
Hungary	8.9	—	3.6	14.3	1.8	1.8	2.7	54.4	3.6	5.3	3.6
Norway	35.6	—	6.7	11.1	13.3	—	—	6.7	—	2.2	24.4
Rumania	4.4	—	2.9	2.9	—	8.8	—	54.4	5.9	8.8	11.8
Japan	40.5	—	45.4	—	13.6	—	—	—	—	—	0.5
Canada	10.9	—	38.0	11.9	9.8	—	—	16.3	—	2.2	10.9

* Data summarized by Dr. P. NICOLLE from the report of the *International Committee of Enteric Phage Typing*, 1958

The value of phage-typing depends primarily on the stability of types *in vivo* and *in vitro*. The constant results with *S. typhi* phage-typing and its usefulness in epidemiological investigations have been confirmed by surveys carried out in various countries. As to the stability of *S. paratyphi B* phage-types, opinions are less uniform. In 1951 FELIX and CALLOW [3] pointed out that type Beccles was not constant. This was later confirmed [6] and on the basis of the examination of 4000 strains found to be true also for several other types (Dundee, Taunton, 3a, 3aI, 3b, Jersey, B. A. O. R.) [7].

During the 5 year period we also attempted to examine the constancy of the various types. Altogether 715 strains were examined, of which 232 had been isolated from patients, 97 from transient carriers and 64 from chronic carriers. The percentage distribution according to this grouping is presented in Table III. All the phage-types isolated from patients were found in chronic and transient carriers as well. The figures also show the incidence of untypable strains sensitive only to phages O_{1-3}, and also that of phage-resistant organisms not sensitive even to anti-O phages. The occurrence of carriers excreting different phage-types at repeated examinations is indicated under "varying results".

Untypable, phage-resistant and varying results cannot be used in epidemiological investigations. According to our data strains giving the above results occurred in 32.8 per cent on typing with FELIX and CALLOW's method. The value of this method was also decreased by the fact that calculated by

foci a high number (on the average 59.5 per cent) of strains belonged to type Taunton.

Table III

Percentage distribibution of phage-types of S. paratyphi B, counted by persons
May, 1956—December, 1960

Phage-type	Examination group		
	chronic carriers	transient carriers	patients
1 Most common	6.3	3.1	5.1
Variation 1	—	—	0.9
Variation 2	—	1.0	0.5
Variation 3, 4	—	1.0	—
2	—	—	—
3a Most common	—	1.0	0.9
Variation 1	1.6	—	1.2
Variation 2	1.6	—	0.5
3aI Most common	4.7	1.0	0.5
Variation 1	—	2.0	0.9
Variation 2	7.8	7.2	3.0
3b Variation 3	—	—	1.0
Variation ?	1.6	—	—
Jersey	1.6	—	0.5
Beccles	—	2.0	3.8
Taunton	32.8	67.4	73.2
B. A. O. R.	4.6	7.2	2.1
Dundee	4.6	4.1	3.5
Untypable	3.1	1.0	1.5
Varying results	26.6	1.0	—
Phage-resistant	3.1	1.0	0.9
Total number of persons	64	97	232

Changes of phage types are shown in Table IV. The strains isolated from the faeces and urine of a Taunton-type harbouring carrier were examined separately. The strain excreted in the faeces belonged to type Taunton, that isolated from the urine corresponded to 3aI. The examination of 30 colonies of each of these cultures showed that both contained Taunton and 3aI type organisms in mixed culture.

From these results it has been concluded that a classification by FELIX and CALLOW's method is not sufficient for epidemiological purposes.

Table IV

Changes in phage-type of S. paratyphi B strains isolated on subsequent occasions from chronic carriers

Examinations performed by FELIX and CALLOW's method

3a	Most common — untypable — Jersey
3a	Most common — untypable
3a	Most common — 3a variation 1 — 3a Most common
3aI	Most common — Dundee
3aI	Most common — untypable
3aI	Most common — Taunton — untypable
3b	— untypable
3b	— Jersey
Beccles	— untypable
Beccles	— untypable — Dundee
Taunton	— 3aI variation 2
Taunton	— phage-resistant
Taunton	— 3aI variation 2 — phage-resistant
Taunton	— phage-resistant — Dundee
B. A. O. R.	— phage-resistant — untypable
B. A. O. R.	— untypable
Dundee	— phage-resistant
Dundee	— untypable
Dundee	— phage-resistant — Taunton

SCHOLTENS [8—11] showed that *S. paratyphi B* can be classified into types on the basis of lysogenicity. Two kinds of phage could be distinguished, namely, phages liberated spontaneously and others liberated by induction from mixed cultures.

SCHOLTENS' natural grouping is based on the following examinations.

1. Determination of lysis with FELIX and CALLOW's typing phages 1, 2, 3a, Jersey, and with the newly adapted phage Beccles-Mepel.

2. Determination of lysis with phages obtained from mixed cultures.

On the basis of reactions 1 and 2 can the strains be grouped.

3. Determination of lysis with spontaneously liberated phages.

4. Detection and determination of phages spontaneously liberated from the strain under investigation.

On the basis of reactions 3 and 4 can the strains within the groups be classified into types. The group and type reactions are tabulated in SCHOLTENS' paper [11].

According to a decision of the *1st Subcommittee on S. paratyphi B* of the *International Committee for Enteric Phage Typing*, the type determination method to be used should be chosen when a sufficient number of results based on epidemiological data will be available with both FELIX and CALLOW's and SCHOLTENS' methods. The purpose of the present investigations was to contribute to the settling of the question.

Table V

Comparison of FELIX *and* CALLOW*'s method with* SCHOLTENS' *natural grouping*

Typing according to FELIX and CALLOW		Typing according to SCHOLTENS		
Phage-type	No. of strains	Phage-type	Phage-group	No. of strains
1 Most common	15	1 Most common	Ungrouped	18
Variation 2	3			
Variation 3, 4	2*	?	?	2*
2	—	2	2	—
3a Most common	8	3a Most common	B	9
Variation 1	1			
3aI Most common	10	3aI Leeuwarden		9
		Taunton-Kampen	B	1
Variation 2	16	3aI Variation 1, 2		16
3b Variation 3	3*	?	?	3*
Variation ?	3	?	B	3
Jersey	11	Jersey	J	1
		J7		10
Beccles	2	Beccles 22	A	1
		Sittard	S	1
Taunton	227	Taunton—Kampen	B	225
		Taunton—the Hague	A	2
B. A. O. R.	19	B. A. O. R.	M	18
		Taunton—Kampen	B	1
Dundee	11	Dundee	A	6
		87	S	2
		Taunton—Kampen	B	2
			Ungroupable	1
Degraded	6*	?	?	6*
Untypable	10	B. A. O. R.	M	4
		Taunton—Kampen	B	6
Phage-resistant	13	Taunton—Kampen	B	12
		Phage-resistant	—	1
Total	360	Total		360

* *S. java*

Table V presents a comparison between results obtained with FELIX and CALLOW's typing and with SCHOLTENS' method introduced in this laboratory in 1959. Altogether 360 strains were examined.

The advantages of SCHOLTENS' method can be summarized as follows.

1. Some of FELIX and CALLOW's phage-types could be divided into further types, viz.

(a) Type Beccles into types Beccles 22 and Sittard;

(b) Type Dundee into types 87 and Dundee;

(c) Type Jersey into types Jersey and J7;

(d) Type Taunton into types Taunton-Kampen and Taunton-the Hague.

Of the new types of SCHOLTENS the following occurred among our strains: Leeuwarden, Beccles 22, Taunton-Kampen, Taunton-the Hague, 87, Sittard. Type J7 described by RISCHE and SCHNEIDER [7] was also met with.

2. Repeated examinations and isolations showed that the SCHOLTENS types were constant.

With the 29 chronic carriers whose phage types proved variable with FELIX and CALLOW's method, the natural grouping yielded uniform results. Some examples are presented as follows.

(a) Of 9 subsequent isolations from a chronic carrier 6 strains belonged to type Taunton, 1 was untypable and 2 were phage-resistant. From all the 9 strains natural phage IVb could be shown. Of the 2 strains isolated from two infections occurring in connection with this carrier, one corresponded to type Taunton, the other to a phage-resistant culture. Natural phage IVb was detected in both strains.

(b) In the course of a Taunton-type outbreak a strain occurred which showed some degree of sensitivity to phage B. A. O. R., but was insensitive to phage Taunton. According to FELIX and CALLOW's method the culture ought to have been classified as type B. A. O. R.; its natural phage was, however, IVb and so, according to natural grouping it had to be regarded as type Taunton.

(c) Of 4 strains isolated on subsequent occasions from a carrier the first belonged to B. A. O. R., the second was phage-resistant and the third and fourth were untypable by FELIX and CALLOW's method. With regard to sensitivity to SCHOLTENS' phages and as to their natural phages the 3 latter strains also belonged to type B. A. O. R.

(d) During a *S. paratyphi B* epidemic in 1960, from all the patients and transient carriers type Taunton was isolated. By FELIX and CALLOW's method on 4 subsequent isolations from a transient carrier types Dundee and phage-resistant strains were revealed alternatively. It should be noted that in the family of this person there were found patients excreting only type Taunton. The natural phages of the 4 strains isolated from the transient carrier corresponded to IVb, which is characteristic of type Taunton.

3. Strains untypable with or resistant to FELIX and CALLOW's typing phages could be typed by SCHOLTENS' method. Typing carried out by the former method yielded 23 untypable or phage-resistant strains. Of the 23 strains 22 were classified into types with the natural grouping method. The correctness of the results was confirmed by epidemiological data.

4. A strain not classifiable into any of the natural groups or types may belong to a new phage-type or may possess some extraordinary properties.

(a) On repeated examination of a person, FELIX and CALLOW's types 3b variation 3,4 and a degraded form had been isolated. Neither of these strains could be included in SCHOLTENS' groups. Biochemical examination revealed that they corresponded to d-tartrate fermenting *S. java* strains. Examination of *S. java* with FELIX and CALLOW's phages showed that with this method the types were not constant and degraded forms with non-characteristic lytic pattern often occurred. In contrast, although these strains could not be included in the natural system, with SCHOLTENS' phages they showed a constant, characteristic lytic pattern (lysis with phages b, c, d, e and *BM)*.

(b) Strains isolated on various occasions from a chronic carrier could not be included by FELIX and CALLOW's typing in either of the variations of type 3b. With SCHOLTENS' phages strains 3b belong to groups A or M; the strains under investigation, however, were members of group B. Later we were able to use BERNSTEIN's newly adapted phage Worksop [12]; it was revealed that the questionable strains corresponded to this very type. The strains were d-tartrate negative.

Discussion

In addition to the method based on sensitivity to adapted phages, investigation of the lysogenic state of bacteria has also been found adequate for type determination. The identification of bacteria on ground of lysogenicity was first described by BURNET [13], without, however, elaborating a practical method. The phage-sensitivity of *S. paratyphi B*, similarly to that of *S. typhi* and other bacteria so far examined depends primarily on the lysogenic state of the strain, *i. e.* on the presence of temperate, latent phages. According to FELIX and CALLOW [3] and NICOLLE *et al.* [14—16] these phages may have type-determining and partly type-determining properties. In *S. paratyphi B* non-type-determining phages can also be found. SCHOLTENS [8] used the difference in lysogenicity of *S. paratyphi B* as a basis of a new typing system. BOYD [17] elaborated a workable method of type determination by the identification of "symbiotic" latent phages of *S. typhi murium*. Although typing based on lysogenicity is generally more circumstantial than that based on the use of adapted phages, it generally proved more reliable and constant.

In our material 11 *S. java* strains occurred, which corresponded by FELIX and CALLOW's typing to types 1 variation 3, 4 and 3b variation 3 and to degraded 3b. Typing of various colonies of the incriminated strains offered no uniform lytic patterns. In contrast, with SCHOLTENS' phages all *S. java* strains behaved uniformly. Within *S. java* BRANDIS and THOMSEN [18] showed with FELIX and CALLOW's method types Beccles, 3b, 1 and untypable strains. These authors explained the identical reactions of *S. java* and *S. paratyphi B* types by the possible identity of latent phages in the two organisms. From the 11 *S. java* strains examined in this study no directly demonstrable natural phages could be isolated.

The present investigations showed that the natural grouping of *S. paratyphi B* can well be applied in practice. It should be noted that as the majority of *S. paratyphi B* strains isolated in Hungary belongs to type Taunton (59.5 per cent according to foci), a further subdivision of this type is needed. On natural grouping 247 of these strains belonged to Taunton-Kampen and only 2 to Taunton-the Hague.

The results obtained by means of natural grouping are of practical importance, being more suitable for epidemiological purposes than thoseyielded by the other method. Moreover, investigation into the lysogenic state of strains may provide data as regards the variation *in vivo* and *in vitro* and also as to the mechanism of phage-type variation.

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AETIOLOGY OF ACUTE RESPIRATORY DISEASES IN MILITARY GROUPS

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Summary. 1. Serum samples were taken from 1201 recruits belonging to three military groups in November, 1959, and two months later. The paired sera were tested for complement fixing antibodies to adenovirus antigen. Significant *(i.e.* 4-fold or greater) rise in titre was demonstrated in 14 per cent.

2. In 18 serum pairs each showing significant rise in CF titre to adenovirus antigen the neutralizing titres to each of the types 1 to 7 of adenoviruses were also established. A significant rise in antibodies to 2 to 4 types of virus was demonstrated in almost every paired serum tested.

3. Of the 168 recruits with significant rise in complement fixing antibody to adenovirus antigen only 9 had respiratory symptoms during the period of observation.

4. None of the 46 cases of acute respiratory diseases (including the 9 cases of adenovirus infection) showed a significant rise in complement fixing antibodies to influenza A, influenza B, or Sendai virus. Mumps was diagnosed serologically in a single case.

5. Of the 14 paired sera showing 4-fold or greater rise in complement fixing antibody to mumps virus, 8 showed a significant rise also to Sendai virus.

Cumulation of acute respiratory disease partly due to adenoviruses has often been observed in military recruits, especially during the winter months. Some of the outbreaks observed in the U.S. Army proved to be caused by the types 3, 4, and 7 of adenoviruses [1, 2]. Similar experience has been reported from Britain [3, 4] Switzerland [5], and Taiwan [6]. In the Netherlands, type 14 adenovirus proved to be responsible for an outbreak affecting military recruits [7].

In Hungary the incidence of adenovirus infections in military groups has not been studied. The aim of the present, mostly serological, work was to obtain data on the possible role of adenoviruses in the acute respiratory diseases occurring among recruits in the Hungarian Army. The paired sera collected for this purpose were also tested for antibodies to influenza, Sendai and mumps viruses.

Materials and methods

Three military groups were involved in the studies. Blood samples were taken from 1201 recruits on the day of their joining the army (November 16, 1959), and two months later (January 20, 1960). The sera were stored at $-40°C$ until used. Each serum pair was tested simultaneously.

Complement fixation (CF) tests. TAKÁTSY's micro method was applied [11]. Adenovirus antigen was produced in HeLa cell cultures. Equal volumes of type 3, type 4, and type 7 culture media were mixed and inactivated at 56°C for 30 minutes. As myxovirus antigens crude allantoic fluids were used. The influenza A antigen was a mixture of PR8 and Singapore 1/57

allantoic fluids; the influenza B, the mumps, and the Sendai virus antigens were prepared from the Lee, the Enders, and the Sendai strains, respectively.

Cell cultures. HeLa cell cultures were used throughout. These had been cultivated and maintained as recommended by BÉLÁDI *et al* [10].

Neutralization tests. Twofold dilution series from 1 : 10 to 1 : 640 were prepared from the sera and tested against 100 $TCID_{50}$ of each of the types 1 to 7 of adenoviruses. To make the results comparable, standard virus suspensions stored at −30°C were used. The cell cultures were observed for 6 days after inoculation.

Faecal samples and throat washings. Properly prepared samples were stored at −30°C until used. Each specimen was inoculated into 3 HeLa cultures. The medium was changed every 4th or 5th day. The tubes were observed for 21 days. Blind passages were done in each case.

Results

Twelvehundred and one serum pairs originating from three military groups were tested for CF antibody to adenovirus antigen (Table I).

Table I

Recruits positive in the adenovirus CF test in the three military groups tested

Group of recruits	No. of recruits tested	Recruits having CF antibodies to adenoviruses			
		November, 1959		January, 1960	
		No.	per cent	No.	per cent
Group I	231	174	75	210	91
Group II	440	359	82	402	91
Group III	530	447	84	473	89
Total	1201	980	81.6	1085	90.3

The incidence of antibodies by group varied between 75 and 84 per cent in the first samples. This agrees with most of the literary data. In Hungary, however, NÁSZ and TÓTH [12] observed a considerably lower percentage for students of the same age.

Table II

Recruits showing 4-fold or greater rise in CF antibody to adenoviruses

Rise in titre	Group I		Group II		Group III	
	No.	per cent	No.	per cent	No.	per cent
4-fold	38	16.5	36	8.2	14	2.6
>4-fold	35	15.2	38	8.6	7	1.3
Total	73	31.6	74	16.8	21	4.0

Number of subjects tested, see Table I

By the end of the two-month observation period approximately the same rate (89—91 per cent) was found in each of the three groups. A remarkable proportion of the recruits showed a significant *(i. e.* 4-fold or greater) rise in CF antibody to adenovirus antigen (Table II).

The incidence of a significant rise in titre was the highest (31.6 per cent) in group I, for which the initial incidence of antibody had been the lowest. Conversely, only 4.0 per cent of the recruits showed a significant rise in group III, in which the highest percentage of recruits had had antibodies. In group II, 16.8 per cent of the recruits showed a significant rise in titre.

Table III

CF titres to adenovirus antigen in 1201 paired sera

Group I (231 recruits)

Second-sample titres	<4	4	8	16	32	64	128
256	1						
128	1	1	4	1			
64		3	9	9	2		
32	4	2	12	11	5	2	
16	9	5	13	18	7	2	
8	10	7	22	12	3		
4	11	10	5	3		1	
<4	21	3		2			

First-sample titres

Group II (440 recruits)

	<4	4	8	16	32	64	128
		1					
		2		3	1		3
	2	2	12	4	10	5	
	8	6	14	15	32	9	
	8	14	25	54	12	1	
	11	17	62	14		1	
	14	17	11	2			
	38	7	1	2			

First-sample titres

Group III (530 recruits)

	<4	4	8	16	32	64	128
			1				2
			1	3	8	7	1
	3	2	4	9	28	5	1
	2	5	34	80	14		
	5	46	79	30	3	1	
	16	51	18	3			
	57	8	2		1		

First-sample titres

In Table III the second-sample titres are plotted against the first-sample titres. The two straight lines show the limits of normal fluctuation. It is seen that mostly those recruits displayed significant rise in titre who had had no CF antibody or a low titre in the first specimen.

To elucidate which type, or types, of virus had been responsible for the antibody responses, 18 paired sera with a significant rise in CF antibody to adenovirus antigen were tested for neutralizing activity against each of the types 1 to 7 of adenoviruses (Table IV).

A significant rise to more than one type of virus was demonstrated in 15 paired sera (a rise from < 5 to 10 was not regarded as significant). Recruits No. 7 and No. 14 showed a rise in antibody to type 7 only, whereas No. 16 did showed no significant rise at all.

From groups I and III 736 serum pairs were tested for CF antibodies to influenza A, influenza B, Sendai, and mumps viruses as well. A rise in antibodies to these viruses was rarely observed; in group I more often than in group III (Table V).

Table IV

CF and neutralizing antibody titres in the paired sera of 18 recruits

No. of recruits	Group of recruits	Clinical disease during observation period	CF titre	Neutralization titres to type of adenoviruses						
				1	2	3	4	5	6	7
1		—	4/64*	320/160	20/20	5/40	0/0	5/5	5/20	0/5
2		—	0/32	20/40	20/80	0/10	0/0	10/20	5/20	0/5
3		—	8/128	10/10	160/160	20/320	10/80	40/40	40/320	10/160
4	I.	—	8/64	320/160	320/320	10/80	NT	20/80	80/640	0/40
5		—	8/64	160/160	80/80	5/40	0/0	160/320	320/320	40/320
6		—	0/128	320/320	0/5	0/10	5/5	0/5	5/80	5/320
7		—	0/256	0/5	160/320	0/10	NT	80/80	0/0	0/320
8		—	0/32	0/0	40/40	20/160	NT	20/20	5/5	5/160
9		+	4/32	160/640	0/20	5/20	0/0	20/40	0/10	20/40
10		+	4/16	80/640	80/640	5/5	0/0	0/0	0/10	10/10
11		+	4/16	5/5	40/640	10/10	0/0	40/40	5/5	40/160
12	II.	+	4/16	5/5	10/40	10/40	0/10	40/40	40/320	5/40
13		+	8/64	0/0	10/320	20/40	0/0	20/80	0/10	40/40
14		+	8/32	320/320	5/5	0/5	0/0	0/0	0/0	20/160
15		—	8/64	320/640	160/160	0/10	0/20	320/320	20/80	10/40
16		—	16/64	320/320	10/10	0/5	NT	0/10	320/320	10/20
17		—	8/64	40/320	320/640	10/10	0/0	5/5	20/80	20/40
18		—	0/64	40/320	160/320	5/5	0/0	40/40	10/40	40/40

* Numerator: reciprocal titre of the first sample. Denominator: reciprocal titre of the second sample (initial serum dilutions).

0 = <4 (CF test); 0 = <5 (neutralization test)

NT = not tested

Table V

Paired sera showing 4-fold or higher rise in CF antibodies to influenza, Sendai and/or mumps antigens

Group	Number of paired sera	4-fold or higher rise in CF antibodies to							
		Influenza A		Influenza B		Sendai		Mumps	
		No.	per cent	No.	per cent	No.	per cent	No.	per cent
I	231	5	2.2	7	3.0	6	2.6	5	2.2
III	505	4	0.8	4	0.8	8	1.6	9	1.8
Total	736	9	1.2	11	1.5	14	1.9	14	1.9

The titres were generally low. The geometric means of the titres are shown in Table VI.

Table VI

Geometric means of reciprocal CF titres to influenza A, influenza B, mumps, and Sendai viruses

Date of sampling	Antibodies to			
	Influenza A	Influenza B	Sendai virus	Mumps virus
November 16, 1959 ...	7.5	8.2	6.2	3.4
January 20, 1960	8.8	7.9	8.2	4.9

The low anti-influenza titres and the lack of rise in antibodies in the great majority of the recruits were in accordance with the fact that no influenza epidemic was observed in Hungary during the period of observation. Most of the rises in antibody were so slight *(e. g.* from < 4 to 8) that their relation to actual infection with the corresponding virus seemed to be unproven.

Mumps antibodies were found in 38.9 and 49.9 per cent of the first samples in group I and group III, respectively. The titres were low (Table V). A fourfold or greater rise was equally found in both groups. Some relation was demonstrable between the anti-mumps and anti-Sendai CF titres. Among the 14 recruits showing a significant rise in anti-mumps CF antibody, 8 gave a significant response to Sendai virus, too.

During the observation period 46 recruits fell ill with acute respiratory symptoms. The clinical picture did not offer sufficient basis for an aetiological diagnosis. Rises in temperature, headache, cough, and injected throat were the most frequent symptoms. Muscular pain, rhinitis, conjunctivitis, and tonsillitis were exceptional. A significant rise in CF antibody to adenoviruses was demonstrated in the paired sera of 9 diseased recruits. The disease of these recruits was clinically indistinguishable from that of the other 37 patients. Neither were the laboratory findings different. No significant rise in CF antibodies to influenza A, influenza B, or Sendai viruses was demonstrated in the patients' paired sera and only one patient showed a 4-fold or greater rise in CF titre to the mumps virus.

Discussion

Since the recruits under study had come from different parts of Hungary, their serological status at the time of their joining the army may reflect the serological status of the 20-year old male population of this country. If 1 : 4 and higher titres are regarded as positive, the percentages of serologically positive recruits were as follows. Adenovirus, 81.2 per cent (calculated from 1201 recruits); mumps, 46.7 per cent (from 736); influenza A, 72.2 per cent

(from 736); influenza B, 72.6 per cent (from 736); Sendai virus, 73.6 per cent (from 750). These data agree with those obtained in other countries [5].

During the two-month military service 168 recruits (14 per cent) showed a significant rise in CF antibody to adenovirus antigen. It is supposed that these subjects had become infected by adenovirus in that period. Similar studies in other countries yielded divergent results. In the U. S. A. HILLEMAN *et al.* [13] demonstrated a rise in titre in 80 per cent of recruits during an eight-week period in the winter of 1954. In the Netherlands VAN DER VEEN and KOK [7] examined 101 recruits in April and May, 1955, and observed a rise in titre in 32 (31.7 per cent). In England, MCDONALD *et al.* [23] demonstrated a rise in 26 per cent of the paired sera tested in 1955—1956, but could not detect any rise in 1956—1957. Our data agree best with those of GRAYSTON *et al.* [14], who examined navy recruits in 1955—1956 and demonstrated a significant rise in CF antibody to adenovirus antigen in 16.2 per cent of the paired sera tested.

Several authors [13, 14, 15] have pointed out that most of the adenovirus infections, being clinically inapparent, are not seen by a physician. It is furthermore supposed that in patients giving a serological response to adenoviruses, simultaneous respiratory symptoms may sometimes be due to other factors. Of the 168 recruits who displayed serological response to adenoviruses in our study, only 9 required medical care. The clinical symptoms and laboratory findings did not distinguish these cases from the 37 cases showing no rise in CF antibody to adenovirus antigen. It could not be decided whether a simultaneous rise in the neutralization titre to several types of adenovirus indicated infection with all the corresponding types of virus. Several authors [6, 16] have reported on heterotypic responses.

In the present study most of the recruits tested had had neutralizing antibodies to the types 1, 2, 5, and 6 of adenoviruses at the time of their joining the army. This is in accordance with the finding of several authors [20, 22] suggesting that the great majority of the population becomes infected by these types of virus in early childhood. The incidence of antibodies to the types 3 and 7 was also high, but their titres never exceeded 1 : 20 and 1 : 40, respectively. Only one of the 18 recruits tested possessed neutralizing antibody to the type 4 virus.

Among the 14 subjects displaying a rise in CF titre to mumps virus only one showed clinical symptoms of parotitis. The others might be regarded as latent infections.

GARDNER [17], DE MEIO and WALKER [18] were the first in reporting on the antigenic relation between mumps virus and Sendai virus. GARDNER [19] examined paired sera of 66 patients suffering from mumps for CF antibodies to both viruses and demonstrated a simultaneous response to Sendai virus in 50 per cent of the cases. In the present study eight of the 14 recruits showing

a significant rise in CF titre to mumps virus gave a parallel response to Sendai virus.

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POLIOVIRUS-CARRYING LINES OF HELA CELLS: THEIR ESTABLISHMENT AND SENSITIVITY TO VIRUSES

By

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(Received February 25, 1961)

Summary. Two virus-carrying lines of HeLa cells were established without the use of immune serum. The HeLa + P_2 line carries the MEF_1 strain of poliovirus type 2, the HeLa + P_3 line the Saukett strain of poliovirus type 3. Both lines possess a reduced sensitivity to the three types of poliovirus and to Coxsackie B3 virus. Their resistance was enhanced by superinfections. The lines which had developed from the cells surviving the second superinfection with homologous virus were resistant to 10 000 TCD_{50} of any of the three types of poliovirus and to about 100 TCD_{50} of Coxsackie B3 virus. In the persistently infected lines the average size of cell nuclei was found to be enlarged and the multiplication rate reduced.

ACKERMANN and KURTZ [1, 2] were the first to observe a persistently infected cell line, namely a line of HeLa cells which had carried poliovirus over 30 passages. Subsequently, several authors have reported on similar lines [3—9]. A calf kidney culture, the L cell line, a chick fibroblast culture, and the Lung-to line carrying WEE, NDV, mumps, and HA_1 viruses respectively, were reported.

In the present paper the establishment and certain properties of two poliovirus-carrying lines of HeLa cells will be discussed.

Materials and methods

Cell cultures. HeLa cells were used throughout. The growth medium contained 10 per cent human serum, the maintenance medium 5 per cent bovine serum, in PARKER's medium 199. The initial cell count was 130 000 cells in 1 ml per tube. Two- or three-day-old cultures were used.

Stained preparations. Cell cultures were prepared by the collodion technique [10], and stained according to May—Grünwald and Giemsa, or with methyl green and pyronine [11].

Nuclei were measured by ocular micrometer. The carrier and the control cultures were of the same age when subjected to this examination. Three hundred nuclei were measured in each line.

Viruses. The Mahoney strain of type 1 poliovirus, the MEF_1 strain of type 2 poliovirus, the Saukett strain of type 3 poliovirus, and a Coxsackie B3 (C B3) strain were used. The Mahoney strain was maintained in monkey kidney cell cultures, the other strains were adapted to HeLa cells. The titres of the viruses in serial passages fluctuated between 10^{-5} and 10^{-6}, as calculated according to REED and MUENCH [12].

Experimental

Establishment of virus-carrying cell cultures. Type 2 and type 3 polioviruses were titrated in HeLa cultures. It was noted that surviving cells were present even in tubes infected with 10 000 TCD_{50} of virus as late as on the

7th day after inoculation. To cultivate the surviving cells tubes containing such cells were washed ten times with PBS and fresh nutrient medium was added to each. During the subsequent five-day period, while the cells were washed and medium was changed every day, some cells began to multiply. On the 7th day two subcultures were made from each culture. By the end of a 48-day period confluent monolayers developed in the subcultures. The cultures so obtained were serially transferred by the mechanical way.

The cell line deriving from cultures infected by type 2 poliovirus grew nearly as well as normal HeLa cells, from the 8th to the 14th passage. In unstained monolayers cytopathic changes were rarely observed. In stained preparations, however, 1 or 2 per cent of the cells always appeared to be damaged. From the 15th passage onward hardly any cells attached to the glass and began to multiply. When the few surviving cells were treated in the same manner as the survivors of the virus infection, monolayers closely resembling those of the 14th passage soon developed. The 19th passage cultures had been destroyed again, but the line was regenerated by the 22nd passage. Since then we have been able to maintain the line without extensive destruction of cells.

In the cell line which had derived from the cells surviving the infection with type 3 poliovirus similar changes occurred. Growth was undisturbed from the 6th to the 11th passage. Then the vast majority of cells died. The surviving cells were treated as described above and the cultures regenerated by the 15th passage. Extensive destruction of cells was observed again at the 18th and 30th passages. The latter affected only 25 to 30 per cent of the cells and was followed by rapid regeneration. Since then the line has grown well.

The 13th, 14th and 21st subcultures of both lines were tested for poliovirus. Three tube cultures of each line were frozen, thawed, and centrifuged, and the supernatants were inoculated into normal HeLa cultures, 0.1 ml per tube. Extensive cytopathic changes developed in each of these cultures on the 5th day after inoculation. Control tubes inoculated with extracts of disrupted normal HeLa cells did not show any cytopathic change by the 10th day.

The tube cultures displaying cytopathic changes were kept at —15°C until used in experiments aiming at the identification of the cytopathogenic agents. In neutralization tests the strain isolated from the line which had derived from cultures infected by type 2 poliovirus was specifically inhibited by the anti-poliovirus 2 immune serum. The other line was neutralized by anti-poliovirus 3 serum only. The two lines thus proved to be virus-carriers. The line carrying type 2 poliovirus was signed HeLa + P_2, that carrying type 3 virus, HeLa + P_3.

Morphologically, the virus-carrying cultures did not remarkably differ from the original cultures. Some differences in the size of nuclei were demon-

strated. Measuring the diameters of 300 nuclei (see Materials and methods) in each line the arithmetic mean for normal HeLa cells was found to be 15 μ, for both virus-carrying lines 17 μ. The standard deviations were 2.2 μ and 2.8 μ, respectively. Statistical analysis showed significant differences in both nuclear size and standard deviation.

Attempts were made to superinfect the 22nd subcultures of both lines with homologous virus. The surviving cells were subjected to the same procedures as the survivors of the primary infections. The lines deriving from these cultures were signed HeLa + P_2 + P_2 and HeLa + P_3 + P_3, respectively. Both lines grew well; extensive destruction of cells had never been observed up to the 7th and 9th passages, respectively, when the cultivation of these lines was interrupted.

The lines HeLa + P_2 + P_2 and HeLa + P_3 + P_3 gave rise to lines HeLa + P_2 + P_2 + P_2 and HeLa + P_3 + P_3 + P_3, respectively, by second superinfection. These cells grew well over 10 passages.

The lines under study were tested for susceptibility to each of the three types of poliovirus and to C B3 virus.

Table I

Sensitivity to viruses of normal and virus-carrying HeLa lines

Experiment No.	Cell line	Titre (– log TCD_{50}/0.1 ml) of			
		Poliovirus type			Coxsackie B3
		1	2	3	
1	HeLa normal	5.75	3.50	3.50	5.75
	HeLa+P_2 carrier	5.00	2.65	2.75	5.50
	HeLa+P_3 carrier	5.25	2.65	2.50	5.25
2	HeLa normal	5.00	3.50	4.50	4.75
	HeLa P_2+P_2 carrier	1.75	1.00	1.50	3.25
	HeLa P_3+P_3 carrier	1.50	1.00	2.00	3.75
3	HeLa normal	4.50	4.25	4.50	5.50
	HeLa P_2+P_2+P_2 carrier	<1.00	<1.00	<1.00	3.25
	HeLa P_3+P_3+P_3 carrier	1.00	<1.00	1.50	3.00

It is seen in Table I that the increase in resistance never exceeded 1.0 logarithmic unit after a single infection. Resistance was, however, further increased by superinfections. The three times infected lines had almost completely lost their sensitivity to polioviruses, but retained a considerable sensitivity to C B3 virus.

Discussion

Cell lines carrying enteroviruses were established by the use of homologous immune serum. In this way were stabilized HeLa cell lines carrying poliovirus, C B3 or C A9 virus and calf kidney cells carrying the virus of foot and mouth disease [1, 5a, 13]. Cell lines carrying viruses other than enterovirus (*e.g.* an L cell line carrying WEE virus [14] and MCN lines carrying NDV or mumps viruses [6]) could be established without the use of immune serum. We succeeded in cultivating HeLa cells carrying the highly cytopathogenic poliovirus, without using immune serum.

The morphological changes shown by the altered cultures are similar to those demonstrated by ACKERMANN [2] in carrier cultures, namely the size of the cell nuclei and the corresponding standard deviations had increased. We have demonstrated, however, that the increase in nuclear size is not a specific phenomenon. Such changes could be induced by adapting a line of HeLa cells to a new nutrient medium (unpublished data).

The resistance to viruses of our virus-carrying lines could be enhanced by superinfection with homologous virus. For this further increase in resistance two factors might have been responsible, (i) the percentage of virus-carrying cells might have increased; (ii) the superinfections may have induced the release of a greater amount of an interferon-like substance. The latter possibility seems to be supported by data published by HO and ENDERS [14] and by HENLE *et al.* [9].

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RAFFINOSE ASSIMILATION OF YEASTS

By

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(Received March 22, 1961)

Summary. Raffinose assimilation has been studied in yeast strains grown from 268 samples of pathological material. In the case of ten species the definition given by LODDER and KREGER-VAN RIJ has been completed with the ability of raffinose fermentation. Raffinose assimilation has been determined with 41 species; in 5 of these our findings agreed with those reported by KUDRJAWZEW. In contrast with LODDER and KREGER-VAN RIJ's data, all the 17 strains of *Candida tropicalis* failed to ferment or assimilate raffinose.

The pigmented yeasts studied have all been found to assimilate raffinose. In the examined material assimilation of raffinose was invariably associated with the assimilation of saccharose.

The identification of yeasts, as also the description of new species and their classification into the yeast system are made on the basis of certain universally accepted morphological and physiological features. The growing number of new species has made it necessary to search for new properties and include them in the technique of identification.

In LODDER and KREGER-VAN RIJ's monograph [8] for the identification of strains a decisive importance is attributed to the fermentative and oxidative breakdown of sugars. With most strains tests for the assimilation of the five sugars: glucose, galactose, saccharose, maltose and lactose, are prescribed. In the case of a few species, the estimation of raffinose fermentation is also required as a criterion for the differentiation of certain species, e. g. *Saccharomyces cerevisiae* and *Saccharomyces carlsbergensis*, *Candida guilliermondii* and *Candida melibiosi*.

KUDRJAWZEW's monograph [7], which so far only includes the description of ascosporogenous yeasts, mentions the capacity of oxidative raffinose assimilation and its ratio with every species.

LODDER and KREGER-VAN RIJ [8], while sometimes prescribing the raffinose fermentation test, refrain from assigning importance to oxidative raffinose assimilation in species determination. Of the authors who described the approximately 100 new species identified since the appearance of LODDER and KREGER-VAN RIJ's [8] monograph, oxidative assimilation of raffinose has been investigated by CAPRIOTTI [1—5], SANTA MARIA [13—15], WICKERHAM [18], REIERSÖL [17], HAJSIG [6], VÖRÖS-FELKAI [20] and others. This

Table I

Name of species	Number of strains investigated	Raffinose			Sugar
		fermentation		assimilation	assimilation
		Lodder & Kreger van Rij	Own finding	Own finding	Lodder & Kreger van Rij
Candida parapsilosis	19	∅	—	—	DGSM
tropicalis	17	+—	—	—	DGSM
krusei	23	∅	—	—	D
pseudotropicalis	15	+	+	+	DGSL
utilis	8	+	+	+	DSM
albicans	17	∅	—	—	DGSM
guilliermondii	5	+	+	+	DGSM
melibiosi	3	+	+	+	DGSM
zeylanoides	5	—	—	—	D
pulcherrima	1	∅	—	—	DGSM
rugosa	1	—	—	—	DG
mycoderma	1	—	—	—	D
stellatoidea	1	∅	—	—	DGM
melinii	1	—	—	—	DSM
claussenii	6	∅	—	—	DGSM
solani	4	∅	—	—	DGSM
aaseri	1	—	—	+	DGSM
Torulopsis famata	7	—	—	—	DGSM
inconspicua	12	—	—	—	D
glabrata	11	∅	—	—	D
candida	1	—	—	—	DGSML
aeria	1	∅	—	+	DGSML
gropengiesseri	1	∅	—	—	DGS
pinus	2	—	—	—	D
Rhodotorula mucilaginosa	18	—	—	+	DGSM
glutinis	1	—	—	+	DGSM
rubra	2	—	—	+	DGSM
flava	2	—	—	+—	DGSML
Cryptococcus neoformans	5	—	—	+	DGSM
laurentii	1	—	—	+	DGSML
albidus	3	—	—	+—	DGSML
diffluens	2	—	—	+	DGSM
Geotrichum candidum	20	∅	—	—	DG
linkii	6	∅	—	—	D
matalense	2	∅	—	+	DGSML
Trichosporon cutaneum	6	—	—	+	DGSML

∅ : no data given

property has been utilised by NOVÁK and ZSOLT [9] in their recently elaborated yeast system.

