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TOMUS V FASCICULUS 1

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1958

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A METHOD TO MEASURE THE PRODUCTION OF ANTIBIOTIC SUBSTANCES BY THE VARIANTS OF STREPTOMYCES GRISEUS

By

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(Received March 12, 1956)

Recent years have witnessed the development of a number of biological methods for the quantitative determination of antibiotic substances produced by fungi and Streptomyces species. While several of them are being generally applied in certain industries to control fermentation processes, and also in clinical laboratories to determine the concentration of antibiotics in blood and other body fluids, they are of little or no avail in the study of certain special problems. Paramount among these is the question of how to select from among the variants those producing a higher quantity of antibiotic substances. Tests for such selection work involve hundreds of variants, and the current methods (shaking apparatus, serial determinations in liquid medium) require very considerable technical equipment and personnel, requirements not available for small laboratories.The simplest method to achieve the above aim would obviously be one permitting a direct determination of the antibiotics as they are produced on solid agar plates. As far as we know, no convenient method has as yet been suggested, although this would be of both theoretical and practical importance.

In the following, a method will be described making it possible to follow quantitatively the production of antibiotics by colonies developed from the individuals of a population. By working up statistically the results, we can on the one hand determine the differences in the antibiotic production of the individual organisms making up the strain, and, on the other hand, to select the individual variants to be transferred.

Experimental

In elaborating the method, an industrially applied *Streptomyces griseus* strain was used.

The medium for production (I), consisted of 900 ml potato extract; 100 ml yeast extract; 10 g Na_2HPO_4, 12 g H_2O; and 1 g KH_2PO_4 this was autoclaved at 1.5 atm. for 20 minutes and adjusted to pH 7.5. As a "titration medium" (II) by which to determine streptomycin production, a meat extract adjusted to pH 8 and containing 0.5 per cent peptone and 1.8 per cent agar, was employed. The *B. subtilis* strain ATCC 6633 served as test organism.

The method originally employed by us to measure the streptomycin production of the individual *Streptomyces griseus* colonies was to cut out the respective colony with a 10 mm borer together with the agar block in the centre which it had grown. This block was then transferred to the surface of a medium previously inoculated with *B. subtilis*. After an incubation of 16 hours at 37° C, the inhibition zone appearing on the agar surface indicated the presence of streptomycin.

To be able quantitatively to evaluate the result of the procedure it appeared necessary to establish the relationship between the size of the inhibition zone on the agar block which carried the colony, and the streptomycin content of that block. To achieve this a standard was required giving rise to inhibition zones at which even within wide concentration limits a linear correlation existed between the logarithm of the concentration and the width of the inhibition zone. This was a prerequisite, because we knew from earlier experience that the antibiotic-producing capacity of the individual *Streptomyces* colonies varied within very wide limits.

The hole-plate and the cylinder-plate methods had to be abandoned as possible standards, for it is only within a very narrow range that they yield a linear correlation between concentration and inhibition zone. In this respect, reference is made to the findings of TETTAMANTI and Mrs. PÉNZES (1952), who on assaying streptomycin with the cylinder-plate method obtained reliable data between 0.5 and 2 U/ml concentrations only.

The filter paper disc method known from the literature (VINCENT and VINCENT, 1944 ; SHERWOOD *et al.*, 1944 ; LOO *et al*, 1945) seemed best to comply with the requirements demanded from a standard method. It indeed yielded reproducible values but we still found that under our experimental conditions it was not suited as the standard. When the antibiotic-producing capacity of the individual colonies developing from the various spores of the test strains are determined by our method, through the agar block layer that carries the colony the antibiotic substance produced diffuses into the agar plate inoculated with the test organism. The diffusion mechanism taking place between two agar surfaces, as well, as the distribution equilibria between them, must obviously differ from those between the paper disc impregnated with antibiotic substance and the agar plate used for titration. For these and similar other reasons the paper disc method was abandoned instead, agar discs equal in diameter and thickness with the colony-carrying agar blocks but containing various streptomycin concentrations, were finally adopted as standards.

In view of the emphasis WAKSMAN *et al.* (1949) and other authors (cf. FLOREY, 1949) had lain upon the part played by the size of the inoculum, the thickness of the agar layer, and the pH of the medium in the quantitative determination of antibiotics by biological methods, we had to be particularly careful in standardising these factors as strictly as possible.

In our method it was of double importance to achieve uniform thickness of the agar layers, because the inhibition zones were affected in their size by the thickness of both the agar blocks carrying the colony and those used as standards, whereby the accuracy of the method was likewise influenced.

To obtain uniform flat-bottomed Petri dishes we applied glass rings* 70 mm in diameter with a ground rim, which were placed on a flat glass plate (14 × 14 cm) and so used instead of Petri dishes (Photo 1). By pipetting 10 ml of molten agar into these rings previously sterilised by dry heat, agar layers identical in thickness were produced. The lids of common Petri dishes served as covers. (In the following, this combination of glass ring and glass plate will be referred to as the standard Petri dish substitute.)

The procedure adopted in determining quantitatively the streptomycin-producing capacity of monocellular cultures was as follows. In common Petri dishes plates were prepared of medium I, and on their surface a spore suspension was spread in a dilution which gave rise to from about 50 to 100 colonies. As soon as after incubation at 27° C the developing colonies had became visible by the naked eye, they were transferred in a sterile room, to the centre of medium I previously prepared in standard Petri dish substitutes. Thereafter the plates were incubated at 27° C until the colonies were fully developed.

A single colony was transferred to each plate, because the colonies were known from our experience to interfere with one another's antibiotic production ; a fact that rendered impossible to evaluate the experiment. After they had reached the appropriate stage of maturity, the colonies together with the agar blocks belonging to them were cut out with a 5.5 mm metal borer, and

* Manufactured by the *Karcag Glass Works.*

the individual blocks were transferred to medium II previously inoculated with *B. subtilis* in a glass pan measuring 17 by 25 cm (Photo 2).

Simultaneously with the cutting out of the colonies to be studied, 10 ml of molten agar containing known concentrations of streptomycin were poured

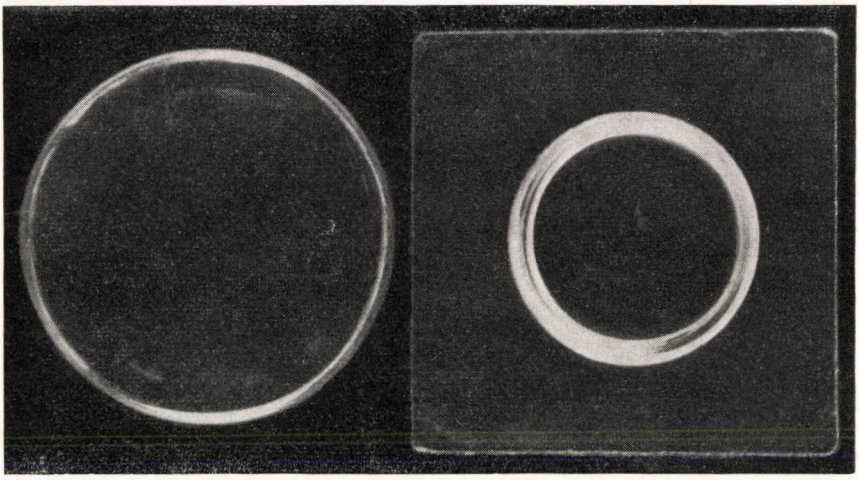

Photo 1
Standard Petri dish substitute, consisting of a 14 × 14 cm glass plate, a glass ring 7 cm in diameter with ground rim, and a common Petri dish lid

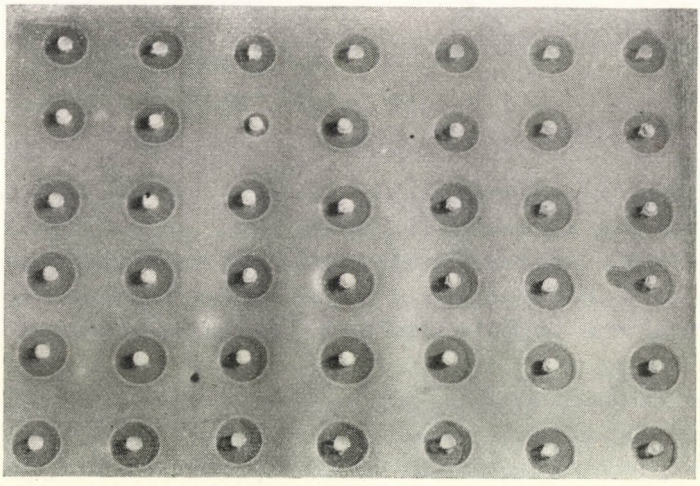

Photo 2
Glass pan measuring 17 × 25 cm. The streptomycin-containing agar blocks in it produced inhibition zones

into each of a number of standard Petri dish substitutes. After the agar had solidified, the blocks required for plotting the standard curve were cut out from the agar plates containing the corresponding streptomycin concentrations

and, like the colony-carrying agar blocks, were transferred to the titration agar plates.

These plates were kept at 37° C for 16 hours before the results were read.

Applying the above method in the analysis of the zones of inhibition produced by blocks containing from 5 to 55 μg of streptomycin per ml, it was found that on plotting the logarithms of the concentration against the values

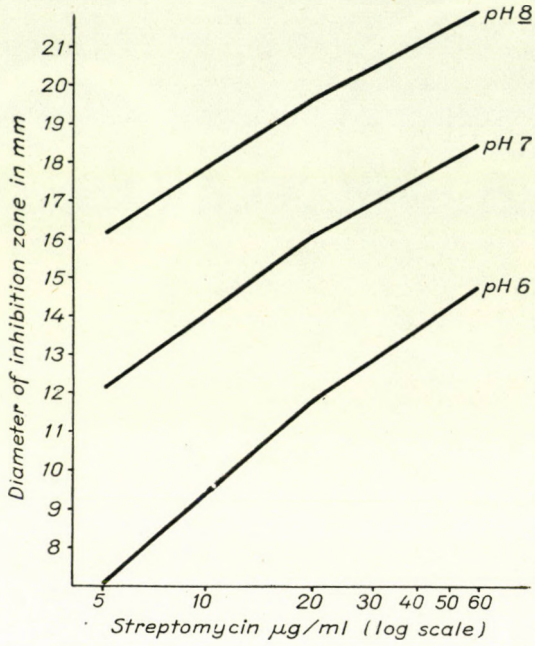

Fig. 1

of the width of the inhibition zones, the curve obtained was concave from below. In one case, examining 11 concentrations, approximation was made with a second order parabola. On the evidence of the results of numerous experiments, the standard deviation of the measured values from the theoretical curve was 0.184 mm, with the maximum deviation amounting to 0.371 mm. On the other hand, these experiments also showed that, between the given limits of concentration, it is the parabola which expresses with the greatest accuracy the relationship of concentration and diameter of inhibition zone.

This fact made the evaluation of the experiments a fairly intricate and cumbersome task. In order to facilitate it, experiments were carried out to see if the deflection of the curve could be eliminated, or at least moderated, by changes in the experimental conditions.

As shown by Figures 1 and 2, changes in the pH between 6 and 8, or a rise in the concentration of the agar from 1.5 to 3 per cent, failed to affect essentially the course run by the curve.

On the other hand, from five experiments carried out under identical conditions, it was established that in part between the concentration limits of 0 to 20 μg/ml, and in part between those of 20 to 55 μg/ml, readily evaluable data can be obtained by substituting for the unwieldy parabola curve a broken line consisting of two straights. Worked up mathematically, the experimental data showed that in accuracy there was hardly any difference between approxi-

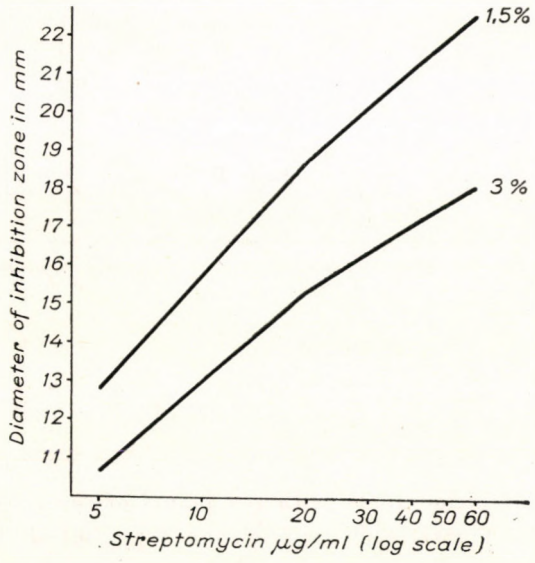

Fig. 2

mation by a broken line and approximation by a parabola of the second order, and that whatever deviation there still was, did not exceed the values of the standard deviation of the measurement itself. This is borne out by the data in Tables I and II.

Accordingly, the essence of our findings is that, given a wide range of concentrations, when the relation between the logarithm of concentration and the width of the corresponding inhibition zone is characterized by a parabola, this parabola can be substituted by a broken line consisting of two straights.

In every one of our routine experiments three points of the curve were determined. For each of the three concentrations selected for this purpose (5, 20, 55 μg/ml) 12 samples were measured, the arithmetic means of which gave the positions of the points of the reference curve. Since the agar surface in the titrating pan held about 42 agar blocks, the blocks of the colonies to be tested had to be placed into another one. However, as the layer of the agar medium in these pans differed in thickness from the layer of the medium used for plotting the reference curve, it was not possible simply to compare the

Table I

Error of the method on the application of one or two straights, respectively

No. of plate	Error of a single straight	Error of the first straight in mm	Error of the second straight in mm
1	0.3	0.21	0.25
2	0.38	0.23	0.21
3	0.33	0.21	0.29
4	0.32	0.23	0.11
5	0.48	0.37	0.17

Table II

Standard error of measurements

Streptomycin μg/ml	Mean of 12 measurements in mm	Standard deviation in mm
5	15.44	±0.27
20	18.54	±0.3
55	20.70	±0.39

diameters of the inhibition zones with the points of the reference curves. For this reason, 12 agar blocks, each representing 20 μg/ml of streptomycin, were placed irregularly upon the agar surface in the titration pan used for the determination of the colonies' streptomycin production. The mean diameter of the inhibition zones produced by these known streptomycin concentrations presented a possibility for the determination of the correction factor relevant to the respective plate. The use of a correction factor was justified by numerous experiments, which proved that on performing the measurements in media of the same composition and under the same experimental conditions, but in different titrating pans, corresponding sections of the straights illustrating the relation of antibiotic concentration and inhibition zone, run parallel with each other. On this basis, it is possible to determine by a simple graphic method the correction factor in millimetres, which is then added to or deducted from the diameter of the inhibition zones produced by the unknown streptomycin concentrations. The antibiotic concentration corresponding to the width of the inhibition zone thus corrected can be read off the reference curve.