We have studied the assimilation of raffinose by various yeast species, in order to complete the existing data and to draw attention to this feature as a help in determination.

Materials and methods

Two hundred and sixty-eight yeast strains isolated from pathological material in our laboratory in the years 1958 to 1960 were studied. Classification of the 1958—1959 material was made according to LODDER and KREGER-VAN RIJ [8], of the 1960 material according to NOVÁK and ZSOLT [10], therefore in the Tables, LODDER and KREGER-VAN RIJ's nomenclature will be used.

The oxidative assimilation of raffinose was estimated by means of the auxanographic method. Fermentation of raffinose accompanied by the development of gas was studied in a fluid medium containing 4 per cent raffinose and covered with paraffin oil or solid paraffin-beeswax. In the case of a positive test the fermented medium was analysed after seven days by paper chromatography [12], to determine the fermentation quotient.

Results

The 268 yeast strains were found to belong to 8 genera and 41 species. Except for 5 species, the strains were members of the Cryptococcaceae family forming no ascospores.

The findings concerning raffinose fermentation were compared with the data of LODDER and KREGER-VAN RIJ (Table I), and data of raffinose assimilation with those of KUDRJAWZEW (Table II). The assimilation of other sugars is shown in our Tables by the usual initials.

Table II

Name of species	Number of strains investigated	Raffinose				Sugar
		fermentation		assimilation		assimilation
		LODDER & KREGER VAN RIJ	Own finding	Own finding	KUDRJAVZEW	LODDER & KREGER VAN RIJ
Saccharomyces fragilis ...	9	+	+	+	+	DGSL
cerevisiae	4	+	+	+	+	DGSM
marxianus	1	+	+	+	+	DGSL
Hansenula anomala	3	+	+	+	+	DGSM
subpelliculosa	1	+	+	+	+	DGSM

Regarding raffinose fermentation, our findings agree with thoo of LODDER and KREGER-VAN RIJ, and also data for assimilation with thse of

KUDRJAWZEW. The only exception was *Candida tropicalis ;* in contrast with the '+—' result registered by LODDER and KREGER-VAN RIJ, none of our 17 strains brought about fermentation of raffinose.

LODDER and KREGER-VAN RIJ gave no data concerning raffinose fermentation for 10 of our species (marked in Table I by the sign ∅). We succeeded in supplying the pertaining results. In the case of three of these, *Torulopsis aeria*, *Torulopsis gropengiesseri*, and *Candida pulcherrima*, despite negative tests for the fermentation of raffinose, and by *Torulopsis aeria* positive result was obtained for its oxidative assimilation.

The pigmented yeasts *(Rhodotorula* and *Cryptococcus* species*)* investigated did not ferment raffinose but assimilated it by an oxidative process.

Discussion

In our experience, investigation of raffinose assimilation should not be neglected in the reliable identification of yeasts, partly because it throws light on the metabolic processes and partly because it allows the differentiation of certain species, such as *Rhodotorula laryngis* [17].

From the pertaining data in the literature it is clear that fermentation of raffinose is associated with fermentation of saccharose or galactose. On the basis of biochemical considerations, NOVÁK and ZSOLT [11] have theoretically established the correlations between fermentation of the above-mentioned carbohydrates, and applied their hypothesis also to the oxidative assimilation of raffinose. On this basis oxidative assimilation of raffinose was to be expected only with the species assimilating saccharose or galactose by oxidative processes. In our material the combination of saccharose and raffinose assimilation was observed among other with *Candida utilis*, *Candida pseudotropicalis*, *Candida aaseri*. In agreement with NOVÁK and ZSOLT's hypothesis, no species was observed to assimilate raffinose without assimilating saccharose or galactose.

According to the above-mentioned theory, assimilation of saccharose may occur only together with decomposition of maltose or raffinose. From this aspect our material again behaved as expected, since all the species assimilating saccharose by oxidation and were not able to assimilate raffinose, gave positive results with maltose, *e. g. Candida melinii*, *Candida parapsilosis*, *Candida tropicalis*, *Candida albicans*, etc.

Apart from the assimilation of raffinose, our findings have confirmed another inference, in that assimilation of lactose was observed only with species that decompose galactose, as *e. g. Candida pseudotropicalis*, *Torulopsis glabrata*, *Torulopsis candida*, *Rhodotorula flava*, *Cryptococcus neoformans*, etc.

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MICROBIOLOGICAL PRODUCTION OF Δ1,4-ANDROSTADIENEDIONE FROM STEROIDS OF DIFFERENT STRUCTURE. THE INTERACTION OF STEROIDS

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Summary. *Fusarium caucasicum* is capable of converting steroids of different structure into Δ1,4-androstadienedione (dienedione). The best yield was obtained by starting from Δ4-androstenedione; with this compound only the double bond Δ1 has to be formed to obtain the product. The number of enzymes required to bring about conversion is in direct proportion, while the yield is in inverse proportion, to the structural deviation of the substrate from androstenedione. This may be ascribed to the uniform rate of enzymatic breakdown of dienedione into Δ1-dehydrotestololactone in the various substrates; the quantity of dienedione accumulating in the culture depends on the relative rate of synthesis and breakdown. The situation is different when saturated steroids of the allo-series are to be converted. These compounds, while converted into dienedione inhibit the activity of the enzyme which decomposes dienedione, and the simultaneous addition of *e. g.* allopregnanedione and Δ4,3-ketosteroids is followed by the accumulation of dienedione in the culture. The mechanism of inhibition has been found to be not competitive.

The microbiological production of dienedione from progesterone is connected with the names of VISHER and WETTSTEIN [16]. With the *Fusarium caucasicum* strain at our disposal, the course of oxidation was not so uniform as with the strain of the Swiss authors whose experimental technique we followed. After an incubation period of 24 hours, Δ4-androstenedione and Δ1-dehydrotestololactone were found in addition to dienedione [17]. Japanese and Dutch authors have also reported on the synthesis of dienedione being accompanied by the appearance of other oxidation products in Fusarium cultures [13, 14].

To throw light on the mechanism of oxidation, the convertibility of various steroids has been studied. *F. caucasicum* has been found to produce widely varying amounts of dienedione from steroids of various structure. Saturated steroids of the allo-series have been found particularly interesting, since these compounds, besides being readily oxidized into dienedione inhibit its oxidative breakdown. This mechanism has been subjected to investigation.

Materials and methods

The microorganism used was a *Fusarium caucasicum* strain obtained from the Baarn collection. As evidenced by preliminary experiments, this organism showed favourable sporulation on potato-agar. Approximately 40 per cent of the developed spores were capable of germination when the spore suspension had been prepared from a 10 to 40 days' culture. The capacity for germination rapidly diminished among spores from older cultures, while growth occurred in only 1 per cent of the spores derived from cultures older than three months.

Culture media. Medium F contained 15 g peptone, 6 g cornsteep-liquor, 0.5 g glucose, in 1 litre of tap-water; prior to sterilization the pH was adjusted to 6.5 with sodium hydroxide. The medium being sensitive to infection, as a selective inoculum medium, RICHARD's medium was employed, consisting of 50 g glucose, 0.5 g KH_2PO_4, 2.5 g $MgSO_4 \cdot 7H_2O$, 0.5 g KNO_3, 5 g NH_4NO_3, in 1 litre of distilled water. 100 ml amounts of the medium were transferred to 500 ml conical flasks and sterilized at 121° C for 25 minutes.

Growth was allowed to proceed in every case at 28° C under shaking at 200 r. p. m. and 2 cm deviation. For inoculation, suspensions with a spore count of $0.5-2.0 \times 10^8$/ml were prepared from slant agar cultures and with 1 ml of this suspension 100 ml amounts of RICHARD's medium were inoculated. From the two-day culture grown on RICHARD's medium, 5 ml were used for the inoculation of 100 ml medium F; to 24 hour F cultures, the steroids to be converted were added, usually in doses of 20 mg, previously dissolved in 1 ml acetone under aseptic conditions.

Methods of determination. The amount of the produced dienedione was determined by KOBER—HAENNI's quantitative test* [11]. The results were controlled by paper chromatography as follows. Macherey-Nagel No 214 paper strips measuring 18 cm by 50 cm were washed for 24 hours with ethanol as overflowing chromatograms. After drying in air, the papers were used one week after washing. Following impregnation with 30 per cent propylene glycol (in methyl alcohol), the superfluous propylene glycol was removed by blotting between filtre papers. The paper was divided into seven equal bands, onto each band 10 to 200 μg of steroid was dropped in a volume of 50 to 200 μl and subjected for approximately 4 hours to descending chromatography with a 1 : 1 mixture of carbon tetrachloride and methyl cyclohexane saturated with propylene glycol as the mobile phase. After drying for two hours at 80° C the standard spots were developed with phthalic acid paraphenylenediamine [2], and the pieces of paper containing the steroid to be determined cut out, transferred to glass-stoppered test tubes calibrated to 10 ml, eluated for an hour with absolute ethanol of analytical grade, while shaken several times. Extinction of the eluate was measured with a Unicam spectrophotometre at 242 mμ maximal absorption, the typical range of the Δ 4, 3-keto structures. The steroid content of the eluates was determined from a standard curve, on the basis of the extinction values.

Results

Investigation of the convertibility of steroids differing in structure was started with the study of the compounds containing a Δ4,3-keto-group. This group includes progesterone and androstenedione, *i. e.* the compounds usually figuring as starting substrates. Androstenedione produced a good yield of dienedione, progesterone a weaker one. Testosterone, which differs from androstenedione only by containing a β-hydroxyl-group instead of a 17-keto-group, yielded even less dienedione. Desoxycorticosterone, on the other hand, was superior to progesterone. In agreement with the findings of VISCHER and WETTSTEIN, 17 α-oxyprogesterone was not converted into androstadienedione. Hardly any dienedione was obtained from 16-α, 17-α-oxydoprogesterone, and slight amounts were yielded by Δ16-dehydroprogesterone. REICHSTEIN's compound S failed to yield dienedione; this had been expected, in view of the 17α-oxy-group. No dienedione was obtained from Δ4,3-keto-bis-norcholene-22-aldehyde, hence the 22 carbon steroid is inadequate as a substrate (Fig. 1).

Comparison of 3 β-oxy, Δ-5 compounds revealed a weaker yield from each substance than from the corresponding Δ4,3-keto compound. Thus dehydroepiandrosterone and pregnenolone yielded less dienedione than andro-

* In the Figures, the extinction values are presented on the ordinate; an extinction of 0.830 corresponds to 200 μg/ml Δ1, 4-androstadienedione.

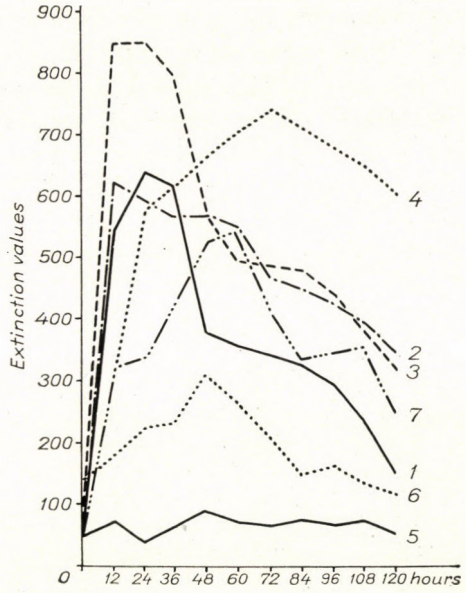

Fig. 1. Convertibility of Δ4,3 ketosteroids into Δ1,4-androstadienedione. Curve 1, 20 mg progesterone; curve 2, 20 mg Δ4-androstenedione; curve 3, 20 mg testosterone; curve 4, 20 mg desoxycorticosterone; curve 5, 20 mg 17-α-oxy-progesterone; curve 6, 20 mg 16,17-α-oxido-progesterone; curve 7, 20 mg Δ16-dehydroprogesterone

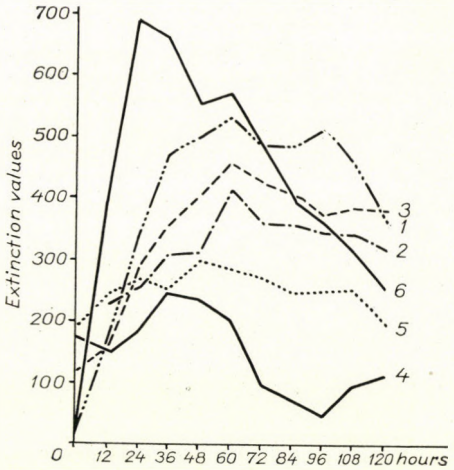

Fig. 2. Convertibility of Δ5—3 β-oxysteroids into Δ 1,4-androstadienedione. Curve 1, 20 mg dehydroepiandrosterone; curve 2, 20 mg pregnenolone; curve 3, 20 mg Δ5,16-pregnadienolone; curve 4, 20 mg 16 α 17 α-oxido-pregnenolone; curve 5, 20 mg Δ5,16-pregnadienolone acetate; curve 6, 20 mg progesterone

stenedione or progesterone, and so did Δ5,16-pregnadienolone. The acetates of the compounds always gave lesser yields than the free alcohols (Fig. 2).

There was an essential difference between steroids of the allo-series and those of the normal series. Allopregnanedione gave better yields of diene-

dione than did progesterone. The adverse influence exerted by the Δ16-double bond on productivity was noticeable also in this group. Δ16-dehydro-allopregnanedione gave a poorer yield than did the basic compound. When the 3-keto-group was substituted with a 3 β-oxy function in allopregnanolone, the yield decreased below that of the corresponding 3-keto compounds. Unlike

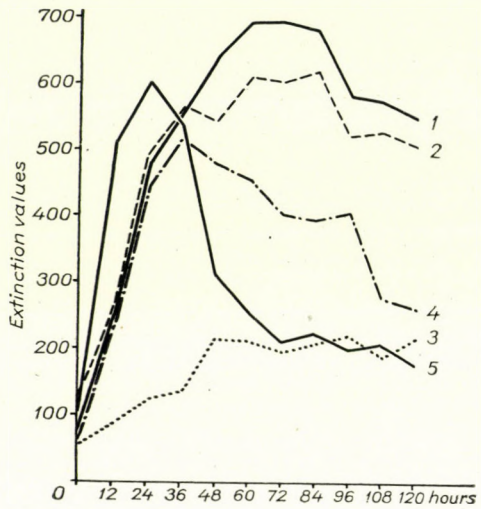

Fig. 3. Convertibility of saturated steroids into Δ1,4-androstadienedione. Curve 1, 20 mg allopregnanedione; curve 2, 20 mg Δ16-allopregnanedione; curve 3, pregnanedione; curve 4, allopregnanolone; curve 5, progesterone

the excellent yield obtained in the case of allopregnanedione, pregnanedione yielded little dienedione (Fig. 3).

The dienedione yield was higher with allopregnanedione than with progesterone, and in cultures containing allopregnanedione the breakdown of dienedione was also slower. Conversion of allopregnanedione into dienedione requires not only the decomposition of the methylketone side-chain and the development of the Δ1-double bond (as *e. g.* in the case of progesterone), but also the presence of the enzyme system suited to build up the Δ4-double bond. Progesterone is oxidized by *Fusarium caucasicum* through the series of reactions illustrated in Fig. 4. The enzymes involved in the oxidation process are of adaptive nature. The possibility has been taken into consideration that in the case of allopregnanedione inhibition of overoxidation may be due to interference with the production of the overoxidizing enzyme by the development of the enzyme responsible for building up the Δ4-double bond. This hypothesis has been controlled by a series of experiments; 20 mg progesterone was added to the first tube, 20 mg allopregnanedione to the second, and 20 mg progesterone together with 20 mg allopregnanedione to the third.

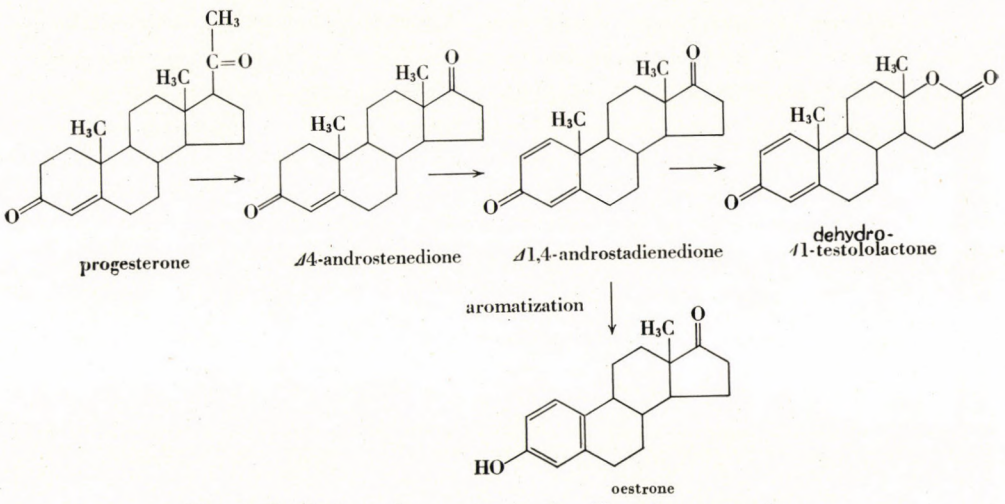

Fig. 4. Oxidation of progesterone by *Fusarium caucasicum*

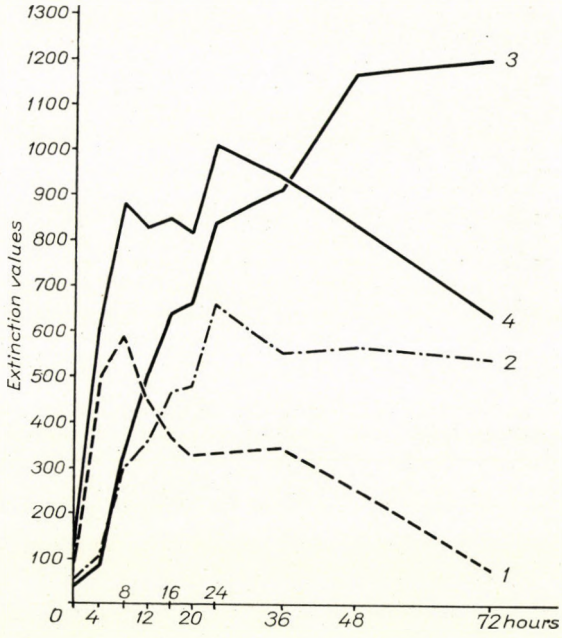

Fig. 5. Oxidation of a mixture of progesterone and allopregnanedione into Δ1,4-androstadienedione. Curve 1, 20 mg progesterone; curve 2, 20 mg allopregnanedione; curve 3, 20 mg progesterone and 20 mg allopregnanedione; curve 4, the sum of curves 1 and 2

The results are presented in Fig. 5. In the third tube increase of the dienedione content was slow to start, but attained a high level, and breakdown of the product was less rapid than in the control tubes, as demonstrated by the values or their sums (curve 4 of Fig. 5). This effect has been termed mixed substrate effect.

Like allopregnanedione, androstenedione is converted into dienedione with a better yield than progesterone. This induced us to perform the experiment illustrated in Fig. 6. Here, too, androstenedione gave a better yield than progesterone; peak production was reached almost simultaneously, but in both cases the produced dienedione was broken down rapidly. On the combined addition of the two substances, the course of the production curve

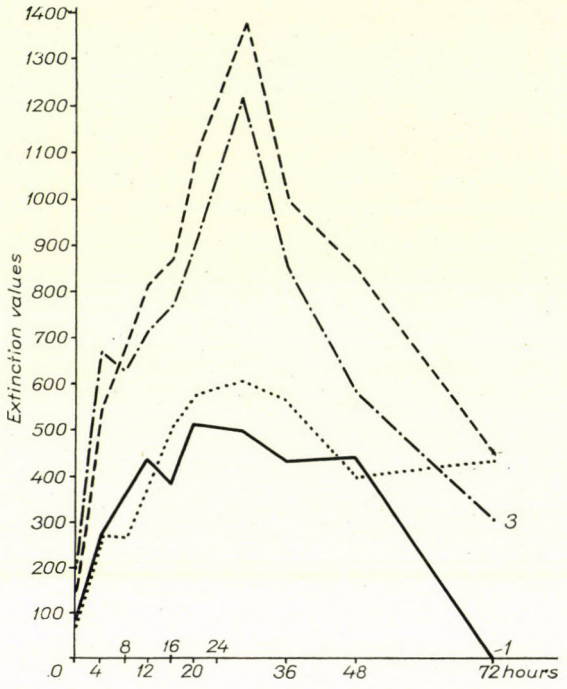

Fig. 6. Oxidation of a mixture of progesterone and Δ4-androstenedione into Δ1,4-androstadienedione. Curve 1, 20 mg of progesterone; curve 2, 20 mg Δ4-androstenedione; curve 3, 20 mg progesterone and 20 mg Δ4-androstenedione; curve 4, the sum of curves 1 and 2

was found to correspond with the sum of the values obtained from the separately applied steroids; thus there was no mixed substrate effect.

Next, we studied the effect of saturated compounds. The mixed substrate effect was demonstrable even when androstenedione and allopregnanedione had been applied together (Fig. 7).

The experiments performed with pregnanedione revealed an important difference between the normal- and the allo-series of saturated steroids. As has been shown before, pregnanedione could be oxidized into dienedione at a poor rate. Nor did it display a mixed substrate effect in the same sense as the allo compounds. Conversion failed to achieve high values, being slower than in cultures containing progesterone or androstenedione (Fig. 8).

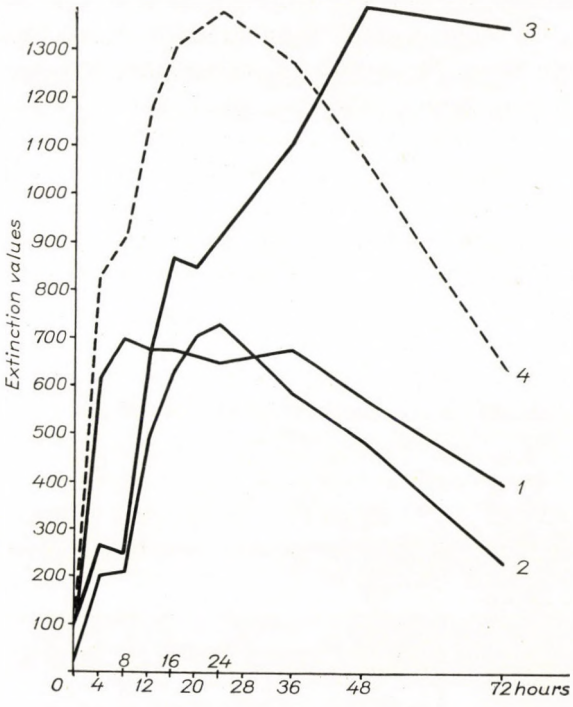

Fig. 7. Oxidation of a mixture of Δ4-androstenedione and allopregnanedione into Δ1,4-androstadienedione. Curve 1, 20 mg Δ4-androstenedione; curve 2, 20 mg allopregnanedione; curve 3, 20 mg Δ4-androstenedione and 20 mg allopregnanedione; curve 4, the sum of curves 1 and 2

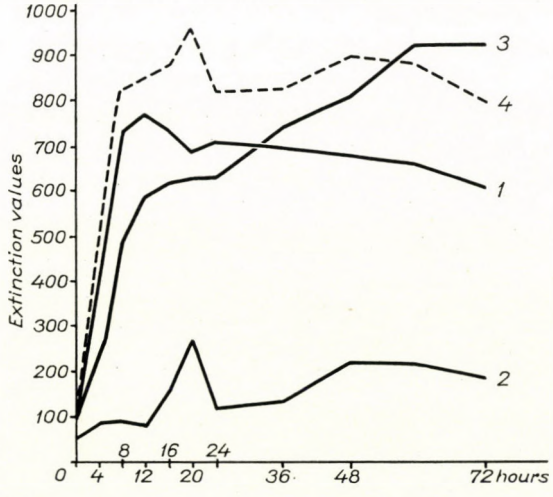

Fig. 8. Oxidation of a mixture of Δ4-androstenedione and pregnanedione into Δ1,4-androstadienedione. Curve 1, 20 mg Δ4-androstenedione; curve 2, 20 mg pregnanedione; curve 3, 20 mg Δ4-androstenedione and 20 mg pregnanedione; curve 4, the sum of curves 1 and 2

Allopregnanolone, on the other hand, exhibited a mixed substrate effect with progesterone and androstenedione; however, since allopregnanolone itself gives a poorer yield, the maximum values thus obtained were inferior to those registered for allopregnanedione (Fig. 9).

Δ16-dehydroallopregnanedione showed a similar mixed substrate effect (Fig. 10) for the development of which the allo configuration was found to be indispensable. This means that the adequate configuration of the rings A and B is a necessary precondition of the effect; the 3-keto group does not appear to be essential (allopregnanolone also exerted the effect), and differences may occur in ring D, since 16-dehydroallopregnanedione, too, gave rise to the mixed substrate effect.

As androstanedione exhibited a mixed substrate effect (Fig. 11), saturated compounds need not contain a methylketone side-chain to give rise to the effect.

Similarly to the allo-compounds, rings A and B are situated on the same plane also in oestrone which equally inhibited overoxidation (Fig. 12). KOBER-HAENNI's reaction having been given more intensely by oestrone than by dienedione, the carbon tetrachloride extract was washed four times with equal volumes of a 4 per cent aqueous sodium hydroxide solution prior to determination, in order to remove the interfering phenol-type steroid.

Subsequently we studied the mechanism responsible for the mixed substrate effect. In a series of shaken flasks, to the first flask were added progesterone and allopregnanedione together, to the remaining flasks the progesterone was given at the beginning of the experiment and allopregnanedione was added 4, 6 or 8 hours later, respectively (Fig. 13). The mixed substrate effect appeared also in the tubes to which allopregnanedione had been added later. As shown by the paper chromatography, in shaken cultures overoxidation could be demonstrated six hours after addition; this means that induction of the adaptive enzyme responsible for oxidation must have taken place within a shorter period of time. The fact that allopregnanedione gave rise to a mixed substrate effect also when added at a later point of time, showed that the assumption forming the starting point of the experiments must have been erroneous. The saturated compounds did not inhibit the synthesis of the inducible enzyme responsible for the overoxidation.

When the progesterone: allopregnanedione ratio was altered, a mixed substrate effect nevertheless developed, even with a 4 : 1 progesterone-allopregnanedione ratio (Fig. 14). When the ratio of androstenedione and allopregnanedione was changed to provide for the presence of a fourfold concentration of androstenedione, the effect was clearly demonstrable (Fig. 15). These findings excluded the possibility of a competitive inhibition.

Twenty mg/100 ml of each progesterone and allopregnanedione, or androstenedione and allopregnanedione were added to the cultures diluted

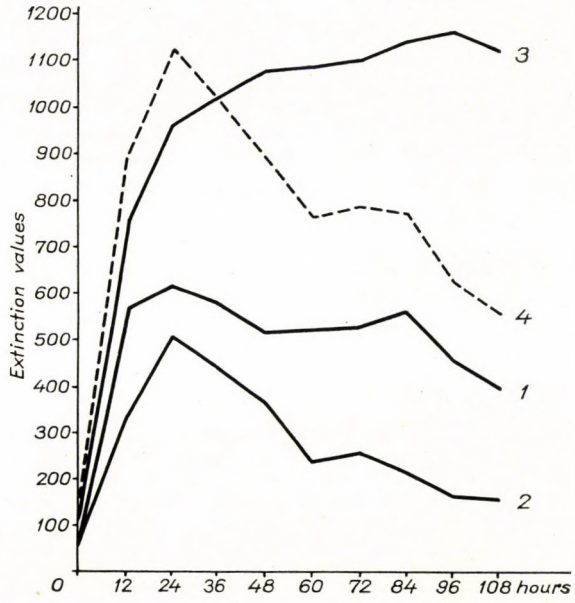

Fig. 9. Oxidation of a mixture of progesterone and allopregnanolone into Δ1,4-androstadienedione. Curve 1, 20 mg progesterone; curve 2, 20 mg allopregnanolone; curve 3, 20 mg progesterone and 20 mg allopregnanolone; curve 4, the sum of curves 1 and 2

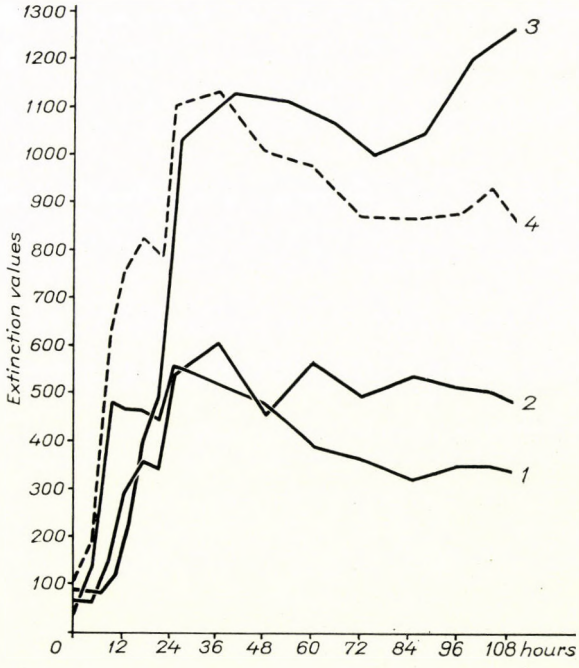

Fig. 10. Oxidation of a mixture of progesterone and Δ 16-allopregnenedione into Δ1,4-androstadienedione. Curve 1, 20 mg progesterone; curve 2, 20 mg Δ16-allopregnenedione; curve 3, 20 mg progesterone and 20 mg Δ16-allopregnenedione; curve 4, the sum of curves 1 and 2

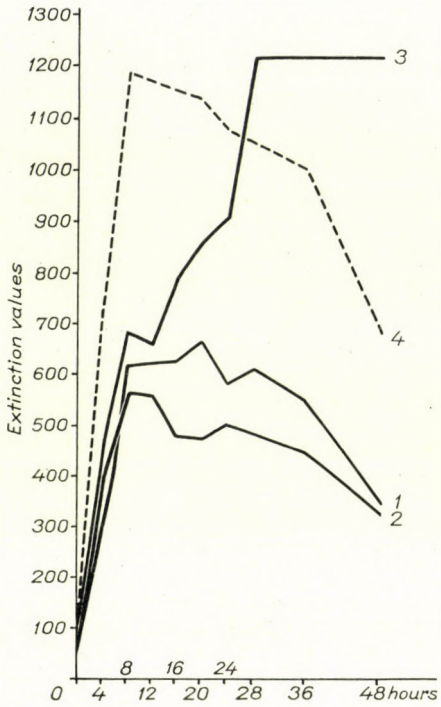

Fig. 11. Oxidation of a mixture of Δ4-androstenedione and androstanedione into Δ1,4-androstadienedione. Curve 1, 20 mg Δ4-androstenedione; curve 2, 20 mg androstanedione; curve 3, 20 mg Δ4-androstenedione and 20 mg androstanedione; curve 4, the sum of curves 1 and 2

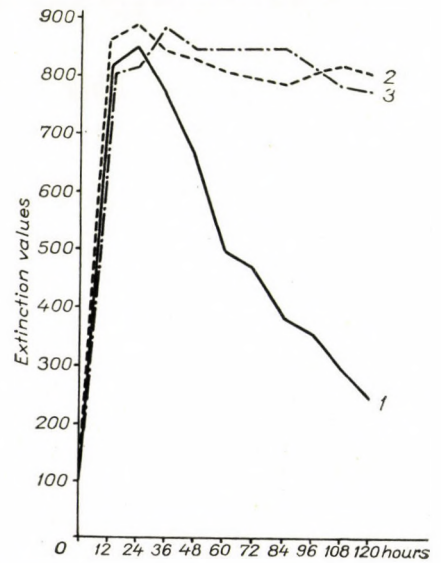

Fig. 12. Oxidation of a mixture of Δ4-androstenedione and oestrone into androstadienedione. Curve 1, 20 mg Δ4-androstenedione; curve 2, 20 mg Δ4-androstenedione and 10 mg oestrone; curve 3, 20 mg Δ4-androstenedione and 5 mg oestrone (cultures diluted 1 : 20)

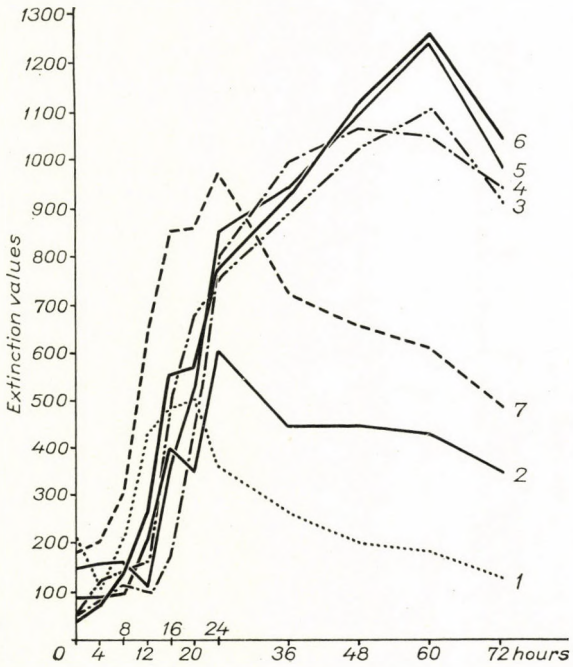

Fig. 13. Oxidation of progesterone into Δ1,4-androstadienedione in the presence of allopregnanedione added at various points of time. Curve 1, 20 mg progesterone; curve 2, 20 mg allopregnanedione; curve 3, 20 mg progesterone and 20 mg allopregnanedione added 4 hours later; curve 4, 20 mg progesterone and 20 mg allopregnanedione added 8 hours later; curve 5, 20 mg progesterone and 20 mg allopregnanedione; curve 6, 20 mg progesterone and 20 mg allopregnanedione added 6 hours later; curve 7, the sum of curves 1 and 2

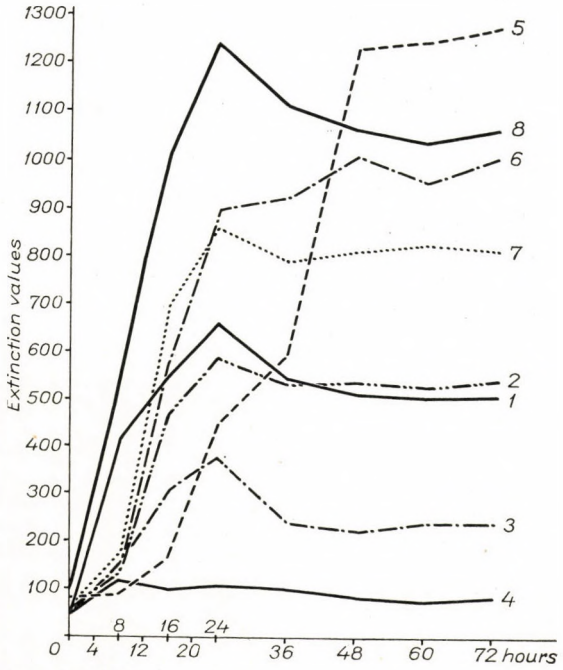

Fig. 14. Oxidation of progesterone into Δ1,4-androstadienedione in the presence of decreasing amounts of allopregnanedione. Curve 1, 20 mg progesterone; curve 2, 20 mg allopregnanedione; curve 3, 10 mg allopregnanedione; curve 4, 5 mg allopregnanedione; curve 5, 20 mg progesterone and 20 mg allopregnanedione; curve 6, 20 mg progesterone and 10 mg allopregnanedione; curve 7, 20 mg progesterone and 5 mg allopregnanedione; curve 8, the sum of curves 1 and 2

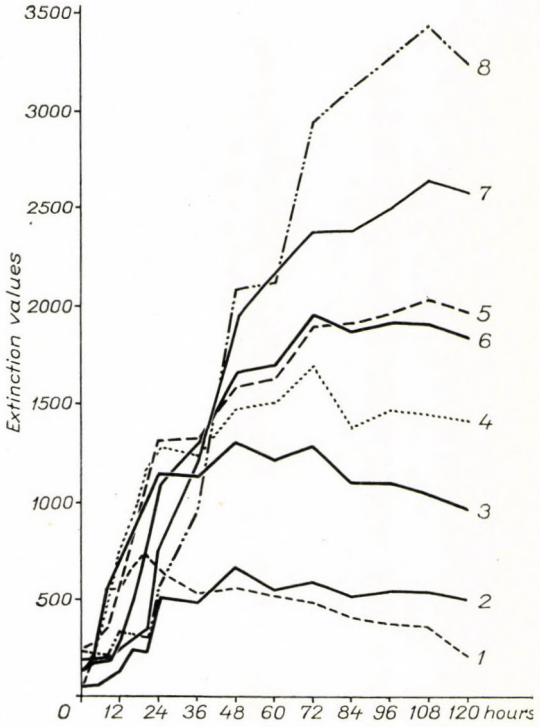

Fig. 15. Oxidation of increasing amounts of Δ4-androstenedione in the presence of allopregnanedione. Curve 1, 20 mg Δ4-androstenedione; curve 2, 20 mg allopregnanedione; curve 3, 40 mg Δ4-androstenedione; curve 4, 60 mg Δ4-androstenedione; curve 5, 80 mg Δ4-androstenedione; curve 6, 40 mg Δ4-androstenedione and 20 mg allopregnanedione; curve 7, 60 mg Δ4-androstenedione and 20 mg allopregnanedione; curve 8, 80 mg Δ4-androstenedione and 20 mg allopregnanedione (Cultures diluted 1 : 20)

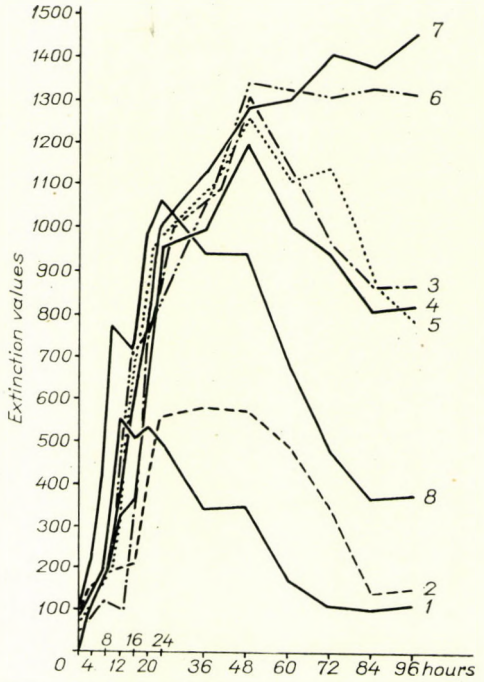

Fig. 16. Effect of diluted cultures on the oxidation of progesterone and allopregnanedione mixtures. Curve 1, 20 mg progesterone in concentrated culture; curve 2, 20 mg allopregnanedione in concentrated culture; curves 3 to 7, 20 mg progesterone and 20 mg allopregnanedione; curve 3, concentrated culture; curve 4, 2-fold diluted culture; curve 5, 4-fold diluted culture; curve 6, 10-fold diluted culture; curve 7, 20-fold diluted culture; curve 8, the sum of curves 1 and 2

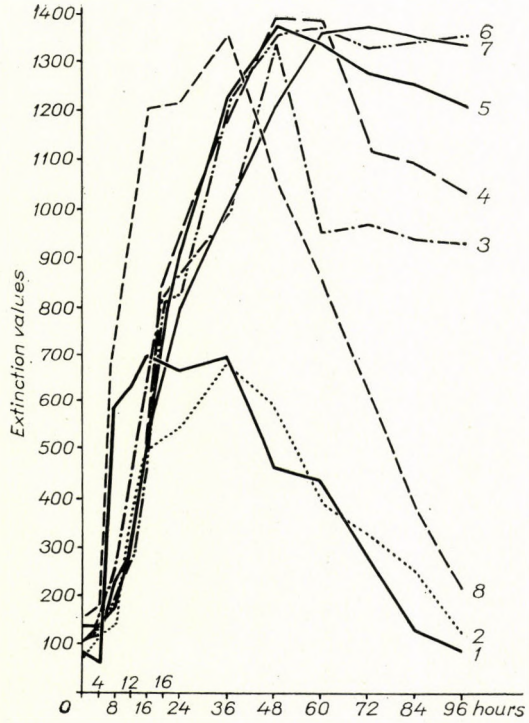

Fig. 17. Effect of diluted cultures on the oxidation of Δ4-androstenedione and allopregnanedione mixtures. Curve 1, 20 mg Δ4-androstenedione; curve 2, 20 mg allopregnanedione; curve 3 to 7, 20 mg Δ 4-androstenedione and 20 mg allopregnanedione; curve 3, concentrated culture; curve 4, 2-fold diluted culture; curve 5, 5-fold diluted culture; curve 6. 10-fold diluted culture; curve 7, 20-fold diluted culture; curve 8, the sum of curves 1 and 2

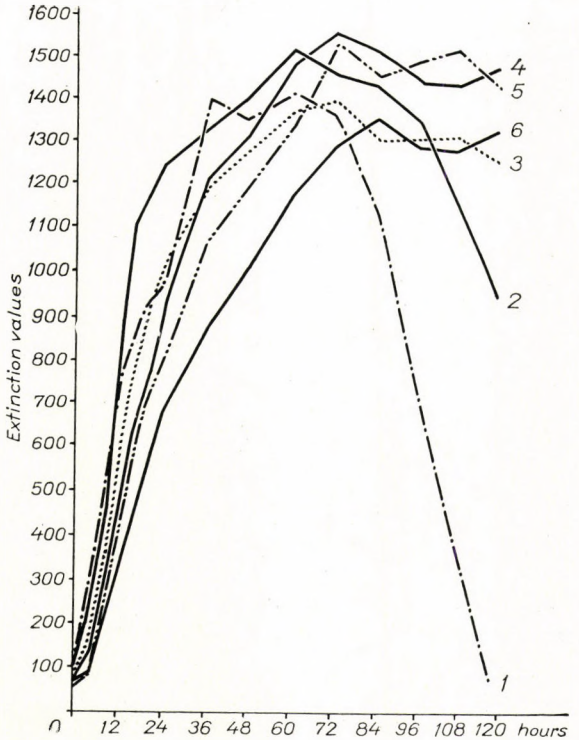

Fig. 18. Effect of dilution on the oxidation of progesterone and allopregnanedione mixtures. Curves 1 to 6, 20 mg progesterone and 20 mg allopregnanedione; curve 1, concentrated culture; curve 2, 10-fold diluted culture; curve 3, 20-fold diluted culture; curve 4, 25-fold diluted culture; curve 5, 40-fold diluted culture; curve 6, 50-fold diluted culture

with sterile distilled water. The mixed substrate effect was found to be in direct proportion to dilution, and with twentyfold dilutions the theoretical yield was attainable (Figs. 16, 17). This enhancing influence of dilution could be detected even in fiftyfold diluted cultures (Fig. 18).

Discussion

As shown by the experiments, *Fusarium caucasicum* is capable of bringing about manifold conversions of steroid structure, introducing the Δ1- and Δ4- double bonds, oxidizing 3β- and 17β-oxy groups into the corresponding ketones, transforming the Δ5-double bond into Δ4, breaking down the methylketone side-chain, saturating the Δ16-double bond, splitting the 16, 17-epoxy-group, changing ring D into a heterocyclic ring in the course of overoxidation. These enzyme reactions proceed at various rates and it is on the rate that the amount of dienedione accumulated in the culture depends. Certain substitutions in the steroid molecule, however, inhibit linkage of the substrate to the enzyme surface. This is the effect of the 17α-oxy group in 21-carbon-steroids or of the 22-aldehyde. Therefore no androstenedione is produced from 17α-oxy-progesterone, REICHSTEIN's compound S, or from Δ-4,3-keto-bis-nor-cholene-22aldehyde. After incubation, these compounds can be recovered unchanged from the culture.

As regards the rate of the various enzyme reactions, no accurate kinetic data have been obtained, since we did not work with isolated enzymes; the experiments nevertheless permit certain conclusions concerning the relative speed of some reactions.

Of all the examined compounds, the best yield was produced in the shortest time by androstenedione. Only one alteration of the molecule is required in order to gain dienedione: the Δ1-double bond has to be built up. The formation of this double bond appears to be the fastest enzyme reaction with the steroid-oxidizing enzyme systems of Fusarium. The developing dienedione is promptly oxidized into Δ1-dehydrotestololactone. In some of our experiments the rate of overoxidation was about four times slower than the conversion of androstenedione into androstadienedione. This rate was not consistently uniform in every experiment, in lack of knowledge of all the conditions influencing the various enzyme reactions. This explains the fluctuating yield of androstenedione, since production undoubtedly depends on the ratio of dienedione formation and breakdown.

Production of dienedione from progesterone is a more complicated process. Here the methylketone side-chain has to be broken down to build up the molecule. This reaction appears to be slower, determining the speed of dienedione formation; since overoxidation may be presumed to take place

at the same rate as in the case of the conversion of androstenedione into androstadienedione, the yields are poorer. Desoxycorticosterone gave better yields than did progesterone; computed per mol, they approached the values obtained for androstenedione. This shows that following oxidation of the methylketone side-chain into an α-ketole side-chain, breakdown of the side-chain is accelerated, creating a more favourable ratio of dienedione synthesis and breakdown.

Saturation of the Δ16-double bond is a slower process than the breakdown of the methylketone side-chain, while removal of the 16α-17α-oxido group proceeds even more slowly. In the case of compounds containing such structures, their rate of conversion becomes the factor controlling the production of dienedione, in agreement with the above considerations. An analytical comparison of the results clearly shows that the production of dienedione is doubtlessly influenced by various alterations of the steroid structure (methylketone side-chain, Δ16-double bond, 16α-17α-oxido group) in every group of compounds under investigation (Δ4,3-keto, 3β-oxy Δ5- and saturated steroids).

Thus in the conversion of testosterone, dehydrogenation of the 17β-oxy group would seem to be the determining factor. TALALAY's studies concerned with *Pseudomonas* β-oxy-steroid-dehydrogenase indicate that oxidation of 3β- and 17β-oxy groups is carried out by the same enzyme [15]. 3β-oxy compounds are converted into 3-keto compounds also by Fusarium which furthermore oxidizes dehydroepiandrosterone and pregnenolone into dienedione, though with a poorer yield than in the case of the corresponding Δ4,3-keto compound. According to the findings of TALALAY, steroid isomerase bringing about the conversion of the Δ5-double bond into Δ4 takes place so promptly that for a long time this step was estimated as a spontaneous reaction. Presuming conditions to be similar in the case of Fusarium, the responsibility for the poor convertibility of 3β-oxy Δ5-steroids might be ascribed to the slow action of 3β-oxysteroid dehydrogenase. This assumption is supported by the results obtained with allopregnane-3β-ol-20-on: the yield of this compound was much worse than that of allopregnanedione.

This raises the problem of the susceptibility of saturated compounds to oxidation. Allopregnanedione gave a better yield of dienedione than did progesterone, and the obtained dienedione was decomposed at a much slower rate than noted in connection with other substrates. This means that the rate of overoxidation was different with dienedione derived from different substrates. Pregnanedione gave very poor yields. Apparently for favourable oxidation of the molecule, rings A and B have to be situated on an equal plane. Saturated compounds of the normal series fail to answer this requirement. A favourable production of the Δ1,4-diene structure by compounds of the allo-series has been described also in connection with *Septomyxa affinis* [10].

TALALAY who obtained similar results with β-oxysteroid-dehydrogenases [9] suggested that in linkage on the enzyme surface, the lower surface structure of the steroid molecule is the decisive factor; he attributed the weaker oxidation of the normal series steroids to the downward bend of ring A, almost forming a right angle to ring B. From human observations, BUSH concluded to the reduction of the 11-oxo group being determined by the stereochemistry of the rings A and B. Reduction invariably takes place with the compounds of the allo-series, whereas hardly any conversion can be demonstrated with compounds of the normal series [4].

The experiments with steroids differing in structure resulted in two observations of practical importance. Androstenedione proved to be the substrate giving the best yield, and the fact that allopregnanedione interferes with the enzyme system responsible for overoxidation.

Induction of dienedione formation from allopregnanedione requires not only decomposition of the methylketone side-chain and development of the Δ1-double bond, but unsaturation of Δ4 must also be brought about in the ring system. The steroid-oxidizing systems of *Fusarium caucasicum* are inductive structures [18]. Thus the oxidation of allopregnanedione involves the development of more enzyme systems than does the oxidation of, *e. g.*, progesterone. Since adaptation to a certain substrate may inhibit the development of an inductive enzyme necessary for the conversion of another substrate [12], development of the enzyme responsible for Δ4-unsaturation might interfere with the formation of the overoxidizing system. This is in harmony with the occurrence of a mixed substrate effect after the combined application of progesterone and allopregnanedione, but the same effect was not produced by the combination of progesterone and androstenedione. However, overoxidation was inhibited also if allopregnanedione had been added to the culture at a stage when the previously added amount of progesterone could already have yielded Δ1-dehydrotestololactone. Hence allopregnanedione inhibited the function but not the development of the overoxidation system.

The data confirming the consistent development of effect regardless of any change in the ratio between the saturated and unsaturated steroids, suggest that inhibition is not of a competitive nature. From the increasingly pronounced mixed substrate effect appearing when progesterone, or androstenedione, together with allopregnanedione had been added to increasingly diluted cultures, the conclusion was drawn that we had to deal here with a special case of irreversible, not competitive, inhibition. While namely allopregnanedione inhibits the oxidative breakdown of dienedione, at the same time it joins the oxidative series, and is converted into dienedione. The concentration of the inhibitive substance is thus reduced in time and finally reaches a limit where, notwithstanding the "irreversible" linkage to the surface of the enzyme system responsible for overoxidation, its concentration

is less than that required for the inhibition of all the overoxidizing enzymes. Thereupon overoxidation sets in.

Competition for the allo-compound nevertheless does take place; not between the substrates, but between the enzyme systems oxidizing allopregnanedione into dienedione on the one hand and breaking down dienedione on the other. So it becomes comprehensible how the mixed substrate effect is enhanced by a dilution of the culture and thus altering the ratio between steroids and enzymes to the advantage of the steroids.

In contrast to the data obtained with allopregnanedione pregnanedione failed to exhibit a mixed substrate effect. In its presence the development of dienedione from Δ4,3-keto compounds proceeded slowly, but the yield did not surpass the control values, hence no inhibition of overoxidation occurred. Allopregnanolone, Δ16-allopregnendione, and androstanedione, on the other hand, showed a marked mixed substrate effect. Therefore the development of the effect really depends on the allo configuration of the rings A and B.

As in the case of allo compounds, with oestrone the rings A and B are also situated on the same plane, and the latter compound — as expected — has been found to inhibit the oxidative breakdown of dienedione.

All our experimental findings agreed on that inhibition of the overoxidizing enzyme could be brought about by steroids with coplanar A and B rings. Similarly to the allo-compounds and oestrone, with Δ4,3-keto compounds, too, rings A and B are situated in one plane. According to our working hypothesis these compounds ought to inhibit the enzyme system responsible for overoxidation. In fact, we possess data confirming that when the Δ4,3-ketosteroid to be converted had been applied at high concentrations, Δ1-dehydrotestololactone appeared in the culture later than in the case of low concentration. Accordingly, the Δ1,4-steroid seemed to have the slightest affinity to the overoxidizing enzyme, whereas Δ4,3-keto- or the saturated steroids of the allo-series, and also oestrone, showed a closer affinity to these surfaces and therefore inhibited the decomposition of Δ1,4-androstadienedione.

Few reports have dealt with the interaction of steroids. Oestrone, testosterone, and hydrocortisone have been shown to accelerate the growth of *Euglena gracilis*, but the simultaneous use of any two of these steroids has been found to slow down growth [3]. The growth of *Neurospora crassa* was found to be inhibited by DOC and a few related compounds; this inhibition was diminished by other steroids, such as ergosterol, cholesterol, and corticosterone [8]. In the supernatant of homogenized rat liver reduction of the Δ4,3-keto group of hydrocortisone was held up by the competitive inhibition of methyltestosterone and ethyniloestradiol [7]. The proliferation of *Tetrahymena piriformis* was inhibited by DOC, progesterone, and cortisone; inhibition was suspended by stigmasterol [6]. In peptone-yeast extract media the growth of *Mycoplasma laidlawii* strain A was promoted by cholesterol; this

effect was inhibited by other steroids [5]. For our work the following two data were of particular interest. In homogenized rat liver the breakdown of DOC was inhibited by glycirrhetic acid [1]. The inhibition due to glycirrhetic acid may be compared to the results obtained with the allo-compounds, the rings A and B of glycirrhetic acid being of allo-configuration. An interaction showing a similarity to the mixed substrate effect was observed by MARCUS and TALALAY [9] in the case of β-oxy-steroid-dehydrogenase, the activity of which was blocked by competitive inhibition due to the oestratrien-3-ol adhering closely to the enzyme surfaces. These findings agree with those of our own in that the compounds of the allo-series proved to be readily convertible substrates, unlike those of the normal series, which were converted at a slower rate, and gave poorer yields.

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CULTIVATION AND HISTOPATHOLOGY OF KIDNEYS FROM MONKEYS SUFFERING FROM DYSENTERY

By

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Summary. The cell yield per gram tissue of 145 kidneys from monkeys with bacillary dysentery was found to be 39.6 per cent less than that from healthy animals.

The low cell yield might be explained by certain histological changes found in the kidneys of sick animals.

In each of the 50 monkeys suffering from dysentery, histological examination revealed changes reminding of toxic nephrosis. No pathological changes were found in the kidneys of 20 healthy monkeys examined as controls.

Bacillary dysentery is one of the frequent infectious diseases in monkeys. The disease is in every respect identical with that observed in humans.

PRESTON and CLARK [1] reported on the incidence of dysentery in monkeys. The aetiology was studied by FAIRBROTHER and HURST [2] and by JANSEN [3]. GALTON *et al.* [4] and CLARK *et al* [5] studied the treatment, KOCH and DEIMEL [6] and DAVID and SCHIRL [7] the pathogenesis and pathology of the disease.

There are, however, no data available on the changes elicited in the kidneys of monkeys suffering from dysentery, although these lesions have a special significance when the preparation of monkey-kidney-tissue-cultures is considered. The problem seems to gain increasing importance with the increasing use of tissue cultures in virus research.

The incidence and severity of Shigella dysentery among the monkeys used in the *Virus Department* of the *State Institute of Hygiene, Budapest* (rhesus and cynomolgus monkeys imported from India and Indonesia) depends mainly on the conditions of transport, and meteorological, dietary and certain other factors. In 1959 out of the 597 rhesus and cynomolgus monkeys imported, 82 died of dysentery.

During dysentery epidemics it may happen that kidneys of clinically healthy infected animals are used for tissue cultures, and economical reasons kidneys from moribund animals were sometimes used for such purpose. According to our experience, however, the cell yield from kidneys of sick or dying animals was usually worse than expected, thus calling our attention to a thorough study of the problem.

Materials and methods

Monkeys. The kidneys of a total of 391 rhesus and cynomolgus monkeys used for tissue culture were studied. The average weight of the animals ranged from 1½ to 2 kg. The kidneys of healthy and dysenteric animals were compared in the same period of observation.

Tissue culture. Monkey kidney epithelial cell monolayer cultures were prepared according to the method of YOUNGNER [8]. Cell count was determined from an 0.5 ml sample withdrawn from the stock cell suspension and stained with 1 ml of 0.1 per cent methyl violet solution. Sample and stain were carefully mixed; the cell count was determined in a Buerker's chamber.

Diagnosis. Dysentery was in every case diagnosed at autopsy, on the basis of the characteristic colonic changes. Fresh faecal samples taken from the lesioned parts of the intestines were examined by the *Department for Bacteriology* and the *Dysentery Laboratory* of our Institute.

Histological examination was made of the kidneys of 50 monkeys died with dysentery or killed in the moribund state. The freshly obtained possibly warm kidney samples were fixed in formaldehyde embedded in paraffine and stained by haematoxylin and eosin. In certain cases ENDES's [9] trichrome staining was also performed. Frozen sections stained with Sudan III were prepared, and for the demonstration of calcium salt deposits the silver-impregnation method of v. KOSSA was used.

Results

Data on the results of trypsinization are summarized in Table I.

Table I

Cell yield of monkey kidneys used for tissue culture preparation in 1959

Monkeys examined			Kidneys used for tissue culture preparation	
Diagnosis	Number	Weight	Average cell count per gram kidney after trypsinization	Cell yield, per cent
Healthy	179	1394.3 g	30.6×10^6	100
Dysenteric	145	1311.4 g	18.5×10^6	60.4
Other diseases .	67	486.0 g	19.6×10^6	64.0

The data presented in Table I show that the total quantity of kidney tissue of 179 healthy monkeys amounted to 1394.2 g and yielded an average of 30.6×10^6 cells per gram kidney tissue on trypsinization. This value appears to be in good agreement with the literary data concerning the average cell count obtained by the same method. The total quantity of kidney tissue from 145 monkeys suffering from bacillary dysentery amounted to 1311.4 g, not much less than that of healthy monkeys. Nevertheless in the latter case the average cell count turned out to be 18.5×10^6 per gram kidney tissue, *i. e.*, if the cell yield of the healthy animals is taken for 100 per cent, the cell yield of dysenteric animals was only 60.4 per cent. Accordingly, the cell yield of dysenteric monkeys showed a decrease of 39.6 per cent.

In order to approach the problem more thoroughly, the above data were compared with the results for 67 monkeys killed because of other diseases (pneumonia, tuberculosis, parasitic infection, apparent sometimes only at autopsy).

In these 67 animals the average cell yield per gram tissue was 19.6×10^6 from 486 g kidney tissue. This corresponded to a cell yield of 64 per cent, slightly higher than the value found with dysenteric monkeys.

As trypsinization, cell counts and preparatory work were carried out according to routine standard methods, it was supposed that the differences in the cell yield might be caused by pathological changes in the kidneys of monkeys infected with bacillary dysentery. Accordingly, the kidneys of these animals were examined histologically.

More or less severe histopathological changes were found in every kidney from monkeys infected with dysentery. As observed in monkeys killed immediatelly after the appearance of the symptoms of acute dysentery, the epithelial cells of the cortical convoluted tubeles were damaged first. These cells appeared to be swollen; the plasma showed homogeneous eosinophilia, the nuclei were either poorly stained by haematoxylin, or fragmented, sometimes even missing (Fig. 1).

In severe cases the epithelial cells of the straight tubules were also attacked (Fig. 3).

The lumen border of the cells was often irregular or severely damaged. Some of the tubular lumens disappeared due to the swelling of cells, and some were filled with cell fragments or serum of homogeneous eosinophilic staining. In the case of an incomplete occlusion the cross section of the tubules showed a star-like shape (Fig. 2).

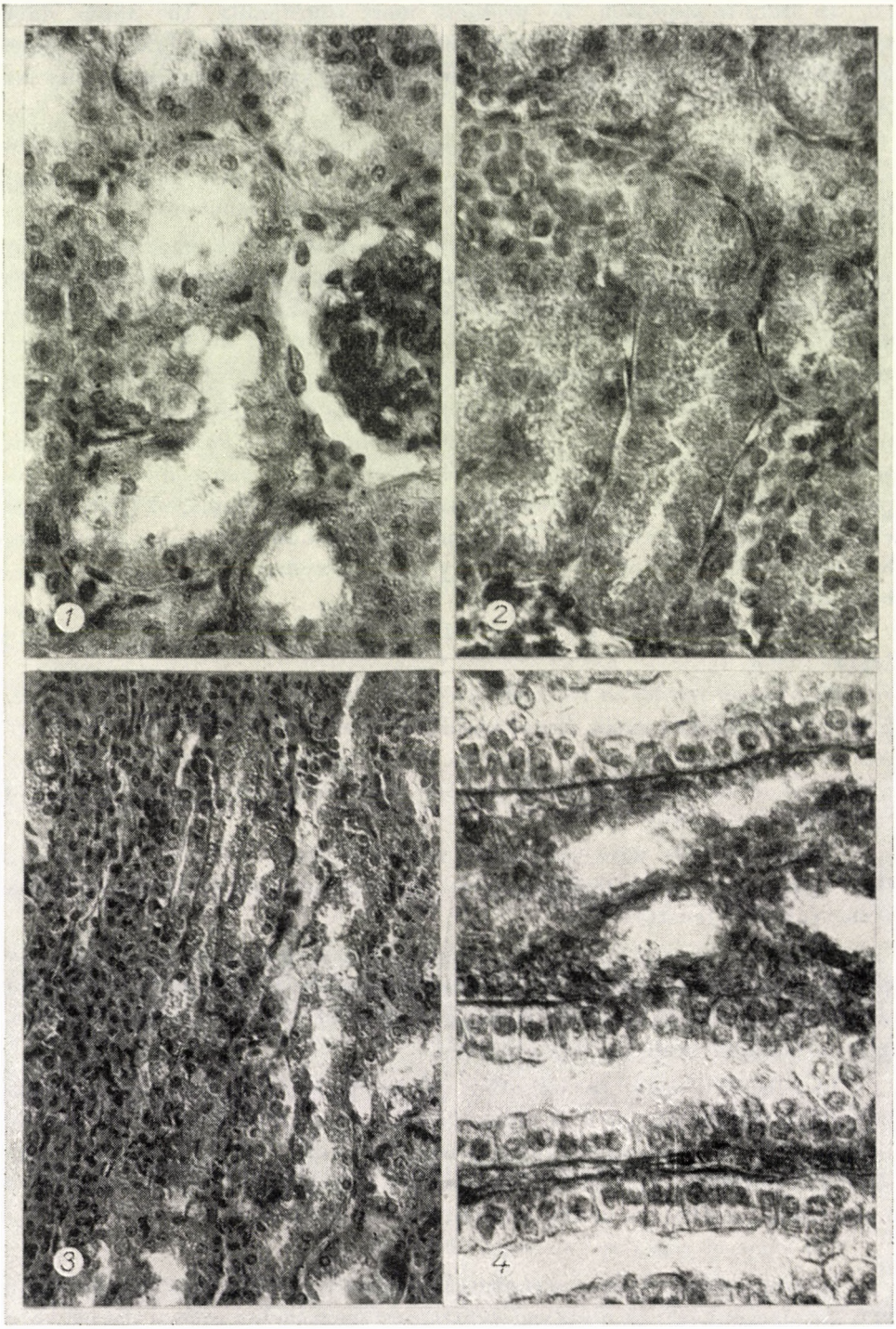
Numerous cells were completely separated from the basal membrane, while some others were desintegrated. This process lead to the destruction of the tubular structure.

Blood vessels were generally wide and filled with red blood cells. No inflammatory cellular reaction was found within the vessels or in the tissue; nevertheless at certain spots the leakage of serum displaying by a homogeneous pink eosinophilic staining was observed.

In subacute cases, vacuolisation of the epithelial cells of the proximal convoluted tubules occurred. In some instances calcium salt granules intensely staining with haematoxylin and showing a positive KOSSA-reaction were found in the tubular epithelium. The calcium salt deposits were most obvious in the basal membranes of the straight tubules (Fig. 4).

No signs of regeneration were observed.

In preparations stained according to ENDES material showing a fibrinoid-type staining was seldom observed, mostly in the epithelial cells of the primary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convoluted tubules. Examinations for fat deposition by the Sudan III stain gave practically negative results.

In the kidneys of the 20 monkeys examined as controls neither changes similar to those described above, nor changes of any other nature were observed.

Discussion

Pathologic changes of the kidneys in human Shigella dysentery were first described by JAFFÉ and STERNBERG [10] who observed vacuolisation of the epithelial cells. OPPENHEIMER found haemorrhagic infarction in the kidney of an infant died with dysentery. The observation of JAFFÉ and STERNBERG was later supported by ANDERSON [12]. The most severe pathologic changes were observed by HARANGHY [13], consisting of lesions similar to those elicited by mercuric chloride poisoning. According to his observations lesions of a wide range of different severity may be associated with dysentery, from moderate calcification of the epithelium of the convoluted tubules and cast formation, to the histological pattern of grave mercury poisoning.

Although the pathological changes observed in the monkeys did not reach that final stage, they should none the less be regarded as "toxic nephrosis" characterized by primary degenerative changes of the tubular epithelium [14, 15].

Most of the infectious fevers are wellknown to induce in the kidneys cloudy swelling, fatty infiltration, hyalin-droplet degeneration and sometimes necrosis of the tubular epithelium. Pathologic changes of that kind were observed in humans with diphtheria, typhoid fever, pneumonia, tuberculosis and other diseases. No such observations have, however, been discribed, in monkeys.

According to our observations, a remarkably decreased number of intact epithelial cells should obviously be expected to be yielded by the kidneys of monkeys suffering from dysentery. The data presented in Table I suggest that a certain decrease of the cell count should be expected also in animals suffering from other diseases, mainly pneumococcal pneumonia, as according to VOLHARD and FAHR [16], and RANDERATH [17] this disease often elicits

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Fig. 1. Degeneration of the epithelial cells of primary convoluted tubules. Haematoxylin-eosin stain. Magnification × 162

Fig. 2. Degeneration of epithelial cells of straight tubules and destruction of tubular structure. Haematoxylin-eosin stain. Magnification × 86

Fig. 3. Complete or partial obstruction of the lumen of convoluted tubules. Haematoxylin-eosin stain. Magnification × 162

Fig. 4. Calcium deposits on basal membrane of straight tubules. Haematoxylin-eosin stain. Magnification × 162

nephrosis in humans. LUND and PETERSEN [18] reported on a high incidence of pneumonia in the monkey (with our monkeys we have a similar experience), the possible occurrence of the above described lesions in monkey kidneys should always be taken into account.

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AN IMPROVED METHOD FOR THE CULTIVATION OF BACTERIA FROM BLOOD

By

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Summary. A new method has been applied for the cultivation of bacteria from blood. In bacteraemia due to different pathogens, the new method yielded 3 times as many positive results as the former technique.

In contrast, for the cultivation of *S. typhi*, the bile medium was found more suitable. In case of patients with suspected typhoid fever, parallel examination with both methods seems advisable, as the new method may reveal bacteraemia caused by pathogens other than Salmonellae, especially when the first blood culture in bile medium has been negative for *S. typhi*.

The use of a diphasic medium in the new technique decreases the hazard of contamination, because bacterial growth can be detected without opening the bottle. Plating from the culture should be made only for further study of the organism.

The disadvantageous effect of natural bactericidal agents of the blood on the results of blood cultures has long been recognized. The introduction of antibiotics into therapy has caused further difficulties. Their action is not limited to blood cultures. Cultivation of bacteria from other materials and especially the evaluation of serological reactions presents considerable difficulties since antibiotic treatment has come into general use [1]. The effective application of aimed drug therapy on the one hand, and the loss of value of some serological reactions (*e. g.* of the Widal reaction) on the other, have made it necessary to ensure optimal conditions for the isolation of the causative agent from blood and other materials. The importance of the question is shown by the fact that a whole day was spent on this subject at the 2nd International Congress of Infectious and Parasitic Diseases (Milan, 1959) [2]. The purpose of the present study was to review our earlier cultivation techniques and, if necessary, to replace them with improved methods.

Materials and methods

The numerous methods described for blood cultures will not be discussed. The methods applied formerly and at present in our Institutes are as follows.

Examination of blood from suspected or diagnosed cases of typhoid fever. The clot is transferred into 6 to 8 ml of ox bile. The tubes are incubated at 37° C for 5 days. The cultures are plated out daily onto brilliant green agar. In the laboratory of the László Hospital from each patient parallel examination of clotted and citrated samples was carried out in bile medium and with the new method, respectively.

The old method for the examination of blood from pathological conditions other than enteric fever. Mostly 10 to 15 ml clotted blood samples were received. These were transferred under sterile precautions into 100 ml of Liebig extract broth containing 0.1 per cent glucose. The flasks were incubated at 37° C for 6 days. From the flasks a loopful of culture was transferred onto blood agar plates after 1, 3 and 6 days of incubation. This procedure decreased but partly the action of natural bactericidal agents and of accidentally present antibiotics and sulphonamides. The opening of the flasks on 3 subsequent occasions increased the hazard of contamination and thus the number of results which could not be evaluated.

The new method. Taking into consideration the simplicity and the effectiveness of the method described by ORTALI *et al.* [3] and the suitability against contamination of the containers recommended by PATOČKA [4], the combination of the two methods seemed advisable. The hospital departments were supplied with sterile 50 ml centrifuge tubes containing 0.5 ml of 3.8 per cent sodium citrate. The tubes were closed with cotton wool and cellophane. From the patients 10 ml blood was withdrawn and injected into the tube through the stopper. In the laboratory the tubes were centrifuged, or, if the blood corpuscles had sedimented, they were used without centrifugation. The blood corpuscles were drawn up from the bottom of the tube with a 100 ml pipette. Immediately after this 80 ml broth was drawn into the pipette and the mixture was transferred into a 300 ml bottle containing a layer of solid nutrient agar. Every day during the 10 day incubation the bottles were gently shaken so as to allow the culture to flow over the agar layer, and at the same time the cultures were inspected as to whether bacterial growth had appeared on the surface of the solid phase. It should be noted that ORTALI *et al.* used 250 and 1000 ml Roux bottles. The growth was transferred onto blood agar or brillant green agar plates. Instead of the 3 per cent tryptose agar recommended by ORTALI *et al.*, the following medium was used.

Agar, 22 g; peptone (Richter), 10 g; beef extract, 10 g; glucose, 1 g; NaCl, 5 g; vitamin B_1, 0.005 g; distilled water, 1000 ml; pH 7.4. With the exception of glucose and vitamin B_1, all ingredients were dissolved by boiling in distilled water. After the medium had been clarified with sedimentation, to the clear supernatant glucose and vitamin B_1 were added. The medium was distributed in 40 ml amounts into 300 ml bottles and autoclaved for 20 minutes at 115° C.* After removal from the autoclave, the bottles were placed on their sides and the agar was allowed to solidify. With the exception of agar, the liquid medium contained the same ingredients as the nutrient agar. The former in 80 ml amounts was sterilized in separate flasks. Before use the media were placed in the incubator for 48 hours for sterility testing.

The efficacy of our medium was proved in the following experiment. A 4 hour broth culture of *Str. viridans* was diluted to 10^{-3}, 2×10^{-3}, 5×10^{-3}, 10^{-4}, 10^{-5}, 10^{-6} and 10^{-7}. As recommended by LÁNYI [5], dilutions were made with 3 per cent glucose. From each dilution an 0.1 ml aliquot was transferred into the diphasic medium and other 0.1 ml samples were inoculated onto blood agar plates. Colony counts showed that the undiluted 4 hour culture contained 3×10^5 cells per ml. Growth was observed in the diphasic medium inoculated with the 10^{-4} dilution, *i. e.* with 3 cells. The advantages of the new method over the formerly used techniques were as follows.

(a) The removal of plasma effectively eliminated the inhibitory action of natural bactericidal agents.

(b) The growth inhibitory effect of unknown factors associated with the blood cells and of the remaining antibiotics was eliminated by dilution of the sample with broth. ORTALI *et al.* used at first P. A. B. and penicillinase; later, however, they simply diluted with 300 ml broth the blood of patients receiving antibiotic therapy. Dilution with broth has been recommended also by FABIANI and DESPAUX [6].

(c) In the diphasic medium bacterial growth can well be distinguished from the autolysis of cellular elements and from the occasional precipitation of proteins due to certain ions, as the growth is clearly visible on the surface of the solid phase.

(d) Accordingly, subcultures are to be made only when the growth has already appeared. This decreases the possibility of accidental contamination.

The blood samples received in the laboratories of the State Institute of Hygiene and the László Hospital originated from patients with septic conditions, endocarditis, typhoid and paratyphoid fever, etc. Parallel examination with the new method and bile cultures was carried out only on the material of the László Hospital including cases of suspected typhoid fever. No parallel examinations were performed with specimens from other patients. Thus in these cases the new and old methods could be compared only on the basis of results given by

* It should be noted that heat treatment at the pH of the medium somewhat decreased the vitamin B_1 content.

the old method during the previous 2 years. A comparison of this type was considered justified, as the distribution of the patients was similar during both investigation periods.

Results

The results obtained with the new method are presented in Table I.

Table I

Result of blood cultures

	László Hospital	State Institute of Hygiene	Tota
(a) *Number of cultures*			
Positive	91	6	97
Sterile	501	37	538
Contaminated	147	22	169
Total	739	65	804
(b) *Percentage of cultures*			
Positive	12	9	12
Sterile	68	57	67
Contaminated	20	34	21
Total	100	100	100

Of the 804 blood samples 97 (12 per cent) yielded pathogenic bacteria. Of the samples 538 (67 per cent) were sterile and 169 (21 per cent) contained contaminating organisms. With the old method (not shown in Table I), out of 944 samples 25 (2.6 per cent) were positive for pathogenic organisms, 483 (51.1 per cent) were sterile and 436 (46.1 per cent) were contaminated.

In Table II is shown the distribution of the isolated bacteria and the minimum time of incubation required for their detection. *Staph. aureus* was cultured in 62 cases, chiefly from the 3rd day onward. This number includes only the coagulase positive strains. The 14 *S. typhi* strains cultured appeared also after 3 days, the only *S. paratyphi B* (Schottmüller) strain grew after 7 days. One *Str. pyogenes* and 10 *Str. viridans* were detected generally on the 10th day. The 3 *Ps. pyocyanea*, 2 *E. coli* and 4 Klebsiella strains became visible on the surface of the solid phase after 2 to 5 days.

Table III shows the comparison of the results yielded by the new method and bile cultures in cases of suspected typhoid fever.

With the new method *S. typhi* was cultured in 15 cases (including the *S. paratyphi B* shown in Table II). In these cases bile cultures were positive

Table II

Distribution of pathogenic organisms in blood cultures according to species and incubation time needed for their detection

Organism	Incubation time, days										Total
	1	2	3	4	5	6	7	8	9	10	
Staphylococcus aureus	2	4	18	9	8	8	3	2	2	6	62
S. typhi	—	—	1	1	3	—	5	1	2	1	14
S. paratyphi B	—	—	—	—	—	—	1	—	—	—	1
Streptococcus pyogenes	—	—	—	—	—	—	—	—	—	1	1
Streptococcus viridans	—	—	2	—	—	—	—	—	1	7	10
Pseudomonas pyocyanea	—	1	1	—	1	—	—	—	—	—	3
Escherichia coli	—	—	1	—	1	—	—	—	—	—	2
Klebsiella	—	—	2	1	—	—	—	—	—	1	4

for Salmonellae on 14 occasions. In 25 cases with the new method pathogenic bacteria other than Salmonellae were isolated, which remained undetected in bile cultures. With the new method no pathogenic bacteria were isolated from 243 samples. The bile culture of these samples yielded 14 additional strains of *S. typhi*. Summing up, from the same material bile cultures gave 28,

Table III

Examination of blood cultures from patients with suspected typhoid fever

Cultivation with the new method		No. of cases	Cultivation in bile medium	
S. typhi	Other pathogens		*S. typhi* positive	*S. typhi* negative
Positive	Negative	15*	14*	1
Negative	Positive	25	—	25
Negative	Negative	243	14	229
Total		283	28	255

* Including the *S. paratyphi B* (Schottmüller) strain indicated in Table II

while the new method only 15 strains of Salmonellae. Negative bile culture and positive result in the new medium occurred on one occasion only. This finding clearly shows the superiority of bile culture in the isolation of Salmonellae. It should be noted that it could not be estimated how many of the 283 suspected cases were really typhoid fever.

Table IV presents the comparison of the results obtained by the new and old methods. Cultures contaminated by saprophytic bacteria have been omitted. The old and new methods could be compared only as to the material indicated as "other cases", since none of the blood samples examined with the old method had originated from patients with suspected typhoid fever. Out of the 508 samples examined with the old method (excluding the 436 contaminated cultures), pathogenic organisms were isolated in 25 cases (4.9 per cent). The new method yielded 57 pathogenic bacteria from 352 samples (16.2 per cent). Accordingly, the new method seemed much more effective; however, a high number of sterile cultures were obtained with both methods (95.1 as compared to 83.8 per cent).