The method described in the foregoing permits the examination, for their respective streptomycin production of colonies derived from individually isolated spores of several kinds of *Streptomyces griseus* populations (treated with UV light, resistant to streptomycin, etc.), and to study the laws which govern the changes of variability within the strain.

The method lends itself to the picking out of the *Streptomyces griseus* variants that produce streptomycin abundantly, because the connection is fairly reliable, which we have established to exist between the behaviour of those variants in shaken cultures and their characterization by our method. The data in Table III furnish evidence that the strains which in shaken cultures

Table III

Production of streptomycin by Streptomyces griseus strains in solid medium and shaken culture

Strain marked	Production on solid medium in U/ml	% *	In liquid medium in shaken culture Samples taken at 24-hour intervals		
			I	II	III
1/2	32.6	53	375	358	446
1/21	32.6	53	286	337	353
2/4	<25		<250	<250	345
2/17	36.8	56	423	395	457
3/5	<25		<250	316	341
3/17	32.4	63	<250	403	536
4/8	26.8	51	<250	<250	<250
4/13	34.8	67	<250	413	362
5/10	<25		<250	380	375
1/15	118	194	386	485	683
2/13	100	154	356	493	610
3/6	100	197	396	523	560
3/110	68	134	250	511	448
3/16	78	153	<250	325	<250 (325)

* The values in column III indicate the productivity in percentage referred to the average streptomycin production of 100 individual colonies.

too produced more streptomycin, all came from among the variants which by our quantitative selection method proved to be better producers than the average of the population. It appears, however, that between the activity measured in a liquid medium and that mesured on a solid medium there exists a correlation which is but unilateral. Accordingly, it cannot be considered a general rule that every strain which on solid media is conspicuous for its antibiotic production will abundantly produce antibiotic substance in shaken culture as well. On the other hand, it is a fact that in our experiments all the strains that were good producers in shaken culture were found to have come from among the variants which at determination on solid media were more active than the average population.

Summary

A quantitative method has been elaborated for the determination of absolute values for the purpose of studying individual *Streptomyces* colonies.

The method is suitable

1. for the quantitative study of the antibiotic-producing capacity of *Streptomyces griseus* individuals (or other microorganisms); for the quantitative study of the variability of that given property.

2. for the study of the interrelations existing between the morphological properties and the antibiotic-producing capacity of the colonies of the individual variants;

3. for the isolation of variants producing streptomycin (or other antibiotic substances) abundantly.

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BIOCHEMICAL STUDIES OF THE SYNTHESIS OF STREPTOMYCIN

I. α-MANNOSIDASE ACTIVITY STUDIED IN STREPTOMYCES GRISEUS CULTURES

By

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(Received April 6, 1957)

In 1948, PERLMAN and LANGLYKKE [1] reported to have found an enzyme in streptomycin-producing *Streptomyces griseus* cultures which was capable of converting streptomycin B into streptomycin A [2]. They gave it the name mannosidostreptomycinase. More recently, HOCKENHULL *et al.* [3] established that cultures capable of splitting mannose off streptomycin B, were also capable of breaking down synthetic *a*-phenylmannose. Since by the their findings breakdown inveriably occurred simultaneously with the two kinds of substrate, these authors assumed that the splitting of both was catalysed by very same enzyme ; this enzyme they named *a*-phenylmannosidase. They also found that under anaerobic conditions the enzyme became reversibly inactivated and that for its activity it depended on active aeration. In their view, with the beginning of cell lysis the enzyme is intracellularly present in the fermentation and is activated by lysis by which it is brought into contact with a higher oxygen tension.

While our own investigations have in part confirmed the observations of HOCKENHULL *et al.* [3], in some respects there is discrepancy between us. Several of our experiments on *a*-mannosidase activity were but duplications, yet since our aim was to clarify the origin of the enzyme and the part it played in the synthesis of streptomycin, they had to be carried out to enable us to estimate later results reliably.

Materials and methods

Two *Streptomyces griseus* variants were used: strain X_1 and strain L. Sz.$_1$. The first was grown on a soya bean-starch-corn-steep liquor, the second on a soya bean-glucose-$(NH_4)_2SO_4$—KH_2PO_4 medium. The experiments were carried out in shake cultures inoculated with vegetative material or with cultures from metal fermentors.

Preparation of mycelial suspension. From cultures of appropriate age the mycelium was centrifuged and, after decantation of the supernatant, removed from the sediment of coarse granulation, to be washed twice in a clean centrifugal tube with 8 to 10 times its volume of ice-colds sterile distilled water. The washed mycelium was then weighed and suspended in 6 volumes by weight of 0.05 M phosphate buffer at pH 7.5. All operations were carried out under aseptic conditions, as far as that was possible, and no experiments were included in the results, in which bacterial or other infections had been noticed.

Determination of enzyme activity. To measure enzyme activity the method of HOCKENHULL *et al.* [3] was applied with slight modification. Enzyme activity was taken as being unity when

under the conditions of the experiment 1 μM of phenol was liberated in 30 minutes. All determinations were carried out at 34° C and an air supply of 5 litres/30 minutes.

α-phenylmannose was prepared by the method of HELFERICH and WINKLER [4], and α-methylmannose by that of FISCHER [5]. Before use, both substrates were recrystallised from absolute methanol.

Streptomycin B was obtained by chromatography with Al_2O_3, and converted to sulphate prior to use. For its determination a method has been elaborated in our laboratory. Streptomycin was bound in two serially connected colums containing Amberlite I R C-50 cation-exchange resin, and from the second eluate, after determination of the total streptomycin, the mannose portion of streptomycin B was determined selectively by means of the anthrone reaction; from this portion was then calculated on a percentage basis the streptomycin-B content of the solution. The maltol reaction was used in measuring the amount of total streptomycin.

Reducing carbohydrate was determined according to SOMOGYI [6]. Polysaccharides were hydrolysed with 1.3 *n* HCl at 100° C for 60 minutes. Respiration was measured in a Warburg apparatus at 28° C.

Results

I. Factors affecting enzyme activity. The cell-free supernatant of 55—80 hour old *Streptomyces griseus* (X_1 and L. $Sz._1$ alike) cultures were used as enzyme source. To 3 ml of this, 0.2 ml of α-phenylmannose were added; thereafter 0.5 ml of the substrate to be tested, and the mixture, were incubated for 30 minutes in the usual manner. Every material was neutralised before use.

In agreement with HOCKENHULL *et al.*[3], it was found that under anaerobic conditions there was very little enzyme activity. When incubated with 0,01—0,1 *M* $K_3[Fe(CN)_6]$, the ferricyanide complex ensured full activity of the enzyme even without aeration. None of a number of other tested oxidizing agents exerted a similarly favourable effect, probably because the experimental conditions had been adverse to that effect. The potassium ferricyanide, dependent for its oxidizing effect on a slightly alkaline medium, presumably acted by ensuring the oxidized state of the active groups of the enzyme. All subsequent activity tests were paralleled in the presence of potassium ferricyanide in order to be able to decide if what we had to do with was a redox or some other specific action.

The optimum temperature for α-mannosidase action was found to be about 40° C, which is in agreement with the findings of HOCKENHULL *et al.* [3], but the optimum pH we established at 7.6 to 7.7.

Various carbohydrates, too, were studied for their effect on enzyme activity; the results agreed fairly with those of HOCKENHULL *et al.* [3], even in the presence of $K_3[Fe(CN)_6]$, but wide discrepancies prevailed between our and their findings for cellular poisons (Table I).

It is noteworthy that HOCKENHULL *et al.* [3] found that cyanide was causing 90 per cent inhibition, and arsenate 40 per cent stimulation in enzyme activity. Analogously with HASSID's hypothesis for saccharose phosphorylase [7], arsenate may be assumed to accelerate the reaction by acting as the acceptor of mannose and forming with it a labile mannose-arsenate, which later spontaneously breaks down into mannose and arsenate.

Table I

Effect of various inhibitors on α-mannosidase activity

Inhibitor		Percentage inhibition	
			in the presence of 0,05*M* K_3 [Fe(CN)$_6$]
KCN	$5 \cdot 10^{-3}$ M	9.5	7.5
KCN	10^{-2} M	23	5.5
NaF	$2.5 \cdot 10^{-2}$ M	6	2.5
NaF	$5 \cdot 10^{-2}$ M	25	26
Na_2HAsO_4	10^{-2} M	— 6	—61
Na_2HAsO_4	$5 \cdot 10^{-2}$ M	—14	—70
Na_2HAsO_3	10^{-2} M	0	0
$NaHSO_3$	$5 \cdot 10^{-3}$ M	92	16
NaN_3	$5 \cdot 10^{-3}$ M	21	22
NaN_3	10^{-2} M	30	33
$CH_2ICOONa$	10^{-2} M	0	0
2,4-DNP	10^{-2} M	38	4
cysteine.....	10^{-2} M	100	0

The figures represent average values from 8 determinations. The — sign indicates the stimulating effect of arsenate.

It can be seen from Table I that while the ferricyanide complex counterbalances the action of the various reducing agents, it doesn't influence the action of azide (or of sugars). These substances assumedly possess other points of attack.

Comparison of the enzyme's power to break down α-phenylmannose and streptomycin B, respectively, showed that within a given period of time it liberated about two and a half times as much mannose from the former than from the latter. This is in contrast to the observation of HOCKENHULL *et al.* [3] according to which the rate of hydrolysis of α-phenylmannose was six times that of streptomycin B. This discrepancy might be due to the fact that the method applied by them for determining streptomycin B is rather unsafe for use with fermentation fluids [8].

Since in our experiments phosphate ions had no effect whatsoever on enzyme activity and since there was no possibility for transglucosidation to take place, we can safely confirm HOCKENHULL's finding that the enzyme asserts its action hydrolytically.

II. *The α-mannosidase activity in shake cultures.* Strain X_1. In 20 to 24 hours after the introduction of 1/50 volume of vegetative inoculum, the mycelium began to grow rapidly ; between the 30th and 35th hour it formed densely interwoven masses in the medium, and the carbohydrate fell in amount to about half the initial value. In this phase, streptomycin was encountered

in the nutrient medium in considerable quantities (from 300 to 500 U/ml) of which as a rule from 40 to 50 per cent consisted of streptomycin B. By between the 60th and 70th hour, all the utilisable carbohydrate was used up, the quantity of streptomycin was doubled, and dilution of the nutrient medium by cell

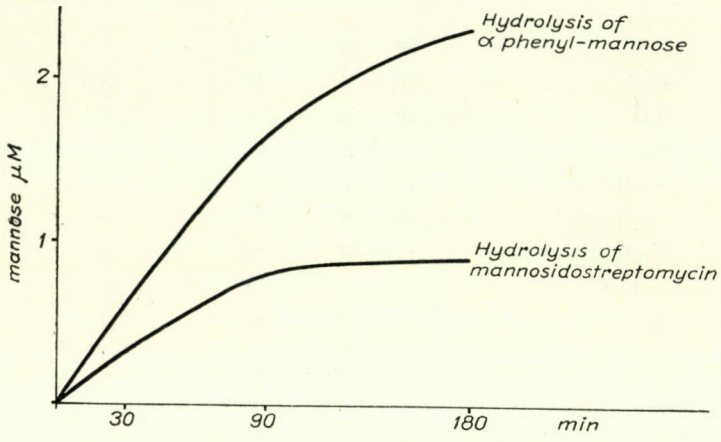

Fig. 1

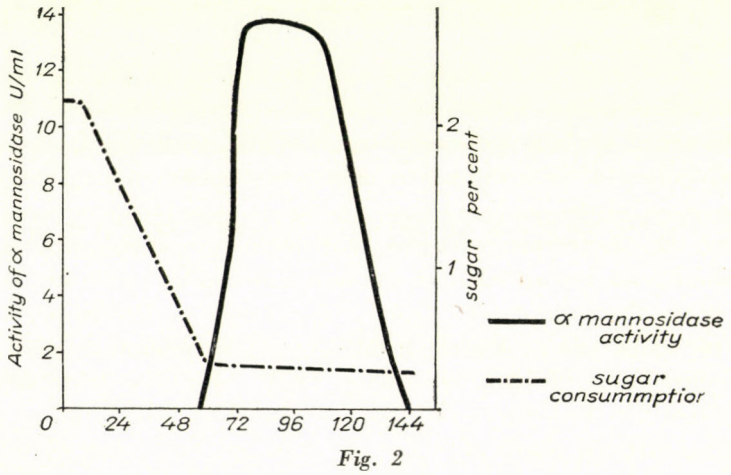

Fig. 2

lysis setting in. α-mannosidase activity usually started at the time the carbohydrate was used up completely, whereafter it intensified violently, attained its peak value and began to decrease, to disappear altogether mostly between the 120th and 140th hour. It should be noted that this activity began to decrease when biosynthesis of streptomycin was at its maximum or beginning to slow down conspicuously. A few hours after α-mannosidase activity had set in, it was no longer possible to demonstrate the presence of streptomycin B in the nutrient medium.