Table IV

Comparison of the results given by new and old type blood cultures
Contaminated cultures are not included in the data

S. typhi	Other pathogens	New method			Old method
		Suspected typhoid cases	Other cases	Total	Other cases
(a) *Number of results*					
Positive	Negative	15	—	15	—
Negative	Positive	25	57	82	25
Negative	Negative	243	295	538	483
	Total	283	352	635	508
(b) *Percentage of results*					
Positive	Negative	5.3	—	2.4	—
Negative	Positive	8.8	16.2	12.9	4.9
Negative	Negative	85.9	83.8	84.7	95.1
	Total	100.0	100.0	100.0	100.0

The present data also show that cultivation in bile medium is more suitable for the isolation of *S. typhi*. From 25 (8.8 per cent) suspected cases of typhoid fever other pathogenic bacteria were namely revealed and thus in these cases the new method helped to settle the correct diagnosis.

Discussion

With the new method the growth inhibitory effect of blood plasma was eliminated. In order to decrease the action of antibiotics, the centrifuged blood cells were diluted with broth. A prolonged incubation time (10 instead

of 6 days) [7] also improved the results of the new technique. In order to eliminate the bactericidal power of blood, MURRAY and KALC [8] recommended a 20-fold dilution.

It is known that plasma and serum, as well as whole blood, are definitely bactericidal [9]. When human blood is incubated at 37°C for ½ hour with an equal volume of the suspension of a sensitive strain, a considerable part of the bacteria is destroyed. Among various bacterial species there is a definite difference in this respect, Gram positives being more resistant than Gram negatives. Human blood is strongly bactericidal for several members of Enterobacteriaceae, especially to Salmonellae and Shigellae and less so to the Proteus and Escherichia groups. Other Gram negatives as *Ps. pyocyanea*, *V. comma* and brucellae are also fairly sensitive. In contrast, *N. gonorrhoeae* is not destroyed in human blood; *N. meningitidis* is somewhat less resistant. Staphylococci are more resistant than streptococci or pneumococci. Vegetative forms of aerobic spore-forming bacilli *(B. subtilis* and *B. anthracis)* are resistant [6]. CARNOT and LAVERGNE [10] on examining the bactericidal action of undiluted serum, found that out of 1500 inoculated *S. typhi* cells only 800 remained viable after an exposure of 5 minutes; after 3 hours all were destroyed. Accordingly, the blood should be inoculated into the medium as rapidly as possible. Unfortunately in practice it often takes several hours until the samples reach the laboratory. This may be an explanation of the high number of sterile samples, although it has been stated that a suitable concentration of sodium citrate eliminates bactericidal action. This very concentration is, however, difficult to ensure, as it widely differs with various bacteria.

The culturing method of ORTALI [11] seems to be superior to ours, as he obtained positive results in 22 per cent. Not including the contaminated cultures, in our material a 15.2 per cent positivity was achieved. The difference may be due primarily to the different distribution of patients. Malta fever and pulmonary or intestinal anthrax is unknown in Hungary. ORTALI isolated *B. melitensis* in 17, *B. anthracis* in 2 cases, while *Str. viridans* only on 4 occasions, in contrast to our 10 *Str. viridans* cultures. Thus the two methods can hardly be compared.

The result of blood cultures is highly influenced by the stage of the disease (febrile condition, bacteraemic period, etc.). Such data have not been collected in the present study.

A considerable improvement was attained with the new method as to the number of contaminated samples. With the old method 46 per cent, with the new method only 21 per cent, of contaminated cultures were encountered. The sample was regarded contaminated when more than one organism or *Staph. epidermidis* or air-borne bacteria had grown. Although in the course of the present investigation the clinicians' attention has repeatedly

been called to careful sampling, the smaller number of contamination may also be due to the fact that unlike the formerly used liquid cultures, the diphasic media do not require the opening of the bottles on three subsequent occasions.

CONRADI and KAYSER [12] in 1906 recommended the use of bile or bile-salts for decreasing the bactericidal action of blood. Enteric pathogens grow particularly well in bile medium. For the cultivation of *S. typhi*, the bile medium was found to be more advantageous than the new diphasic medium, although into the former clotted blood had been inoculated and, as opposed to 10 days, incubation had lasted for 5 days only and the sample had been diluted less. Nevertheless, by means of the new technique a correct diagnosis was achieved in 25 cases; this suggests that blood samples from patients with suspected typhoid fever should be examined with both methods.

Summing up, the use of the diphasic medium for blood cultures from patients suffering from diseases other than enteric fever, is more advantageous than the old method, both to obtain a higher number of positive results, and to decrease the number of contaminated samples.

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THE STUDY OF ATTENUATED TUBERCLE BACILLUS STRAINS ON RABBITS

By

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Summary. Attenuated tubercle bacillus strains (BCG substrains and strain No. 115) administered intravenously to rabbits caused severe lesions mainly in the lungs. Histologically the lesions corresponded to disseminated epitheloid cell tubercles with occasional central necrosis and partly to non-specific granulation tissue. Ten to 20 mg doses of a suspension prepared from 9-day cultures caused the death of the majority of the animals.

In rabbits which survived after 40 days the lesions gradually disappeared, with simultaneous development of immunity.

The pathogenic effect of intravenously administered attenuated tubercle bacillus strains is more marked in rabbits than in guinea pigs. This might be ascribed to the higher primary sensitivity of rabbits to the cell constituents of the tubercle bacillus.

Live vaccines bring about a process similar to natural infection, but not malignant, resulting in an immunity of higher order than that caused by killed vaccines. A certain degree of residual virulence is needed for the vaccine strains to be effective. Residual virulence allows multiplication of the bacteria in the organism and so ensures the vaccination process to bring about immunity. This explains the importance of studying residual virulence. The residual virulence of the attenuated vaccine strains is variable in different species and races of animals; of prime interest is of course its knowledge in man.

The BCG strain is also known to have a certain residual virulence. Guinea pigs develop two to three weeks after subcutaneous inoculation with 0.1 to 1.0 mg of BCG an abscess and changes in the regional lymph nodes which later regress. JENSEN recommended to study the reaction of guinea pigs to intracutaneous inoculation with various amounts of BCG; this method is now widely used [3]. ROSENTHAL suggested intraperitoneal inoculation of guinea pigs with five to ten mg [5]; three to six weeks after such inoculation alterations appear in the mesenterial lymph nodes; the extent of this alteration gives the measure of the residual virulence of the substrain used. SUTER and DUBOS studied the residual virulence of BCG strains on white mice inoculated intravenously [6]; the lesions were comparatively slight and disappeared in four weeks. The residual virulence of BCG strains in rabbits was investigated by COULAUD and LURIE. COULAUD studied the effect of intravenous inoculations with 5, 10 and 15 mg and found that doses up to 15 mg caused focal lesions in the lungs, liver and spleen; caseation did not occur and complete histological recovery took seven to ten months [2].

LURIE studied the histological changes and the multiplication of the BCG strain after the intravenous inoculation of 1 mg [4].

So far the sensitivity of rabbits to BCG substrains and other attenuated tubercle bacillus strains has not been studied. We therefore investigated in rabbits the residual virulence of two BCG substrains received from the *Pasteur Institute, Paris*, and from the *State Serum Institute, Copenhagen*, respectively. Experiments were also carried out with strain No. 115, cultivated by one of us [8]. As different species of rabbits display a different sensitivity to tuberculosis, we investigated the sensitivity to the strains in question of rabbits of different species (grey and albino), age and weight.

Materials and methods

Eighty-three grey and albino rabbits of 200 to 2000 g weight were used, in every experiment at least three animals of the same species and weight. They were inoculated intravenously and if still alive after 30 to 40 days, they were killed and their organs examined. The lesions in the lungs and spleen were recorded at autopsy and these organs were weighed, based on the fact that the degree of lesion is proportional to the weight increase of the lung and spleen.

In addition to the mentioned BCG substrains and strain No. 115, the effect of the virulent human strain $H_{37}Rv$ was studied for comparison.

The animals were inoculated intravenously with 5, 10 and 20 mg of bacterial suspension of 9-day old cultures grown on SAUTON's medium and semi-dried on sterile filter paper.

Results

The results of the experiments are summed up in Table 1. First the residual virulence of the BCG strain from the Pasteur Institute (BCG Paris) was studied, as this strain may be taken as an international standard.

After inoculation with 20 mg of BCG, 10 out of 12 grey rabbits died in 10 to 34 days, 17.5 days on the average. The lungs weighed 27.3 g on the average, as compared with 9 to 12 g in healthy animals. All the 13 albino rabbits inoculated with the same dose died in 16.6 days. When inoculated with 10 mg of bacteria, 50 per cent of the animals died in the experimental period.

The effect of 5 mg of BCG was studied in 14 animals. Out of 7 grey rabbits none, while out of 7 white rabbits 2 died within 32 days. These experiments indicate that white rabbits are more sensitive than grey ones. Out of 25 grey rabbits 10, out of 26 white rabbits 19 died. In our experience animals which have survived for 40 days after the inoculation are no longer in danger and the lesions caused by BCG will gradually regress.

According to histological examinations, the lungs were the organs most severely affected. The lesions consisted of multiple, sometimes coalescing inflammatory foci, the major part of which were made up of epitheloid cells, while the rest of granular tissue without any specific character. In the centre of some tubercles homogenization and necrosis were seen. Similar, but considerably milder lesions were observed in the spleen and liver.

Table I

Experiments with attenuated Tbc and $H_{37}Rv$ strains on rabbits

Strain	Dose (mg)	Rabbit species	Average weight of animals (g)	Number of animals	Died	Average survival days	Average weight of lungs (g)
BCG Paris	20	grey	1260	12	10	17.5	27.3
		white	1010	13	13	16.6	27.1
	10	grey	1500	6	2	38.0	21.5
		white	1300	6	4	14.0	
	5	grey	1370	7	0	—	24.0
		white	1360	7	2	28.6	
BCG Copenhagen	20	white	430	3	2	15.0	14.1
	10	white	440	3	2	20.5	18.7
	5	white	330	3	0	—	8.7
No. 115.	20	grey	1330	7	5	18.2	30.1
		white	970	3	0	—	
	10	white	575	2	1	18.0	14.0
	5	white	430	3	1	25.0	9.1
$H_{37}Rv$	20	white	1125	2	2	14.0	39.8
	10	white	1100	3	3	12.3	41.9
	5	white	930	3	3	15.3	39.1

The histological changes found in two rabbits inoculated with the Paris strain were the following.

1. White rabbit No. 4,300 g. Died 14 days after infection with 20 mg. In the lungs, uniformly distributed proliferative inflammation spreading over several acini. In several places coalescing tubercles consisting mostly of epitheloid cells, with an accumulation of alveolar phagocytes at the periphery of the tubercles, and fibroblasts and lymphocytes in the alveolar wall (Fig. 1). Some scattered tubercles consisting of epitheloid cells were observed in the liver and the spleen.

2. White rabbit No. 806, 1500 g. Infection with 10 mg. Died 17 days after infection. A great number of proliferative tubercles consisting mostly of epitheloid cells was found in the lungs; some of them displayed necrosis (Fig. 2). Some lymphoid foci were found in the liver.

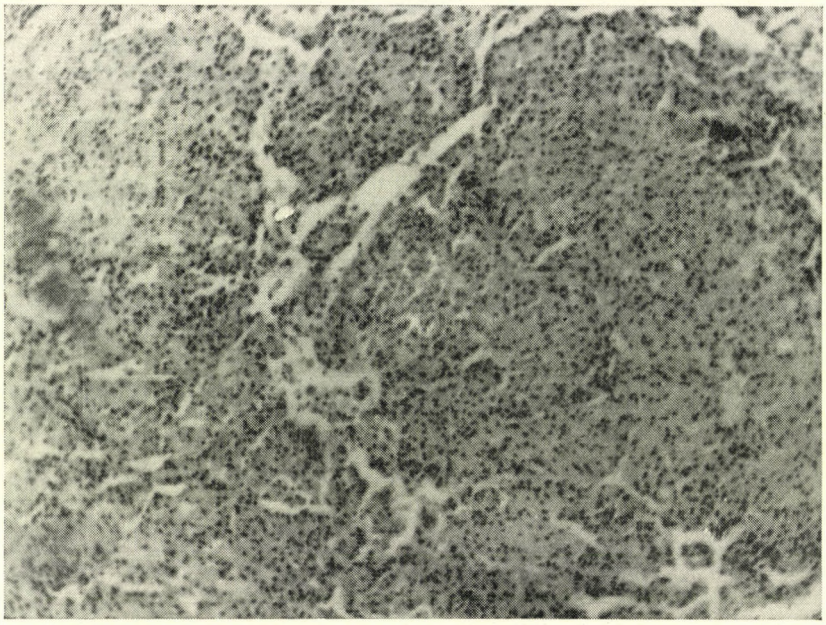

Fig. 1. White rabbit No. 4, 300 g, died 14 days after intravenous inoculation with 20 mg of BCG Paris strain. *Lung.* Proliferative tubercle consisting mainly of epithelcid cells. Haematoxylin-eosin, × 80

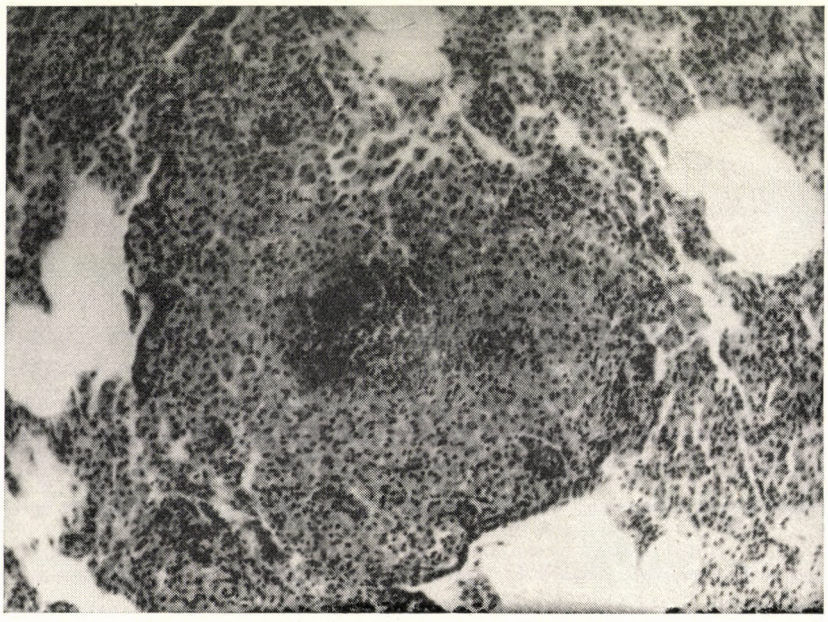

Fig. 2. White rabbit No. 806, 1500 g, died 17 days after intravenous inoculation with 10 mg of BCG Paris strain. *Lung.* Tubercle consisting of epitheloid cells, with central necrosis. Haematoxylin-eosin, × 80

In the experiments with the Copenhagen substrain only white rabbits ranging in weight between 320 and 470 g were used. On inoculation with 20 mg 2 out of 3 animals died within 15 days, the third animal was killed 25 days after inoculation.

After inoculation with 10 mg out of 3 animals 2 died within 24 days, the third one was killed 37 days after inoculation.

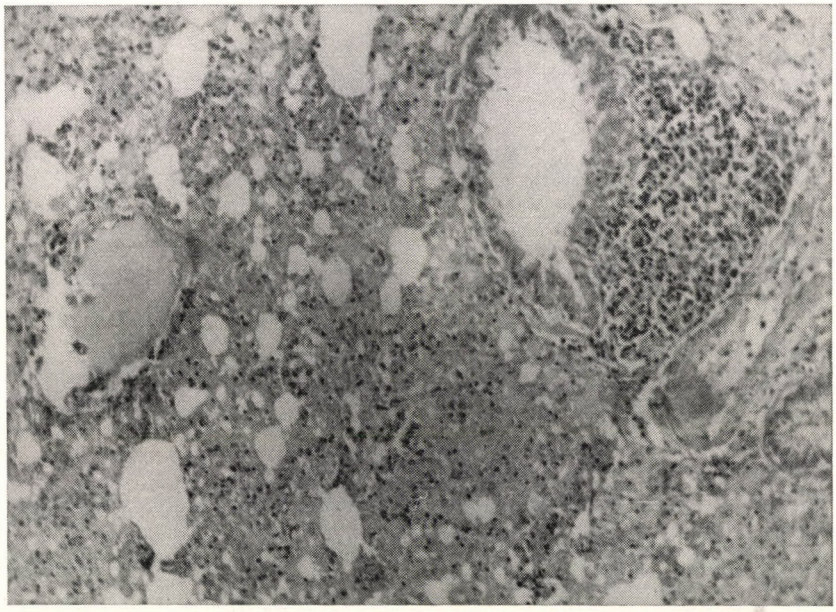

Fig. 3. Grey rabbit No. 635, 1850 g, killed 35 days after intravenous inoculation with 20 mg of strain No. 115. *Lung.* Oedema. Lymphoid cell infiltration. Haematoxylin-eosin, × 80

When inoculated with 5 mg the animals were still alive after 61 days, when they were killed. Only slight changes were found in their organs. The slight pathogenic effect of this dose was proved beside the histological pattern also by the weight of the lungs which was not more than 8.7 g on the average.

The gross and histological changes in these animals were similar to those found on inoculation with the Paris substrain.

Twenty mg of strain No. 115 killed 5 grey rabbits out of 7 in 30 days, but none of 3 white rabbits. When treated with 10 and 5 mg, respectively, 2 out of 5 white rabbits died.

The histological changes found in two rabbits inoculated with strain No. 115 were as follows.

1. Grey rabbit No. 635, 1850 g. Killed 35 days after infection with 20 mg. The lungs showed oedema, thick alveolar walls and tubercles consisting of lymphoid cells (Fig. 3).

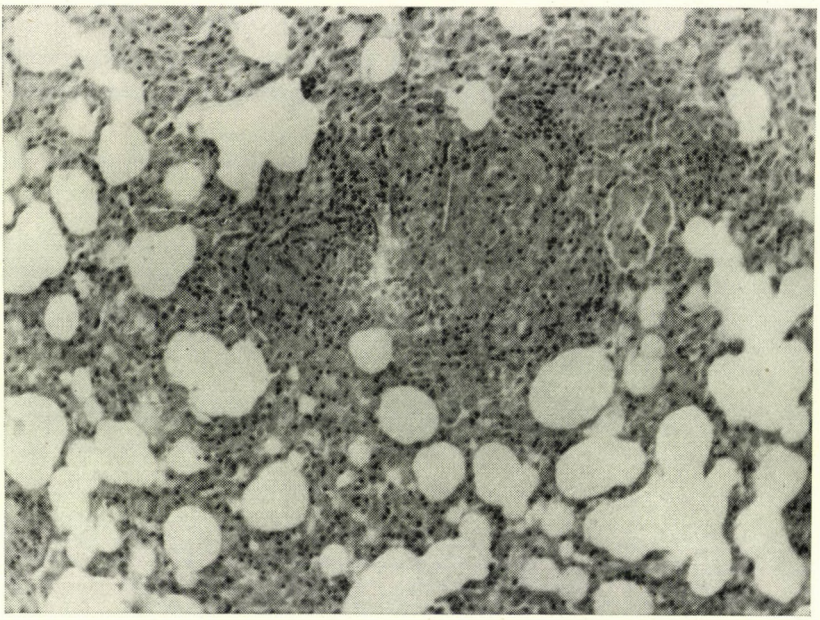

Fig. 4. White rabbit No. 679, 550 g, killed 32 days after intravenous inoculation with 10 mg of strain No. 115. *Lung.* Circumscribed tubercle consisting of epitheloid cells. Haematoxylin-eosin, × 80

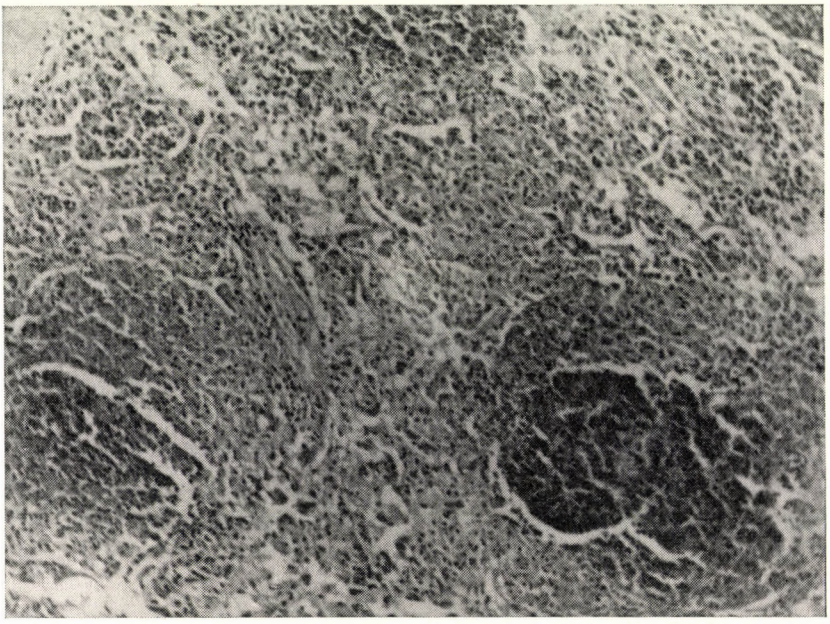

Fig. 5. White rabbit No. 606, 800 g, died 14 days after intravenous inoculation with 5 mg of strain $H_{37}Rv$. *Lung.* Coalescing proliferative tubercles with extensive necrosis. Haematoxylin-eosin, × 80

2. White rabbit No. 639, 550 g. Killed 32 days after infection with 10 mg. A number of solitary, in some places coalescing tubercles consisting of epitheloid cells with no central necrosis were found in the lungs (Fig. 4). There were tubercles in the spleen and liver, consisting mainly of epitheloid cells, side by side with Langhans type giant cells.

Accordingly, strain No. 115 caused severe, sometimes lethal lesions in the rabbits.

The most severe lesions were induced by the virulent human $H_{37}Rv$ strain which caused even in a dosage of 5 mg the death of all experimental animals within 14 to 17 days. The weight of the lungs was also much greater than in the animals which died after inoculation with attenuated tubercle bacillus strains.

Histological examination revealed grave changes in the organs, especially the lungs. The changes covered a larger area than in the three previous groups and characteristic necroses were found in most of the nodules.

The histological examination of No. 606 white rabbit of 800 g weight, which died 14 days after inoculation with 5 mg of $H_{37}Rv$, revealed coalescing proliferative inflammatory foci with extensive necroses and desquamative pneumonia around them (Fig. 5). In certain follicles of the spleen epitheloid granulation was found.

Discussion

Our observations have shown the fallacy of the generally accepted view that BCG induces no lethal process in the animals. CALMETTE's and COULAUD's studies were considered to have clarified the effect of BCG in rabbits, but COULAUD's statement that 10 to 20 mg of BCG produce in rabbits nothing but benign reversible lesions lacks the necessary foundation.

Ten to 20 mg doses of the Copenhagen substrain caused the death of rabbits. The experiments carried out so far are not sufficient to establish the difference between the pathogenic effect of the Paris and Copenhagen substrains.

Strain No. 115, though of human origin, causes changes similar to those produced by BCG.

According to our observations, white rabbits are more sensitive than grey ones, and young white rabbits, weighing 300 to 600 g, die somewhat earlier than old ones. The results have shown that the rabbit is well suited for the study of the pathogenic effect of attenuated tubercle bacillus strains.

Nine-day old bacterial cultures were used in our experiments. As one of us had pointed out in 1940, young, 9 to 12-day old, cultures possess a higher immunogenic capacity than old cultures [7]. It is quite possible that the fact that COULAUD observed no lethal effect from 10 to 20 mg of BCG in rab-

bits, was due to the fact that he has studied the pathogenic effect of 21 to 28-day old cultures, as originally suggested by CALMETTE.

Our experience seems to indicate that the changes caused by BCG gradually disappear, provided the animal is still alive 30 to 40 days after inoculation. The progression of the changes may be explained by the growth of the BCG bacilli, while regression is probably due to the immunity caused by the same, and this immunity leads to the gradual destruction of the BCG bacilli in the organism [9]. The immunity level resulting from the regression process is naturally not reached within the same time in different individuals.

It is remarkable that 20 mg of intravenously administered BCG or strain No. 115 does not kill guinea pigs and causes milder changes in the lungs of these animals than in rabbits. This is the more surprising as guinea pigs are considered particularly sensitive to tuberculosis. The fact that guinea pigs are more sensitives to tuberculosis than rabbits, while rabbits are more sensitive than guinea pigs to large doses of intravenously administered attenuated tubercle bacilli may be explained by assuming a higher primary sensitivity of rabbits, mainly of their lungs, than of guinea pigs towards the cell constituents of tubercle bacilli.

In 5 mg doses the pathogenic effect of the $H_{37}Rv$ human strain was considerably higher than that of the attenuated strains. This high virulence manifested itself on the one hand with the fact that all animals died in 14 to 17 days after inoculation, and also with the grave pulmonary lesions. Thus rabbits display a high primary sensitivity to virulent human tubercle bacillus strains.

On the basis of our experiments the study of the residual virulence of the attenuated tubercle bacillus strains in rabbits seems justified. White rabbits up to 1000 g weight seem to be best suited for such experiments.

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THE PHAGE RECEPTORS OF BACILLUS ANTHRACIS

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Summary. In the cell wall of *Bacillus anthracis* the presence of distinct receptors specific for certain groups of A phages has been demonstrated.

Individual receptors are bound to different structural elements of the cell wall. The receptors could not be identified with the immune-specific polysaccharide of *B. anthracis*.

The immune-specific polysaccharide, a galactose-glucosamine-polymeric peptide, is a characteristic component of the cell wall which cannot be isolated by mild chemical treatment but goes into solution on enzymatic autolysis.

We have recently reported about the isolation of phages from soil samples [1, 2], which had specific lytic activity on *B. anthracis*. Some representatives of these phages were extensively studied. For the designation of this group of agents the name "A phage" was suggested. According to their sensitivity to heat and UV irradiation, furthermore to some other characteristics, especially their antigenic structure, the A phages could be divided into 3 groups [2]. In this classification the *a*-mutant of phage W isolated by MCCLOY [3] from a lysogenic strain of *B. cereus* was used as reference agent. Group Al was represented by phage W which has been known to be highly specific for *B. anthracis*. A sharp difference was found in the antigenic structure of phages belonging to groups Al and A3, while certain serologic relationships were found between group Al and A2.

The mutants of *E. coli* resistant to T phages exhibit a remarkable host specificity which may serve as a basis for a differentiation of the T system. To allow a more thorough differentiation of A phages, it was attempted to isolate hosts which are resistant to individual A phages. Except for phage W this attempt has failed. Thus resistant mutants of *B. anthracis* which determine the specificity of receptors for individual groups of phage were only available for Al phage. The characterization of the phages was also attempted by the isolation of A-phage receptors. This attempt has also failed as we did not succeed in solubilisation of the receptors specific for A phages. In spite of the failure some observations have been made which might prove valuable for a further characterization of A phages. The present paper will deal with these observations.

Materials and methods

Bacterial strains. Most of our experiments were performed with an atypical asporogenous *B. anthracis* strain Davis. A non-capsulogenic mutant of the Vollum strain (VC^-) was also used. The non-capsulogenic strain resistant to W*a* phage was isolated in connection with the lysogenization experiments carried out with the Vollum strain [4].

Cultivation of bacteria. It was carried out in a fluid medium under constant shaking in YP bouillon as described earlier. Growth and cell count of the cultures were determined by measuring their optical density, as described previously [2].

Phage strains were described and characterized in a previous paper [2]. The groups and the trains of phages were:

Phage A1,	strain W*a*
Phage A2,	strains 26 *cr* and 27 *cr*
Phage A3,	strains 26 *cs*, 26 *tl*, 27 *tl*, 28 *tl*,*cg*, 29 *cg*.

The phage material was produced on strain Davis cultivated in fluid medium. The lysates were centrifuged and sterilised by shaking with chloroform for a few minutes. Titres of the lysates were $10^9 - 10^{10}$/ml.

Phage assays were made according to ADAMS [5].

Preparation of bacterial suspensions for receptor studies. Cell suspensions were usually prepared from 16-hour cultures of strain Davis and sometimes from those of strain Vollum (VC^-), both cultivated on YP agar. The cells were suspended in chilled saline and washed twice by centrifugation in the cold. The sediment was re-suspended in saline and diluted to contain 20 mg/ml dry weight of bacteria, as determined by optical density.

Cell-wall preparations. Cell-wall preparations were obtained from washed suspensions as described by PARK and HANCOCK [6]. Fifty ml of the bacterium suspension was centrifuged and re-suspended in 20 ml of 5 per cent trichloroacetic acid (TCA) at 4° C. The material was allowed to stand for 30 minutes at this temperature. This was followed by centrifugation and the sediment was washed in 75 per cent ethanol, re-suspended in 5 per cent TCA and heated to 90° C for 6 minutes. After centrifugation the sedimented bacteria were washed in water and re-suspended in 0.05 *M* ammonium chloride solution. The pH of the latter was adjusted to pH 7.6 by NH_4 OH. After addition of 1 mg/ml of crystalline trypsin the material was digested for 2 to 3 hours at 37° C.

The process was followed by examination under a phase-contrast microscope. If the treatment did not result in removing the cell content, trypsin digestion was repeated. Digestion was followed by washing in water three times. The final suspension was stored in a refrigerator.

Even after successful trypsinisation it was possible to identify inside the cell wall a shrunk dark central part, apparently a residue of the cytoplasmic membrane. This mass was remarkably electron dense, as demonstrated in Fig. 1. It could not be removed by solvents or enzymes (lipase, lysozyme, chymotrypsin, pepsin, ribonuclease). Extraction of the cell-wall preparations with 90 per cent phenol resulted in further shrinkage of the electron dense body, and in further increase of its density (Fig. 2.). The electron micrographs revealed wrinkling of the cell wall at the poles.

Determination of the receptor substance. The quantity of receptor substance in bacterial suspensions and cell-wall preparations was estimated on the basis of their phage adsorbing capacity. Living, appropriately treated bacteria or cell-wall preparations were suspended in YP broth to a density of 5×10^7 cells or cell walls per ml. An equal amount of YP broth containing 10^7 phage particles was added to the suspensions (multiplicity 0.2), the mixtures were allowed to stand at 37° C for 30 minutes, then diluted 1 : 100 by cold diluent (one volume YP broth and 4 volumes saline). The diluted suspensions were centrifuged at 3000 rpm for 10 minutes. The supernatants were again diluted 1 : 100, and 0.1 ml of this dilution mixed with indicator organisms was overlayered in soft agar.

The receptor content of cell-free extracts was determined by their phage-inactivating activity. As described by WEIDEL [7], this reaction is pseudomonomolecular and irreversible in the presence of an excess of dissolved receptor. To secure an appropriate receptor excess, phage was added to preparations corresponding 5×10^7/ml bacteria in 0.001 multiplicity.

Chemical examination of bacterial and cell-wall preparations. Nitrogen content was determined by nesslerization according to MILLER and MILLER [8]. The method of FISKE and SUBBAROW [9] was used for phosphorous determination. Anthrone-positive materials were determined as suggested by SEIFTER *et al.* [10].

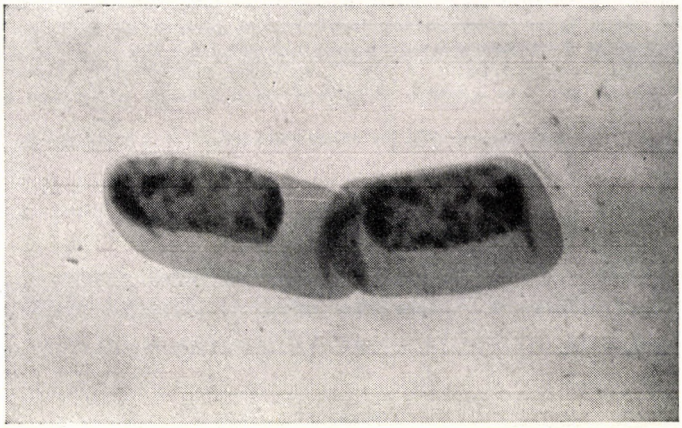

Fig. 1. Cell-membrane preparation from strain Davis (according to PARK and HANCOCK) Magnification × 10 000

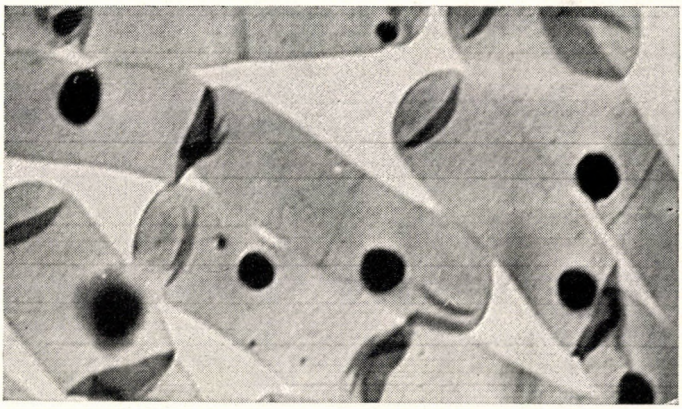

Fig. 2. Cell-membrane preparation after phenol extraction. Magnification × 18 000

For the examination of the cell-wall components, samples containing 20 mg of dry substance were mixed with 1 ml 2*N* HCl, sealed in ampoules, and kept at 100° C for 2 hours. The bulk of hydrochloric acid was removed by distillation under reduced pressure, while the residual acid was removed in an NaOH exsiccator.

The hydrolysate thus obtained was used for the determination of the total reducing substance by the method of HAGEDORN and JENSEN [11]. The results obtained were expressed in glucose values. The amount of hexosamine present was determined according to NEUHANS and LETZRING [12]. The individual monosaccharide components of the hydrolysates were determined by one dimensional ascending paper chromatography, on Whatman's paper No. 1. Two solvents were used in parallel, (a) butanol-pyridine-water (6 : 4 : 3), and (b) pyridine--ethyl-acetate-water (7 : 10 : 3). The chromatograms were developed either by aniline phosphate or by ammonium silver nitrate solution.

For the analysis of the amino-acid component the preparations were hydrolysed by 6 *N* HCl at 105° C for 8 hours. After having removed the acid, 20 μg amounts were used for paper chromatography. Two dimensional chromatograms were run in pyridine-water (4 : 1), and N-butanol-acetic acid and water (6 : 1 : 2) solvents, respectively. The chromatograms were developed by 0.1 per cent solution of ninhydrine in acetone and dryed at 105° C for 5 minutes.

The lipid content was determined by ether extraction of a hydrolysate in 2*N* HCl.

Results

The sensitivity of VC$^-$/*a, a Wa phage resistant strain, to heterologous phages.* Various dilutions of phages were dropped onto the surface of indicator plates prepared with strains VC$^-$ and VC$^-$/*a*. The results are presented in Table I.

Table I

Titre of different phages on sensitive VC$^-$ *and resistant* VC$^-$/*a strain*

Phage group	Phage strain	Phage titre (logarithmic value of dilution)	
		VC$^-$	VC$^-$/*a*
A1	W*a*	7	0*
A2	*26 cr*	7	5
	27 cr	6	6
A3	*26 cs*	6	5
	26 tl	7	7
	27 tl	7	6
	28 cs	7	6
	28 tlcg	6	6
	29 cg	7	6

* Plane lysate had no effect.

As revealed by Table I strain VC$^-$/*a* resistant to phage W*a* was just as sensitive to heterologous phages as the original strain VC$^-$. In adsorption experiments strain VC$^-$/*a* failed to adsorb phage W*a*, while no changes could be observed in the adsorption of some representants of the other phage types. We did not succeed in obtaining mutants resistant to other phages, thus no cross experiments could be performed.

Attempts to solubilize the receptor substance. Suspensions of *B. anthracis* were extracted under different conditions and the phage-binding capacity of the bacteria and the phage-inactivating activity of the extracts was estimated. Usually phage W*a* was applied, while in some experiments certain representants of groups A2 and A3 (27 *cr* and 29 *cg* strains) were also employed to detect the presence of the receptor.

Samples of the moist bacterial suspension were treated for 30 minutes at room temperature with 0.1 *N* NaOH, 90 per cent phenol, ethylene glycol or glacial acetic acid. None of these treatments had any effect on the phage-adsorbing capacity of the bacteria. Similarly, no decrease in the adsorbing capacity was found on treatment with different enzymes at pH 7 in phosphate buffer. Crystalline trypsin, chymotrypsin, ribonuclease, lysozyme and a lipase concentrate proved equally ineffective.

Washed bacteria were autolysed in the presence of trypaflavine (20 μg/ml) in pH 8 phosphate buffer (*M* 0.02) under a toluene layer at 37°C for 48 hours. The Gram negative cell debris thus obtained was sedimented at 3000 rpm for 10 minutes. The slightly opalescent supernatant had a remarkable inactivating effect on phage Wα, while against the other two it was ineffective. This supernatant, after 30 minutes' centrifugation at 10 000 rpm yielded a little sediment and a clear supernatant. The latter was deprived of inactivating activity, thus apparently the sedimentable bacterial detritus was responsible for the inactivation of phage Wα.

The cell wall of *B. anthracis* could be lysed only by relatively rough methods, e. g. by alkaline NaOCl solution [13] which, however, does not seem to leave intact the original chemical structures. Extraction of the receptor by formamide solution was attempted under different conditions. Lyophilised bacteria were suspended in concentrated formamide and heated. This was followed by repeated washing and testing for phage-binding capacity. No decrease in the adsorbing capacity for phage A3 took place, even after extraction at 170°C for 30 minutes. This procedure, however, remarkably reduced the adsorbing capacity for group A1 and A2 phages. Nevertheless, no active solubilised receptors were demonstrable in the formamide supernatant. Bacteria extracted with formamide were found to have undamaged contours in preparations stained according to GUTSTEIN. The extract contained only traces of the immune specific polysaccharide of *B. anthracis* [14]. Thus even extensive formamide treatment did not result in remarkable solubilization of the cell-wall component. The specific receptors of phages A1 and A2 on the other hand were inactivated on formamide treatment at 170°C.

An 0.1 per cent solution of the immune specific polysaccharide of *B. anthracis* was definitely ineffective against all the phages tested. Thus the receptor substances could not be identified with the galactose-acetyl-glucosamine mucopeptide.

We did not succeed in solubilising the receptors by lysozyme treatment though it is well known that this enzyme is able to lyse completely the bacteria under appropriate experimental conditions. Young cultures grown in YP broth (0.5 o. d.) were centrifuged, the sediment was washed and resuspended in a pH 8 tris-buffer, and 50 μg/ml of lysozyme was added. This treatment resulted in a remarkable decrease of the optical density of the suspension

and no intact bacteria could be detected by microscopic examination. The clear supernatant obtained on centrifugation was added to equal amounts of phages in YP broth and the mixture was assayed for phage at intervals. The extract was found to be ineffective, in inactivating phage; this indicated the absence of active receptor substance in the lysozyme extract.

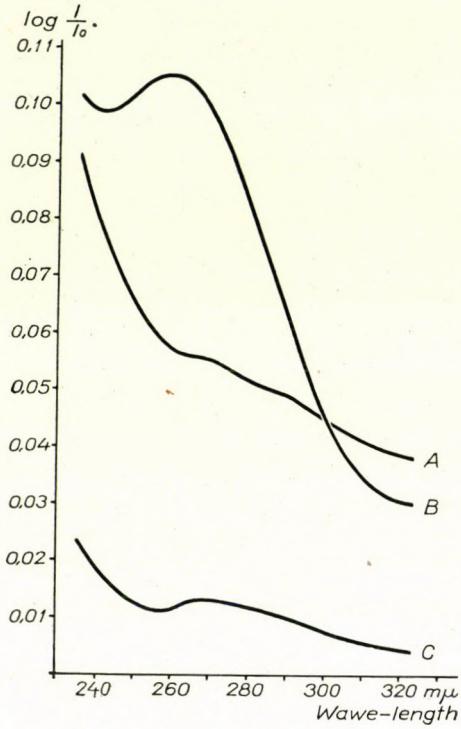

Fig. 3. Changes in the optical density of Davis suspension on different treatments. *A* live untreated suspension; *B* after boiling TCA extraction; *C* trypsin-digested suspension after TCA treatment

Examination of the phage-binding capacity of cell-wall preparations. Fig. 3 demonstrates the UV adsorption of a suspension obtained from strain Davis by extraction with boiling trichloroacetic acid and trypsin digestion.

Extraction with boiling TCA resulted in a remarkable drop of the extinction values in the range between 255 and 266 mμ. The very low adsorption observed for the cell-wall preparations obtained by trypsin digestion revealed a slight inflection at 260 to 280 mμ. Fig. 4 contains the data on the phage adsorption (A1, A2 and A3 group) of the cell-wall preparations at the different phases of PARK and HANCOCK's procedure.

Living, not washed bacterial suspension adsorbed all the 3 phages to 90 to 98 per cent. The rate of adsorption of A1 (W*a*) and A2 *(27 cr)* phages

decreased remarkably during the consecutive treatment, while the adsorbtion of A3 *(29 cg)* was essentially unchanged. The difference in adsorption of individual phages appeared after treatment with boiling TCA, and increased, on further treatment with trypsin and lysozyme.

The experiment demonstrated above was performed with one representative of each phage group. Similar results were obtained in another experiment where all the phage strains belogning to the 3 phage groups were examined.

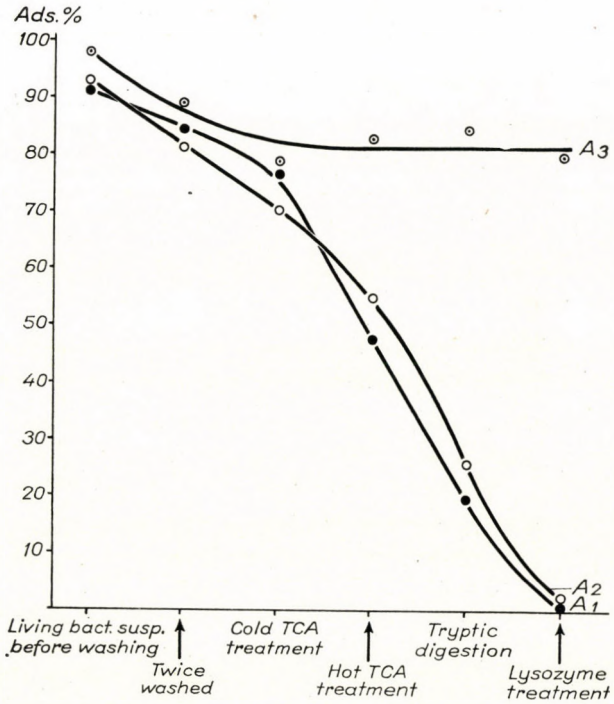

Fig. 4. Phage binding of a suspension of strain Davis (per cent) after different treatments

In this experiment the suspensions of strain Davis were examined for phage adsorption following different treatments.

Short heat treatment caused a moderate decrease in the adsorption of all the phages. Extraction with boiling TCA and trypsin treatment ("cell-wall") resulted in a remarkable decrease in the adsorption of Wα and A2 phages. The same treatment was, however, ineffective on the receptor of phages belonging to group A3, while this receptor was mostly damaged at autolysis. A short heat treatment of bacteria suspended in saline resulted in a slight decrease in adsorption of group A3. Nevertheless this receptor appeared to be resistant to boiling formamide or boiling TCA.

Table II

Phage binding of the suspension of B. anthracis strain Davis after different treatments

Suspension	Grade of adsorption in per cent								
	Members of the individual phage groups								
	A1	A2		A3					
	Wα	26 cr	27 cr	26 cs	26 tl	27 tl	28 cs	28tlcg	29 cg
Live washed bacteria (1)	97	88	90	80	94	91	94	90	98
Heat treated bacteria (2)	88	88	70	70	92	73	83	62	70
Cell wall[636] (3)	10	51	44	85	98	96	92	89	97
Autolysed bacterium (4)	72	46	52	43	8	0	28	23	41

(1) Live Davis suspension.
(2) Five ml of Davis suspension in test tube, kept for 1 minute in a 100° C water bath and cooled at room temperature.
(3) After hot trichloroacetic acid and trypsin treatment according to PARK and HANCOCK.
(4) The suspension was autolysed in the presence of 20 μg/ml trypaflavine in a pH 8 phosphate buffer at 37° C for 48 hours and the phage adsorption of the centrifuged sediment was determined.

Chemical analysis of the cell-wall preparations. Some chemical characteristics of live bacteria of strain Davis and those of different preparations obtained from strain Davis are given in Table III.

Table III

*Some chemical characteristics of washed Davis suspension and cell-wall preparation, calculated according to dry material content**

Suspension No	Nitrogen		Phosphorus		Sugar (anthron)		Reducing material		Hexosamine		Lipid	
	Per cent											
Davis I	9.3		1.2		3.8		4.0		1.8		2.2	
Davis II	11.0	10.7	1.5	1.4	4.8	4.0	4.4		2.3	2.1	3.4	2.8
Davis III	12.0		1.6		3.5		4.3		2.2		2.8	
Cell-wall I	3.5		0.5		8.0		13.6		7.2		2.9	
Cell-wall II	3.6	3.6	0.8	0.6	8.6	7.6	13.4	13.5	7.9	7.2	2.7	3.0
Cell-wall III	3.7		0.6		6.8		13.7		6.6		3.4	

* Results and means of 3 different examinations.

The obtained cell-wall preparations amounted on the average to 25 per cent of the bacterial weight. Only about half of the N and P content of the original bacterial cells was present in the cell-wall preparations. This is in accordance with the decrease in the UV adsorption of the cell-wall

suspension, indicative of a marked decrease in nucleic acid and protein. The carbohydrate content of the preparations appeared to be comparatively higher than that of the bacterial suspension, as measured by the total amount of anthrone-positive and reducing substance. The carbohydrate content of the cell-wall preparations as calculated from the anthrone-positive substances and hexosamine was found to be about 15 per cent. It should be stressed that the hexosamines are anthrone-negative substances. The total amount of reducing substances as determined by the method of HAGEDORN and JENSEN and expressed in glucose gave somewhat lower values. This might be explained by differences in the reducing value of individual carbohydrate components.

Paper chromatography of the cell-wall preparations revealed the presence of galactose and glucosamine. This is in agreement with the chemical composition of the polysaccharide isolated from *B. anthracis* [14]. In certain hydrolysates a faint spot was found of higher R_f than those of glucosamine and galactose. This spot could not be identified. It might have been muramic acid as a faint ninhydrine-positive spot could also be found in the same area. The presence of alanine, glutamic acid, glycine, diamino-pimelic acid and aspartic acid could be demonstrated in hydrolysates of cell wall.

Discussion

Our experiments have yielded certain data concerning the different receptors specific for the different groups of A phages. A good agreement could be observed between the differences in the receptors and the grouping of the A phages based on their antigenic structure and other characteristics (UV, heat and citrate sensitivity). These results have justified our earlier proposal to divide phages A into at least 3 groups.

A mutant of *B. anthracis* resistant to phage W*a* (A1) exhibited unaltered sensitivity to phages A2 and A3. This phenomenon in itself proved the distinctness of the receptors of phage A1. The receptors of phages A2 and A3 can be differentiated on the basis of their behaviour on treatment with hot (170°C) formamide. On autolysing the cells, the receptors of phages A1 and A2 were present in remarkable amounts in the cell debris, while similarly obtained debris were practically ineffective in adsorbing phage A3. This, however, did not mean that the glucose-galactose polymer solubilised on autolysis had any receptor effect on A3 phages.

The cell-wall preparations obtained according to PARK and HANCOCK exhibited an undamaged receptor activity only against A3 phages. Trichloroacetic acid and trypsin treatment was found remarkably to inactivate the other two receptors. On further treatment with lysozyme the A1 receptor was totally destroyed. Extraction with boiling trichloroacetic acid has been known to solubilize ribityl phosphatides (theicoic acid) [6]. This substance,

however, could not be identified with either of the receptors. On the basis of our experiments we may therefore state that the localisation of A1 and A2 receptors is different from that of the A1 receptor.

The immune-specific polysaccharide which is a glucose and galactose polymer containing a peptide moiety [14] was found to be only a part of the structure of the cell-wall preparations obtained from *B. anthracis* by the method of PARK and HANCOCK. The structure of the cell wall of *B. anthracis* appears to be highly resistant to different chemical treatments. The mucopeptide, a structural element of the cell wall, could not be solubilised by any of the chemical methods applied. Enzymatic treatment (lysozyme, autolysis), however, readily dissolved this material from the cell wall.

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TITRATION OF DIPHTHERIA TOXIN, ANTITOXIN AND TOXOID IN TISSUE CULTURE

By

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Summary. The cytopathogenic effect of different amounts of diphtheria toxin and the possibilities of a comparative testing of different batches of diphtheria toxin, antitoxin and toxoid preparations were examined in cynomolgus endocardial cell cultures.

Cell cultures were found to be very sensitive to the effect of diphtheria toxin both in tube cultures and in colour test. The toxins used destroyed the cells at a concentration as low as about 1/1000 Lf. The cytopathogenic effect of toxins could be used for the comparison of the absolute and binding values of different toxin, toxoid and antitoxin preparations. In the case of antitoxins and toxoids, beyond the quantitative determination of their antibody and antigen contents, also information on their respective avidities could be gained.

The order of magnitude of the sensitivity to individual toxins of the cell cultures did not change during the one and half year period of observation. Because of the easy maintenance and handling of the cell strain, its use is recommended for examinations of this type.

The effects of toxic bacterial products damaging the tissues of the human organism were first examined on tissue cultures by LEVADITI and MUTTERMILCH [1] half a century ago. Similar examinations were described later by BURROWS and SUZUKI [2], SUZUKI [3], OKABE and TERUUCHI [4] and OKABE [5]. The studies of this type were greatly promoted by the successful cultivation of cells directly on glass and other improvements in tissue culture techniques. Of the examinations performed with more advanced tissue cultivation methods we mention those of PLACIDO SOUSA and EVANS [6], PENSO and VICARI [7, 8], and of Hungarian authors those of BACKHAUSZ, VETŐ and HORVÁTH [9] and HORVÁTH [10].

The cytopathogenic effect of bacterial toxins shows the following characteristics. (i) The symptoms of cell damage manifest themselves only after a certain incubation period characteristic of the individual toxins (with large quantities of diphtheria toxin the incubation period lasts for 24 hours); (ii) the cell damage is neutralized by homologous antitoxin and thus the toxin can be identified.

The present examinations performed with diphtheria toxin, antitoxin and toxoid on cynomolgus heart cell cultures are regarded as preliminary to detailed study on the possibilities to develop an improved titration method.

Materials and methods

Cell culture. The cynomolgus heart culture provided by the *Virus Vaccine Control* and *Virus Departments* of our Institute was used throughout. The strain was maintained in 2-litre Roux flasks. Passages were performed every 9th or 10th day with 5 to 6 million cells. The quantity of fluid medium was 150 to 180 ml per Roux flask. Cells were released by versenisation.

Examinations were performed chiefly in stationary tube cultures inoculated with 150 000 cells each, and incubated at 35° C for 7 to 8 days.

Examinations were also carried out by the colour test as described by SALK, YOUNGNER and WARD [11].

Medium. For maintenance and experiments the following medium was used. Solution No. 199, 75 per cent; Hanks' solution, 15 per cent; calf serum, 10 per cent. The medium contained penicillin and streptomycin. The calf serum was free of diphtheria antibodies.

Diphtheria toxins, antitoxins and toxoids. Toxins Di 85, 85/a and 83 produced in our laboratory with PW8 Weissensee strain in Pope's bouillon were used. The crude toxin was concentrated by ammonium sulphate precipitation and preserved in glycerol.

As antitoxins, 2 sera of known value were used, standard serum No. 45 (Copenhagen), containing 10 AU/ml; and a commercial purified and concentrated therapeutic preparation H. No. 584/1 containing 3000 AU/ml.*

In a few experiments the purified and concentrated toxoid preparations Di II and Di III produced in our laboratory were examined. The titration of sera from guinea pigs immunized with two doses of different, combined, precipitated diphtheria-pertussis-tetanus vaccine preparations was also attempted.

The titres of toxins and antitoxins used were determined by Ramon's flocculation test [12]. The antibody content of guinea-pig sera was tested by the intracutaneous rabbit inoculation method of JENSEN [13].

Inoculation of the cell cultures. Inoculation of tube cultures was performed at the time when a ripe confluent cell layer had developed. Before inoculation the medium was poured off. The test material was mixed with the fresh medium and then distributed to the tubes, 1.0 ml each. Thus the final dilution of toxin (antitoxin, toxoid) in the medium was 1 : 10. In neutralisation (fixation) tests the antitoxins and antigens were appropriately diluted and mixed. Before adding to the medium the mixtures were incubated at room temperature. Mixtures of toxins and antitoxins were incubated for 2 hours, those of antitoxins and toxoids for 24 hours. The pH of the test materials was adjusted so as not to interfere with that of the medium. For the different levels 5 tubes were inoculated each, and incubated at 35° C.

According to the nature of the test the following controls were used: tissue control, medium control (intact sterile medium used for toxin production, concentrated and diluted in the same way as the lowest dilution of the toxin used in the experiment), serum control and anatoxin control.

Evaluation of results. Two limit values were checked usually; (i) direct toxin value m. c. d. (minimal cytotoxic dose) *i. e.* the smallest quantity of toxin bringing about destruction of the cell layer and decomposition of more than 50 per cent of the cells up to the 4th day [6]; (ii) indirect toxin value or "test dose" (LC_{50}), *i. e.* the lowest quantity of toxin which, when mixed with 0.02 IU, was able to elicit decomposition of 50 per cent of the cells up to the 4th day [6].

In addition, binding tests (L^+ value determination) were performed at different levels.

Results

First, the sensitivity of the cells to diphtheria toxin was established, inoculating stationary cultures with decreasing quantities of diphtheria toxin. The results are presented in Table I.

* Produced by the "*HUMAN*" *Institute for Serobacteriological Production and Research, Budapest.*

Table I

Titration of toxin Di 85 in stationary cultures and in colour test

Toxin Lf/tube	Grade of damage in stationary cultures	Colour of the indicator on 4th day (Colour test)
0.01	5/5	L L L L L
0.005	5/5	R R R R R
0.0025	5/5	P P P P P
0.00125	5/5	P P P P P
0.000625	0/5	O O O O O
0.00031	0/5	Y Y Y Y Y
M.c.d. =	0.00094 Lf	0.000625 Lf

L = lilac, R = red, P = pink, O = orange, Y = yellow

Similar examinations were performed by means of the colour test. The results of a representative experiment are shown in Table I.

According to the results, inoculation of cell cultures with diphtheria toxin appeared to be an appropriate method for the demonstration of small quantities of toxin and the tube culture method and the colour test were sensitive to similar amounts of toxin.

Next, the LC_{50} values were determined in stationary cultures. The results are demonstrated in Table II.

Table II

Determination of the LC_{50} value of toxin Di 85

Stationary culture Toxin Lf/tube	0.02 IU antitoxin/tube. Grade of total damage
0.32	5/5
0.16	5/5
0.08	5/5
0.04	3/5
0.02	0/5
0.01	0/5

LC_{50} = 0.04 Lf

The method proved suitable for the demonstration the action of diphtheria toxin in the presence of antitoxin as well as for the estimation of the protective effect of slight quantities of antitoxin.

In these experiments toxin Di 85 was used. Other diphtheria toxin products were also examined comparing toxins Di 85, Di 85/a and Di 83. The reproducibility of the results after an interval of one month was also studied. These examinations were performed chiefly by the determination of m.c.d. and/or LC_{50} values, both in stationary tube cultures and by the colour test. At the same time, preliminary experiments for the determination of different L^+ values were carried out using the Di 85 toxin preparation. The data of the above series of experiments have been summarized in Table III.

Table III

Titration of different Di toxins in repeated experiments

Experiment No	Examined in	Designation of toxin	Toxin values in Lf				
			M. c. d.	LC_{50}	$L^+/10$	$L^+/100$	$L^+/1000$
I	stationary culture	Di 85	0.0009	0.04	0.23	0.03	0.005
II	stationary culture	Di 85	0.0009	0.4	.	.	
		Di 85/a	0.003	.	.	.	.
		Di 83	0.0016	.	.	.	.
III	colour test	Di 85	0.0006	0.04	.	.	.
		Di 85/a	0.0016	.	.	.	.
		Di 83	0.0006	.	.	.	.

The results for the three different toxin preparations at different points of time showed good agreement. A close correlation was found between the results obtained in stationary tube cultures and the colour test. Examination of the L^+ values showed the cell culture used by us to be well suited for the examination of the binding values of diphtheria toxin and antitoxin on a $L^+/1000$ level. No. unequivocal results were obtained at the $L^+/10\,000$ level (these have been omitted from Table III).

In these examinations serum No. 45 was used for the determination of the binding values. In further experiments the neutralising effect of identical dilutions of other sera on the $L^+/10$ dose of toxin Di 85 was studied. For this purpose parallel serial dilutions of sera No. 45 and H. 584/1 were prepared. The different serum dilutions were mixed with $L^+/10$ toxin doses each and the mixture was inoculated into cell cultures. The results are presented in Table IV.

Table IV

Titration of anti-diphtheria sera No. 45 and Human 584/1 in the presence of 023 Lf/tube toxin quantities

AU/tube	Designation of serum									
	No. 45					No. 584/1				
	0.8	0.4	0.2	0.1	0.05	0.8	0.4	0.2	0.1	0.05
Grade of damage	0/5	0/5	0/5	3/5	5/5	0/5	0/5	0/5	3/5	5/5

As seen in Table IV both antitoxins neutralised the $L^+/10$ dose of toxin perfectly at 0.2 IU/tube and partially at 0.1 IU/tube. Thus the $L^+/10$ dose of toxin Di 85 proved to be the same when measured by two different sera.

Using the same two antitoxin preparations, further titrations were carried out at different L^+ levels. The results obtained are presented in Table V.

Table V

Comparison of $L^+/10$, $L^+/100$ and $L^+/1000$ doses of toxin Di 85 using No. 45 and H, 584/1 antidiphtheria sera

Stationary culture

Serum examined	Different L^+ values of the toxin as expressed in L.		
	$L^+/10$	$L^+/100$	$L^+/1000$
No. 45	0.23	0.03	0.005
No. H.584/1	0.23	0.03	0.005

The fixation values obtained by the parallel examination of the two antitoxic sera proved that the results of titrations at different levels with two different sera yielded appropriately fitting, well reproducible and clear-cut results in tissue cultures. Both sera exhibited the same avidity at high dilutions, thus the quality of the two preparations was equally good.

In the preceeding experiments horse sera of good avidity were used. In further experiments we examined the possibility of titrating guinea-pig sera of poor avidity in tissue cultures. Pooled sera obtained from groups of six guinea-pigs immunised with combined diphtheria vaccine were used. The antibody content was determined by JENSEN's method. The results of comparative titrations in tissue cultures are given in Table VI.

As compared with the results obtained with JENSEN's method titration in cell cultures yielded lower values for all the serum mixtures used. The ratios of the different values in the two kinds of experiment were, however, identical. Essentially similar results were obtained in repeated experiments with the same sera.

The possibility of substituting the binding test in cell cultures for the flocculation test for estimating the value of diphtheria antitoxins was also examined. The pertaining results are given in Table VII.

Table VI

Comparative study in cynomolgus heart culture and by JENSEN's *titration of sera of guinea pigs immunised against diphtheria*
Stationary culture; toxin Di *85*

Designation of serum	IU/ml	
	JENSEN's method	Tissue culture method
Group "a"	9.2	5.5
,, "b"	5.3	4.35
,, "c"	5.5	4.1
,, "d"	4.92	3.8

Table VII

Comparison of diphtheria toxoids by flocculation, and binding test at two levels in cynomolgus heart cell cultures

Designation of anatoxin	Anatoxin values			
	flocculation		tissue culture	
	Lf/ml	Kf_{25}	$L^+/10$	$L^+/100$
Di II	1700	105	>1280 <2560	>160 <320
Di III	3700	57	>2560 <5120	>1280 <2560

The flocculation test and the binding test in tissue cultures on $L^+/10$ basis showed good agreement. On the $L^+/100$ basis lower values were obtained for both toxoids. In the decrease of value there was a significant difference between the two toxoids. The decrease for Di III toxoid was one dilution step. The decrease for Di II was remarkably less. In connection with the lower avidity of Di II toxoid in tissue culture titration prolonged Kf value observed in the flocculation test appeared to be interesting.

Discussion

The high sensitivity to toxic bacterial products of tissue cultures has repeatedly been reported. PLACIDO SOUSA and EVANS [6] have found no difference in the sensitivity to diphtheria toxin between monkey kidney tissue cultures and the guinea-pig skin. In our studies the cell line examined

was sensitive to Lf/1000 both in stationary cultures and in the colour test. Though there was no difference in the sensitivity of the two methods, we still think that stationary cultures should be preferred in similar studies. The exactness of evaluating the results is namely greatly improved by the direct observation of the toxin's cytopathogenic effect. The colour test has its special advantages in studies carried out with routine methods.

The limit values of the toxic effect of toxins can readily be determined in tissue cultures. A certain difference in comparison with the cytopathogenic effect of viruses is the fact that toxins may produce partial effects. This difficulty, however, may be overcome and reading of the results be achived with high accuracy if appropriate amounts of toxin are used. In routine serological and biological methods the estimation of diphtheria antigens and antibodies is comparative, evaluation being based on international standards. With the use of standard serum tissue cultures suit themselves well for such comparative measurements. It appears that when using the same cell line, the same standard serum and the same method in every detail, the results obtained in different laboratories will be more readily comparable than those yielded by animal experiments subject to variations resulting from strain and individual differences. For the development of standard methods the selection of the appropriate cell line, the determination of the appropriate cell number and other factors should precisely be determined.

After appropriate standardisation of the technical details a wide range of practical experience would be necessary to decide whether the tissue culture methods could replace the procedures now generally used for the determination of the main characteristics of diphtheria antigens and antitoxins. At present the use of cell cultures can only be regarded as yielding supplementary data to the results obtained by the classical methods. According to our experience, the tissue culture method is particularly suitable for the examination of the binding capacity of antigens and antitoxins. The examination (particularly at low levels) of antidiphtheria therapeutic sera might yield results not only concerning the actual antibody titre but also as to the therapeutically highly important avidity. The binding test performed at different levels may offer information on the avidity (antigenicity) of anatoxins used for the preparation of vaccines. With the tissue culture method information might be obtained not only as to the antibody content of sera of animals vaccinated for potency tests with anatoxins but the fixation characteristics of the developed antibodies may also be examined. Cell cultures being highly sensitive to diphtheria toxin, they might be used for certain other examinations of practical importance, e. g. for safety testing of diphtheria anatoxins, as well as for the study of different research problems.

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DIFFERENCES IN THE CYTOPATHOGENIC EFFECT OF SOME ADENOVIRUS STRAINS ON HUMAN AMNIOTIC TISSUE CULTURES

By

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Summary. The cytopathogenic effect of 28 adenovirus strains belonging to the serological types 1 to 16, together with earlier examined 14 strains belonging to types 1 to 7, *i. e.* a total of 42 strains was studied in primary human amniotic tissue cultures. According to the character of the cytopathogenic effect the strains could be divided into 2 well-defined groups. The first group was characterized by a network-like structure forming grape-like cell aggregates on further degeneration. In the second group fragmentation of the cell monolayer took place resulting in the loosening of the individual cells. Finally the cytoplasm disappeared and only the nuclei were left, surrounded by some faint cytoplasmic remnants. The cytopathogenic effect characteristic of certain strains proved to be a stable feature in some cases even after 3 years of laboratory maintenance.

The cytopathogenic effect of viruses on cell cultures has a basic importance in studies on the virus host-cell relation. Some of our earlier observations concerning about the cytopathogenic effect of adenoviruses on primary human amniotic tissue cultures have already been reported [9, 11]; on the basis of the cytopathogenic effect the virus strains could be divided into 2 groups denoted by us as type 3 and type 5 degeneration, as the difference appeared to be most characteristic of type 3 and type 5 strains. Our further investigations were made in order to decide whether a similar type of degeneration might be elicited by other types of adenovirus strains, too; whether strains belonging to the same serological type might elicit the same cytopathogenic effect; and whether there ensued changes in the cytopathogenic effect of the strains examined before 3 years and kept in passage since that time.

Materials and methods

A total of 42 adenovirus strains was examined. Two belonged to type 1, three to type 2, seven to type 3, two to type 4, five to type 5, two to type 6, four to type 7, one to type 7/a, two to type 8 and also two to type 9, three to type 10 and also three to type 11, one to type 12, two to type 13; to types 14, 15 and 16 belonged one strain each.

Strains. The individual strains were designated by fractions using the No. of the individual serotypes as numerator and the series number of the single strains of different origin in the same serological group as denominator.

The strains examined were partly type strains kindly supplied by Dr. U. Krech (1/1 to 7/1), Dr. R. S. Dreizin (7a/1, 8/1, 9/1, 10/2, 11/2, 13/1), Dr. J. Vilcek (1/2, 2/3, 3/7, 4/2, 5/5, 6/2, 7/4, 8/2, 9/2, 10/3, 11/3, 12/1, 13/2, 14/1, 15/1, 16/1) Dr. I. Béládi (5/4, 7/3, 10/1, 11/1) and Dr. S. Horváth (7/2), and partly strains isolated by Vilcek *et. al.* [13] (3/4, 3/5, 3/6), Béládi and Kahán [14] (3/2, 3/3) and ourselves [16] (2/2, 5/2, 5/3).

Tissue cultures were usually infected with 10^3 to 10^4 TCID/50. Some experiments were made with smaller inocula.

Tissue cultures. Virus suspensions were harvested and titrated on rabbit serum adapted Detroit-6 tissue cultures and/or primary human amniotic tissue cultures. The use of Detroit-6 cells and their adaptation to rabbit serum was described earlier [15]. Primary human amniotic tissue cultures were prepared as follows. Amniotic membranes separated from fresh and aseptically kept placentas were repeatedly washed in saline containing penicillin, streptomycin and mycostatin until the fluid was clear and incubated in an 0.25 per cent solution of trypsin under gentle shaking in a water bath of 35° to 36° C for 30 minutes. The trypsin solution was changed every 15 to 20 minutes until a satisfactory cell yield had been obtained. All fractions were immediately centrifuged (at about 1500 rpm.) and the cellular sediment was re-suspended in tissue culture medium. After making a cell count the suspension was diluted to contain 200 000 to 300 000 cells per ml. Stationary cultures were prepared using 1 ml of the suspension per tube. The cultures grew to a monolayer in 4 to 8 days. The tissue culture medium consisted of 90 per cent Hanks' solution, 10 per cent calf serum and 0.4 per cent lactalbumin hydrolysate. After infection a maintenance solution containing 5 per cent rabbit serum, 95 per cent Hanks' solution and 0.25 per cent lactalbumin hydrolysate was used.

The infected tissue cultures were examined first at 1 to 2, later at 4 to 5 days intervals usually for 25 to 30 days under low power (× 150), to permit inspection of extensive areas.

The neutralisation tests were performed in primary human amniotic tissue cultures with immune sera produced in rabbits, as described earlier [10].

Results

After the examination of the 14 adenovirus strains belonging to types 1 to 7, the cytopathogenic effect on primary human amniotic tissue cultures of 28 further strains belonging to the types 1 to 16 was studied; *i. e.* a total of 42 strains was examined.

Similarly to our previous results [9, 10, 11, 12] our recent investigations have shown that the additional strains examined might also be divided into two well-defined groups according to the nature of the cytopathogenic effect produced. In contrast to our earlier studies [6, 11] in the present work interest was focussed not to single cells but more to the general aspect of the total cell population.

The types of cytopathogenic effects thus observed could again be denoted as "type 3" and "type 5" degeneration, as the recently studied strains have also been characterized by one of these two types of degeneration.

These degeneration types were described earlier [9, 10, 11, 12], but let us mention that type 3 degeneration manifests itself in a rounding up, shrinking and increased refraction of the cells. Separation of the degenerated cells is not complete, thus a coherent network of degenerated cells is formed. This network is characteristic of the early phase of type 3 degeneration (Figs. 1 to 4). During the further process of degeneration the continuity of the network is gradually broken up and of the persisting groups of 3 to 10 cells grape-like degenerated clumps are formed. These are characteristic of the late phase of tpye 3 degeneration (Fig. 5).

Type 5 degeneration is characterized by a gradual centripetal development of refractory matter and granules in the infected cells; the margins become indistinct and separation of the individual cells ensues. The culture

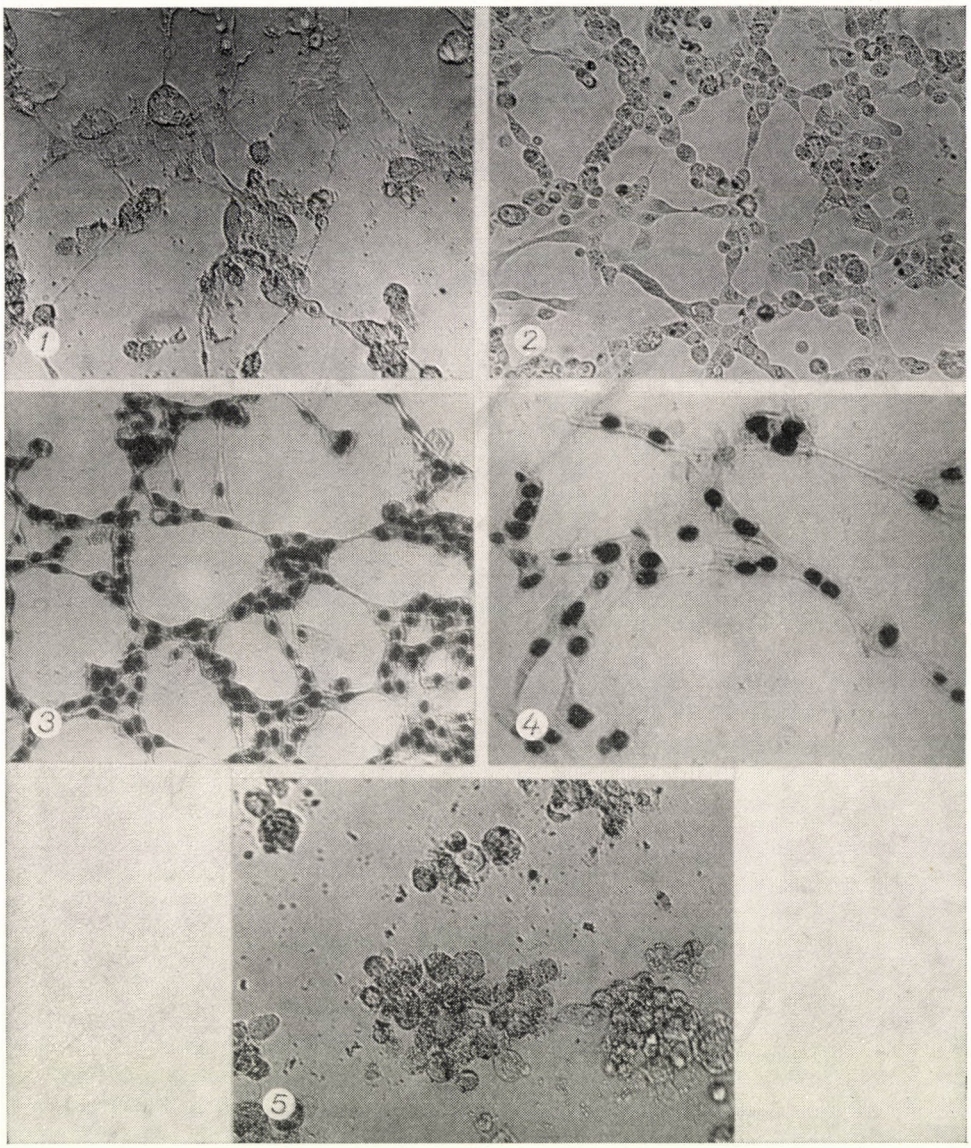

Fig. 1. Amniotic tissue culture on 4th day after infection with adenovirus strain 11/3. Native preparation

Fig. 2. Tissue culture on 3rd day after infection with strain 16/1. Native preparation

Fig. 3. Tissue culture on 4th day after infection with strain 14/1. Giemsa stain

Fig. 4. Tissue culture on 3rd day after infection with strain 12/1. Giemsa stain

Fig. 5. Tissue culture on 5th day after infection with strain 10/3. Native preparation

breaks up into individual degenerated cells distributed at random (Figs. 6, 7 and 8). Subsequently cytoplasm becomes loosened up and more granulated to disappear gradually until the whole culture appears to consist of individual nuclei coated with faded cytoplasmic residues (Figs. 9, 10 and 11).

Table I

Grouping of the adenovirus strains examined according to the type of cytopathogenic effect

Cytopathogenic effect	
"type 3"	"type 5"
Strain 3/1+	Strain 1/1+
" 3/2+	" 1/2
" 3/3+	" 2/1+
" 3/4	" 2/2+
" 3/5	" 2/3
" 3/6	" 5/1+
" 3/7	" 5/2+
" 4/1	" 5/3+
" 4/2+	" 5/4+
" 7/1+	" 5/5
" 7/4	" 6/1+
" 8/2	" 6/2
" 9/1	" 7/2+
" 9/2	" 7/3
" 10/3	" 7a/1
" 11/3	" 8/1
" 12/1	" 10/1
" 13/2	" 10/2
" 14/1	" 11/1
" 15/1	" 11/2
" 16/1	" 13/1

Table I shows the distribution of the 42 virus strains according to their cytopathogenic effect (type 3 or 5 degeneration). In the strains denoted by + the cytopathogenic effect has not undergone changes during nearly 3 years of laboratory maintenance except for strain 7/2 which in the previous experiments had elicited a type 3 degeneration but recently observed to produce type 5 degeneration. Most strains within one and the same serological type exhibited the same type of cytopathogenic effect, nevertheless different cytopathogenic effects within one serological group were also observed.

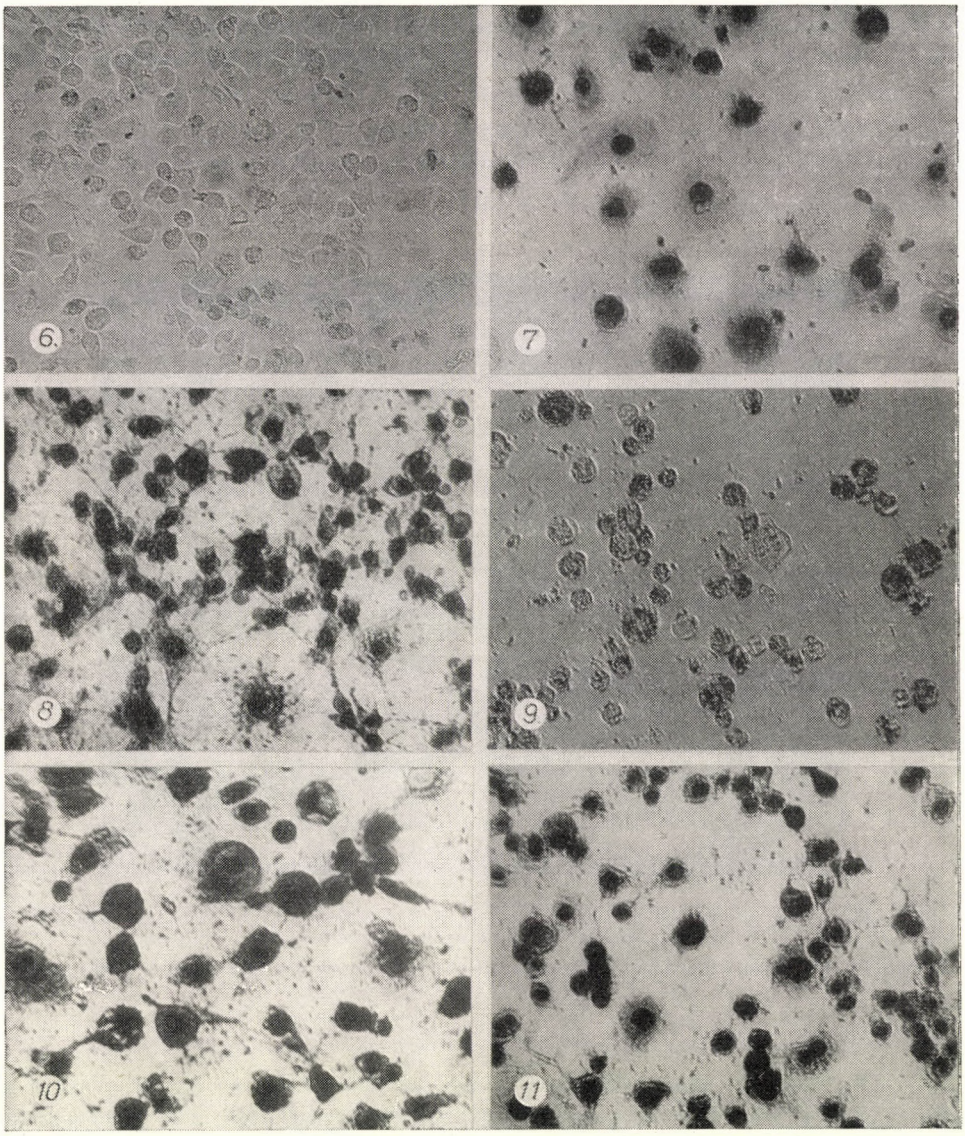

Fig. 6. Tissue culture 60 hours after infection with strain 10/1. Native preparation
Fig. 7. Tissue culture on 5th day after infection with strain 5/5. Giemsa stain
Fig. 8. Tissue culture on 5th day after infection with strain 13/1. Giemsa stain
Fig. 9. Tissue culture on 7th day after infection with strain 11/2. Native preparation
Fig. 10. Tissue culture on 6th day after infection with strain 10/1. Giemsa stain
Fig. 11. Tissue culture on 6th day after infection with strain 7a/1. Giemsa stain

It might be stated that the cytopathogenic effect of adenoviruses on primary human amniotic tissue cultures is typical in relatively young (4 to 10-day old) cultures. The type of the cytopathogenic effect was less easy to differentiate in older cultures; these often showed intermediary forms, particularly in the case of type 5 degeneration. On reducing the quantity of the inoculum the type of the cytopathogenic effect remained the same but degeneration ensued only at the periphery.