Strain L. Sz_1. The morphological and chemical changes were similar to those observed in strain X_1, but followed each other at a greater pace. A decisive difference from X_1 manifested itself in that from the very onset of streptomycin production, the presence of streptomycin A only was practically demonstrable in the nutrient medium, although α-mannosidase activity could doubtlessly be shown to require 50 to 55 hours to set in, quite like in the case of the other strain.

Immersed cultures of both strains grown in experimental fermentors behaved exactly like shake cultures, only took slightly shorter time to develop.

On the evidence of the findings of HOCKENHULL *et al.* [3] and our own observations made in various types of culture, we risk the following suggestions to explain the enzyme activity.

(i) An inactive form of α-mannosidase is present in the mycelium until cell lysis sets in, when it becomes activated in some way or other.

(ii) The enzyme is present within the cells in the active form, but presumably because of possible differences in the intracellular organization from strain to strain, it is unable to assert *in situ* action in the case of every strain.

(iii) Owing to variation, a mutant possessing α-mannosidase activity arises in the culture.

(iv) After a certain inhibition has ceased, the enzyme becomes synthesised in the cells inductively.

In the case under (i) the enzyme may become activated by the proteases making their appearance during cell lysis, or, as is the view of HOCKENHULL *et al.* [3], by being brought into contact with a higher oxygen tension, or lastly by the joint action of both these moments, analogously to a zymogenic transformation.

With a view to testing these possibilities, 30-hour shake cultures of strain L. $Sz._1$, which had not as yet shown signs of α-mannosidase activity, were centrifuged ; the mycelium was twice washed with ice-cold distilled water, suspended in 7 vol. 0.05 *M* phosphate buffer at pH 7.8, and distributed in 25 ml portions in 100 ml Erlenmeyer flasks. Acetone powder containing protease was then added in quantities varying from 25 to 250 mg. The acetone-dried substance was prepared with the method of SIMON [9] from L. $Sz._1$ culture. One mg of the preparation decomposed 22 mg of casein in 30 minutes at 28° C. After addition of the protease, the test tubes were shaken for 24 hours. Despite an almost complete cell lysis by the end of the experiment, in no case was it possible to obtain evidence of α-mannosidase activity. Similarly negative results were obtained when pepsin was used as a proteolytic enzyme source. Experiments with 40-hour mycelium likewise failed to yield positive results, notwithstanding that marked mannosidase activity developed in the 56th or 58th hour in cultures which for control purposes were kept shaking for a longer stretch of time. Nor was any activity observed in our experiments in which incubation of the

suspension was carried out with $K_3[Fe(CN)_6]$ at a concentration of 0.05 *M*, at which the ferricyanide complex ensured maximum activity even without aeration. All the results obtained suggested that in α-mannosidase activity no role is played neither by a proteolytically controlled zymogenic transformation nor by molecular activation by some other factors. Accordingly, the assumption under (i) was rejected.

To deal with (ii), cell-free extracts were prepared from centrifuged mycelium of α-mannosidase-inactive 24- to 48-hour cultures, using various methods (trituration with quartz sand at 0° C ; treatment with 8 *M* carbamide or with concentrated sodium chloride ; freezing followed by extraction with phosphate buffer ; etc.). However, all these extracts proved to be inactive, though with the extraction methods applied it was possible to prepare very active extracts from the mycelium of cultures displaying enzymic activity. On incubation for a longer period of time under aeration with α-phenylmannose or in the presence of 0.05 *M* potassium ferricyanide, a very slight increase in the amount of phenol was observed in the mycelial extract of strain L.Sz.$_1$, but no change whatever in that of strain X_1.

Since the very moderate activity in the L. Sz.$_1$ extracts was 2—3 magnitudes below the activity developing later, it cannot possibly be conceived of as the corresponding endocellular form of the latter. The results of the extraction experiments make it seems improbable that the enzyme be present in the endocellular form before a measurable activity makes its appearance in the culture.

The third assumption, the one postulating a new mutant, we had to reject at once, for we had observed the α-mannosidase activity to set in at a developmental stage when there was practically no longer any cell division, whereas cell division is an indispensable prerequisite of the arisal of a new morphological variant. Besides, its rapid development seems to exclude the possibility that the enzyme activity was that of a new mutant.

Summary

1. Experimental evidence has been found in support of HOCKENHULL *et al.*'s [3] assumption that in *Streptomyces griseus* cultures the same α-mannosidase is responsible for the cleavage of streptomycin B and α-phenylmannoside.

2. Various compounds (carbohydrates, inhibitors) have been examined for their effect on enzyme activity, and the optimum pH and temperature have been established.

3. A number of hypotheses to explain α-mannosidase activity have been analysed ; the conclusion is that the appearance of enzyme activity cannot be explained by some activation process, mutation, or the liberation of the endocellular form. The conditions under which the enzyme arises point to an inductive character.

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BIOCHEMICAL STUDIES OF THE SYNTHESIS OF STREPTOMYCIN

II. FORMATION OF AND ROLE PLAYED IN THE BIOSYNTHESIS OF STREPTOMYCIN BY STREPTOMYCES GRISEUS α-MANNOSIDASE

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In an earlier study [1] we examined the α-mannosidase activity of *Streptomyces griseus*. The present report deals with our investigations into the synthesis of the enzyme and the interrelations between it and the synthesis of streptomycin.

In describing our experiments we follow the principles proposed in 1953 by COHN, MONOD, POLLOCK, SPIEGELMAN and STAINER [2] for the nomenclature of the phenomenon referred to as "enzyme induction". By doing this we hope to avoid the frequent misunderstandings due to the use of the earlier and not always unambiguous designations.

There appears to be no justification of concluding enzyme formation to be of inductive nature, unless two principal criteria are present, viz. : —

(i) Synthesis of the enzyme is inducible by means of the corresponding substrate.

(ii) The rate of enzyme synthesis is a multiple of the velocity of cell division.

As will be seen below, the system examined by us fulfilled both these conditions and the results of our studies confirmed the assumption of an inductive character of the enzyme.

Materials and methods

Two *Streptomyces griseus* variants were involved in our experiments X_1 and L. Sz.$_1$. As regards the substances and methods employed, they were the same as those described in our earlier communication [1].

Results

Enzyme activity induced by α-mannosides in washed mycelium suspensions. Of a phosphate-buffered cell suspension prepared from 24-hour cultures of strain L. Sz.$_1$, 20 ml portions were measured into 100 ml Erlenmeyer flasks. While to a number of them α-phenylmannoside and α-methylmannoside, respectively, was added at an end concentration of 0.005 *M*, the rest was brought to equal volumes by the addition of distilled water, whereafter all the flasks were placed

in a shaking apparatus for 22 hours. In all the test tubes containing α-mannoside, marked activity was observed to develop, while no activity whatever was detectable in the control flasks.

Table I

Inductive effect of α-mannosides on α-mannosidase activity

No.	Substrate	Enzyme activity in 16 hours	U/ml in 24 hours
1	Control	0.27	0.69
2	Control	0.25	0.72
3	+0,005 *M* α-phenylmannose	0.58	9.67
4	+0,005 *M* α-phenylmannose	0.59	8.28
5	+0,005 *M* α-methylmannose	0.60	7.50
6	+0,005 *M* α-methylmannose	0.58	8.00

We also examined the effect of various disaccharides (maltose, saccharose, melibiose, cellobiose, lactose) on enzyme formation, but found that none of them gave rise to α-mannosidase activity.

Experiments carried out with strain X_1 yielded quite similar results, yet for activity to develop at the velocity obtained with 24—26-hour mycelium of the L. Sz.$_1$ strain, the mycelium of the X_1 strain had to be prepared from cultures at least 48—50 hours old. In mycelium suspensions derived from 24—30-hour X_1 cultures enzyme activity failed to develop upon the effect of α-phenylmannose, or became manifest after a long period of time only (in from 30 to 34 hours).

Attempts were made to induce enzyme synthesis by means of streptomycin B (in sulphate form), but failed throughout. Since it seemed obvious that in nature streptomycin B might be the inducer of α-mannosidase synthesis, we gave the matter closer attention. Into 100 ml Erlenmeyer flasks 35 ml portions were measured of a mycelial suspension starved for 16 hours and prepared from α-mannosidase-inactive 24-hour cultures of strain L. Sz.$_1$. To a number of these test tubes α-phenylmannoside, to the rest α-mannosidostreptomycin sulphate was added at 0.001 *M* concentration, and all flasks were placed in the shaker. Samples taken from time to time were then centrifuged, and the α-phenylmannoside and mannosidostreptomycin contents of the supernatant were determined ; the former by means of the phenol reaction following hydrolysis (Table II).

The fact that during the experiments streptomycin B did not change its concentration in the solution, is an indication of its inability to penetrate the cells. α-phenylmannoside, on the other hand, is taken up in considerable quant-

Table II

Changes in the concentration of α-phenylmannose and mannosidostreptomycin, respectively, in the dissolved phase of aerated mycelium suspensions

No.	Substrate	Changes in concentration in μg/ml				
		0^h	2^h	4^h	8^h	10^h
1	α-phenylmannose mg/ml	1024	1020	998	776	696
2	α-phenylmannose mg/ml	1014	1012	966	832	734
3	mannosidostreptomycin mg/ml ...	1010	980	990	1012	1005
4	mannosidostreptomycin mg/ml ...	988	995	992	1004	1000

To eliminate simultaneous induction, the examinations were carried out in the presence of 10^{-3} *M* dinitrophenol

ities even in the presence of dinitrophenol, without which enzyme activity sets in as early as in 3 to 5 hours. When working with mycelial suspension prepared from 24-hour cultures of strain X_1 and starved in the usual manner for 16 hours, it was interesting to observe that although the uptake of *α*-phenylmannose from the solution proceeded at the normal rate, enzyme activity, nevertheless, failed to set in. This indicates that in the young mycelium the inhibition of enzyme synthesis is connected not with permeability phenomena but endogenous metabolism.

When before the introduction of the inducer the mycelial suspension had been kept starving for some longer stretch of time (shaking for 16 to 20 hours), enzymic activity was observed to set in as early as in 4 to 5 hours after the administration of the inducer, while in non-starved suspensions this took from 16 to 20 hours. Since, unlike in fresh suspensions where the mycelium continued to grow slowly for some hours, in starved suspensions practically no cell division (growth) was ever observed, in all our subsequent investigations on enzyme formation we only used suspensions previously starved for 16 hours.

Our earlier investigations [1] already confirmed the view of HOCKENHULL *et al.* [3] that the very same enzyme, a typical *α*-mannosidase, was responsible for the cleavage of both streptomycin B and *α*-phenylmannose. We now established that, neglecting minor differences, the conditions under which the enzyme was synthesised, were the same for both the strains we studied. In the following we therefore discuss only the experimental results obtained for strain L. $Sz._1$, except where the data for the two strains differ.

Role of oxygen in enzyme formation. On the evidence of data in the literature, oxygen is a factor of greatest importance for inductive enzyme formation. In experiments carried out with yeast cells, SPIEGELMAN [4] found that under anaerobic conditions the synthesis of galactozymase either failed to set in at all or started but slowly. STIER and STANNARD [5] showed that yeast cells dissimilate their carbohydrate reserves exclusively by means of an aerobic mecha-

nism, and that consequently, under anaerobic conditions, lack of dissimilation of the reserves renders it impossible for the synthetic process to find an adequate source of energy. The work of COCHRANE *et al.* [6, 7, 8] furnished evidence that the principal routes by which carbohydrates are decomposed in Streptomyces species, are the hexosemonophosphate shunt and aerobic glycolysis; under anaerobic conditions these microorganisms are hardly capable of any dissimilative action at all [9].

In experiments carried out with washed mycelium suspensions (of X_1 or L. Sz_1) in Warburg vessels in a nitrogen atmosphere, we too found that in the absence of oxygen there was practically no dissimilation by the mycelium. This marked aerobic character of the experimental organism foreshadowed that aerobiosis will again turn out to be an indispensable condition of the formation of α-mannosidase.

To washed phosphate-buffered mycelium suspension, prepared in the usual manner and distributed into 100 ml Erlenmeyer flasks, α-phenylmannoside was added at a concentration of 0.004 *M*. A number of these flasks was directly placed on the shaker table, while the rest, with potassium ferricyanide added at 0.005—0.05 *M* concentration, was allowed to stand at an identical temperature (Table III).

Table III

Effect of active aeration on enzyme activity

No.	Substrate	Activity U/ml		
		3^h	12^h	24^h
1	Control (shaken)	0.40	0.50	—
2	+0.004 *M* α-phenylmannose (shaken)	2.94	14.10	—
3	Control (unshaken).....	0	0	0
3	+0.004 *M* α-phenylmannose (unshaken)	0	0	0
5	+0.004 *M* α-phenylmannose + 0.005 *M* $K_3[Fe(CN)_6]$	0	0	0

As was to be expected, in the absence of active aeration no enzyme activity set in, nor was potassium ferricyanide able to replace aeration. It will be seen later that at this concentration potassium ferricyanide inhibits enzyme activity to about 50 per cent, but this cannot of course be responsible for that complete suppression of activity which was observed in the above described experiments in the presence of a ferricyanide complex. While SPIEGELMAN [4] found that aerobic incubation for a short time sufficed to start continuous synthesis of galactozymase, in our experiments the synthesis of α-mannosidase discontinued instantly after aeration had ceased. Interrupted for a short time only, the effect proved to be reversible, and if the flasks were again subjected to shaking,

enzymic action continued to increase at almost the original intensity; but with an anaerobic interval of longer (16 to 20-hour) duration, synthesis no longer continued (Fig. 1). Since anaerobic incubation for 16 hours at 20° C was found greatly to diminish respiratory activity (94—98 per cent), it appeared obvious that the said negative effect was due to large-scale cellular destruction.