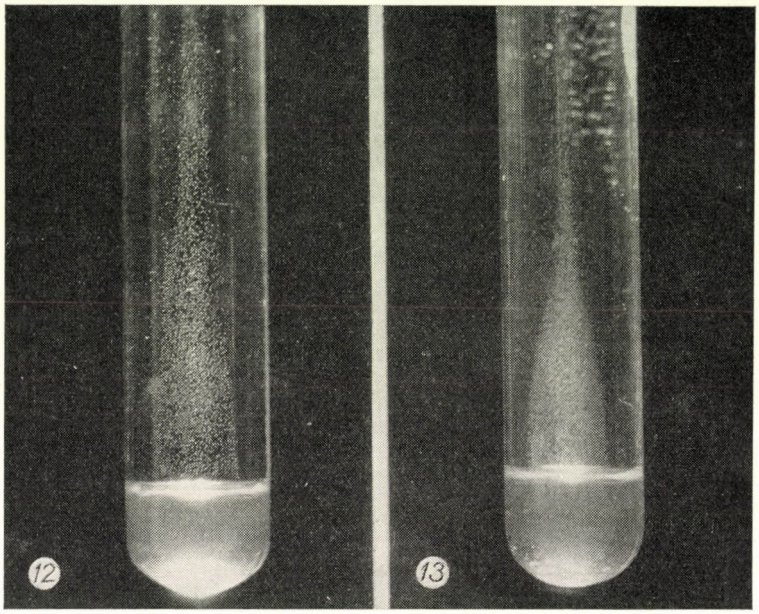

Fig. 12. Gross appearance of degenerated tissue culture on 4th day after infection with strain 3/5. Native preparation

Fig. 13. Gross appearance of degenerated tissue culture on 5th day after infection with strain 10/1. Native preparation

The type of degeneration was not influenced by previous passage of the virus on Detroit-6 or human amniotic tissue cultures. The infected and degenerated cells were observed to adhere for a long time to the glass wall and the type of the cytopathogenic effect could be identified even after incubation for 1 month at 37°C or room temperature. The cytopathogenic effect could be neutralised by appropriate immune serum.

The difference between the two types of cytopathogenic effect was marked and could be established by the naked eye (Figs. 12 and 13) on the basis of the granulated appearance of type 3 as contrasted with the homogeneous aspect of type 5 degeneration.

Discussion

The cytopathogenic effect of adenoviruses was studied on the basis of the morphological and biochemical changes observed in individual cells [1—8]. Among others BOYER *et al.* [3] examined the different types of adenoviruses in HeLa cell cultures; they observed different cytopathogenic changes in individual cells. We succeeded in demonstrating differences in the individual cells of cultures infected with type 3 or type 5 strains [11]. In the present experiments, on the other hand, the general appearence of cultures has been compared after infection. Naturally, the changes in the whole culture cannot be examined separately from those of the individual cells, both being due to the same determining factors. Still, examination of the culture as a whole has been supposed to yield additional information concerning the dynamics of infection.

Studying the total infected cell population allowed to gain a more complete picture of the destructive effect, in view of the fact that the changes in the individual cells can only be studied in preparations fixed and stained at a certain phase, while the total infected population may be examined throughout all stages of the whole cytopathogenic process.

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THE OCCURRENCE AND SIGNIFICANCE OF PHOSPHATASE IN ENTERIC BACTERIA

By

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Summary. The phosphatase activity of 929 Enterobacteriaceae strains has been examined. The distribution of strains according to phosphatase production was as follows. Of 67 Providencia strains 12 positive, 55 negative; of 140 *Proteus hauseri* strains 139 positive, 1 negative; of 125 *Proteus morganii* strains 4 positive, 121 negative; of 38 *Proteus rettgeri* strains 8 positive, 30 negative. No phosphatase activity was observed in other enteric bacteria (150 *E. coli*, 153 Klebsiella, 115 Shigella, 45 Salmonella, 72 Citrobacter, 23 Arizona, 2 *Cloaca cloacae* and 1 Hafnia cultures).

The uniform positivity of the phosphatase reaction with *Proteus hauseri* strains is indicative of the relation between *Proteus vulgaris* and *mirabilis*.

The relative frequency of positively reacting strains within the genus Providencia seems to confirm the conception that this genus belongs to the tribe Proteae.

No antigenic relationship was shown among *Proteus hauseri* and other phosphatase positive Proteae strains.

The detection of bacterial enzymes has a considerable diagnostic and pathogenetic importance. Certain enzyme reactions (carbohydrate fermentation, urease, phenylalanine desaminase, amino acid decarboxylase reaction, etc.) can be used for differentiation among groups or subgroups of bacteria. Other enzymes (coagulase, hyaluronidase, lecithinase, etc.) are supposed to be associated with pathogenicity. Their estimation affords information of the causative role of microorganisms isolated from pathological conditions.

In staphylococci, in addition to coagulase and hyaluronidase, phosphatase is considered to be characteristic of pathogenicity. BARBER *et al.* showed that pathogenic cocci are producing phosphatase [1, 2]. This observation was confirmed by RAMGAN and KAJDARE, although these authors obtained no such uniform results [3].

As regards the spore-forming bacilli, phosphatase activity in *B. cereus* was described by IVÁNOVICS and FÖLDES [4].

For the detection of bacterial phosphatase the phenolphthalein phosphate method was first applied by BRAY and KING in 1942 [5]. For the examination of staphylococci BARBER *et al.* used an agar medium containing 0.01 per cent of sodium phenolphthalein phosphate. They showed that no influence was exerted on the reaction either by 10 per cent blood or by 5 to 10 per cent sodium chloride. With media containing the latter substance, as growth was scanty, readings were recommended to be performed only after 48 hours [1, 2].

Instead of an agar plate medium, HALLMANN in 1954 recommended the use of a fluid medium containing the same concentration of the substrate[7].

The purpose of the present work was to examine the occurrence of phosphatase-producing strains among enteric bacteria, and to ascertain whether the reaction was suitable for systematic differentiation. It was also studied whether phosphatase production was merely a property of freshly isolated cultures, or it remained stable also on subculturing the strain.

Materials and methods

Test strains. Of the 929 strains 472 were international type cultures, 457 were isolated from materials sent for routine examination to this laboratory.

Of the international type cultures the following were examined (bracketed names and figures indicate the name of the collector and the number of the examined strains).

Providencia (EVING, 76), *Proteus hauseri* (KAUFFMANN, 76), *Proteus morganii* (RAUSS, 31), *Proteus rettgeri* (NAMIOKA—SAKAZAKI, 36), *Escherichia coli* (KAUFFMANN, 49), Klebsiella (KAUFFMANN, 30), Shigella (WEIL—CARPENTER, Flexner and Large-Sachs group 501—505, 42), Salmonella (KAUFFMANN, 44), Arizona (KAUFFMANN, 1), Ballerup (KUFFMANN, 1), *Cloaca cloacae* (KAUFFMANN, 1), Hafnia (KAUFFMANN, 1), Citrobacter (SEDLAK, 71), Arizona (SEDLAK, 22).

Isolation of bacteria from routine materials was performed on Endo and desoxycholate citrate agar. The systematic position of the strains was determined on the basis of biochemical tests; the characteristic colonial morphology was also taken into consideration. By use of the polytropic media I and II [8, 9], the following reactions were carried out. Fermentation of lactose, glucose, sucrose, production of urease and H_2S, utilization of citrate, production of indole and gelatinase. Shigellae and *E. coli* types associated with infantile enteritis were identified by slide agglutination in O and OB sera, respectively.

From routine material 457 strains were selected. According to biochemical and serological properties these were grouped as follows.

Proteus hauseri 64, *Proteus morganii* 94, *Proteus rettgeri* 2, *E. coli* 101, Klebsiella 123, Shigella 69, Salmonella 1, Citrobacter 2, *Cloaca cloacae* 1.

Among 69 Shigella strains 28 *Sh. flexneri* and 41 *Sh. sonnei* were encountered.

Of 16 *E. coli* strains associated with infantile enteritis 11 belonged to O111 : B4, 3 to O55 : B5, 1 to O112 : B13 and 1 to O126 : B16.

Medium. The substrate was prepared as described by FISHMAN *et al.* [6].

Instead of the agar medium employed by BARBER *et al.*, HALLMANN's broth containing 0.01 per cent sodium phenolphthalein phosphate was used [7]. The medium was distributed in 3 ml amounts into sterile Widal tubes. After the sterility test had been performed, the tubes were inoculated with a needle from 24 hour agar slant cultures. Incubation lasted for 24 hours at 37° C. In all series of examinations one uninoculated, one positive and one negative control were included.

A positive reaction was indicated by a red colour after the addition of 2 or 3 drops of 0.1 *N* or *N* NaOH.

The strains were tested immediately after isolation, then after 2 weeks and finally after 3 months.

Results

Among the type strains phosphatase activity was distributed as follows. Of 67 Providencia strains the enzyme was produced by 12 cultures. No phosphatase was formed by 55 Providencia strains. Of 76 *Proteus hauseri* strains 75 reacted positively, 1 negatively. Of *Proteus morganii* 1 strain was positive 30 strains were negative. Of *Proteus rettgeri* 8 were positive, 28 negative. No phosphatase production was found in 49 *E. coli*, 30 Klebsiella, 42 Shigella,

44 Salmonella, 23 Arizona, 72 Citrobacter, 1 *Cloaca cloacae* and 1 Hafnia cultures (Table I).

Table I

Phosphatase activity of type strains

	Positive	Negative	Total
Providencia	12	55	67
Proteus hauseri	75	1	76
Proteus morganii	1	30	31
Proteus rettgeri	8	28	36
Escherichia coli	—	49	49
Klebsiella	—	30	30
Shigella	—	42	42
Salmonella	—	44	44
Arizona	—	23	23
Citrobacter	—	72	72
Cloaca cloacae	—	1	1
Hafnia	—	1	1
Total	96	376	472

Strains isolated from routine material behaved as follows. All the 64 *Proteus hauseri* cultures reacted positively. Of 94 *Proteus morganii* 3 were positive, 91 were negative. Other strains exhibited no phosphatase activity (2 *Proteus rettgeri*, 101 *E. coli*, 123 Klebsiella, 69 Shigella, 1 Salmonella, 2 Citrobacter, 1 *Cloaca cloacae)* (Table II).

Table II

Phosphatase activity of freshly isolated strains

	Positive	Negative	Total
Proteus hauseri	64	—	64
Proteus morganii	3	91	94
Proteus rettgeri	—	2	2
Escherichia coli	—	101	101
Klebsiella	—	123	123
Shigella	—	69	69
Salmonella	—	1	1
Citrobacter	—	2	2
Cloaca cloacae	—	1	1
Total	67	390	457

Table III summarizes the phosphatase reaction of the 929 examined strains.

Table III

Phosphatase activity of 929 Enterobacteriaceae strains

	Positive	Negative	Total
Proteus hauseri	139	1	140
Proteus morganii	4	121	125
Providencia	12	55	67
Proteus rettgeri	8	30	38
Escherichia coli	—	150	150
Klebsiella	—	153	153
Shigella	—	111	111
Salmonella	—	45	45
Citrobacter	—	74	74
Arizona	—	23	23
Cloaca cloacae	—	2	2
Hafnia	—	1	1
Total	163	766	929

Of 140 *Proteus hauseri* cultures 139 were phosphatase positive and only 1 was negative, of 125 *Proteus morganii* 4 were positive and 121 were negative. Of 67 Providencia strains 12 reacted positively, 55 negatively. Of 38 *Proteus rettgeri* 8 produced phosphatase. Uniformly negative reactions were obtained with 150 *E. coli*, 153 Klebsiella, 111 Shigella, 45 Salmonella, 74 Citrobacter, 23 Arizona, 2 *Cloaca cloacae* and 1 Hafnia strains.

The phosphatase test was carried out in addition to freshly isolated cultures on the corresponding subcultures after 2 weeks and 3 months. The results obtained with subcultures were always in agreement with those obtained with the fresh organisms.

Phosphatase-producing Providencia, *Proteus morganii* and *rettgeri* strains showed no antigenic relationship to *Proteus hauseri*.

Discussion

The occurrence and significance of phosphatase in various groups of enteric bacteria has not so far been investigated. Because of its simplicity, the phosphatase reaction is well suited for practical purposes. Phosphatase-producing strains gave consistently positive reactions on subsequent examinations and the subcultures of strains giving a negative reaction at the first

examination continued to yield negative results. Accordingly, phosphatase production in subcultures has been found a property stable for at least 3 months.

No association was found between phosphatase production and other biochemical reactions. With the exception of urease and H_2S production, no parallelism was revealed with any of the employed biochemical tests. It was striking that among enteric bacteria phosphatase-producing organisms occurred exclusively in the tribe Proteae.

On the basis of KAUFFMANN and PERCH's studies on the antigenic structure of these bacteria, *Proteus mirabilis* and *Proteus vulgaris* have been included in one genus *(Proteus hauseri)* [10]. In the present paper it has been shown that within the tribe Proteae phosphatase production is characteristic of the genus *Proteus hauseri*. Within the genera *Proteus morganii* and *rettgeri* this enzyme occurred infrequently, a fact supplying a biochemical confirmation of the relationship between *Proteus vulgaris* and *mirabilis*.

The phosphatase reaction may be considered a test equal in rank with the H_2S reaction.

Among 125 *Proteus morganii* strains 4 phosphatase-positive cultures were found (3 per cent). This finding does not affect the above consideration. The test, as well as other reactions, seems to be subject of certain variability. Of 38 *Proteus rettgeri* strains 8 were phosphatase-producing organisms (21 per cent). These strains showed no antigenic relationship to *Proteus hauseri*. From the results it may be concluded that with the tribe Proteae the test is well suited for the differentiation of *Proteus hauseri*.

It should be noted that in the genus Providencia a considerable number (approx. 18 per cent) of phosphatase-positive cultures occurred. The fact that outside the tribe Proteae no such enteric bacteria have been encountered, supports the view that the genus Providencia belongs to the tribe Proteae.

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DETECTION OF THE ROUTE OF NOSOCOMIAL ESCHERICHIA COLI INFECTIONS BY PHAGE-TYPING

By

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Summary. The phage-type, flagellar antigen and β-phenyl propionic acid (PP) reaction of 483 *E. coli* O111 : B4 and O55 : B5 strains isolated from hospitalised infants have been examined.

Strains belonging to group O111 : B4 fell mostly into phage-type 111/2, contained flagellar antigen H2, and gave a positive PP reaction. Strains O55 : B5 belonged generally to phage-type 55/2, contained H antigen 6 and were PP negative.

No close association was found between these properties (phage-type, serotype and biotype) and pathogenicity or spreading potency.

The route and source of nosocomial *E. coli* infections could adequately be followed and traced by phage-typing.

In the search for the source of nosocomial infections caused by *E. coli* associated with infantile enteritis, in addition to biochemical and serological examination, phage-typing of the cultures is considered important.

The pathogenicity of *E. coli* strains associated with infantile enteritis has been confirmed all over the world. Several epidemiological questions concerning this problem have not been elucidated. In order to achieve a reliable tracing of the source and following up the route of infection, in other words to make possible the introduction of effective preventive measures, in addition to serological and biochemical examinations phage-typing of the pathogenic agent was also necessary.

In 1952 EÖRSI *et al.* [1, 2] reported that, with newly isolated phages, they were able to divide into types *E. coli* O111: B4 and O55: B5 strains. The typing-phages were specific for the corresponding serological groups. With 4 phages strain O111 : B4, and with 3 phages strain O55 : B5 were classified into 4 and 3 types, respectively. An association was found between phage-sensitivity and flagellar antigen. Strains containing the same H antigens (e.g. O111 : B4 : H2 and O55 : B5 : H6) were subdivided into phage types [3, 4].

In subsequent work our set of phages was supplemented with newly isolated or adapted phages and the examinations were extended to group O26 : B6.

By means of typing-phages, biochemical and serological reactions, strain O111 : B4 has been divided into 7, strain O55 : B5 into 5, strain O26 : B6 into 4 types [5].

When the elaboration of our phage-typing method was in progress, NICOLLE *et al.* [6] also worked with the phage-typing of *E. coli* strains associated with infantile enteritis. At first with 14 then with 25 phages they were able to classify the O111 : B4 and O55 : B5 strains into 11, strain O26 : B6 into 7 types. The phages of NICOLLE *et al.* were not specific for the serological groups, but similarly to our phages, they showed an association to flagellar antigens. A connection was also found between certain biochemical properties and phage-sensitivity. According to the above authors, the ß-propionic acid reaction (in the following PP reaction) described by D'ALESSANDRO and COMES confirms and completes the results obtained by phage-typing [7, 8].

Accordingly, for the differentiation of our strains serological typing and the PP reaction were performed in addition to phage-typing. (Examinations concerning NICOLLE's phages are to be described in a subsequent paper.)

The distinguished phage-types of group O111: B4, their flagellar antigens and their PP reaction are presented in Table I.

Table I

Phage pattern of E. coli O111 : B4

Phage-type	Serotype	PP reaction	Lysis with typing-phages				
			a	a1	aF42	b	b2
111/1	O111 : B4 : H—	+	+	±	+	+	+
111/5	O111 : B4 : H—	+	—	+	—	—	±
111/7	O111 : B4 : H—	+	±	—	—	+	+
111/2	O111 : B4 : H2	+	—	—	—	+	+
111/4	O111 : B4 : H2	—	—	—	—	+	+
111/3	O111 : B4 : H12	—	—	—	—	±	±
111/6	O111 : B4 : H12	—	—	—	—	—	—

+ = confluent lysis
± = semi-confluent lysis

With 5 typing-phages, strains belonging to this serogroup fall into 7 phage-types. Of the 5 typing phages "a", "a1", "b" and "b2" are specific for group O111 : B4; phage "aF42" lyses also phage-types belonging to groups O55 : B5 and O26 : B6. The phage-types are grouped according to their flagellar antigens. Types 111/1, 111/5 and 111/7 are non-flagellated. Thus, as to phage-sensitivity, non-flagellated 111 : B4 strains can be divided into 3 types; these types are all PP positive. Strains with antigen H2 belong to a separate phage-type and can be divided into PP positive and PP negative variants. Strains with antigen H12 are PP negative and at present can be distinguished as phage-sensitive and phage-resistant variations. Within group

O55 : B5, by the use of 3 phages, 5 types were so far differentiated (Table II). Based on their sensitivity to phages, "k" and "l" strains 55/1, 55/2 and 53/3 may be recognized. These types usually contain H antigen 6. In addition to phages "k" and "l", which are specific for the O55 : B5 group, strains sen-

Table II

Phage-pattern of E. coli O55 : B5

Phage-type	Serotype	PP reaction	Lysis with typing-phages		
			k	l	aF42
55/1	O55 : B5 : H6	−	+	+	−
55/2	O55 : B5 : H6	−	+	−	−
55/3	O55 : B5 : H6	−	−	+	−
55/4	O55 : B5 : H2 or H−	+	+	±	+
55/5	O55 : B5 : H6	−	−	−	−

+ = confluent lysis
± = semi-confluent lysis

sitive to phage "aF42" are distinguished as a separate type, 55/4. These strains, although unfrequently occurring, are well distinguishable from the other phage-types. The flagellar variants of these strains contained H antigen 2, *i.e.* a flagellar antigen identical with that found in group O111 : B4. The relationship to group O111 : B4 is shown by the sensitivity to phage "aF42", which lyses O111 : B4 strains as well. That this phage-type differs from other O55 : B5 strains is proved by the fact that while phage-types 55/1, 55/2, 55/3 and 55/5 are PP negative, strains belonging to 55/4 give a posiuve reaction.

Division of group O26 : B6 strains was also performed. As in the examined materials this serogroup was rare or absent its phage-typing will be discussed in a subsequent paper.

In the present paper we shall report on the phage-typing of O111 : B4 and O55 : B5 strains isolated from infants admitted to various hospitals.

The original purpose of the present work was (i) to examine the phage-type distribution of the isolated strains in epidemic and non-epidemic periods in different hospitals; (ii) to reveal the association between phage-types and the severity of the disease and the spread of the infection; (iii) to examine whether our phage-typing method elaborated in recent years was suitable for the following up of *E. coli* infections, *i.e.* for the detection of the infective source.

Materials and methods

Cultures. Altogether 483 *E. coli* strains associated with infantile enteritis were examined. All the cultures had been isolated in 1959. Of the strains 438 belonged to group O111 : B4, 135 to group O55 : B5. The cultures were obtained from 5 different paediatric hospitals. Hospitals designated with A and B were situated in country centres in Hungary. Hospital C was an East German childrens' hospital, D and E were Budapest hospitals. In addition, during a search for the source of nosocomial enteric infections, strains isolated from an infants' hostel were also included. With some exceptions, the strains originated from enteric infections; strains from other individuals were examined only on the condition that they had been isolated from contacts.

Typing phages. Typing was carried out by means of 8 phages, of which 5 (a, b, c, k, l) were isolated in the past years from the faeces of infants suffering from enteritis. The remaining 3 phages were obtained by adaptation, namely phages "al" and "aF42" from phage "a", phage "b2" from phage "b".

All phages were propagated on the strain on which they had been indicated at the time of isolation. In case of adapted phages propagation was carried out on the culture to which the adaptation had been performed. All phages were used undiluted.

Culture media. For the enrichment of phages Hartley's broth, for typing Hartley's agar was used. With the exception that instead of beef digest horse flesh digest was employed, the media were the same as originally described [4].

Phage-typing. In order to avoid the variation to R forms, which often causes the decrease or loss of phage-sensitivity, typing was preferably carried out 1 to 2 days after isolation. Hartley's agar plates were flooded with a 2 to 3-hour broth culture of the strain to be examined, so as to obtain an even spread on the surface of the medium. The excess of suspension was pipetted off. After drying the plates for approximately 15 minutes at room temperature with open lids, the typing phages were dropped onto the plates. After a subsequent drying the plates were incubated at 37°C for 6 hours, then readings were performed.

Serological examinations. Serological grouping of the strains was made with slide agglutination in OB sera; the flagellar antigens were determined with the method of RAUSS and UJVÁRY [10] with sera obtained from the *Institute of Microbiology, Pécs* (H2, H6, H11 and H12).

Biochemical examinations. Of the biochemical reactions the test described by D'ALESSANDRO and COMES was of especial value.

PP reaction. On an agar medium containing 1 g β-phenyl propionic acid per 5000 ml, the colonies of positively reacting strains become pink, red and finally brown in colour. The reaction may be read after 8 hours; it reaches the highest intensity after 24 to 36 hours.

Results

Distribution in various hospitals of phage-types in epidemic and non-epidemic periods. The phage-type distribution of the isolated strains is presented in Table III and Table IV. Group O111 : B4 strains belonged mostly to type 111/2 (80.2 per cent). Of O55 : B5 strains type 55/2 emerged (77.8 per cent). In Hospitals A, C, D and E type 111/2 predominated, in Hospital B type 111/1 occurred in a high precentage (90.6). This type was met with also in Hospitals A and C. In Hospitals D and E no 111/1 strains were observed. The most frequent O55 : B5 type (55/2) was found chiefly in Hospitals C and D.

Association between phage-types and the severity of enteritis or the spread of infection. Observations were made in Hospitals A, B and C, as into these institutions strains belonging to the same serotype but differing in phage-type were introduced simultaneously.

Table III

Phage-type distribution of O111 : B4 strains isolated from different hospitals

Origin of strains	O111 : B4			
	111/1	111/2	111/4	Total
Hospital A	23	131	6	160
Hospital B	29	3	—	32
Hospital C	7	23	—	30
Hospital D	—	82	—	82
Hospital E	—	37	—	37
Infants' Hostel N	1	3	3	7
Total	60	279	9	348

Table IV

Phage-type distribution of O55 : B5 strains isolated from different hospitals

Origin of strains	O55 : B5					
	55/1	55/2	55/3	55/4	55/5	Total
Hospital A	1	1	2	3	—	7
Hospital B	3	—	—	—	—	3
Hospital C	5	71	—	1	6	83
Hospital D	3	23	—	—	3	29
Hospital E	—	10	—	—	—	10
Infants's Hostel N	3	—	—	—	—	3
Total	15	105	2	4	9	135

Between January and December, 1959, 160 O111 : B4 and 7 O55 : B5 strains isolated from Hospital A were typed. Between January and March there was a severe epidemic of enteritis in this hospital. During this period 107 strains isolated from 91 infants were examined. All strains belonged to group O111 : B4. Of the strains 91 were members of phage-type 111/2, contained antigen H2 and were PP positive. The remaining 16 strains belonged to phage-type 111/1, were non-flagellated and PP positive.

During the whole epidemic period in different departments of the hospital (Infants' Department 1 and 2, Infectious Disease Department) type 111/2 (H2) predominated. Part of the cases was mild, but not unfrequently medium and severe and grave toxic cases also occurred. Three of the examined infants

who had been excreting type 111/2, died. In the course of the epidemic caused by type 111/2, type 111/1 also appeared; the latter occurred but rarely in Department 1 and in the Infectious Disease Department in association with enteric infections undoubtedly of an external origin. In Infants' Department 2, however, in addition to the severe enteric infections caused by 111/2, type 111/1 spread and caused severe infections.

In the post-epidemic period in the various deparments of Hospital A the following types were found. In Department 1 in April the non-flagellated type 111/1; later phage-type 111/2 (H2) predominated. Type 111/1 had been introduced by two infants admitted with severe enteritis. In the same department in November severe cases of enteritis were again observed; these were caused by type 111/2. In Department 2 and in the Infectious Disease Department in the post-epidemic period 111/2 was the type most frequently encountered. During May in Department 2 type 111/4 (H2, PP negative) occurred. This type was isolated from 3 infants admitted with severe enteritis.

From the Infants' and Premature Departments of Hospital B during the period January to August, 1959, 32 O111 : B4 and 3 O55 : B5 strains were isolated. In these departments between January and March there were several cases of enteritis.

Most of the examined strains were isolated during this period, some were cultured during a non-epidemic period in the summer. In the epidemic period, of the 20 strains isolated from 20 infants, 19 corresponded to type 111/1 or degraded 111/1 (non-flagellated, PP positive). Only one type 111/2 (H2, PP positive) strain occurred during the period indicated. Apart from the infants admitted with enteric symptoms, the course of nosocomial infections was generally mild. During the summer months (July—August) an outbreak occurred in the Premature Department. Of 12 strains isolated from 12 patients 10 belonged to type 111/1 (non-flagellated, PP positive) and 2 to type 111/2 (H2, PP positive). Both types caused severe enteritis.

It was remarkable that while in Hospital B throughout and after the epidemic type 111/1 (non-flagellated) predominated and caused relatively mild infections, in Hospital A during the outbreak chiefly type 111/2 strains occurred. Unlike type 111/1, the latter caused severe, toxic enteric infections.

Type 111/4 was isolated during a non-epidemic period from severe cases of sporadic enteritis. Different phage-types of group O55 : B5 were encountered mostly in Hospital C. In this institution type 55/2 (H6, PP negative) predominated, although also other O55 : B5 and O111 : B4 strains occurred. Similarly, in Hospitals D and E type 55/2 was most frequently met with. Type 55/1 (H6, PP negative) and 55/4 (H2 or H—, PP positive) were isolated from some sporadic cases and from a smaller nosocomial outbreak.

The applicability of our phage-typing method for the detection of the infective source. Investigations have shown that phage-types of *E. coli* associated with

infantile enteritis are less stable both *in vivo* and in vitro than those of *S. typhi* and *S. paratyphi B.* Strains isolated on repeated occasions from the same individual may more or less vary in phage-sensitivity. Nevertheless, according to our experience, the different phage-types may be regarded practically stable *in vivo.*

Repeated examinations were carried out on 50 infants. From 10 of them O111 : B4 and O55 : B5 strains were simultaneously or subsequently isolated. From one infant harbouring type 111/2 the next examination showed type 111/1. In the latter case, however, probably a nosocomial cross-infection occurred, since in the ward a spread of 111/1 as well as that of 111/2 was observed. In 45 cases repeated isolations revealed the same phage-type.

It should be noted that because of the lability of *E. coli* phage-types *in vitro*, typing should be made shortly after isolation. The route and source of the infection could adequately be followed up in Hospital A and B, where O111 : B4 strains belonged to two different phage-types.

As noted above, in Hospital A during an outbreak due to type 111/2, in the wards of the Infectious Disease Department type 111/1 appeared. This type was first isolated from the faeces of a premature baby admitted with bronchopneumonia. The infant was transferred from country hospital M to the Infectious Disease Department. One week later a new infant and one month later another infant, both excreting type 111/1, were admitted with severe enteritis. These infants had been referred to us by Infants' Hostel N. In Department 2 of the same hospital during an outbreak due to type 111/2 only one infant was shown to harbour a non-flagellated type 111/1 strain. The latter patient was transferred with severe enteritis from Nursery P. In Department 1 all strains cultured until the beginning of March belonged to type 111/2. The infants were admitted partly with enteritis and partly with parenteral diseases. The first 111/1 strain was isolated from the faeces of an infant referred to us with severe enteritis by Infants' Hostel N. Some days later another 111/1 strain was cultured from an infant lying in the same ward. This baby had arrived from Hospital M with severe enteric infection. The causative agent spread rapidly first to a premature baby until then free from enteric symptoms, then to a newborn infant lying in the same bed and finally to 4 further infants. All the incriminated patients became gravely ill; the premature baby died.

As the examinations indicated Infants' Hostel N as the source of infection, a search for carriers was performed in that institution. Among the healthy infants one type 111/1 excretor was found. The strain which caused severe infections in the hospital, did not spread among the healthy infants, and the host was also symptomless. (It may be assumed, of course, that the latter had passed through an enteritis.)

In the non-epidemic period type 111/1 was again introduced from Infants' Hostel N and Hospital M by two infants admitted with severe enteritis.

The severe cases of enteritis due to type 111/2 occurring in November could be traced back to the Maternity Department of the hospital.

Our investigations have shown that by means of phage-typing it is possible to detect strains belonging to the same serogroup but differing in phage sensitivity and to follow up the nosocomial or interhospital spread of the causative agent.

The original purpose of the present study was to elucidate the three questions mentioned. During the work and the evaluation of the results a frequent incidence of nosocomial infections was noted. As this problem arose

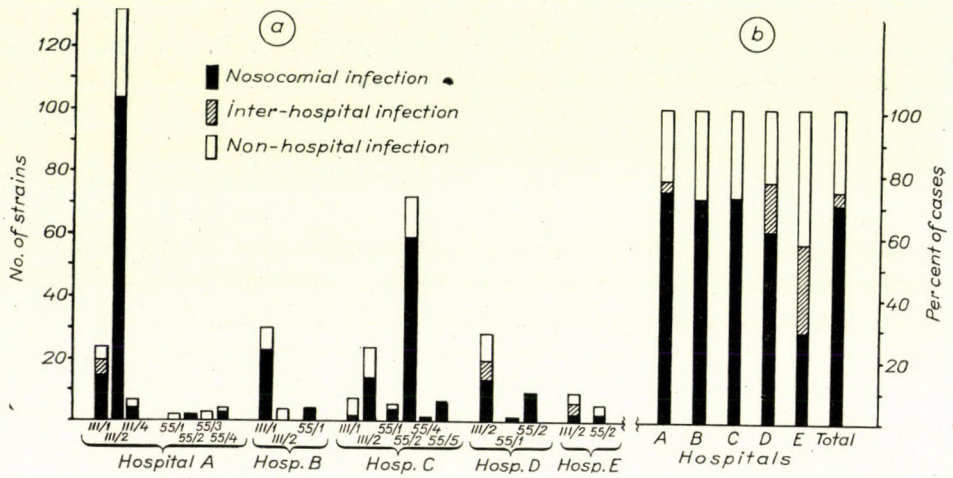

Fig. 1. (a) Phage-type distribution of O111 : B4 and O55 : B5 strains according to origin of cultures
(b) Origin of enteritis cases according to hospitals

only later, the question whether the examined infants had acquired enteritis in or outside the hospital could be answered only partly. The results can be summarized as follows.

Hospital A. Of the 91 infants examined during the epidemic, 24 had been admitted with enteritis; the remaining infants had suffered originally from other, chiefly respiratory diseases, and acquired enteritis in the hospital. Thus the percentage of nosocomial infection amounted to 73.1.

Hospital B. Patients treated in the Infants' Department had been admitted mostly with diseases of the respiratory organs, displaying no enteric symptoms. The enteritis manifested itself on the 6th to 12th day after admission. During the epidemic period out of 20 infants only 5 had been admitted with enteric symptoms. Altogether 71.4 per cent of the strains originated from infants who had acquired the infection in the hospital. It should be noted that

of the infants admitted with enteric symptoms, 4 had previously been under hospital treatment; 2 of them in another department of the same hospital, 2 had been referred from Hospital N.

Hospital C. Of the examined strains 71.7 per cent was isolated from infants who had been infected in the hospital.

Hospital D. Of the strains 60.5 per cent originated from nosocomial infections. In addition, nearly one half of the infants admitted with enteritis was previously treated and probably infected in other hospitals.

Hospital E. Only few of the data were available. Of the examined strains 28.5 per cent was isolated from nosocomial infections; however, more than one half of the infants admitted with enteritis was transferred from other hospitals.

The phage-type distribution and origin (nosocomial or external infection) of O111 : B4 and O55 : B5 strains isolated in different hospitals is shown in parts 1a and 1b of Fig. 1.

On the basis of the available data the origin of 367 strains could be estimated. Of the strains 255 (69.5 per cent) were isolated from nosocomial enteric infections. Strains cultured from cases admitted with enteritis amounted to 112 (26.2 per cent). The incidence of strains isolated from patients who had been trasferred from other institutions, was 3.4 per cent. Thus more than 70 per cent of the strains originated from nosocomial infection.

Discussion

The results obtained with phage-typing of *E. coli* strains isolated from patients under hospital treatment have confirmed our previous observation of a close connection between phage-sensitivity and flagellar antigen. NICOLLE *et al.* [7] also mentioned that differences in phage-sensitivity may indicate differences in antigenic structure.

According to RAUSS *et al.* [11, 12], GYENGÉSI and BODÓ [13], KAUFFMANN and DUPONT [14], LAURELL *et al.* [15], at the same time in the same hospital strains of the same serotype and biotype occur. The examinations of RAUSS *et al.* at the *Paediatric Department, Pécs* revealed chiefly the non-flagellated variant of group O111 : B4 during epidemics [11, 12], an observation confirmed by KAUFFMANN and DUPONT [14], LAURELL *et al.* [15] and EÖRSI *et al.* [2]. RAUSS *et al.* [12] suggested the possible role of a special property of the microorganism in the development of the outbreaks.

The occurrence of different serotypes belonging to the same O group was noted by WRIGHT and RODEN [16] and by BRAUN and SPECHT [17]. In both papers the simultaneous occurrence of group O55 : B5 strains containing flagellar antigens H6, H2 and H7 was described. Recently KRÖGER *et al.* [18] at the *Clinic of Paediatrics, Göttingen* has shown the simultaneous occurrence of strains belonging to one serotype but to different phage-types.

In the present study performed in the infants' department of 5 different hospitals revealed the simultaneous occurrence of type 111/1 (non-flagellated) and 111/2 (H2) of group O111 : B4 in Hospital A. In the same hospital the former type was more predominant than the latter and caused often severe symptoms.

A similar situation was found in Hospital C.

In contrast, in Hospital B non-flagellated type 111/1 predominated and caused severe cases of enteritis, although type 111/2 (H2) was also present.

From these findings it may be concluded that in the development of an outbreak certain factors play a greater part than does the individual property of the infecting strain. Part of these factors are undoubtedly provided by a possible rapid fall in the hygienic standards of the hospital, by conditions favourable for the chain-spread of the disease when a patient representing a new infecting source is admitted. The immunological state and other diseases of the host should also be taken into consideration. In addition, however, the existance of other, unknown factors may also be assumed.

The importance of nosocomial infection in enteric disease has been stressed by many authors in recent years. GILES *et al.* [19] noted that 58.9 per cent of enteritis cases caused by O111 and O55 strains were due to nosocomial infections. The importance of interhospital infections was postulated by ROGERS and KOEGLER [20] who described the route of infection from one hospital to others. In Hungary, SOLT *et al.* [21] and LOSONCZY [22] reported on pertaining data and showed the way of a practical solution of the problem.

Acknowledgements. The authors wish to thank the *Public Health* and *Hospital Laboratories* for sending the isolated strains, the *Hospital Departments* for providing clinical and epidemiological data, DR. G. BARSY, *State Institute of Hygiene, Budapest*, for help in statistical analysis, and *Mr.* I. VARGA for skilled technical assistance. Thanks are also due to *Prof.* H. HUDEMANN, *Institute of Hygiene, Magdeburg, Germany*, for a constant supply of strains and records, and to *Prof.* K. RAUSS, *Institute of Microbiology, Pécs*, for a supply of H sera.

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EXAMINATION OF CLOSTRIDIUM PERFRINGENS WITH THE FLUORESCENT TRACER TECHNIQUE

By

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(Received July 7, 1961)

Summary. The applicability of fluorescent immunological staining for the rapid identification of *Clostridium perfringens* has been examined. The high titre antibacterial serum was labelled with the fluorescent dye *Lissamine Rhodamine RB*-200. Homologous and control smears were stained directly and indirectly. About 50 *Cl. perfringens* cultures stained with the homologous labelled serum yielded preparations showing a bright orange fluorescence of ++++ intensity. The numerous Gram positive and Gram negative control bacteria stained with the same serum gave no fluorescence. No fluorescence was obtained when *Cl. perfringens* was stained with Rhodamine-labelled non-immune rabbit serum.

Spores of *Cl. perfringens* showed a fluorescence similar to that of the vegetative forms.

The method was found suitable for the differentiation between the Clostridium types A and F. With adequately conjugated sera, the identification and typing of *Cl. perfringens*, which require several days and often 1 or 2 weeks by the usual methods, have been accomplished within 1 hour.

The causative agent of gas gangrene, *Clostridium perfringens* is widely distributed in nature. It was isolated from 100 per cent of soil samples originating from different areas and from 80 to 100 per cent of infected war casualties. The organism was described as the causative agent of pulmonary gangrene, infections of the uterus following delivery or abortion, respiratory diseases and enteritis.

Cultivation, isolation, examination of biochemical, pathogenic and serological (A or F type) properties of *Cl. perfringens* require several days, often some weeks. In the present study the use of the fluorescent tracer technique has been attempted. This technique is known for its simplicity, rapidity and high specificity.

Materials and methods

An antigen containing 1000 million cells per ml was prepared from *Cl. perfringens* strain No. 168, which had been isolated from pathological material.

Rabbits were immunized intravenously with rising doses ranging from 0.5 to 5.0 ml for a 3-week period. Each week 3 injections were given. The agglutination titre of the antibacterial serum was 1 : 10240. As a fluorochrome, *Lissamine Rhodamine RB*-200 was used, which gave an orange fluorescence under the fluorescence microscope [3]. The globulin fraction of the immune serum was precipitated with saturated ammonium sulphate.

Absorption of the serum was carried out with mouse liver powder. The smears were fixed by heat. After staining, the preparations were examined under a Leitz model fluorescence microscope, generally at a magnification of 400.

Rabbit antiglobulin was prepared in a goat by use of Freund's adjuvant.

Staining of smears was performed with both the direct and the indirect method described by Coons *et al.* [1, 2].

Results

Cl. perfringens and control Gram negative organisms were stained with unconjugated dye. Attachment of the dye to *Cl. perfringens* was distinct and the bacteria showed a bright ++++ degree of fluorescence. Gram negatives, however, exhibited a hardly visible fluorescence.

Comparison of the staining property of fluorochromized immune serum and globulin was carried out by direct staining of *Cl. perfringens* and control bacteria. Both serum and globulin showed ++++ staining with the homologous bacteria, while the control organisms gave uniformly negative results.

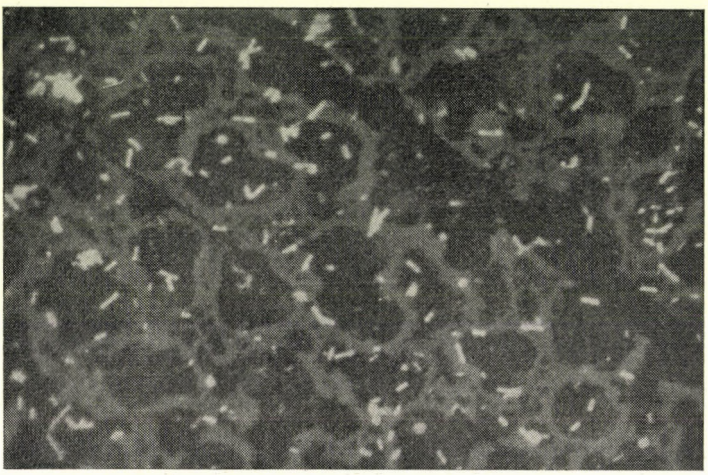

Fig. 2. Mixed smear of *Cl. perfringens* and *E. coli* stained with *Lissamine Rhodamine RB-200* labelled *Cl. perfringens* immune serum
Magnification × 400

In subsequent experiments the immune serum and globulin were absorbed with mouse liver powder as described by COONS *et al.* [2]. The staining capacity of serum and globulin for *Cl. perfringens* remained unchanged. Control bacteria showed no fluorescence.

Cl. perfringens and *E. coli* suspensions were mixed at a proportion of 1 : 10 and stained with fluorochromized serum and globulin. In the smears only bacteria corresponding in size to *Cl. perfringens* showed fluorescence; no fluorescence was exhibited by *E. coli*, only the edge of the conglomerates formed by these bacteria was visible.

With homologous serum and globulin *Cl. perfringens* and *Cl. oedematiens* were stained together. *Cl. perfringens* exhibited a ++++ degree fluorescence, while the control strain gave a negative result. Consequently, the two members of the gas gangrene group could be distinguished on the basis of fluorescence.

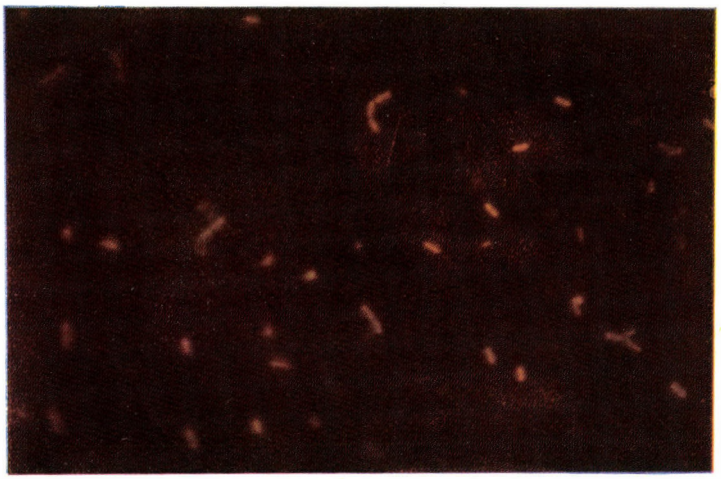

Fig. 1. Cl. perfringens stained directly with *Lissamine Rhodamine RB-200* labelled immune serum
Magnification × 400

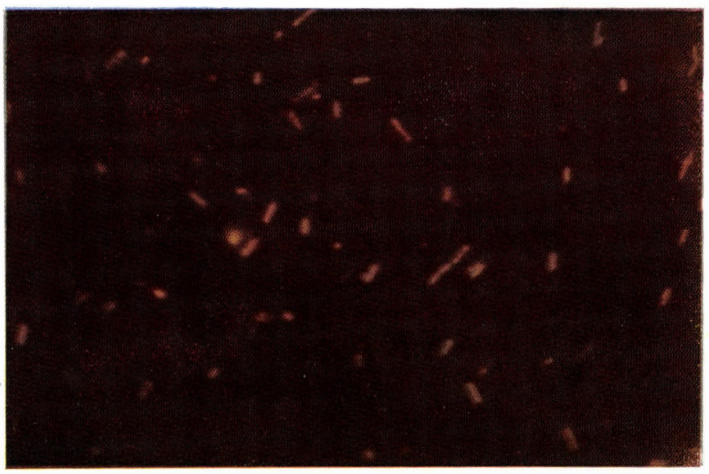

Fig. 3. Cl. perfringens indirectly stained with *Lissamine Rhodamine RB-200* labelled immune globulin
Magnification × 400

Examination of the staining capacity of labelled serum prepared from *Cl. perfringens* strain 168 for other *Cl. perfringens* cultures was considered important. Twelve F type *Cl. perfringens* strains were stained by the direct method. Other *Cl. perfringens* strains gave fluorescence of the same intensity as strain No. 168.

The fluorescent tracer method was found suitable for the differentiation between human pathogenic A and F types of *Cl. perfringens*. Typing of 50 strains was carried out with the spore-thermoresistance test. Staining with F type fluorochromized immune serum or globulin revealed that strains regarded on the basis of the thermoresistance test as type F organisms showed a ++++ positivity, while type A bacteria did not take the stain and only their faint outlines were visible.

As controls, *Cl. botulinum* type A and B, and *Cl. sporogenes* were examined. Fluorescence was exclusively shown by the homologous strains [4].

The direct staining recommended by COONS *et al.* is disadvantageous in that it requires the laborious, time and material consuming labelling of every specific immune serum.

Therefore in subsequent experiments the indirect method of COONS and KAPLAN [1] was applied. *Cl. perfringens* immune serum did not stain *Cl. oedematiens*, *Cl. botulinum* A and B, and *Cl. sporogenes*, *i.e.* the results were similar to those obtained with direct staining.

It may be concluded that the indirect method is a simple, sensitive and specific procedure for the detection of *Cl. perfringens*. The method is suitable for the identification of relatively small numbers of *Cl. perfringens* not only in pure but also in mixed cultures.

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ACTA MICROBIOLOGICA

ТОМ VIII

РЕЗЮМЕ

E. K. Novák: STUDIES ON THE OLIGOSACCHARIDE-DECOMPOSING ACTIVITY OF YEASTS

ИССЛЕДОВАНИЕ СПОСОБНОСТИ ДРОЖЖЕЙ РАСЩЕПЛЯТЬ ОЛИГОСАХАРИДЫ

Е. К. НОВАК

Авторы исследовали первичные фазы брожения рафинозы, лактозы, сахарозы и мальтозы при помощи дрожжевых грибов *Saccharomyces carlsbergensis* и *Candida pseudotropicalis*. Для исследования применялись живые клетки, подверженные обработке ацетоном и свободные от клеток гомогенизаты. Процесс ферментации проверялся методом хроматографии на бумаге.

Было установлено, что *Saccharomyces carlsbergensis* внеклеточно расщепляет рафинозу и сахарозу, причем живые клетки усваивают только моносахариды. Расщепление рафинозы оказалось этапным, а расщепление мелибиозы началось только после полного расщепления рафинозы. Внеклеточного расщепления мальтозы не удалось выявить. Расщепление мальтозы наблюдалось только в том случае, если для экспериментов применялись обработанные ацетоном дрожжи *Saccharomyces carlsbergensis*. Клетки *Candida pseutdotropicalis* усваивали лактозу без расщепления. Только в случае применения обработанных ацетоном или гомогенизированных клеток наблюдалось расщепление лактозы.

I. Mécs, E. Szőllősy, Eszter Kukán, Ilona Béládi: AETIOLOGICAL AND EPIDEMIOLOGICAL STUDIES OF POLIOMYELITIS IN SZEGED, 1956—59.

ЭТИОЛОГИЧЕСКОЕ И ЭПИДЕМИОЛОГИЧЕСКОЕ ИССЛЕДОВАНИЕ ПОЛИОМИЕЛИТА В Г. СЕГЕД В 1956—59 ГГ.

И. МЕЧ, Е. СЁЛЛЁШИ, Э. КУКАН, И. ЕЛАДИ

Излагаются опыты по изолированию энтеровируса, проведенные в 1956—1959 гг. в г. Сегед. Удалось изолировать 190 штаммов, из которых 116 оказались вирусами полиомиелита. Сообщаются данные о заболеваемости полиомиелитом жителей г. Сегед, на основании проб нейтрализации вирусов.

K. Ujhelyi, L. Ormay: A SIMPLE AND PRACTICAL METHOD FOR THE PURIFICATION OF DIPHTHERIA TOXOID

ПРОСТОЙ, ПРАКТИЧЕСКИЙ МЕТОД ДЛЯ ОЧИЩЕНИЯ ДИФТЕРИЙНОГО АНАТОКСИНА

К. УЙХЕЛЬИ, Л. ОРМАИ

Излагается способ очищения дифтерийного анатоксина, основанный на практических, простых методах. Добавление формалина к полученному на мясо-пептонном агаре в стационарных культурах, отделенному в оптимальный срок токсину более высокой концентрации проводится выбором количества, температуры и продолжительности воздействия примененного формалина. Полученный таким путем анатоксин осаждается в присутствии ионов трихлоруксуснокислого натрия с помощью трихлоруксусной кислоты при pH около 4.0. Немедленно растворенное вещество вновь осаждается трихлоруксусной

кислотой в присутствии роданистого калия. После растворения преципитата он подвергается в течение короткого времени диализу, и центрифугированием и слабым формалинированием производится окончательная детоксикация и стерилизация. Полученный таким путем антиген имеет в среднем чистоту 1000—1300LF (мг N.) Исходя из неочищенного анатоксина хорошего качества антиген может быть на 90% получен обратно. Очищенный анатоксин можно в концентрированном виде хранить в течение выше одного года без потери антигенных свойств. Связанный с преципитатом фосфата алюминия очищенный анатоксин оказывает весьма хороший иммунизирующий эффект, как в опытах на животных, так и в ходе прививок людей. Соответственно большой чистоте, изготовленная из этого анатоксина вакцина вызывает только весьма слабую реакцию на прививку.

I. Mihályfi, É. Kende, E. Jónás, Gy. Vámos:

SALMONELLA-UNTERSUCHUNGEN IN BUDAPEST IN DEN JAHREN 1956—58

ИССЛЕДОВАНИЕ МИКРОБОВ ГРУППЫ САЛЬМОНЕЛЛ В БУДАПЕШТЕ (1956—1958 гг.)

И. МИХАЛЬФИ, Е. КЕНДЕ, Е. ЙОНАШ, ДЬ. ВАМОШ

1. Авторы сообщают об исследованиях микробов группы сальмонелл, проведенных в течение трех лет (1956—58 гг.) в бактериологической лаборатории Будапештской санитарно-эпидемиологической станции. За этот период в связи с профессиональными профилактическими исследованиями и исследованиями больных из стула было изолировано 1847 штаммов сальмонелл.

2. Зараженность здорового населения определилось в среднем в 0,32%.

3. Сообщается годовое распределение случаев зараженности микробами группы сальмонелл в годы 1956, 1957 и 1958.

4. Возраст 43% больных оказался меньше 2 лет.

5. 1847 штаммов относились к 15 типам. Половина штаммов оказалась *Salmonella saint paul.*

6. Выделение возбудителя в 80% случаев прекратилось в пределах одного месяца.

L. Váczi, M. Fodor:

THE RELATION OF SURFACE PROPERTIES AND ANTIBIOTIC-RESISTANCE IN STAPHYLOCOCCUS AUREUS. III. THE EFFECT OF UNSATURATED FATTY ACIDS ON THE RESISTANCE TO ANTIBIOTICS

СВЯЗЬ МЕЖДУ ПОВЕРХНОСТНЫМИ СВОЙСТВАМИ И ЧУВСТВИТЕЛЬНОСТЬЮ К АНТИБИОТИКАМ ШТАММОВ *STAPHYLOCOCCUS AUREUS* III. ВЛИЯНИЕ НЕНАСЫЩЕННЫХ ЖИРНЫХ КИСЛОТ НА ЧУВСТВИТЕЛЬНОСТЬ К АНТИБИОТИКАМ

Л. ВАЦИ, М. ФОДОР

Авторы в присутствии антибиотиков исследовали чувствительность к ненасыщенным жирным кислотам — олеиновой кислота, линолевой кислоты, линоленовой кислоты — штаммов *Staphylococcus aureus*, обладающих различной антибиотической чувствительностью.

1. На агаровой питательной среде олеат натрия в концентрации свыше 0,12%, линолевая кислота и линоленевая кислоты в концентрации свыше 0,03% задерживают развитие чувствительных и резистентных к антибиотикам штаммов *Staphylococcus aureus*. В бульоне эти вещества обладают бактериостатическим действием только при значительно более высокой концентрации.

2. Ненасыщенные жирные кислоты уже в количестве 4 мг% значительно повышают чувствительность к пенициллину резистентных к пенициллину и полирезистентных штаммов *Staphylococcus aureus.*

3. В присутствии субстатической концентрации пенициллина олеиновая кислота, линолевая кислота и линоленовая кислота снижают потребление кислорода клеток резистентных только к пенициллину, на 48%, а полирезистентных клеток на 59%. Действие этих веществ на чувствительные к антибиотикам штаммы *Staphylococcus aureus* гораздо менее выраженное.

4. Эти ненасыщенные жирные кислоты не оказывают непосредственного действия на пенициллиназу.

5. Причиной значительного синергизма между пенициллином и ненасыщенными жирными кислотами, наблюдаемого в случае резистентных к антибиотикам штаммов *Staphylococcus aureus*, является повышенная чувствительность клеток, обладающих этими биологическими свойствами, по отношению ненасыщенным жирным кислотам.

6. Указывается на отклонение поверхности чувствительных и резистентных к антибиотикам клеток и на практическое значение этого наблюдения.

K. Rauss, I. Kétyi, A. VerténYi, S. Vörös:

STUDIES ON THE NATURE OF PHASE VARIATION OF SH. SONNEI

ИССЛЕДОВАНИЯ ИЗМЕНЕНИЯ ФАЗЫ БАКТЕРИЙ *SH. SONNEI*

К. РАУШ, И. КЕТИ, А. ВЕРТЕНЬИ, Ш. ВЁРЁШ

1. Авторы сравнивали особенности II-ой фазы **Sh. sonnei** со свойствами вариантов-R кишечных бактерий и полученного искусственным путем варианта-R. **Sh. sonnei** (отмеченного знаком Rl).

2. II-ая фаза **Sh. sonnei** в отношении морфологических и культурных особенностей совпадает с характерными особенностями вариантов R. Она в физико-химическом отношении дает одинаковую с вариантами реакцию Миллона, кислотную-агглютинацию, она чувствительна к трипафлавину, но в физиологическом растворе поваренной соли агглютинирует только в кипяченном состоянии. Следовательно II-ая фаза **Sh. sonnei** в физико-химическом отношении не обособляема от R вариантов.

3. В отношении антигенной структуры авторы подтверждают обособленность I-ой и II-ой фаз. Вариант находился только в односторонней связи со II-ой фазой, поскольку он снизил титр сыворотки II-ой фазы, а не редуцировал сыворотки Rl в титре II-ой фазы.

4. В иммунобиологическом отношении токсичность на мышах II-ой фазы была в три раз, а ее вирулентность на мышах без муцина был в пять раз ниже чем у I-ой фазы. При применении муцина наблюдалась прибл. 10 миллионнократная разница в пользу I-ой фазы. В исследованиях инвазивности внутрибрюшинно введенная I-ая фаза **Sh. sonnei** быстро размножалась, и по истечении короткого времени наступила прочная бактериемия, в то время как II-ая фаза скоро стала жертвой фагоцитов, а бактериемия наблюдалась только изредка и лишь временно. Иммунобиологический характер-R II-ой фазы подчеркивается также тем наблюдением, что у больных дизентерией Зонне — в противоположность ко II-ой фазе — не удалось выявить ни агглютинина, ни повышения титра защиты мышей.

5. Литературные данные, указывающие на обратное развитие II-ой фазы в I-ую фазу, на основании результатов авторов можно объяснить тем, что одна часть типичных колоний II-ой фазы на самом деле является смешанной культурой, причем диссоциация I-ой фазы наблюдается **in vitro** и **in vivo** также во 2-ом и 3-ем поколениях. Обратное развитие из чистой культуры II-ой фазы невозможно.

6. В ходе обсуждения авторы устанавливают, что полученный ими Rl, как и известный из литературы «третий тип колонии» предположительно являются S вариантами.

7. На основании исследований авторов изменение фазы **Sh. sonnei** следует рассматривать вариантом S—R, а II-ую фазу — вариантом R.

F. Ördögh, K. Szende:

TEMPERATE BACTERIOPHAGES ISOLATED FROM RHIZOBIUM MELILOTI

ТЕМПЕРИРОВАННЫЕ БАКТЕРИОФАГИ, ИЗОЛИРОВАННЫЕ ИЗ КЛУБНЕЙ КОРНЯ RHIZOBIUM MELILOTI

Ф. ЭРДЭГ, К. СЕНДЕ

При помощи комбинации метода тестирования Фиска с методом Ледерберга исследовались 152 линии **Rhizobium meliloti**, полученных из свежих клубней, во всех возможных комбинациях, причем 103 из исследованных линий оказались носителями бактериофагов, или лизогенными. Из нефильтрированного лизата удалось изолировать 63 фага, из которых 44 фага оказались умеренными, а —11 вирулентными, в то время как 8 фагов дали отрицательный результат тестирования. При исследовании соотношения фага-хозаина 8 из умеренных фагов оказались поливалентными, 10 обладали средней вирулентностью, а 23 — незначительной вирулентностью. Три чувствительных линии **Rh. meliloti** были заражены 40 различными умеренными фагами в целях лизогенизации. У 13 из полученных лизогенных линий исследовались изменения, происходившие в соотношении фага-хозяина. На основании исследований 17 фагов удалось отнести к 7 родственным группам или определить их тожественность.

M. Járai:

GENETIC RECOMBINATION IN STREPTOMYCES AUREOFACIENS

ГЕНЕТИЧЕСКАЯ РЕКОМБИНАЦИЯ STREPTOMYCES AUREOFACIENS

М. ЯРАИ

Автор наблюдал у **Streptomyces aureofaciens** генетическую рекомбинацию. Для опытов использовались 10 однократных и двукратных ауксотрофных штаммов. Ауксотрофные штаммы можно было на основании их происхождения отнести к 3 различным по линии группам.

Для наблюдения генетической рекомбинации применялась смесь комплементарных ауксотрофных штаммов в соотношении 1:1, на дополненной питательной среде, содержащей только определенные факторы роста, в совсем незначительном количестве. На этой питательной среде рекомбинированные ауксотрофные штаммы образовали хорошо разграниченные колонии.

Исследовалась антибиотическая активность рекомбинированных ауксотрофных штаммов. В ряде случаев наблюдалось значительное повышение производства антибиотика, по сравнению с исходными прототрофными штаммами на 40—60%.

При анализе происхождения рекомбинированных ауксотрофных штаммов с высокой антибиотической активностью, наблюдалось, что из комплементарных ауксотрофных штаммов с различной линией получаются в бóльшем количестве рекомбинированные штаммы с хорошим производством, чем из биохимических парных мутантов одинаковой линии. Это указывает на то что рекомбинация происходит у различных групп биохимических мутантов неодинаковым способом, и следует предполагать определенностью взаимосвязь между механизмом генетической рекомбинации и антибиотической активно полученных рекомбинированных штаммов.

M. JÁRAI:

TRANSFORMATION IN STREPTOMYCES

ТРАНСФОРМАЦИЯ У STREPTOMYCES

М. ЯРАИ

Автор наблюдал с очищенными препаратами дезоксирибонуклеиновой кислоты Streptomyces трансформацию ауксотрофных штаммов **Streptomyces aureofaciens**. При помощи примененного им метода удалось выявить несколько количественных взаимосвязей.

Проводились опыты с дезоксирибонуклеиновой кислотой прототрофных и ауксотрофных штаммов **Streptomyces aureofaciens** на минимальной питательной среде с однократными и двукратными ауксотрофными штаммами. Было установлено, что для возникновения одного трансформированного штамма необходимо приблизительно 10^{-4} μг дезоксирибонуклеиновой кислоты. При сравнении с количеством дезоксирибонуклеиновой кислоты, необходимом для трансформирования двукратных ауксотрофных штаммов, наблюдалось, что для трансформации 1-ого двукратного ауксотрофного штамма требовалось не в два раза, а в 7,1 раза больше дезоксирибонуклеиновой кислоты. Не прямое соотношение между числом трансформированных свойств и количеством дезоксирибонуклеиновой кислоты предположительно можно объяснить тем фактом, что прорастающий конидий труднее осваивает дезоксирибонуклеиновую кислоту.

Существует логарифмическое соотношение между количеством примененной дезоксирибонуклеиновой кислоты и числом трансформированных штаммов. Доза ультрафиолетового облучения дезоксирибонуклеиновой кислоты также показывает экспоненциальную связь с инактивизирующим действием облучения на активность трансформации.

В серии опытов удалось наблюдать межспецифическую трансформацию с дезоксирибонуклеиновой кислотой **Streptomyces griseus** у двух ауксотрофных штаммов **Streptomyces aureofaciens**. Для возникновения межспецифической трансформации необходимо около 10^{-3} μг дезоксирибонуклеиновой кислоты, значит больше, чем для внутривидовой трансформации. Не удалось наблюдать межспецифической трансформации с дезоксирибонуклеиновой кислотой **Streptomyces rimosus** у ауксотрофных штаммов **Streptomyces aureofaciens**, ни с дезоксирибонуклеиновой кислотой **Streptomyces griseus** у нескольких ауксотрофных штаммов **Streptomyces aureofaciens**.

GY. VÖRÖS-FELKAI, E. K. NOVÁK:

INCIDENCE OF YEASTS IN HUMAN MATERIAL

ДРОЖЖЕВЫЕ ГРИБКИ В МАТЕРИАЛЕ ИССЛЕДОВАНИЯ, ВЗЯТОМ ОТ ЛЮДЕЙ

ДЬ. ВЁРЁШ-ФЕЛКАИ, Е. К. НОВАК

Авторы выращивали, из материала исследования 1200 больных с подозрением на грибковое заболевание, 443 штамма дрожжевых грибков. При идентификации полученных штаммов методами Лоддера и Крегер-Ван-Рия, их можно отнести к 9 родам и 32 видам. Большая часть изолированных штаммов происходит главным образом из выделений зева и бронхов, и тождественные с болезнетровными видами **Candida albicans, Candida pseudotropicalis, Candida tropicalis, Candida Crusei**. Из выделений дыхательных путей и других выделений в большем количестве были изолированы также дрожжевые грибки, рассматриваемые до сих пор как сапрофиты.

В выделениях дыхательных путей в большом количестве были обнаружены виды **Candida**, штаммы **Geotrichum**: **Rhodotorula mucilaginosa** и **Torulopsis inconspicua**. Из крови удалось изолировать **Candida albicans, Cryptococcus diffluens** и **Rhodotorula mucilaginosa**. Из желчи были выращены кроме **Candida albicans** главным образом виды **Rhodotorula**. В желудочном содержимом преимущественно встречались виды **Candida** и **Geotrichum**. В секционном материале чаще всего встречался вид **Candida crusei**. Патогенность изолированных штаммов исследовалась на белых мышах, которым делались прививки внутрибрюшинно. Патогенными для мышей оказались известные болезнетворные виды **Candida**, штаммы **Geotrichum** и **Cryptococcus neoformans**. У мышей патологические изменения и гибель животных вызывали также виды **Candida utilis, Rhodotorula mucilaginosa, Hansenula subpelliculosa, Saccharomyces marxianus, Saccharomyces fragilis**.

Gy. Vörös-Felkai:

STUDIES ON ARTHROSPOROUS YEASTS

ИССЛЕДОВАНИЕ АРТРОСПОРОВЫХ ДРОЖЖЕВЫХ ГРИБКОВ

ДЬ. ВЁРЁШ-ФЕЛКАИ

Дополнением метода исследования Лоддера и Крегер-Ван-Рия с пробой на восстановление визмута, разжижжением желатины и исследованием чувствительности к актидиону удалось идентифицировать 26 артроспоровых штаммов дрожжевых грибков. Эти 26 штаммов относятся к одному кругу. Среди обнаруженных штаммов 14 были **Geotrichum candidum**, 4 **Trichosporon cutaneum**, 2 **Geotrichum matalanse**, 6 **Geotrichum linkii n. sp.**

Автор предлагает переместить род **Geotrichum**, причисляемый раньше к семейству **Mucedinaceae** серии **Hyphomycetes**, в подсемейство **Trichosporoideae** семейства **Cryptococcaceae.**

Дается описание нового вида, под названием **Geotrichum linkii n. sp.**

O. Rudnai, G. Barsi:

THE RESULTS OF SALK VACCINATION IN HUNGARY AS MEASURED ON THE 1959 POLIOMYELITIS EPIDEMIC

РЕЗУЛЬТАТЫ ВАКЦИНАЦИИ ПО САЛКУ В СВЕТЕ ЭПИДЕМИИ ПОЛИОМИЕЛИТА 1959 ГОДА В ВЕНГРИИ

О. РУДНАИ, Г. БАРШИ

Из 1830 случаев заболевания полиомиелитом во время эпидемии 1959 года в Венгрии удалось выяснить данные вакцинации 1769 больных. На основании разработки полученного материала можно было установить, что

1. только 35,4% больных получили перед заболеванием 3 или 4 прививки. Ввиду того, что 80% затронутой популяции были привиты три раза, то вышеприведенное соотношение уже указывает на эффективность предварительной прививки.

2. Поражает высокое, почти 57%-ное соотношение лиц, заболевших вопреки трехкратной прививки, получившие третью вакцинацию свыше года до заболевания.

3. Наибольший эффект предохранительной прививки дает прививка, полученная за восемь месяцев до эпидемии. Поразительно, что почти равноценным оказалось действие первых двух прививок, полученных за три-четыре месяца до вспышки эпидемии. Наименьшее защитное действие наблюдалось у лиц получивших третью прививку за 16 месяцев до начала эпидемии. Следовательно, результаты исследований говорят за то, что предохранительная ценность полной иммунизации по истечению одного года уже заметно снижается.

4. При помощи проводимых с 1957 года прививок по Салку в 1959 году удалось достигнуть по крайней мере 60%-ной иммунизации.

5. Смертность больных, получивших предохранительные прививки была значительно меньше той непривитых лиц.

J. Földes:

SOME OBSERVATIONS ON THE MECHANISM OF THE MIDDLEBROOK-DUBOS HAEMAGGLUTINATION TEST

О МЕХАНИЗМЕ ГЕМАГГЛЮТИНАЦИИ ПО МИДДЛБРУК-ДЮБО

Й. ФЁЛЬДЕШ

Автор изучал активность полисахаридов и протеинов, изолированных из **Mycobacterium tuberculosis** на основании теста по Миддлбрук—Дюбо. Полисахарид с обозначением PI способен адсорбироваться красными кровяными тельцами, в то время как полисахарид PII не обладает этой способностью. Обработка эритроцитов трипсином, пепсином, папаином или таннином (при 37° C) не влияло на адсорбцию полисахаридов. Обработанные таннином эритроциты при комнатной температуре в течение 10 минут не адсорбируют вытяжку PI.

Вытяжки туберкулезной палочки протеинового характера проявляют на основании адсорбции эритроцитами однородное поведение. Адсорбционная способность эритроцитов повысилась больше всего после обработки таннином, но эффект обработки папаином также был весьма выраженным.

В случае применения клеток, обработанных таннином, а затем сенсибилизированных протеином, способность вытяжек протеинового характера задерживать гемаглютинацию, соразмерна содержанию полисахаридов, связанных с протеином.

Реакция Шмидт Таннгаузера прекращает сенсибилизирующую способность вытяжки PI.

На основании достигнутых результатов автор дает объяснения реакции Миддлбрук—Дюбо.

T. Angyal:

STUDIES ON THE CLASSIFICATION, PATHOGENITY AND ANTIGENIC STRUCTURE OF STAPHYLOCOCCI. I. THE OCCURRENCE AND PATHOGENICITY OF MICROCOCCACEAE

ИССЛЕДОВАНИЕ СИСТЕМАТОЛОГИИ И АНТИГЕННОЙ СТРУКТУРЫ СТАФИЛОКОККОВ. I. ИССЛЕДОВАНИЯ ЧАСТОТЫ МИКРОКОККОВ И ПРОБЫ НА ПАТОГЕННОСТЬ

Т. АНДЬАЛ

Автор в ходе повседневной работы изолировал 311 штаммов, относящихся к семейству **Micrococaceae** среди которых 233 оказались стафилококками, а 75 микрококками. Систематологического места 3 штаммов не удалось выяснить.

Систематологическое определение сравнительно часто выделенных **Micrococcus ureae, Micrococcus luteus** и **Micrococcus flavus** при помощи простого морфологического исследования наталкивается на трудности, ввиду возможности их смешивания со стафилококками.

У видов микрококков удалось выявить гиалуронидазу.

У штаммов **Micrococcus** и **Staphylococcus (epidermidis)** не удалось выявить коагулазной активности. На этом основании коагулазную активность можно рассматривать обязательным свойством патогенных стафилококковых штаммов.

Коагулазо-отрицательного штамма **Staphylococcus aureus** удалось выделить в 13 случаях. Они были изолированы в связи с сепсисом.

Если из крови выращивается коагулазо-отрицательный штамм, то следует провести также другие пробы на патогенность (гиаза, фосфатаза).

G. Ujváry, T. Angyal, L. Tóth:

STUDIES ON THE CLASSIFICATION, PATHOGENICITY AND ANTIGENIC STRUCTURE OF STAPHYLOCOCCI. II. SEROLOGICAL TYPING OF STAPHYLOCOCCUS AUREUS.

ИССЛЕДОВАНИЯ СИСТЕМАТОЛОГИИ И АНТИГЕННОЙ СТРУКТУРЫ СТАФИЛОКОККОВ. II. СЕРОЛОГИЧЕСКИЕ ИССЛЕДОВАНИЯ ШТАММОВ STAPHYLOCOCCUS AUREUS

Г. УЙВАРИ, Т. АНДЬАЛ, Л. ТОТ

Авторы при помощи метода Эдинга определили серотип 192 штаммов **Staphylococcus aureus** происходящих из гноеродных инфекций.

Чаще всего обнаруживались штаммы четвертой группы.

Обособлялись 42 серотипа, из которых 22 оказались новыми серотипами.

В случаях фурункула и флегмоны встречались главным образом штаммы группы 4.

Между серотипами, патогенностью и чувствительностью к антибиотикам не удалось выявить взаимосвязи.

Авторы обращают внимание на тот факт, что при эпидемиологических исследованиях кроме определения фаготипа и чувствительности к антибиотикам весьма ценным методом может быть также определение антигенной структуры.

T. Angyal:

STUDIES ON THE CLASSIFICATION, PATHOGENICITY AND ANTIGENIC STRUCTURE OF STAPHYLOCOCCI. III. EXAMINATION OF STAPHYLOCOCCI ISOLATED FROM FAECES

ИССЛЕДОВАНИЯ СИСТЕМАТОЛОГИИ, ПАТОГЕННОСТИ И АНТИГЕННОЙ СТРУКТУРЫ СТАФИЛОКОККОВ. III. ИССЛЕДОВАНИЕ СТАФИЛОКОККОВ, ИЗОЛИРОВАННЫХ ИЗ КАЛА

Т. АНДЬАЛ

Автор из 539 образцов кала изолировал 174 штаммов **Staphylococcus aureus**, 104 штамма **Staphylococcus epidermidis** и 12 штаммов **Micrococcus**. В пробах на патогенность фекальные штаммы **Staphylococcus aureus** показали одинаковое поведение со штаммами, происходящими из гноеродных инфекций. Резистентные к антибиотикам штаммы стафилококков встречались среди фекальных штаммов гораздо чаще, чем среди штаммов, изолированных из гнойных процессов.

У штаммов **Staphylococcus aureus** автор обособлял 51 серотип, а у штаммов **Staphylococcus albus** 25 серотипов.

Автор приходит к тому заключению, что выращивание одного штамма **Staphylococcus aureus** из кала в связи с энтеритом не означает, что данный бактерий находится в этиологической связи с этим заболеванием.

B. Serény:

VARIANTS OF SH. SONNEI PHASE I.

ВИДОИЗМЕНЕНИЯ I ФАЗЫ SH. SONNEI

Б. ШЕРЕНЬ

Автор при косом освещении и при постепенном изменении угла впадения света проводил исследование морфологии колоний, причем он в случае культур **Sh. sonnei** дифференцировал 6 форм колоний, каждая из которых содержала антигены I-ой фазы. Между вирулентностью этих вариантов была выявлена значительная разница. Путем примененного автором метода, становится возможным выбрать культуры соответствующей вирулентности. Один вариант, содержащий почти исключительно антигены I-ой фазы, оказался невирулентным, а самой большой вирулентностью обладал вариант, в колониях которого обнаруживались антигены I-ой и II-ой фаз. В соответствии с этими наблюдениями по мнению автора вирулентность культур **Sh. sonnei** вызывается не исключительно только антигенами I-ой фазы.

E. Scheiber, T. Kákosy, E. T. Gláz:

EFFECT OF ANTIFUNGAL AGENTS ON THE FAECAL YEAST FLORA OF MICE

ДЕЙСТВИЕ ФУНГИЦИДНЫХ СРЕДСТВ НА ФЛОРУ ДРОЖЖЕВЫХ ГРИБКОВ В КИШЕЧНОМ ТРАКТЕ МЫШЕЙ

Е. ШЕЙБЕР, Т. КАКОШИ, Е. Т. ГЛАЗ

Авторы исследовали действие различных фунгициодных веществ на флору дрожжей в кишечном тракте мышей, развивавшуюся во время кормления окситетрациклином. Введение через рот нистатина, трихомицина, трипафлавина, трихотецина, флавофунгина, витамина K_5 и пентахлорфенола в возрастающей в порядке перечисления мере снизило число дрожжевых клеток в испряжнении мышей, по сравнению с исходной величиной. Генцианвиолет, оксибен B, 8-окси-хинолин в примененной дозе оказались неэффективными. Оказавшиеся in vivo эффективными вещества исследовались также in vitro против Candida albicans и Torulopsis glabrata.

G. Cseh, I. Szabó:

STUDIES ON THE MECHANISM OF PROPERDIN-ZYMOSAN REACTION. THE ROLE OF POLYSACCHARIDE STRUCTURE IN COMBINATION WITH PROPERDIN

ИССЛЕДОВАНИЯ МЕХАНИЗМА РЕАКЦИИ ПРОПЕРДИН-ЗИМОЗАН. РОЛЬ ПОЛИСАХАРИДНОЙ СТРУКТУРЫ В СВЯЗЫВАНИИ ПРОПЕРДИНА

Г. ЧЕХ, И. САБО

Авторы исследовали способность зимозана, полисахарида клеточной стенки Saccharomyces cerevisiae, связывать пропердин, по сравнению с ее неспецифической способностью к адсорбции белков. На основании сравнения поведения различных полисахаридов (в том числе участвующих в построении зимозана глюкана, маннана и гликогена, далее целлюлозы, крахмала, инулина, а также и ацетилового производного зимозана) и поведения неорганических адсорбентов (активный уголь, каолин, гель фосфата кальция) авторы старались сделать заключения о значении структуры поверхности полисахаридов в деле связывания пропердина.