Effect of hydrogen-ion concentration. Mycelium suspended in phosphate buffers of various pH values was used in studying the optimum pH for enzyme

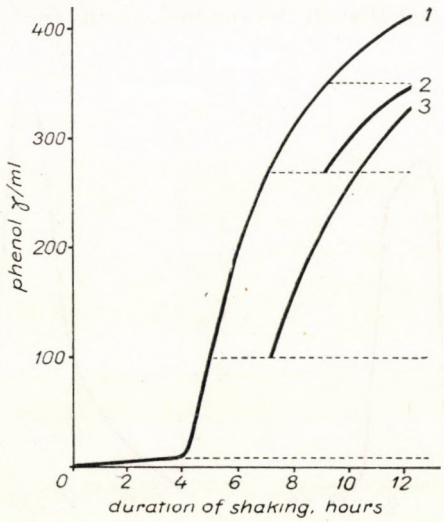

Fig. 1. Effect of aeration on α-mannosidase activity. 1 Normal induction curve. 2 Induction curve obtained after a 2-hour interruption following 7 hours of shaking. 3 Ditto, following 5 hours of shaking.

———— = phenol concentration in shaken suspension
- - - - - - - - - - = phenol concentration in suspension at rest

synthesis. To measure α-mannosidase activity, samples were adjusted to pH 7.6 and were, after the addition of 0.002 *M* α-phenylmannoside, incubated in the presence of 0.05 *M* potassium ferricyanide for 30 minutes at 34° C without aeration. Under these conditions enzyme synthesis was found to stop altogether, while the enzyme activity that had already developed proved readily measurable.

α-mannosidase synthesis attains its peak value at about pH 7.4—7.5. (Fig. 2). This comes to lie very near the optimum pH for the activity of this enzyme (7.6—7.7), wherefore by the system of GALE [10] Streptomyces griseus α-mannosidase can be classed with the group of enzymes whose optimum pH for synthesis coincides closely with that for activity.

Effect of temperature. To study the effect of temperature, 100 ml gas wash bottles fitted with sterile air filters were used; in these, 40 mg of α-phenylmannoside were added to 40 ml of phosphate-buffered mycelial suspension,

and air was passaged through them at a supply of 20 litres per hour. Samples were measured for activity in the presence of 0.05 *M* potassium ferricyanide without aeration. The system synthesising α-mannosidase was found to react with excessive sensitiveness to changes in the temperature (Fig. 3).

Synthesis was a slow process at low temperatures, it attained its peak at 27—29° C, and stopped altogether above 38° C ; moreover, incubation at 40° C for 60 minutes irreversibly inactivated the synthesising system. Rather remarkably, the optimum temperature for enzyme action was found to be about 40° C. Generally, it was observed that in the studied strains the optimum temperature

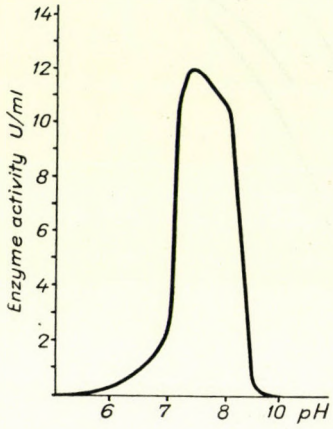

Fig. 2

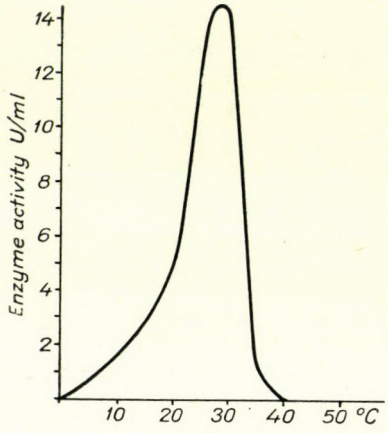

Fig. 3

for action by the enzymes which participated in the dissimilation (α-mannosidase, amylase, phosphatase, meta-phosphatase, protease), was 39—40° C, and that respiratory activity too was the most marked at 38—40° C. On the other hand, assimilation processes (e. g. the synthesis of α-mannosidase or streptomycin) required lower (28—30° C) temperatures for their optimal activity, higher ones inhibiting markedly the synthetic processes.

Effect of inhibitors on enzyme synthesis. The effects of various inhibitors on the synthesis of α-mannosidase and, to serve comparative purposes, their influence upon the respiratory activity, are illustrated in Table IV.

The data obtained for sodium azide and dinitrophenol were in agreement those for the effect which these inhibitors are well-known to exert upon the synthetic reactions [11, 12]. Perfectly similar results were obtained in the presence of sodium azide and dinitrophenol for the inhibition of respiration in the *Streptomyces griseus* strain studied by HOCKENHULL *et al.* [13]. It was remarkable however, that while potassium cyanide and sodium mono-iodoacetate did not too strongly influence respiration, they completely inhibited the synthesis of

Table IV

Effect of inhibitors on α-mannosidase synthesis and endogenous respiration

Inhibitor	Phenol liberated, μg/ml		Oxygen consumption	
	6^h	10^h	μl/h	%
Control	213	340	304	100.0
NaN_3 $5 \cdot 10^{-3}$ *M*	0	0	260	85.5
2,4-dinitrophenol 10^{-3} *M*	0	0	338	112.1
KCN $5 \cdot 10^{-4}$ *M*	0	0	222	73.0
$CH_2ICOONa$ $5 \cdot 10^{-4}$ *M*	0	0	227	74.7
Na F 10^{-2} *M*	225	345	300	98.6
Na F $5 \cdot 10^{-2}$ *M*	0	0	175	57.6
Na_2HAsO_4 10^{-2} *M*	210	350	299	98.4
Na_2HAsO_4 $5 \cdot 10^{-2}$ *M*	0	15	158	52.0
Na_2HAsO_3 $5 \cdot 10^{-3}$ *M*	0	0	50	16.5
$K_3[Fe(CN)_6]$ $5 \cdot 10^{-2}$ *M*	53	175	158	52.0
$K_3[Fe(CN)_6]$ 10^{-2} *M*	38	115	275	90.5
Streptomycin SO_4 10^{-2} *M*	35	192	152	50.1
Streptomycin SO_4 $5 \cdot 10^{-3}$ *M*	43	317	252	82.9

The figures represent average values from at least 5 determinations. — Percentage deviation was nowhere in excess of ±10%.

α-mannosidase even at a concentration of $5 \cdot 10^{-4}$, presumably because they specifically suppressed one or the other of the enzymes participating in enzyme synthesis. Fluoride and arsenate were found to inhibit *α*-mannosidase synthesis at fairly high concentrations only, and since they exerted a similar effect upon respiration, their inhibitory action was probably due to denaturation following high ion concentration. Arsenite intensely inhibited all the life processes. The effect of streptomycin was presumably not specific either, but asserted itself through inhibiting respiration. In the case of potassium ferricyanide the fact interfered materially with evaluation, that in dependence on concentration, the ferricyanide complex influenced enzyme activity as well.

Inhibitor and inducer were administered simultaneously, and the inhibitory action was measured in proportion to the difference between the phenol liberated from the inhibitor-containing and the control suspension, taking of course due account of the influence which the applied inhibitor exerted on enzymic action.

Effect of amino acids on enzyme synthesis. Results contrary to those in the literature were obtained in our experiments concerned with enzyme synthesis in the presence of various amino acids.

On the evidence of our investigations, the inhibitory action of amino acids specifically affects enzyme synthesis ; those that display such action do not

Table V

Effect of amino acids on enzyme synthesis

No.	Substrate	Phenol liberated μg/ml						Respiration O_2	
		3^h	%	6^h	%	10^h	%	μl/h	%
1	$4 \cdot 10^{-3}$ α-phm.*	122	100	258	100	530	100	220	100
2	$4 \cdot 10^{-3}$ α-phm. + 2% casein hydr. ..	12	10	15	6	32	6	450	204
3	$4 \cdot 10^{-3}$ α-phm. + 0,01 *M* glycine ...	136	112	305	118	459	87	238	108
4	$4 \cdot 10^{-3}$ α-phm. + 0,01 *M* alanine....	87	71	223	18	483	91		
5	$4 \cdot 10^{-3}$ α-phm. + 0,01 *M* valine	38	31	46	18	81	15	206	94
6	$4 \cdot 10^{-3}$ α-phm. + 0,01 *M* arginine ...	94	77	252	98	542	102		
7	$4 \cdot 10^{-3}$ α-phm. + 0,01 *M* ornitine ...	32	26	60	23	111	21		
8	$4 \cdot 10^{-3}$ α-phm. + 0,01 *M* glutamic acid	29	24	29	11	59	11		
9	$4 \cdot 10^{-3}$ α-phm. + 0,01 *M* histidine...	115	94	230	98	450	85		
10	$4 \cdot 10^{-3}$ α-phm. + 0,01 *M* asparagine	146	120	201	78	508	96		
11	$4 \cdot 10^{-3}$ α-phm. + 0,01 *M* aspartic acid	136	112	225	87	446	84		
12	$4 \cdot 10^{-3}$ α-phm. + 0,01 *M* glutamine .	134	110	228	88	403	76		
13	$4 \cdot 10^{-3}$ α-phm. + 0,01 *M* methionine	38	31	70	27	175	33	295	134

* α-phm. = α-phenylmannose

influence respiration, neither one by one, nor in a complex; on the contrary, they may even stimulate it; nor do they suppress enzyme activity. It seems that in the synthesis of α-mannosidase the microorganism is unable to utilise the amino acids of inhibitory effect. This in itself does not constitute a reason why there should be inhibition, but it seems possible that these amino acids themselves induce some kind of an enzyme synthesis, by which the α-mannosidase synthesis is suppressed ("diauxie"). Future research is required to clarify this point.

Effect of inhibitors on the production of streptomycin B in shake cultures. In 500 ml Erlenmeyer flasks, 100 ml of nutrient medium were inoculated with 2 ml of a 48-hour vegetative culture of strain L. $Sz._1$, and subjected to shaking at 25° C for 64 to 88 hours. The inhibitors were introduced at intervals ranging from 0 to 48 hours. After shaking for 64 and 88 hours, respectively, the total streptomycin and the streptomycin B contents of the flasks were determined, and the α-mannosidase activity was measured (Table VI). Most of the inhibitors failed to give satisfaction, for, in addition to the synthesis of α-mannosidase, they inhibited the production of streptomycin and even the development of microorganisms. Nevertheless, the difference in time between the commencement of streptomycin production and α-mannosidase synthesis, provided some footing for us to distinguish the two inhibitory processes from each other; in the case of dinitrophenol and $CH_2ICOONa$, mostly with succes.

Table VI

Selective inhibition of α-mannosidase synthesis

No.	Inhibitor	Inhibitor introduced after hours	Streptomycin U/ml			Streptomycin B %			α-mannosidase activity U/ml		
			40h	64h	88h	40h	64h	88h	40h	64h	88h
1	0	—	310	865	1260	0	0	0	0	10.82	8.66
2	0	—	319	800	1255	0	2	0	0	11.69	10.18
3	2,4-DNP ... $8 \cdot 10^{-3}$ *M*	0	0	0	0	—	—	—	0	0	0
4	2,4-DNP ... $8 \cdot 10^{-4}$ *M*	24	100	166	196	70	65	—	0	0	0
5	2,4-DNP ... $8 \cdot 10^{-4}$ *M*	40	239	395	574	0	31	47	0	0.59	0
6	2,4-DNP ... $8 \cdot 10^{-4}$ *M*	48	340	508	616	0	0	0	0	1.49	2.86
7	$CH_2ICOONa$ $5 \cdot 10^{-3}$ *M*	0	0	0	0	—	—	—	0	0	0
8	$CH_2ICOONa$ $5 \cdot 10^{-3}$ *M*	24	0	0	0	—	—	—	0	0	0
9	$CH_2ICOONa$ $5 \cdot 10^{-3}$ *M*	40	280	373	576	0	14	21	0	0.59	0.62
10	$CH_2ICOONa$ $5 \cdot 10^{-3}$ *M*	48	295	585	616	0	1	2	0	2.28	1.40

Fig. 4 shows that the production of streptomycin was fairly advanced when synthesis of α-mannosidase just set in, while the data in Table VI illustrate that when introduced after the starting of streptomycin synthesis but before that of α-mannosidase activity, the inhibitor did not wholly suppress the production of streptomycin, but did prevent enzyme formation, or allowed it to

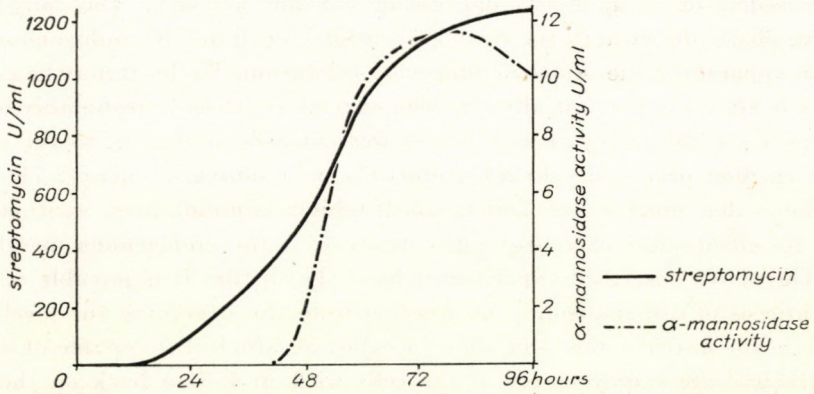

Fig. 4

set in but late and a low intensity. Cultures with an inhibitor invariably contained streptomycin B up to a certain percentage, but in controls practically streptomycin A only was found. On these grounds it appeared that introduced at the right time, the inhibitor which suppressed streptomycin synthesis up to 50 or 60 per cent only, brought α-mannosidase formation to a complete stop,

and that for the lack of an adequate quantity of α-mannosidase, a part of the streptomycin produced after the introduction of the inhibitor failed to undergo conversion into streptomycin A. In the same experiments carried out with strain X_1 there was only production of streptomycin B, provided the inhibitor had been introduced at the right time.

Because of appreciable differences in the vitality of the inoculation materials at our disposal, and therefore in the rate of development of the experimental cultures, it was very difficult to choose the right time for the introduction of the inhibitor, yet the streptomycin B appearing in most of the experiments testifies to an actual connection between the presence of α-mannosidase in fermentation and the ratio of streptomycin A to streptomycin B.