При добавлении определенного количества упомянутых соединений к сыворотке с известным содержанием пропердина выявилось, что эти соединения связывают весьма различные количества пропердина и что размер неспецифической адсорбции белков не протекает параллельно с этим. Зимозан связывает самое большое количество пропердина, причем специфическая способность зимозана связывать пропердин также весьма высока.

Авторы выявили, что из растворимых в воде полисахаридов в присутствии двувалентных катионов маннан, в соответствии с ингибицией гаптена, задерживает связывание пропердина к зимозану. Гликоген не оказывает такого действия.

Реакция глюкана коренным образом отличается от реакции зимозана, так как глюкан не требует присутствия двувалентных катионов и образует очень лабильную связь с пропердином (его, вместе с другими сывороточными белками, уже при ионной силе 0,15 можно извлекать из глюкана).

Результаты исследований указывают на то, что полисахаридная структура зимозана предоставляет возможность для сравнительно очень специфического связывания пропердина, и что это свойство связано со структурной особенностью компонента маннана.

I. Dömök, E. Molnár:

ENTEROVIRUS STUDIES IN CONNECTION WITH THE 1959 POLIOMYELITIS EPIDEMIC IN HUNGARY

ИССЛЕДОВАНИЯ ЭНТЕРОВИРУСОВ В СВЯЗИ С ЭПИДЕМИЕЙ ПОЛИОМИЕЛИТА В 1959 ГОДУ В ВЕНГРИИ

И. ДЁМЁК, Е. МОЛЬНАР

В 1959 г. во время одной из самых тяжелых эпидемий полиомиелита в Венгрии, авторы пытались выявить энтеровирусы на 2329 материалах исследований 1822 лиц. Из 800 материалов исследований 746 лиц удалось изолировать вирусные штаммы. Из изолированных штаммов 630 относились к типу полио 1, три штамма к типу полио 2, и 31 штамм к типу полио 3; 39 штаммов входили в группу Коксаки, 53 в группу ECHO, в то время как 27 штаммов еще неидентифицированы. Из материала исследования 17 больных изолировали штаммы, относящиеся одновременно к нескольким типам вирусов. Авторы сообщают результаты изолирования вирусов в группировке по клиническому диагнозу, по возрасту больных, их месту жительства и сроку заболевания. Среди исследованных лиц у 625 больных окончательным диагнозом был полиомиелит (34,1% всех случаев, зарегистрированных в 1959 году). Из 73,6% материала исследований больных авторы выращивали вирусные штаммы типа полио 1.

Среди 16 больных полиомиелитом и 89 больных асептическим менингитом, у которых не удалось изолировать вируса, в крови 10 больных удалось выявить повышение титра антител 1. типа вируса полиомиелита.

В одном будапештском детском коллективе, в течение 14 дневного периода после одного случая заболевания полиомиелитом, вызванным вирусным типом полио 1, полиовирусом заражались 81% детей, в то время как занесенный одновременно в коллектив штамм Коксаки типа B2 не разносился в коллективе, предположительно вследствие интерференции.

L. Váczi, L. Farkas:

ASSOCIATION BETWEEN LIPID METABOLISM AND ANTIBIOTIC SENSITIVITY I. THE LIPID COMPOSITION OF ANTIBIOTIC SENSITIVE AND RESISTANT STAPHYLOCOCCUS AUREUS STRAINS

ВЗАИМОСВЯЗИ МЕЖДУ ЛИПОИДНЫМ ОБМЕНОМ И ЧУВСТВИТЕЛЬНОСТЬЮ БАКТЕРИЙ К АНТИБИОТИКАМ. I. СОСТАВ ЛИПОИДОВ ЧУВСТВИТЕЛЬНЫХ И РЕЗИСТЕНТНЫХ ШТАММОВ STAPHYLOCOCCUS AUREUS

Л. ВАЦИ, Л. ФАРКАШ

Авторы исследовали состав липоидов и компонентов жирной кислоты двух штаммов **Staphylococcus aureus** с различной чувствительностью к антибиотикам.

Чувствительный к антибиотикам штамм **Staphylococcus aureus** содержал 3,6% липоидов, а полирезистентный штамм — 5,3% липоидов. Значительное отклонение было найдено у двух штаммов также в содержании фосфатидов. Общее содержание фосфатидов полирезистентного штамма почти в два раза больше, а содержание фосфатидов кефалинового типа почти в 2,5 раза больше содержания в чувствительных штаммах.

Общее содержание липоидов весьма чувствительного к антибиотикам штамма **Streptococcus haemolyticus** и в пределах этого также содержание фосфатидов оказались весьма низкими (2,5% или же 0,7%).

Между фракциями жирной кислоты исследованных штаммов **Staphylococcus** не было найдено достоверной разницы. В твердой фракции жирной кислоты удалось выявить 5 компонентов (арахиновая кислота, стеариновая кислота, пальмитиновая кислота, миристиновая кислота и одна точно еще неопределенная жирная кислота с 19 углеродными атомами). В жидкой фракции жирных кислот также были обнаружены 5 главных компонентов, не являющихся ненасыщенными жирными кислотами У обоих штаммов **Staphylococcus aureus** ненасыщенные жирные кислоты были выявлены только в весьма незначительном количестве.

L. Váczi, M. Fodor, L. Farkas:

ASSOCIATION BETWEEN LIPID METABOLISM AND ANTIBIOTIC SENSITIVITY. II. THE INFLUENCE OF ESTERASE INHIBITORS ON THE ANTIBIOTIC SENSITIVITY OF STAPHYLOCOCCUS AUREUS STRAINS

ВЗАИМОСВЯЗЬ МЕЖДУ ЛИПОИДНЫМ ОБМЕНОМ И ЧУВСТВИТЕЛЬНОСТЬЮ БАКТЕРИЙ К АНТИБИОТИКАМ. II. ДЕЙСТВИЕ ИНГИБИТОРОВ ЭСТЕРАЗЫ НА ЧУВСТВИТЕЛЬНОСТЬ К АНТИБИОТИКАМ ШТАММОВ STAPHYLOCOCCUS AUREUS

Л. ВАЦИ, М. ФОДОР, Л. ФАРКАШ

Авторы исследовали действие ингибиторов эстеразы на чувствительность к антибиотикам штаммов **Staphylococcus aureus**, и установили, что липолитическая активность стафилококков, чувствительных к антибиотикам и резистентных только в отношении пенициллина, повышена, по сравнению с активностью полирезистентных штаммов. Среди ингибиторов эстеразы параоксон, метилпаратиооксон и олеат натрия в количестве 0,5 мг% повышают чувствительность к пенициллину и в меньшей мере чувствительность к террамицину стафилококков, резистентных к антибиотикам. Метилпаратиооксон даже в большой концентрации только в незначительной мере снижает активность эндопенициллиназы стафилококков, однако, он весьма сильно задерживает синтез фермента. Содержание фосфатидов у полирезистентных стафилококков, выращенных в присутствии метилпаратиооксона, сильно сокращается, и состав их содержания липоидов подобен составу липоидов у стафилококков, чувствительных к антибиотикам.

A. M. Kelemen, A. Simon:

VITAMIN B_{12} ANTAGONIST ISOLATED FROM PROPIONBACTERIUM SHERMANII I. ISOLATION, STRUCTURE AND MICROBIOLOGICAL EXAMINATIONS

АНТАГОНИСТ ВИТАМИНА B_{12}, ИЗОЛИРОВАННЫЙ ИЗ PROPIONBACTERIUM SHERMANII. I. ИЗОЛИРОВАНИЕ, СТРУКТУРА И МИКРОБИОЛОГИЧЕСКОЕ ИССЛЕДОВАНИЕ

А. М. КЕЛЕМЕН, А. ШИМОН

Из **Propionbacterium shermanii** было изолировано вещество (фактор X), которое в противоположность витамину B_{12}, задерживает рост E. coli. Это вещество кислой природы авторы выкристаллизировали.

Авторы определили инкубационный показатель фактора X и установили, что он является компететивным ингибитором витамина B_{12}.

Структура фактора X была определена на основании ультрафиолетового спектра, далее отожествления с искусственными продуктами гидролиза и обратным превращением в витамин B_{12}.

A. M. Kelemen, A. Simon

A VITAMIN B_{12} ANTAGONIST ISOLATED FROM PROPIONBACTERIUM SHERMANII II. INVESTIGATIONS ON THE ORIGIN OF FACTOR X.

АНТИГОНИСТ ВИТАМИНА B_{12}, ИЗОЛИРОВАННЫЙ ИЗ PROPIONBACTERIUM SHERMANII. II. ИССЛЕДОВАНИЕ ПРОИСХОЖДЕНИЯ ФАКТОРА X.

А. М. КЕЛЕМЕН, А. ШИМОН

Авторы в опытах, проведенных с радиоактивным витамином B_{12} и путем изолирования при нейтральной реакции доказали, что монокарбонокислое производное витамина B_{12}, изолированное из **Propionbacterium shermanii** не является артефактом, а оно ферментативного происхождения.

Количество монокарбоновой кислоты B_{12} в процессе ферментации постепенно уменьшается, и следовательно, можно предполагать, что она получается не из витамина B_{12}, а является промежуточным продуктом биосинтеза витамина B_{12}.

A. M. Kelemen, A. Simon:

MICROBIOLOGICAL EXAMINATION OF A NATURAL VITAMIN B_{12} ANTAGONIST

МИКРОБИОЛОГИЧЕСКОЕ ИССЛЕДОВАНИЕ ЕСТЕСТВЕННОГО АНТАГОНИСТА ВИТАМИНА B_{12}

А. М. КЕЛЕМЕН, А. ШИМОН

Из культуры одного штамма **Propionbacterium shermanii** удалось изолировать значительное количество вещества с антагонистическим действием по отношению к витамину B_{12}. Авторы доказали, что данное вещество является компететивным антагонистом витамина B_{12} и разработали простой метод для исследования компететивного антагонизма.

Для количественного определения антагонистического фактора разработан агар-диффузионный метод. Авторы пытаются теоретически объяснить явления, наблюдаемые при экспериментах, проводимых этим методом.

J. Böszörményi:

ANTISTREPTOLYSIN-O TITRE OF HEALTHY ADULTS IN BUDAPEST

ТИТР АНТИСТРЕПТОЛИЗИНА-О У ЗДОРОВЫХ ВЗРОСЛЫХ БУДАПЕШТСКИХ ЖИТЕЛЕЙ

Й. БЁСЁРМЕНЬИ

Автор установил, что средний титр антистрептолизина-О 600 здоровых доноров равняется 125,6 ед., в пределах рассеяния 63,8—247,2 ед. У 16,66% исследованных лиц титр был выше 247,2 ед. а титр 70,5% исследованных лиц оказался в пределах рассеяния.

Численное распределение случаев около исчисленной средней величины с единицей титра в 30—470 придерживается нормального распределения по Гауссу, но титр с 25—30 и 555—800 единицами встречался в значительно большем количестве, чем это можно было ожидать на основании нормального распределения по Гауссу.

Средний титр по отдельным возрастным группам начиная с 18—20 летнего возраста до более старшего, постепенно снижался. Средний титр у женщин более высокий, чем у мужчин, но разница недостоверная.

Титр у лиц с высокими показателями с весеннего времени в течение нескольких месяцев незначительно снижается, но в осеннее время титр вновь повышается.

Авторы проводили титрование видоизмененным методом. Метод технически проще и дает более точные и математически лучше разрабатываемые результаты, чем примененные до сих пор методы титрования, так как расстояние между отдельными цифрами при титровании характеризуются всегда коэффициентом $\sqrt[4]{2}$. Поэтому статистические исчисления проводились не фактическими цифрами титрования, а простыми порядковыми числительными («ступени величин») и только конечные результаты переводились на величину титра.

M. Kecskés, E. Manninger:

ÜBER DIE EMPFINDLICHKEIT DER BODENMIKROORGANISMEN GEGEN ANTIBIOTIKA. V. DIE WIRKUNG VERSCHIEDENER ANTIBIOTIKA AUF DAS WACHSTUM DER RHIZOBIEN

ЧУВСТВИТЕЛЬНОСТЬ ПОЧВЕННЫХ МИКРООРГАНИЗМОВ К АНТИБИОТИКАМ. V. ВЛИЯНИЕ РАЗЛИЧНЫХ АНТИБИОТИКОВ НА РОСТ РАЗЛИЧНЫХ ШТАММОВ RHIZOBIUM

М. КЕЧКЕШ, Е. МАННИНГЕР

Авторы исследовали чувствительность к антибиотикам (неомицин, хлортетрациклин, гидрат тетрациклина, эритроциклин, эритромицин и грубилин натрия) 8 бактериальных штаммов **Rhizobium**, изолированных из трех различных мотыльковых растений взятых с различных районов страны. На основании экспериментов установлено, что самыми эффективными антибиотиками на исследованные микроорганизмы оказались неомицин и хлортетрациклин.

L. Nyiri:

VARIABILITY OF STREPTOMYCES RIMOSUS

ИССЛЕДОВАНИЯ ИЗМЕНЧИВОСТИ STREPTOMYCES RIMOSUS

Л. НЬИРИ

Автор сравнивал культуры, морфологические, физиологические и биохимические особенности естественных и искусственных вариантов вида **Streptomyces rimosus**, синтезирующего окситетрациклин. На примененным автором питательных средах консервативными свойствами оказались форма колоний, форма спороносцев и споры, способности этого вида к синтезу антибиотика и окраска вегетативных мицелий. Разница проявлялась в росте на различных питательных средах, в окраске воздушных мицелий и в способности к спорообразованию. Изменчивость наблюдалась в отношении физиологических и биохимических свойств. Отдельные видоизменения не оказывали антагонистического действия по отношению друг к другу.

E. Szabó, J. Csongor, B. Csaba, L. Kocsár, L. Kesztyűs:

DIE VERTEILUNG VON COLI-ENDOTOXIN IM KANINCHENORGANISMUS WÄHREND DER SCHWARTZMANNSCHEN REAKTION

РАСПРЕДЕЛЕНИЕ ЭНДОТОКСИНА КИШЕЧНОЙ ПАЛОЧКИ У КРОЛИКОВ В ХОДЕ РЕАКЦИИ ШВАРЦМАНА

Е. САБО, Й. ЧОНГОР, Б. ЧАБА, Л. КОЧАР и Л. КЕСТЬЮШ

Согласно прежним исследованиям авторов хромированный эндотоксин непригоден для подготовки феномена Шварцманна. Для получения феномена Шварцманна они применяли меченый P^{32} эндотоксин кишечной палочки и установили, что хромированный эндотоксин не влияет в существенной мере на распределение эндотоксина в животном организме. Наибольшее количество его после подготовки феномена связывается в печени, а затем в легких, в почках и сердце, точно так же как это было установлено с другими макромолекулярными антигенами. На основании определения специфической активности авторам удалось выявить, что способность подготовленной поверхности кожи связывать эндотоксин повышается — по сравнению с нормальной кожей — на 40%. Кожа в течение первого часа связывает то количество эндотоксина, которое выявляемо в момент появления геморрагической реакции (4 часа после внутривенной инъекции). Спустя 24 часа содержание эндотоксина прибл. одинаковое, как в подготовленной, так и в нор-

мальной коже. Предполагается, что введенный при разрешении феномена Шварцманна эндотоксин попадает в геморрагическое пятно вместе с лейкоцитами. Было установлено, что примененный для подготовки реакции Шварцманна эндотоксин с места препарирования в преобладающей части всасывается и не накапливается, и следовательно он оказывает свое действие на развитие феномена Шварцманна не прямым путем, а через подробно еще невыясненный механизм, действующий непосредственно на клетки или капилляры подготовленной области.

J. Szathmáry:

A STANDARDIZED METHOD FOR THE DETERMINATION OF STAPHYLOCOCCAL ALPHA-ANTITOXIN

СТАНДАРТНЫЙ МЕТОД ДЛЯ ОПРЕДЕЛЕНИЯ СТАФИЛОКОККОВОГО АЛЬФА АНТИТОКСИНА

Й. САТМАРИ

Автор проводил исследования в целях повышения точности гемолитического метода, применяемого для определения стафилококкового антитоксина. При помощи разработанного на основании полученных результатов метода, величины титра даже при низком уровне титрования (1/100 I. E.) показывают рассеяние лишь ± 10%. Метод сводится к нижеследующему:

Разведение сыворотки и токсина, и изготовление суспенсии эритроцитов производятся физиологическим раствором поваренной соли, содержащем 0,1% желатины, при pH 7,2. Желатина хорошо стабилизирует легко разрушаемое разведение токсина, но кроме того, все манипуляции с токсином: разбавление, размер и число встряхиваний после добавления суспенсии эритроцитов, производятся весьма осторожно.

Эритроциты берутся, по возможности, всегда у одних и тех же кроликов, или у таких кроликов, чувствительность которых к токсину почти одинакова, и сыворотка крови которых не содержит стафилококкового антитоксина.

Гемолиз определяется невооруженным глазом после инкубации при температуре 37° C, либо после немедленного центрифугирования, либо 20—30 мин. после выемки из водяной бани, причем предельной величиной считается самый первый признак гемолиза.

Постановка контрольной серии из пяти пробирок повышает точность титрования, причем средняя пробирка содержит из стандартной сыворотки количество антитоксина, соответствующее уровню титрования, а остальные пробирки содержат на 10 и 20% меньше или больше антитоксина. Если в контрольной серии самый первый признак гемолиза появляется не в средней (3) пробирке, то полученный в результате исследования «титр» следует умножить на 0,8—1,2 чтобы получить фактический титр исследованной сыворотки.

I. Gadó, A. Szentirmai, K. Steczek, I. Horváth:

METABOLIC STUDIES WITH STREPTOMYCES RIMOSUS

ИССЛЕДОВАНИЯ ОБМЕНА ВЕЩЕСТВ У ВАРИАНТОВ

И. ГАДО, А. СЕНТИРМАИ, К. ШТЕЦЕК, и И. ХОРВАТ

Хранение споры штамма **BS-21 Streptomyces rimosus** при температуре —10° C или же серийная перевивка этого штамма вызывает уменьшение синтеза окситетрациклина. Снижение производства сопровождается изменением морфологических свойств и активности обмена веществ. На питательной среде, содержащей глюкозу и различные аминокислоты (глицин, DL-аланин, β-аланин, и DL-серин) между видоизменениями хорошо и плохо вырабатывающих окситетрациклин, наибольшая наблюдаемая разница имеется в отношении обмена пировиноградной кислоты. Снижение производства антибиотика наибольшее на питательной среде с содержанием серина. Одна часть вариантов, дающих на других питательных средах еще неизменный синтез окситетрациклина, но проявляющих большую склонность к снижению выработки, на питательной среде с содержанием серина дают низкое образование антибиотика. При снижении производственной способности конститутивная сериндеаминазная активность **Streptomyces rimosus** повышается, но в то же время их способность к использованию пировиноградной кислоты снижается.

L. Lovrekovich, Z. Klement

SPECIES-SPECIFIC ANTIGENS OF PSEUDOMONAS TABACI

ВИДОСПЕЦИФИЧЕСКИЕ АНТИГЕНЫ PSEUDOMONAS TABACI

Л. ЛОВРЕКОВИЧ, З. КЛЕМЕНТ

На основании величин титра агглютинации сыворотка, полученная применением живых бактерий, **Ps. tabaci**, не обособляет необработанных штаммов **Ps. tabaci** и **Ps. angulata** от штаммов **Ps. lachrymans**, **Ps. mors-prunorum** и **Ps. mori** Однако, штаммы **Ps. tabaci** и **Ps. angulata** хорошо обособляются от вышеприведенных видов, если до агглютинации содержать бактерии в водяной бане при температуре 100° С в течение 60 мин.

На основании исследований авторов можно установить, что после соответствующей термообработки **Ps. tabaci** можно серологическим путем надежно дифференцировать от родственных видов. Для этой цели хорошо применимы как агглютинация, так и гель-диффузия. Однако, гель-диффузия более специфический метод и она удобнее проводима чем агглютинация. Объяснением того, что эта дифференциация проводима таким простым методом, является тот факт, что исследованные виды **Pseudomonas** обладают общим термолабильным А-антигеном, специфическим для этой группы, в то время как видоспецифический антиген устойчив к теплу. Это установление имеет то значение, что как известно авторам, это первый случай, при котором антиген с поверхностной термолабильностью имеет не видо- или типоспецифический, а группоспецифический характер.

Предполагается, что эти методы можно применять не только для дифференциации **Ps. tabaci**, но и других видов **Pseudomonas**.

H. Milch, V. G. László

PHAGE-TYPING OF SALMONELLA PARATYPHI B.

РЕЗУЛЬТАТЫ ОПРЕДЕЛЕНИЯ ФАГОТИПА ШТАММОВ SALMONELLA PARATYPHI B

Х. МИЛЬХ, В. Г. ЛАСЛО

С 1956—1961 гг. проводились определения фаготипа по Феликс—Калдоу 715 штаммов **Salmonella paratyphi B** и на основании эпидемиологической разработки полученных результатов было установлено, что они непригодны для эпидемиологических целей. Поэтому авторы внедряют «естественную группировку по Шолтенсу, метод распределения типов на основании лизогенного состояния штаммов.

Авторы исследовали 360 штаммов одновременно методом Феликс—Каллоу и методом Шолтенса. На основании группировки Шолтенса в ходе повторных исследований бациллоносителей отдельные типы оказались постоянными. При помощи естественной группировки Шолтенса из 12 новых типов удалось обнаружить 7 новых типов (**Leeuwarden, Beccles 22, Taunton-Kampen, Taunton-the-Hague, 87, Sittard & J7**).

Á. Jancsó, M. Simon:

AETIOLOGY OF ACUTE RESPIRATORY DISEASES IN MILITARY GROUPS

ИССЛЕДОВАНИЕ ЭТИОЛОГИИ ОСТРЫХ ИНФЕКЦИЙ ДЫХАТЕЛЬНЫХ ПУТЕЙ В ВОИНСКИХ ЧАСТЯХ

А. ЯНЧО, М. ШИМОН

В ходе двухмесячного периода наблюдения авторы проводили обследование трех групп призывников, с целью определить вирусную этиологию заражений дыхательных путей. В ходе серийных исследований связывания комплемента против аденовирусов, проведенных с 1201 парой сывороток, в 14% сывороточных пар было установлено четырехкратное или большее повышение титра.

В 18 парах сывороток с целью определения типа циркулирующего аденовируса проводились установления нейтрализующего титра против аденовирусов типа 1—7. Ввиду гетеротипичных результатов титра не удалось определить тип аденовируса, ответственного за инфекцию.

В 46 случаях серологически и клинически исследованных острых заболеваний дыхательных путей у 9 лиц удалось обнаружить повышение титра связывания комплемента против аденовируса, у одного лица против вируса свинки; в остальных случаях не удалось доказать вирусной этиологии. Согласно полученным результатам преобладающая часть инфекций аденовирусом протекла незамеченно.

Из 14 пар сывороток, проявивших повышения титра связывания комплемента против вируса свинки в 8 случаях удалось выявить также повышение титра связывания комплемента против вируса.

S. Pácsa:

POLIOVIRUS-CARRYING LINES OF HELA CELLS: THEIR ESTABLISHMENT AND SENSITIVITY TO VIRUSES

СОЗДАНИЕ КЛЕТОЧНОЙ КУЛЬТУРЫ HeLa, ЗАРАЖЕННОЙ ПОЛИОВИРУСОМ, И ЕЕ ЧУВСТВИТЕЛЬНОСТЬ К ВИРУСАМ

Ш. ПАЧА

Автор создал без применения иммунной сыворотки две клеточных культуры **HeLa** зараженных вирусами. Клеточная линия с обозначением **HeLa** + P_2 была носителем полиовируса 2. типа с обозначением MEF_1 а клеточная линия **HeLa** + P_3 была заражена полиовирусом 3. типа с обозначением **Saukett**. Обе клеточных линии обладали меньшей чувствительностью в отношении всех трех типов полиовируса, и против повторного заражения вирусом Коксаки В3. Повторным заражением удалось повысить резистентность культур. Клеточные культуры из клеток оставшихся в живых после двухкратного повторного заражения, оказались резистентными против 10 000 TCD_{50} всех трех типов полиовируса и против 100 TCD_{50} вируса Коксаки. Повысился средний размер ядер клеток в клеточных вирусных культурах, а интенсивность размножения клеток снизилась.

Gy. Vörös-Felkai, E. K. Novák:

RAFFINOSE ASSIMILATION OF YEASTS

ИССЛЕДОВАНИЕ АССИМИЛЯЦИИ РАФФИНОЗЫ ДРОЖЖЕВЫМИ ГРИБКАМИ

ДЬ. ВЁРЁШ-ФЕЛКАИ, Е. К. НОВАК

Исследовалась ассимиляция раффинозы 268 штаммами дрожжевых грибков, выращенными из материала больных. В отношении 10 видов имеющиеся в монографии *Лоддера* и *Крегер ван Рия* данные были дополнены данными брожения раффинозы. У 41 вида дается описание ассимиляции раффинозы. Исследованные автором 17 видов **Candida tropicalis** — в противоположность данным *Лоддера* и *Крегер ван Рия* — не ассимилировали и не вызывали брожения раффинозы. Автор проводил исследования с дрожжевыми грибками, содержащими цветное вещество, которые ассимилировали раффинозу. Ассимиляция раффинозы во всех случаях сопровождалась ассимиляцией сахарозы.

G. Wix, K. Albrecht:

MICROBIOLOGICAL PRODUCTION OF Δ 1,4-ANDROSTADIENDIONE FROM STEROIDS OF DIFFERENT STRUCTURE. THE INTERACTION OF STEROIDS

МИКРОБИОЛОГИЧЕСКОЕ ПОЛУЧЕНИЕ Δ 1,4-АНДРОСТАДИЕНДИОНА ИЗ СТЕРОИДОВ РАЗЛИЧНОЙ СТРУКТУРЫ И ВЗАИМОДЕЙСТВИЕ СТЕРОИДОВ

Г. ВИКС, К. АЛБРЕХТ

Fusarium caucasicum способен превратить стероиды различной структуры в Δ 1,4-андростадиендион (диендион). Самая большая продукция получилась из андростендиона. У этого соединения приходится только создать Δ 1-ую двойную связь для достижения полезного продукта. Чем более отдален субстрат по своей структуре от андростендиона, тем больше ферментативной активности требуется для его превращения, и тем меньшее образование диендиона. Причиной этого является тот факт, что ферментативная система, расщепляющая диендион на Δ 1-дегидротестололактон, функционирует в случае различных субстратов с неизменной скоростью, причем количество накопляющегося диендиона в культурах зависит от относительной скорости синтеза и расщепления. Иное положение наблюдается если превратить насыщенные стероиды алло-ряда. Эти соединения, хотя и превращаются в диендион, но они задерживают активность энзима, расщепляющего диендион, и если добавлять одновременно с Δ 4,3-кетостероидами напр. аллопрегнандион, то в культурах накопляется диендион. Исследование механизма задержки показало, что он не компететивного типа.

D. Karasszon, P. Ruzicska:

CULTIVATION AND HISTOPATHOLOGY OF KIDNEYS FROM MONKEYS SUFFERING FROM DYSENTERY

КУЛЬТИВИРОВАНИЕ И ГИСТОПАТОЛОГИЧЕСКИЕ ИЗМЕНЕНИЯ ПОЧЕК ОБЕЗЬЯН, СТРАДАЮЩИХ ДИЗЕНТЕРИЕЙ

Д. КАРАССОН, П. РУЗИЧКА

В почках 145 обезьян, страдающих бациллярной дизентерией, использованных для тканевых культур, число клеток в граммах оказалось в среднем на 40% меньше числа клеток у здоровых обезьян. Доказанные гистологические изменения данных экземпляров объясняют низкое число клеток, полученное из почек дизентерийных обезьян.

У 50 дизентерийных обезьян при гистологическом исследовании выявлены патогистологические изменения, соответствующие патологическому понятию токсического нефроза. В почках 20 контрольных, здоровых обезьян изменений не было выявлено.

J. Szita, K. Czéh, S. Bognár:

AN IMPROVED METHOD FOR THE CULTIVATION OF BACTERIA FROM BLOOD

НОВЫЙ МЕТОД ДЛЯ ВЫРАЩИВАНИЯ БАКТЕРИЙ ИЗ КРОВИ

Й. СИТА, К. ЦЕХ, Ш. БОГНАР

Авторы применяли новый метод для выращивания бактерий из крови. Новый метод в случае бактериемий, вызванных различными патогенными палочками, оказался в три раза лучше, чем применяемый раньше метод. Разработанный авторами метод, — по сравнению с прежним, — снижает также возможность последующего загрязнения питательной среды, так как на примененном двухфазовом субстрате рост бактерий хорошо видим, и пересев оказался необходимым только в целях идентификации выращенного штамма.

J. Weiszfeiler, V. Karassova, I. Földes, E. Vincze, G. Gyenes:

THE STUDY OF ATTENUATED TUBERCLE BACILLUS STRAINS ON RABBITS

ИССЛЕДОВАНИЕ НА КРОЛИКАХ ШТАММОВ ТУБЕРКУЛЕЗНЫХ ПАЛОЧЕК С ОСЛАБЛЕННОЙ ВИРУЛЕНТНОСТЬЮ

Й. ВЕЙСФЕЙЛЕР, В. КАРАСОВА, И. ФЁЛЬДЕШ, Г. ДЬЕНЕШ

Внутривенное введение кроликам штаммов туберкулезных палочек (подштаммы BCG и штамм № 115) с ослабленной вирулентностью вызывает тяжелые изменения, прежде всего в легких. Очаги изменений гистологически соответствуют творожисто-некрозным, эпителиоидным клеточным очагам и часто грануляционным тканям неспецифического характера. 10—20 мг-овые дозы 9-дневных культур вызывали гибель большой части животных.

У кроликов, не погибших в течение 40 дней, в результате развившегося иммунитета, изменения тканей показали постепенное обратное развитие.

Патогенность штаммов туберкулезных палочек с ослабленной вирулентностью при внутривенном введении кроликам больше, чем при их введении морским свинкам. Авторы объясняют этот факт повышенной чувствительностью кроликов к клеточным веществам туберкулезных палочек.

L. Lantos, G. Iványovics:

THE PHAGE RECEPTORS OF BACILLUS ANTHRACIS

ФАГОРЕЦЕПТОРЫ ПАЛОЧКИ СИБИРСКОЙ ЯЗВЫ

Й. ЛАНТОШ, Г. ИВАНОВИЧ

В клеточной стенке палочки сибирской язвы удалось выявить специфические рецепторы отдельных групп А фагов. Эти рецепторы связаны с различными структурными элементами клеточной стенки. Рецепторы эти не удалось отожествить с иммуноспецифическим полисахаридом **B. anthracis**. Иммуноспецифический полисахарид — пептид полимера галактозо-глюкозамина — представляет собой характерный компонент клеточной стенки, его нельзя освободить из стенки слабыми химическими воздействиями, но при энзиматическом воздействии он путем автолиза переходит в раствор.

L. Ormay, K. Ujhelyi:

TITRATION OF DIPHTHERIA TOXIN, ANTITOXIN AND TOXOID IN TISSUE CULTURE

ИССЛЕДОВАНИЕ ДИФТЕРИЙНОГО ТОКСИНА, АНТИТОКСИНА И АНАТОКСИНА В КЛЕТОЧНЫХ КУЛЬТУРАХ

Л. ОРМАИ, К. УЙХЕЛЬИ

Прививкой культур клеточных штаммов **cynomolgus** эндокарда авторы исследовали: а) поражающее действие на клетки различного количества дифтерийного токсина; б) возможности сравнительной оценки дифтерийных токсинов, антитоксинов и анатоксинов различного производства. Было установлено, что клеточные культуры как в стационарных культурах, так и при применении «Colour test», весьма чувствительны к действию дифтерийного токсина. В случае исследованных авторами токсинов уже количество прибл. в 1/1000 **Lf** токсина вызывает гибель клеток. Поражающее действие токсинов на клетки можно предотвратить антитоксином. Клеточные культуры оказались пригодными для сравнительных исследований в целях оценки абсолютных величин и величин связывания токсинов различной выработки, далее для оценки анатоксинов и антитоксинов различной выработки. В случае исследования антитоксинов и анатоксинов кроме количественного определения единиц антител и антигенов, можно получить также данные относительно их активности.

Чувствительность примененных авторами клеточных культур за период наблюдения (полтора года) оставалась без изменения.

I. Nász:

DIFFERENCES IN THE CYTOPATHOGENIC EFFECT OF SOME ADENOVIRUS STRAINS ON HUMAN AMNIOTIC TISSUE CULTURES

РАЗНИЦЫ В ЦИТОПАТОГЕННОСТИ ШТАММОВ АДЕНОВИРУСА В АМНИОТИЧЕСКИХ ТКАНЕВЫХ КУЛЬТУРАХ ЛЮДЕЙ

И. НАС

В первичных человеческих амниотических тканевых культурах исследовалось цитопатогенное действие 42 штаммов аденовируса, относящихся к серотипам 1—16. На основании природы цитопатогенного действия исследованные штаммы можно отнести к двум группам. Для цитопатогенного действия, вызванного вирусными штаммами первой группы, характерно, что клеточные культуры вначале приобретают сетчатую структуру, а затем возникают гроздевидные вырожденные клеточные острова. После заражения вирусными штаммами второй группы дегенерирующиеся клетки разобщаются, единая структура клеточных культур разрыхляется, клеточная плазма исчезает, и на стенке сосуда остаются в большей части окруженные остатками плазмы одиночные клеточные ядра. Природа цитопатогенного действия, в случае отдельных вирусных штаммов после пассажей и повторных исследований, оказалась одинаковой.

S. Vörös, T. Angyal, V. Németh, T. Kontrohr:

THE OCCURENCE AND SIGNIFICANCE OF PHOSPHATASE IN ENTERIC BACTERIA

НАЛИЧИЕ И ЗНАЧЕНИЕ ФОСФАТАЗЫ В ГРУППАХ КИШЕЧНЫХ БАКТЕРИЙ

Ш. ВЁРЁШ, Т. АНДЬАЛ, В. НЕМЕТ, Т. КОНТРОР

Исследовалась фосфатазная активность 929 штаммов кишечных бактерий. Распределение 67 штаммов рода **Providencia** было следующее: 12 положительных, 55 отрицательных штаммов. Среди 140 штамма рода **Proteus hauseri** 139 штаммов дали положительную, а 1 штамм отрицательную реакцию. В случае 121 штамма **Proteus morganii** получилась отарицтельная проба, в то время как 4 штамма дали положительную реакцию. Среди 38 штаммов **Proteus rettgeri** в 8 случаях наблюдалось наличие фермента. Все остальные штаммы (150 **E. coli**, 153 **Klebsiella**, 115 **Shigella**, 45 **Salmonella**, 72 **Citrobacter**, 23 **Arizona**, 2 **Cloaca cloacae**, 1 **Hafnia**) показали отрицательную реакцию.

Наблюдаемая в пределах рода **Proteus hauseri** фосфатазная активность повидимому подтверждает, что **Proteus vulgaris** и **Proteus mirabilis** принадлежат к одной и той же группе.

Сравнительно часто получаемые положительные реакции у штаммов рода **Providencia** повидимому обосновывают причисление рода **Providencia** в **Protea tribus**.

Между членами рода **Proteus hauseri** и прочими, показывающими фосфатазную активность штаммами **Proteus morganii**, **Proteus rettgeri** и **Providencia** не удалось выявить родственной антигенной структуры.

H. Milch, Zs. Deák:

DETECTION OF THE ROUTE OF NOSOCOMIAL ESCHERICHIA COLI INFECTIONS BY PHAGE-TYPING

ИССЛЕДОВАНИЕ БОЛЬНИЧНОЙ ИНФЕКЦИИ, ВЫЗВАННОЙ БАКТЕРИЯМИ ESCHERICHIA COLI DISPEPSIAE, ПРИ ПОМОЩИ ОПРЕДЕЛЕНИЯ ФАГОТИПА

Х. МИЛЬХ, Ж. ДЕАК

Исследовались фаготипы, жгутиковые антигены и реакция с β-фенилпропионовой кислотой (PP) 483 штаммов **E. coli** группы 0111:B4 или же группы 055:85, выращенных из материала больных, находящихся на больничном лечении.

Большинство штаммов группы 0111:B4 обладает фаготипом 111/2, жгутиковым антигеном H2 и дает положительную реакцию PP. Преобладающая часть штаммов группы 055:B5 имеет фаготип 55/2, содержит жгутиковый антиген H6 и дает отрицательную реакцию PP.

Между фаго-серо-биотипом и патогенностью не наблюдалось взаимосвязи.

При помощи определения фаготипа хорошо можно проследить путь больничных заражений, вызванных **E. coli dispepsiae** и найти источник инфекции.

P. Geck, R. Szántó:

EXAMINATION OF CLOSTRIDIUM PERFRINGENS WITH THE FLUORESCENT TRACER TECHNIQUE

ИССЛЕДОВАНИЕ CLOSTRIDIUM PERFRINGENS ПРИ ПОМОЩИ МЕТОДА ФЛЮОРЕСЦЕНЦИИ

П. ГЕКК, Р. САНТО

Авторы при помощи описанного Кунсом и сотрудниками метода флюоресценции изучали возможность быстрой идентификации **Clostridium perfringens**. Антибактериальная сыворотка с высоким титром была мечена флюоресцирующим красителем **Lissamine-Rhodamin RB 200**. Гомологи и контрольные мазки окрашивались прямым и косвенным методами. Около 50 штаммов **Clostridium perfringens**, после окрашивания с гомологичной меченной сывороткой, флюоресцировали красно-оранжевым цветом. Ряд грамположительных и отрицательных контрольных штаммов не давал флюоресценции. Споры **Cl. perfringens** флюоресцировали одинаковым образом, как и вегетативные формы. Этот метод оказался пригодным также для разобщения типов **A** и **F Clostridium**.

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