Mention has already been made of our observation that in the early active growing period the cell did not react to the presence of the inducer (i. e., no enzyme synthesis set in), and a longer inactive phase (of 16 to 20 hours) preceded enzymic action. If, on the other hand, the cells had previously been starved and the inducer had been added to the mycelial suspension not earlier than the 16th hour, activity showed in 3 to 4 hours. Extension of the starvation time to 24—26 hours mostly failed to bring about induction, but even in such cases enzyme synthesis was successfully elicited; for instance, on the addition of 1 per cent glucose to the suspension. Exactly the opposite effect was called forth by adding the glucose to a freshly prepared suspension, for then enzymic action set in late in relation to the glucose-free control; moreover, with a higher initial concentration (4 per cent) or the continued administration of glucose, we succeeded in completely suppressing enzyme activity. The long-lasting negative phase observed in the fresh suspension consuming its endogenous reserves, was apparently due to some endogenous inhibition. To this it may be ascribed that while glucose given initially retarded enzyme synthesis, presumably because in its presence the endogenous reserves were utilised but later, to the starved cells it ensured practical synthesis, probably as a source of energy. This in its turn shows that glucose does not by itself inhibit α-mannosidase synthesis, but exerts its effect indirectly through a decrease in the endogenous metabolism.

The above described experiments have shown that it is possible to retard the synthesis of α-mannosidase by keeping from the beginning the level of the glucose in the nutrient medium above a value, at which it is capable of meeting the carbohydrate requirements of the cells without falling back on the endogenous reserves. With shaken experiments there were two ways of proving this. One was to use a nutrient medium in which the microorganisms would grow leisurely and consume the carbohydrate slowly; the other was to supply glucose continuously.

In the first experiment a synthetic glucose-KH_2PO_4-$(NH_4)_2SO_4$ medium was used, in which both strains were very slow in growing and the glucose was not consumed until the 6th or 7th day. To the nutrient in the test tube α-phenyl-

mannose at a concentration of 0.002 *M* was added (excepting the controls) (Table VII).

Table VII

Decomposition of α-phenylmannose in synthetic medium with accompanying inhibition of induction

No.	Strain	Medium					Activity U/ml				Streptomycin U/ml	
			40^h	64^h	88^h	112^h	40^h	64^h	88^h	112^h	88^h	112^h
1	L. Sz.$_1$	Synth. I + $4 \cdot 10^{-3}$ *M*	54,2	180	220	254	0	0.21	0	0	200	227
2	L. Sz.$_1$	α-phenylmannose	63	202	237	—	0.12	0	0.1	0	117	—
3	X_1		0	15	21	27	0	0	0	0	—	158

The data in Table VII show that, as was to be expected, no inductive α-mannosidase synthesis had set in, though in the tubes inoculated with strain L. Sz.$_1$ the α-phenylmannoside had in a few days nevertheless undergone slow decomposition; whereas in the cultures of strain X_1, phenol was observed to have been liberated in quite insignificant quantities only; there was no measurable phenol oxidase activity in either culture.

In the second experiment, with a continued supply of glucose, the results obtained were essentially the same.

The findings in these two experiments indicated that the mycelium of strain L. Sz.$_1$ contained a small amount of α-mannosidase which had not been produced inductively in the cells. From the amount of phenol liberated we calculated that the enzyme present in the cells in our first experiment would have sufficed to decompose about 900 μg of streptomycin B in four and a half days; i. e., more than the total the cells produced during the same period of time. It should be noted that to decompose even 3000 μg streptomycin B during that time would have required an enzymic activity too slight to be demonstrated in 30 minutes by our method. This at any rate be regarded as the explanation of the continued production of streptomycin A in strain L. Sz.$_1$. Ultimately, our experimental results indicate that this strain possesses a small amount of constitutive α-mannosidase which during mycelial development arises without induction and ensures the breakdown *in situ* of all the streptomycin B produced. Strain X_1, on the other hand, seems to have inductive α-mannosidase only, or at the utmost a negligible quantity of the enzyme of constitutive character, and enzyme formation is much more strongly inhibited than in strain L. Sz.$_1$.

The part of streptomycin in the synthesis of α-mannosidase. In addition to the phenomena already described, Fig. 4 reveals the otherwise regularly observable fact that under natural conditions, without induction from outside the activity of α-mannosidase is invariably preceded by the synthesis of streptomycin, and that the rate of that activity does not begin to diminish until the

streptomycin synthesis is markedly abating. It was found that whenever in shaken and fermentor cultures or mycelial suspensions it was possible to demonstrate α-mannosidase activity without external induction, streptomycin was already present in the nutrient medium.

Direct experiments, too, were undertaken to prove the part played by streptomycin. It is known from the literature[13], and we ourselves have repeatedly observed that, besides causing rapid mycelial growth and intensified metabolism in *Streptomyces griseus* cultures, high inorganic phosphate concentrations (500 to 1000 μg/of P per ml) completely suppress streptomycin production. It appeared an intriguing task to investigate to what extent in the otherwise abounding and vigorous cultures the absence of this production affected α-mannosidase formation. Experiments carried out in shaken cultures or in 10 l glass fermentors containing 500 μg/ml inorganic phosphorus, furnished unanimous evidence that whenever streptomycin ceased to be produced, there was no α-mannosidase formation either. At the same time, on adding to such cultures 1 mg/ml α-phenylmannose, α-mannosidase activity was found to develop in 8 to 10 hours (Table VIII); in other words, the cells were not lacking the potential to synthesise the enzyme, what was required was only an inducer.

Table VIII

Interrelation of the synthesis of streptomycin to that of α-mannosidase

No.	Substrate*	Shaking for 48h		Shaking for 96h	
		Streptomycin U/ml	α-mannosidase U/ml	Streptomycin U/ml	α-mannosidase U/ml
1	Control**	480	12.1	1250	11.2
2	+ 500 μg/ml inorg. P	0	0	0	0
3	+ 500 μg/ml inorg. P + 0,004 *M* α-phenylmannose in the 86th hour	0	0	0	10.7

* The values represent results obtained for strain X_1, but similar results were obtained for strain L. Sz.$_1$.

** Normal nutrient medium containing soya bean—glucose—KH_2PO_4—$(NH_4)_2SO_4$.

This observation allows the conclusion that under natural conditions either streptomycin B or some intermediate α-mannoside, which forms during its synthesis, may serve as the inducer of the production of α-mannosidase. By our findings this "internal" induction is weaker than the "external" induction elicited with synthetic α-mannosides but, like the latter, is under the control of the endogenous inhibition discussed above. It is a general observation that in cultures producing streptomycin in low concentrations, α-mannosidase activity is weak or fails to set in at all, while in abundantly producing cultures it is intense

and particularly develops at the time streptomycin production is at its peak. The latter phenomenon seems to warrant the assumption that the difference in efficiency between the "internal" and the "external" induction results from the difference in the concentration of the natural internal and the applied external inducer.

Discussion

The following of our experimental findings would appear to support the statement that in *Streptomyces griseus* cultures the enzyme α-mannosidase is the product of inductive synthesis in the mycelium. (i) no active enzyme extract was obtainable from the mycelium until α-mannosidase activity had become demonstrable by the usually applied method ; (ii) proteolysis or an increase in the redox potential were of no effect upon enzyme activity ; (iii) in previously starved and practically no longer growing mycelial suspensions α-phenylmannose or α-methylmannose induced a marked α-mannosidase activity.

Strain L. $Sz._1$ was invariably found to produce streptomycin A, and from the L. $Sz._1$ mycelium, even before the appearance of an activity measurable with the usual method, extracts were obtainable [1] which displayed very slight yet still demonstrable α-mannosidase activity. These two facts permit the inference that in the case of strain L. $Sz._1$ it is not an inductive process alone which is responsible for enzyme formation. This was borne out by those of our experiments, from which we had precluded the possibility of induction, and yet observed the release of phenol from phenylmannose.

A different situation prevailed with strain X_1, the other variant studied ; with α-mannosidase activity failing to set in, streptomycin B only was observed to be produced throughout.

By YUDKIN [17] it was noticed in 1938, and later by SPIEGELMANN [18], that the same enzyme may be partially inductive and partially constitutive in character. Studying the question in β-galactosidase and amylomaltase, respectively, LEDERBERG [19] and COHEN—BAZIRE and JOLIT [20] more recently came to the conclusion that for an enzyme it was possible to assume even within one and the same strain either a constitutive or an inductive character. It is common knowledge that ultraviolet or X-ray irradiations exert a marked influence on these characters.

The foregoing would seem to justify the assumption that, to all intents and purposes, strain X_1 possesses inductive α-mannosidase only, while in strain L. Sz_1, which is but a variant derived by repeated ultraviolet irradiation, a part of the α-mannosidase is stabilised to a constitutional enzyme.

The fact that under normal conditions α-mannosidase activity manifests itself in the cultures of both strains, is an indication that during their life cycles both produce a substance which induces α-mannosidase synthesis. Considering that α-mannosidase formation can take place spontaneously (internal induction),

without the action of an external inducer, though perhaps at a different intensity, the enzyme ought really to be regarded as a constitutive one. Inconsistent with this are, on the other hand, the conditions under which enzyme activity develops, since they are typical of the inductive enzymes. The dimorphism noticeable in α-mannosidase formation within strain L. $Sz._1$ likewise indicates that enzyme synthesis is not a uniform process throughout. Data in the literature, too, support the view that no sharp line of demarcation can be drawn between consitutive and inductive enzymes.

On the evidence of our experimental findings, the development of enzymic activity is retarded by a powerful internal inhibition closely connected with endogenous metabolism, which cannot, however, be brought into harmony neither with the earlier theory of "glucose diauxie" [14, 15], nor with the more recent view of COHN and CAPDUPUY [16] that glucose inhibits the inductive synthesis of β-galactosidase by preventing the inducer (the corresponding β-galactoside) from permeating the cell wall. Since by our above mentioned experimental results the permeability of the cell wall is of no prime importance in induction, we must assume either that active endogenous metabolism in some way affects the intracellular receptors, or that there are one or more among the reserve substances of the cell (such as an amino acid, a carbohydrate or one of its derivatives) which inhibit the synthesis of α-mannosidase. This inhibition only affects α-mannosidase activity developing rapidly and in large quantities (inductively), but does not interfere with the continuous production of streptomycin A in strain L. $Sz._1$.

It still awaits clarification what compounds can be inducers of α-mannosidase synthesis *in vivo*. The mannosidostreptomycin itself or some intermediate α-mannoside forming during its synthesis, are the most likely natural inducers. Support is afforded for this assumption by our observation that under natural conditions (without an external inducer) no α-mannosidase activity was noticed unless streptomycin had already formed in the culture. In cultures where by the application of high phosphate concentrations streptomycin production had been prevented, there was never any α-mannosidase activity, although it was not inhibited, since upon the introduction of an inducer it developed rapidly. Investigations carried out with streptomycin B introduced from outside have so far failed to yield satisfactory results, presumably because of the impermeability of the cell wall. The details of this question remain to be elucidated by future research.

In our experiments we were only able to elicit induction with α-mannosides, and thus, with due regard to the circumstances, the presence of the enzyme was of necessity an indication that in the synthesis of streptomycin an α-mannoside (streptomycin B) formed first, and streptomycin A, the aglycone, was but a secondary product. Accordingly, the last step in the synthesis of streptomycin is mannosidostreptomycin → streptomycin + mannose.

On the evidence of our investigations, the above reaction would seem to be the only route for the last step in the synthesis of streptomycin A, nevertheless the possibility that the reaction follows some other pathway, by which that last step is evaded, should not be altogether excluded.

No conclusions can of course be drawn from the experiments we have carried out so far, but from the inductive character of the enzyme catalysing the last step it seems reasonable to infer that the enzymes in the preceding steps are of the same character, and that the synthesis of streptomycin, wholly or partially, takes place *via* a chain of inductively forming enzymes, like the galactozymase system in yeast, or the anaerobic chain of enzymes induced by oxygen [21].

A solution of the problem would not only bring us nearer to an understanding of the biosynthesis of streptomycin ; it might also open up new prospects in the production of it and, perhaps, some other antibiotics.

Summary

1. In studying α-mannosidase activity in *Streptomyces griseus* cultures it has been found that the enzyme is inductively synthesised in the mycelium upon the action of various α-mannosides.

2. The optimum temperature and pH for enzyme synthesis have been determined, and the action of various compounds (cellular poisons, amino acids, carbohydrates, etc.) on the synthetic process has been examined.

3. Enzyme synthesis has been found to be retarded by an internal inhibition connected with endogenous metabolism ; the part played by streptomycin synthesis in the formation of α-mannosidase has been investigated.

4. The conclusion has been reached that the synthesis of α-mannosidase is not exclusively inductive in character, at least not with every strain. In small quantities the enzyme may form constitutively in the mycelium. For α-mannosidase no sharp line of demarcation can be drawn between the two characters.

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VACCINATION AGAINST PERTUSSIS. AN APPRAISAL OF ITS EFFICACY

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Vaccination against pertussis has now passed the experimental stage and the number of reports evaluating its practical results is increasing. Some authors, expressing the efficacy of vaccination numerically, claim to have achieved 80 to 90 per cent success [1—4], whereas others [5—8] report of more modest, but still "significant" results.

In Hungary, G. OLÁH [9] has carried out the most detailed studies on vaccination against whooping cough, but others [10—12] also performed investigations in this field. More recently, ÚJHELYI [13] has reported 80 to 90 per cent success from his own.

Apart from the vaccinations made by the authors referred to and by some of the practising physicians in Hungary, vaccination against pertussis is carried out in combination with that against diphtheria and tetanus since 1954, after having done it for one year in combination with vaccination against diphtheria alone. Vaccination against these diseases is compulsory and is carried out twice a year (spring and autumn) throughout the country. Infants aged 6 to 11 months are inoculated twice at 4-week intervals and are re-inoculated one year later, i. e. at the age of 18 to 23 months. In addition, the children aged 6 years are inoculated in the first grade of elementary school. During epidemics, before collective holidays, etc. occasional inoculations are made, if so necessary.

The pertussis component of the vaccine used in the period discussed in this paper was produced by the "*Human State Institute for Vaccine Production and Research*" by the method of UJHELYI. It is a bacterial extract, prepared from 3 or 4 strains of S-phase H. pertussis, grown in a solid semisynthetic medium containing casein hydrolysate, salts, yeast extract and cystine. On completion of cultivation the cultures in Roux flasks are washed off with saline, the pooled suspension are centrifuged without delay and the sediment is resuspended in distilled water. This suspension is frozen and thawed 9 times in succession in solid dry ice-acetone and in a water bath of 37° C, respectively, and is centrifuged. To the supernatant, which is a slightly viscous, opalescent fluid containing the pertussis extract antigen is added 0.3 per cent formaldehyde. After 4 days at 37° C in an incubator the antigen can be stored for a long time under refrigeration. The final product contains in each ml an amount of extract antigen equivalent to 10 000 million germs.

The combined (Di-Per-Te) vaccine contains, in addition, 15 Lf purified diphteria toxoid and 5 B. U. purified tetanus toxoid per ml, as well as $Al(OH)_3$ as an absorbent and merthiolate (1 : 10 000) as a preservative.

Before trying to evaluate the effectiveness of the vaccinations against pertussis carried out since 1953, it had to be established which age groups had been

inoculated in what rate during the test period. The data obtained are presented in Fig. 1.

The ordinata shows the date of birth, the abscissa the calendar dates. The diagonal lines may be called the age axis. The areas delineated by the age

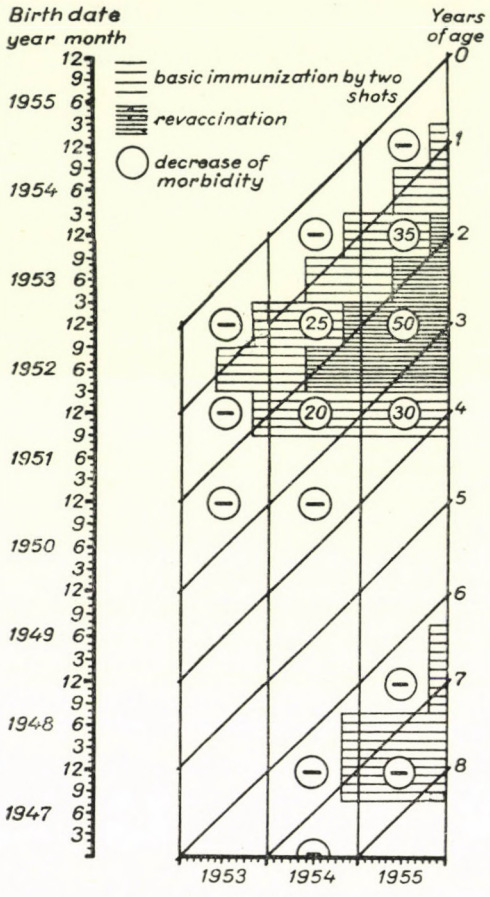

Fig. 1

axis and the lines for the calendar years shown on the abscissa include the members of 1 age group each. Fig. 1 should be interpreted as follows. The test subjects born in April, 1954, are at that time 0 year of age, thus, they start from the age axis for the 0 year old, parallel with the abscissa. For one year, i. e. until April, 1955, they are under 1 year of age and in April, 1955, they reach and cross the diagonal line indicating 1 year of age. Basic immunizations carried out at the times indicated on the abscissa are shown by shading in the corresponding age groups, whereas the re-inoculations are designated by denser shading. Reading along the straight lines parallel with the ordinata allows to

determine without difficulty for any point of time the rate of vaccination in any age group. For example, in April, 1955, none of those under 1 year of age had been vaccinated, babies 1 year old were given the basic immunization, the 2 year old children were re-vaccinated, part of the 3 year old was given basic immunization, and so forth.

It can be seen that the immunizations made in 1953 could hardly have any significant effect that year, except perhaps among those 1 year old. On the

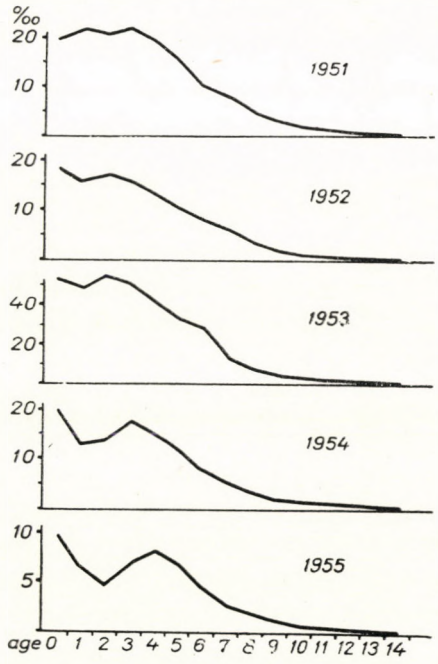

Fig. 2

other hand, in 1954, the entire 1 year old group, as well as most of the 2 year old had been vaccinated. In 1955, the immunity of the 1 year old group moved at about the same level as in 1954, whereas that of the 2 year old group had increased considerably, because that age group as a whole had been re-vaccinated. Due to vaccinations made in earlier years, a considerable percentage of the 3 year old children developed immunity by that time. As to the school-children, the 6 year old group was insignificantly immunized in 1954 and 1955. On the other hand, 7 years old children were inoculated in considerable numbers in 1955.

The first gross estimate of the effectiveness of vaccination can be made from the age curve. In Fig. 2 are presented the values of specific morbidity per thousand. To facilitate comparison, different scales were used in successive years, in order to eliminate interference by changes in morbidity. It is seen that the age curve for 1953 did not differ from that for 1952, and in 1953 the

inoculations made that year had no noticeable influence. In 1954 the shape of the curve has somewhat changed, the value for the 1 year and 2 year old groups having obviously decreased in comparison to the other age groups. The change in the shape of the curve was even more conspicuous in 1955, when a remarkable relative decrease occurred not only in the 1 year and 2 year groups but also in the 3 year one.

When comparing the age curves it is assumed that the relation of morbidity between the different age groups has remained, ceteris paribus, unchanged. This applies to pertussis just as to some other infectious diseases. Plotted in

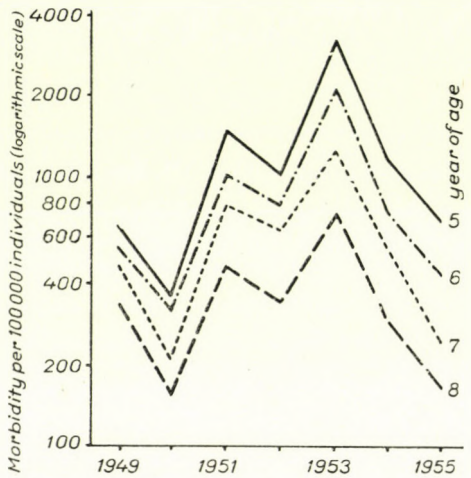

Fig. 3

the logarithmic scale, the morbidity curves for the different age groups run parallel. This can be seen in Fig. 3, in which the morbidity of the 5, 6, 7 and 8 year age groups is shown for the period 1949—1955. Except for minor, insignificant deviations, the four curves are parallel. It is to be born in mind that, according to Fig. 1, the 5 years and 8 years old had not been vaccinated until the end of 1955. A small percentage of the 6 year old group had been vaccinated in 1954 and 1955, and in 1955 a considerable percentage of the 7 year old could be considered immune. Had this vaccination been effective, the curve for the 7 year old group would not have remained parallel with the other curves in 1955, but would have markedly decreased.

Fig. 4 illustrates clearly the relative decrease of morbidity. The 4 to 6 year age group was unvaccinated, the other have been gradually immunized during the test period. The morbidity curves for the 0 and the 4 to 6 year groups remained parallel throughout, indicating that vaccination had no effect on the 0 year old. In the period 1949—1953 the curve for the 1 year old had been parallel with that for 4 to 6 years old, but this parallelism ceased in 1954 and

1955. Accordingly, morbidity has relatively decreased. An even more marked decrease is evident from the morbidity curve for the 2 year group, it having sunk far below that for the 4 to 6 year group. Finally, in 1955 the curve for the 3 year old children shows some relative decrease.

Thus, vaccination has brought evaluable results only in the 1 to 3 year age group. It is, however, desirable to express the decrease in morbidity also numerically. Such a determination cannot be as precise as a comparison of the morbidity rates in homogeneous groups. All we can do is to compare the morbidity rates for the vaccinated age groups to those for the unvaccinated ones near to them. A simple way to do so is to express year for year the morbidity

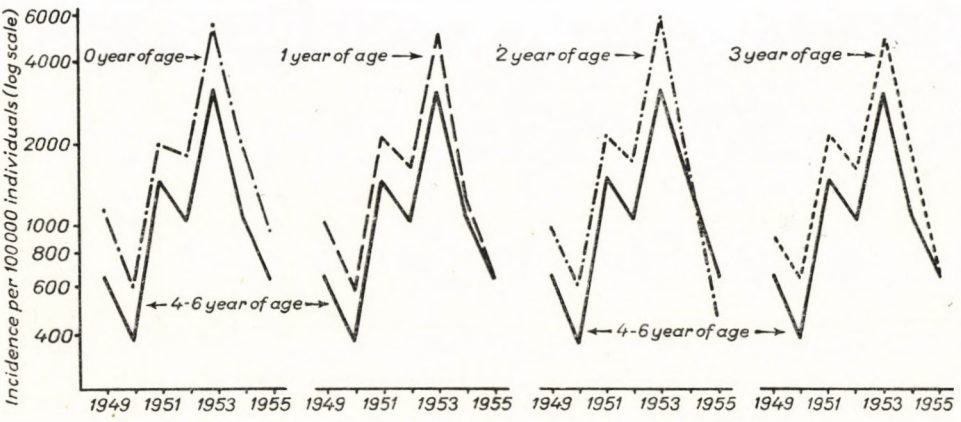

Fig. 4

rates for the vaccinated age groups in percentage of that for the unvaccinated age groups. This is shown in Fig. 5, in the first part of which (a) the morbidity for the 0, 1, 2 and 3 year age groups is given in percentage of the values obtained for the 4 to 6 year old.

According to Fig. 5, the morbidity rates for the youngest age groups, as expressed in percentage of the morbidity rates of the 4 to 6 year age group underwent little change in the period 1949—1953. This means that the effectiveness of vaccination can be determined with a high degree of accuracy by comparing morbidity rates in the vaccinated and the non-vaccinated groups. In 1954 the curves for the 0 and the 3 year old remained at the level of the previous year, but those for the 2 and the 1 year old showed a marked decline. In 1955 the curve for the 0 year group was unchanged, that for the 1 year group declined as compared to the previous year, the curve for the 2 year old showed a further decrease and that for the 3 year old a decline for the first time.

The effectiveness of vaccination, *i. e.* the decrease in the morbidity rates of the vaccinated age groups, can be determined numerically also by comparing

the results for the period after 1953 to the average for the previous period. The results of this comparison are presented in the second (b) part of Fig. 5. In 1953, none of the age groups showed any evidence of an effective immunization. In 1954 there was no change in the morbidity rates under 1 year of age and in the group of the 3 year old. The relative morbidity rates for the 1 and 2. year age groups had definitely decreased, by about 25 and 20 per cent, rel spectively. In 1955, the 0 year age group still did not show evidence of successfu-

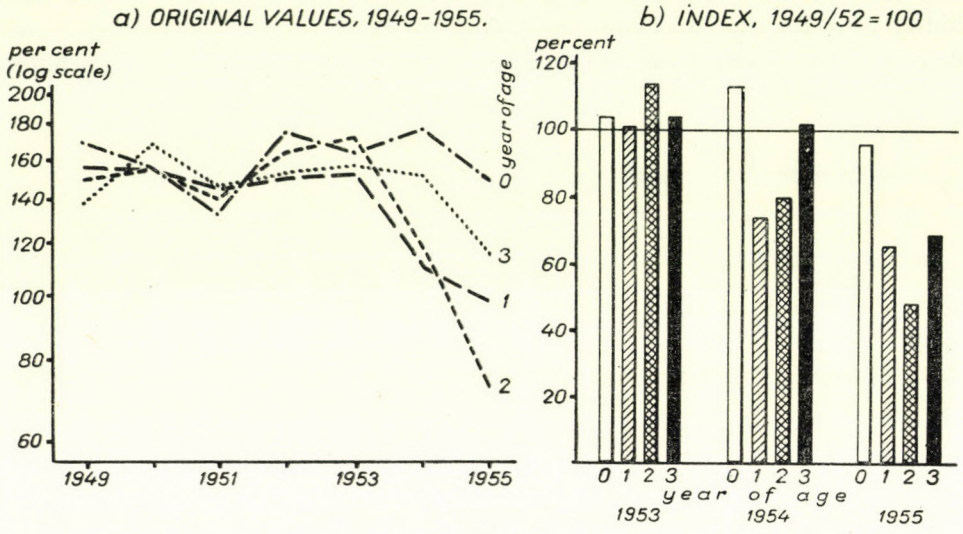

Fig. 5

vaccination. Among the 1 year old, morbidity rate decreased by 35 per cent, and among those 3 years old by about 30 per cent. In 1955, the 2 year group showed the best results, inasmuch as its morbidity rate decreased by more than 50 per cent.

In view of the high morbidity and mortality rates of infantile pertussis we have analysed separately the higher age groups of infants. None of these groups showed any evidence of a decrease in morbidity ascribable to vaccination.

The data thus obtained for the effectiveness of vaccination against pertussis were then compared with the results of immunization in the single age groups. This comparison is represented in Fig. 1.

In Fig. 1 beside the shading indicating the state of immunity, the percentage decrease in the morbidity rate is also presented. The best results were obtained in 1955, among the 2 year old. This is absolutely logical, as by that time almost all of the 2 year old children had been revaccinated. The 1954 and 1955 values for the 1 year old group, the 1954 ones for the 2 years old and the 1955 values for the 3 years old are about the same. This, too, is logical, as the

1 year old group had been subjected to at least basic immunization in both years, and a small percentage was even revaccinated. A small proportion of the 2 year old in 1954, and of the 3 year old in 1955, had not been vaccinated. Another small proportion were even re-vaccinated. However, we are unable to explain the absence of results among the 7 year old children in 1955. As regards the duration of immunity, the decrease in the morbidity rate of the 3 year group in 1955 has to be considered a most favourable sign. These children has received basic immunization in 1953 ; some of them were not even re-vaccinated and yet the immunization made 2 years earlier still had a marked effect.

Thus, among infants the present method of immunization against pertussis fails to bring the desired results, as only a small percentage of the infants are vaccinated and even this vaccination means a basic immunization only. In the age groups showing evidence of an effective basic immunization (in 1955 the 1 year old and some of the 3 year old) the immunity rate is about 20 to 30 per-cent. The best results were obtained in the age groups vaccinated twice. For example, in 1955, 50 to 60 per cent of the 2 year old were immune. The protective value of the vaccine is presumably higher, but in the course of compulsory immunizations 10 to 15 per cent of the children usually remain unvaccinated for some reason or other and the higher morbidity rate of the unvaccinated subjects cannot be evaluated separately.

The above data make it possible to compute the decrease in general pertussis morbidity due to vaccination. According to our calculations, this amounted to 6.5 per cent in 1954, and to 17.4 per cent in 1955. If immunizations are continued systematically for years, and if infants under 1 year of age are vaccinated earlier, the decrease should attain 40 to 45 per cent, this being the best effect expectable from the present method. It seems that we possess a vaccine affording 50 per cent immunity, but this does not suffice to solve the problem of pertussis.

In order further to enhance the effectiveness of immunization, better protection of the most exposed age group, that of infants, is necessary. In this group not only morbidity, but also the mortality rate is the highest. It is true that a decrease of morbidity at older age may, by a reduction in the number of sources of infection, have a favourable effect on the morbidity of infants. It seems nonetheless desirable to increase the number of immune infants. According to most authors, immunization has no untoward effect in the youngest infants, but it has been suggested that in view of the uncertain immune body production vaccinations should be carried out only after the age of 3 months [14—20].

In some countries, vaccination against pertussis is begun with Di-Per-Te inoculation at the age of 3, 4 or 5 months, and is repeated later. Such inoculations can be carried out only continuously.

Thus, to achieve a significant reduction in the morbidity rate of pertussis immunization at the age of 3, 4 or 5 months, and continuous vaccinations are necessary, perhaps with anti-pertussis vaccine alone.

Summary

Vaccination against pertussis has been compulsory in Hungary since 1953. The present method is to immunize infants at the age of 6 to 11 months with Di-Per-Te vaccine by 2 inoculations made at an interval of 4 weeks, and to re-vaccinate 1 year later. The compulsory inoculations are made every spring and autumn. In addition, children attending the first grade of elementary school, *i. e.* practically those 6 years old, are also inoculated with the above vaccine.

The effectiveness of vaccination against pertussis has been analysed by comparing the decrease in the morbidity rate of the vaccinated age groups to the morbidity rate for the non-vaccinated age groups.

The results thus far obtained show immunization to have been effective in 20 to 30 per cent of the 1 and 3 year old (who had been inoculated once) and in about 50 per cent of the 2 year old, who had been vaccinated twice. No effect of vaccination was observed in the 6 and 7 year old children. To reduce significantly the morbidity rate of pertussis, immunization must to be begun in early infancy, at least at 3, 4 or 5 months of age.

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ISOLATION OF PHAGES ACTIVE AGAINST MYCOBACTERIA

By

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Although it was more than 40 years ago that TWORT first noted the existence of bacteriophages [1], phages active against mycobacteria have been studied only in the last 10 years. GARDNER and WEISER [2], in 1947, were the first to describe a definite phage action on mycobacteria. Similar phenomena had been observed earlier by other authors, who, however, failed to notice that the lysis was the result of phage action, i. e. due to virus. All that had been presented was a description of the phenomenon, explained rather ambiguously. GARDNER and WEISER made more precise observations, ascribed the phenomenon of lysis to phage action and succeeded in isolating phage from soil.

Later HAUDUROY and ROSSET [3] reported the isolation of phage from garden soil.

PENSO and ORTALI [4, 5] also isolated phages from fertilized soil and obtained 6 kinds of phage active against the more important mycobacteria.

Recently, the number of reports dealing with the isolation of phages active against mycobacteria has increased [6, 7, 8, 9]. PENSO et *al.* demonstrated the presence of acid-fast bacteria and of phages active against them in fertilized soil. A symbiosis has been suggested between phages and the acid-fast bacteria and it has been supposed that the organisms are to be found most often in soil. This is why research workers studying phages usually examine samples of soil.

Investigations

We have examined 28 samples of soil. In 5 of them the presence of phage was demonstrated by two different methods. The samples originated from different places. In one case phage was isolated from fertilized soil and in the other 4 cases it was isolated from fertilized garden soil. Attempts at isolating phage from sewage-sludge or from a mixture of sewage and fertilized soil ended in failure.

The strains used were *Mycobacterium phlei*, *smegmatis*, *friburgensis* and *minetti*.

Methods of isolations

The samples of soil were inoculated with the above bacterial suspensions twice a week, over 4 to 6 weeks. The bacterial suspension were prepared by the following method. The 5-day, 5 ml 2 per cent glycerol broth cultures were centrifuged, the bacterial sediment was washed twice in physiological saline, then suspended in 10 ml physiological saline and pipetted to the soil samples. This method differs from that described by GARDNER and WEISER only in the duration

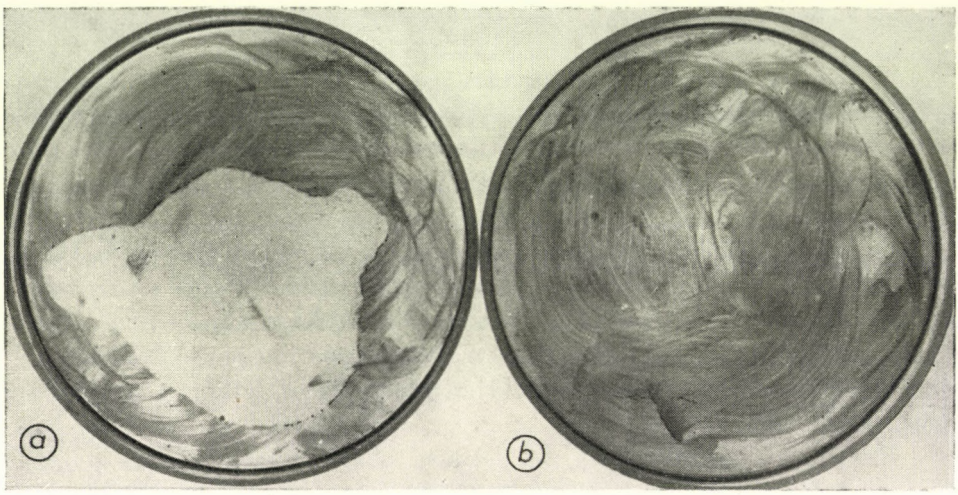

Fig. 1. a) Lytic area of *Mycobacterium phlei* phage
b) Phage-free control

of incubation. By this method we succeeded to isolate on one occasion a phage active against *Mycobacterium phlei.*

After the concentration time had been complete the fluid was decanted from the samples of soil and filtered through gauze, then through a sterile G_5 filter. One or two drops of the filtrate were placed onto the surface of the culture medium, which had been previously infected with the strain used for inoculating the sample of soil. The culture media were 2 per cent agar and broth containing 2 per cent glycerol, poured into Petri dishes and allowed to stand at 37° C for 2 days (sterility test). The pre-cultures were grown in 2 per cent glycerol broth for 24 to 48 hours. The agar plates were infected with this material, taking care to ensure even distribution of the inoculum over the surface of the culture medium. Subsequently, a certain amount of the filtrate containing the phage was added drop-wise to the inoculated medium. Using a suitable control, the plates were placed into an incubator for 24 to 48 hours (at 37° C), making the reading according to the rate of growth of the strain. Depending upon the amount of fil-

trate added, lytic areas of varying size appeared on the culture medium. The surface of the lytic area was gently streaked with a glass rod, taking care not to damage the surface of the medium. The thin film thus obtained was homo-

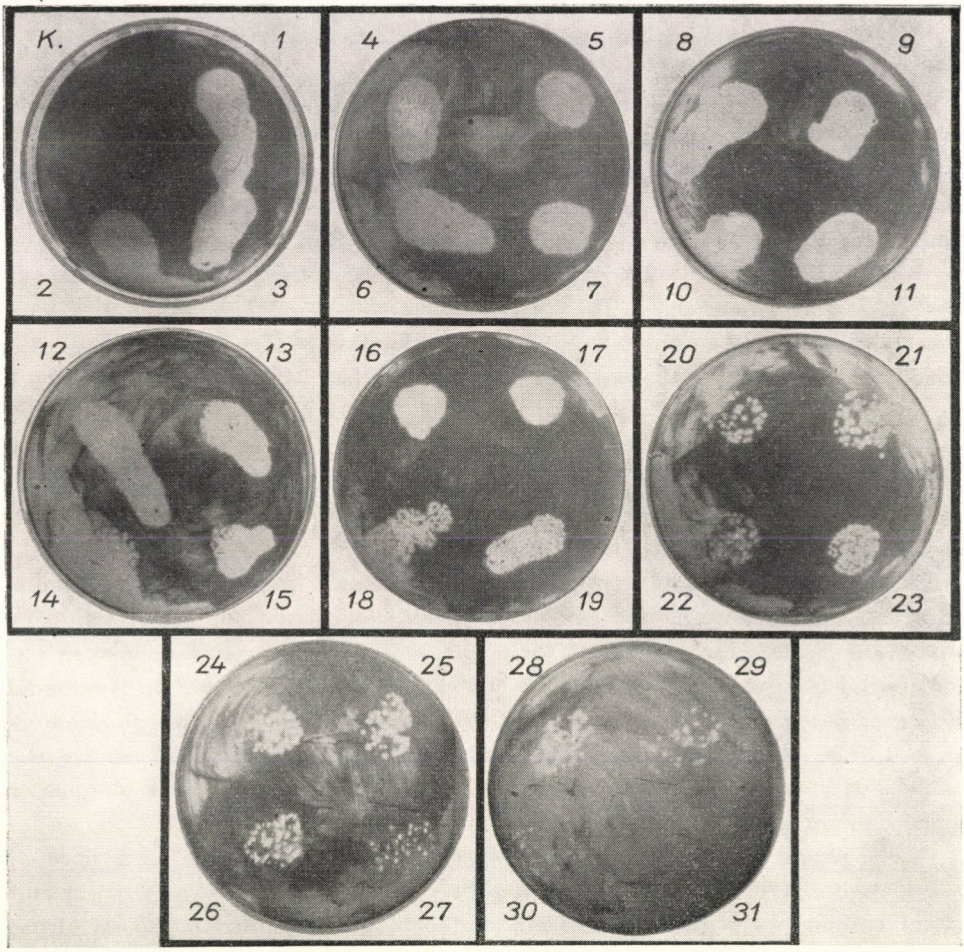

Fig. 2. Titration of *Mycobacterium friburgensis* phage on agar plate.
Twofold serial dilution series. At a phage concentration of 10^{-9} (sign 31) 2 to 3 plaques are still visible

genized with broth in a braying mortar, poured into a Petri dish and allowed to stand for 24 hours in a refrigerator. After filtering through gauze and a sterile G_5 filter, the filtrate was stored in sterile tubes, in a refrigerator (Fig. 1).

Subsequently, we tried HAUDUROY's concentration technique, which since then we have been using in our experiments.

In this case the fertilized garden soil was added to the culture medium, i. e. to 2 per cent glycerol broth, incubated at 37° C for 45 hours and filtered through gauze and a sterile G_5 filter. This filtrate was brought together with bacteria, in the following way. Five ml of glycerol broth culture medium was inoculated with equal amounts of a 24-hour 5 ml glycerol broth culture. To half of the medium was added 0.5 ml of the filtrate, adding nothing to the other half, which served as control.

After 3 days of incubation at 37° C, a slight difference in intensity appeared between the controls and the cultures to which filtrate had been added. The latter were filtered and the filtrate was again brought together with the same strain of mycobacterium. By repeating this procedure 4 to 5 times, a complete inhibition of the strains was effected. We have succeeded to isolate phage by this method in four cases, obtaining phages for *Mycobacterium phlei*, *minetti*, *smegmatis* and *friburgensis*. The *Mycobacterium phlei*, *smegmatis* and *friburgensis* strains were completely inhibited in the 4th to 5th passages, whereas complete lysis of *Mycobacterium minetti* did not result until the 10th passage.

All four phages are almost completely inactivated by exposure to 75° C for 5 to 10 minutes. To determine specificity, the phages were placed on agar plates inoculated with other mycobacteria. They were found to be active only against the original strain.

The isolated phages were titrated in solid and in liquid culture media. Informative titration on solid culture medium was carried out in the following way. Agar plates were inoculated with 24 to 48-hour cultures. The culture was distributed evenly on the medium by streaking with a glass rod. On the media thus inoculated 0.1 ml volumes of the different dilutions of the phage were applied. The phage was diluted in increasing twofold steps and usually the 5.10^{-8} — 10^{-9} concentrations were the highest still showing 1 to 4 plaques or appearence of growth (Fig. 2).

The plaque obtained with the highest dilution was harvested and filtrated as specified above. Increasing twofold dilutions were made of this filtrate and 0.1 ml volumes were added to the inoculated plates. In addition, 0.5 ml of the filtrate was propagated in glycerol broth and the titre of the material thus obtained was also determined. A significant difference in titre, 5000 or 10 000-fold, was found between the filtrates obtained with and without propagation.

The liquid medium used for titration was 5 ml of glycerol broth. To this was added 1 ml of the suspension containing 10^7 microbes per ml. The suspension was prepared by homogenization, from a 48-hour culture. Tenfold dilutions were made of the phage filtrate, adding 1 ml of each dilution to the culture media. The results were read after incubation at 37° C for 3 days (Fig. 3). The highest dilution still giving complete lysis was 10^{-7} or 10^{-10}. At higher dilutions growth increased gradually and finally a surface layer appeared.

As regards the phage effect, there was no difference either in behaviour or in titre between the suspectible strains and those of *Mycobacterium phlei*, *friburgensis* and *minetti* made resistant to streptomycin and isonicotinic acid hydrazyde *in vitro*.

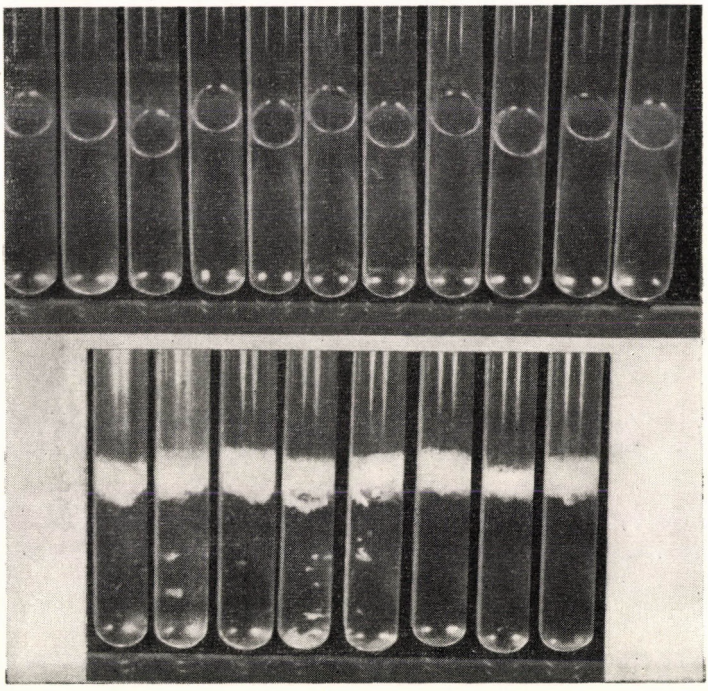

Fig. 3. Titration of *Mycobacterium friburgensis* in liquid medium. Tenfold dilution series. There is complete lysis at 10^{-10} phage dilution. With increasing dilution growth reappears gradually, the surface membrane increases in thickness. The two last tubes are phage-free controls

We have also inoculated samples of soil with $H_{37}Rv$ and with a strain of *Mycobacterium tuberculosis* grown from pathologic material for six months, without any result. The various samples of soil and this method are apparently not suited for the isolation of phages against pathogenic acid-fast organisms.

Summary

From 28 samples of soil, phage was isolated by two different methods in 5 cases. In 2 cases phage was active against *Mycobacterium phlei* and in the other 3 cases against *Mycobacterium minetti*, *smegmatis* and *friburgensis*, respectively.

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REACTIVATION OF ULTRAVIOLET INACTIVATED STREPTOMYCES GRISEUS AT LOW TEMPERATURE

By

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It is well-known that ultraviolet inactivated microbial cells may regain their ability to multiply and to form colonies under certain conditions. There are data in the literature on reactivation by light [1, 2], by intermediary metabolites [3], by thermal influences [4, 5, 6], and by storage in the dark in saline [7].

As to the reactivation of ultraviolet inactivated Streptomyces strains, KELNER [1], further WAINWRIGHT and NEVILL [8, 9, 10,] were successful in reactivating by light and by addition of different metabolic antagonists, respectively. There are no data available on the possibility of a thermal reactivation of these organisms.

In the present report, experiments will be presented as to the effects of incubation at different temperatures on ultraviolet inactivated spores of *Streptomyces griseus*.

Materials and methods

Spores of a *Streptomyces griseus* strain used for industrial purposes were studied. For cultivation of the organism the following medium was prepared: 900 ml potato extract; 100 ml yeast extract; 10 g $Na_2HPO_4 \cdot 12\ H_2O$; 1 g KH_2PO_4; and 2 per cent agar.

Irradiation of 10 ml amounts of washed spore suspensions in normal Petri dishes was made under constant stirring, from 40 cm distance, by using an "*Original Hanau*" type S 300 ultraviolet lamp. After 10 min. of irradiation the ratio of organisms killed was over 99 per cent. A heat effect comparable with that of the irradiation did not affect the viability of the cells. All the experiments were made in triplicate.

Experimental

Spores of *Streptomyces griseus* after irradiation by ultraviolet light as described above, were incubated for 1 to 5 days at low temperatures, *i. e.* at 0° C, 10° C and 17° C, respectively. Care was taken to avoid any possibility of photoreactivation. As a control, cultures incubated at 27° C were used. The results presented in Table I seem to prove that at low (0° C and 10° C) temperatures reactivation occurred.

Table I

Reactivation of UV inactivated Streptomyces griseus spores by incubation at different low temperatures, for 6 days

Temperature of incubation (°C)							
27		0		10		17	
Number of colonies	in per cent of cells inoculated	Number of colonies	in per cent of cells inoculated	Number of colonies	in per cent of cells inoculated	Number of colonies	in per cent of cells inoculated
28**	0.0006	306	0.007	74	0.0018	28	0.0006

** Mean of experiments in triplicate

The number of cells reactivated increases with time (Table II). It is remarkable that the rate of reactivation was higher at 0° C than at 10° C.

Table II

Increase in the number of cells reactivated, in relation to the length of incubation

Temperature of preliminary incubation	No. of colonies grown at 27° C after			
	0	2	4	6
	days of preliminary incubation			
27° C	28	—	—	—
0° C	—	27	76	306**
10° C	—	23	60	74**

** Mean of experiments in triplicate

Discussion

A characteristic feature of the experiments was that reactivation did not occur in every parallel culture. This reminds the observation of CHARLES and ZIMMERMANN [7], who found reactivation only in one of their E. coli strains examined.

STAPLETON *et al.* [6] found that after irradiation of bacteria there is an optimum temperature which exerts the maximum reactivating effect. In our studies on *Streptomyces griseus* maximum reactivation occurred at 0° C in the temperature range tested. The possible existence of a temperature with an even better reactivating effect cannot be excluded.

The facts that the rate of reactivation is higher at low temperatures and after protracted incubation makes it probable that reactivation might be a result of the elimination of certain toxic substances produced by ultraviolet irradiation in the spores.

Summary

Part of ultraviolet irradiated *Streptomyces griseus* spores regains its ability to grow and form colonies when incubated at a low temperature for a certain period. The rate of reactivation is higher at 0° C than at 10° C. At 17° C no reactivation occurs.

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DIE LEPTOSPIRENINFEKTION DER HUNDE IN BUDAPEST

Von

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(Eingegangen am 6. Mai 1957)

Die Leptospireninfektion der Hunde und ihre ansteckungsverbreitende Rolle sind ziemlich lange bekannt. Bereits zur Zeit der Erforschung der Wirtstiere der *L. icterohaemorrhagiae* wurde nachgewiesen, daß dieser Leptospirentyp nicht nur in Ratten, sondern stellenweise auch in Hunden vorkommt [1]. Größeres Interesse erweckte jedoch die Frage, als Klarenbeek und Schüffner aus erkrankten Hunden einen neuen Leptospiratyp isolierten [2], der sich unter Hunden als weit verbreitet erwies und — da er nur bei Hunden anzutreffen war — den Namen *L. canicola* erhielt. Lange schien es, daß in der Ätiologie der Hundeleptospirose lediglich diese beiden Leptospirentypen von Bedeutung sind, und es erschienen zahlreiche Mitteilungen, die sich mit der Bedeutung der *L. canicola* und *L. icterohaemorrhagiae* teils vom Gesichtspunkt der veterinärmedizinischen, teils der menschlichen Infektionen beschäftigten. Inzwischen wurden jedoch, anfangs nur vereinzelt, später jedoch immer häufiger, bei Hundeuntersuchungen auch andere Leptospirentypen festgestellt, die bisher nur in Nagetieren oder anderen Haustieren vorhanden gewesen waren. So hat man in Europa bis 1956 außer *L. canicola* und *L. icterohaemorrhagiae* folgende Leptospirentypen beobachtet : *L. grippo-typhosa*, *L. australis*, *L. pomona*, *L. sejrő*, *L. saxkoebing*, *L. bataviae*, *L. mitis*, auf anderen Kontinenten *L. hebdomadis*, *L. autumnalis*, *L. medanensis*, *L. javanica*, *L. congo-belge* und *L. kabura* [3, 4, 5, 6, 7, 8]. Die Bedeutung dieser Leptospirentypen kennen wir noch nicht genügend. Einzelne wurden neuerdings stellenweise häufig angetroffen, in gewissen Gegenden öfter als *L. canicola*. Bei mehreren wurde festgestellt, daß die Leptospiren nach der Ansteckung entleert werden, ja es wurden auch menschliche Erkrankungen beschrieben, wo die *L. saxkoebing*-Infektion vermutlich von Hunden ausging [9]. Aus den neueren Wahrnehmungen kann demnach geschlossen werden, daß Hunde wahrscheinlich im Zusammenhang mit zahlreichen Leptospirentypen als Infektionsquelle in Frage kommen ; die praktische Bedeutung der Frage wird sich jedoch erst nach weiteren experimentellen und epidemiologischen Untersuchungen beurteilen lassen.

In Ungarn wurde die Aufmerksamkeit auf die Frage des Leptospirenbefalls der Hunde durch die ersten Fälle von menschlichem Canicola-Fieber gelenkt [10]. Die erste Reihenunter-

suchung nahmen wir 1954 in einer nordungarischen Gemeinde vor, wo im vorangegangenen Jahr menschliche Canicola-Infektionen vorgekommen waren [11]. Wir untersuchten 54 Hunde serologisch (Agglutination—Lysis—Reaktion) und stellten beträchtliche (55,5%ige) Verseuchung fest. Am häufigsten war der Canicola-Befall zu beobachten, doch kamen auch *L. pomona* und *L. sejrő* vor. Mit folgenden Typen fiel die Reaktion negativ aus: *L. grippo-typhosa*, *L. hebdomadis*, *L. icterohaemorrhagiae*, *L. bataviae*, *L. australis-B*, *L. ballum*. Nach diesen Untersuchungen im dörflichen Milieu entschlossen wir uns zur serologischen Reihenuntersuchung der Budapester Hunde, einerseits um die epidemiologische Bedeutung der Hunde genauer festzustellen, anderseits um zu ermitteln, ob sich aus der städtischen und dörflichen Umgebung Differenzen ergeben.

Im Laufe des Jahres 1955 wurde die Agglutination-Lysis-Reaktion im Serum von insgesamt 104 Budapester Hunden durchgeführt. Bei den Untersuchungen benutzten wir folgende Serotypen: *L. grippo-typhosa*, *L. canicola*, *L. icterohaemorrhagiae*, *L. hebdomadis*, *L. sejrő*, *L. pomona*, *L. mitis* (JOHNSON), *L. bataviae*, *L. australis-A*, *L. australis-B*, *L. ballum*. Als positiv wurden die bei 1 : 100 oder höherer Serumverdünnung zustande gekommenen Reaktionen bewertet.

Tabelle I

Altersverteilung der seropositiven Hunde

Alter	Anzahl der untersuchten Hunde	Anzahl der seropositiven Hunde
Unter 1 Jahr.....	12	—
1—4 Jahre	47	7
4—8 Jahre	34	9
Über 8 Jahre	11	5
Insgesamt:	104	21

Von den Sera der 104 Tiere ergaben 21 (21,1%) positive Resultate, und zwar mit *L. canicola* 16, mit *L. sejrő* 3 und mit *L. pomona* 2. Die anderen Leptospirentypen reagierten nicht bzw. gaben sie in einigen Fällen schwache Mitagglutination. Bei den seropositiven Tieren fanden wir keinerlei leptospirosisverdächtige Symptome, sämtliche Tiere waren gesund. — Wie bereits aus unseren vorangegangenen Untersuchungen [11] hervorging, ist es bei der Beurteilung der globalen Ansteckungszahl wichtig, auch die Alterszusammensetzung des Tierbestandes zu berücksichtigen. Diese ist auf Tabelle I wiedergegeben. Wie ersichtlich, erwiesen sich die weniger als 1 Jahr alten Tiere sämtlich als negativ, während die Seropositivität mit dem Alter zunimmt. — Bei dem vorigen Untersuchungsmaterial hatten wir auch die Aufteilung des Befalls nach Geschlechtern in Betracht gezogen, weil mehrere Autoren die häufigere Seropositivität der männlichen Tiere betonen. Die vorliegenden Untersuchungen geben in dieser Hinsicht kein bewertbares Resultat, da die Anzahl der Hündinnen im Vergleich zu den Männchen verschwindend gering war.

Besprechung

Vergleichen wir die Resultate mit den Untersuchungsergebnissen in Nordungarn im Jahre 1954 [11], so sehen wir, daß der Befall der hauptstädtischen Hunde im wesentlichen das gleiche Bild zeigt. An beiden Stellen ist zu beobachten, daß die Tiere die Infektion in einer mit dem Alter zunehmenden Häufigkeit überstehen und die gleichen Leptospirentypen vorkommen. Die Dorfhunde sind jedoch stärker befallen, was auch für die Canicola-Ansteckung, noch mehr aber für den Sejrő- und Pomona-Typ gilt. Im Zustandekommen des stärkeren Befalls auf dem Lande spielt sicherlich eine Rolle, daß sich die Leptospirose in dörflicher Umgebung leichter verbreitet, mehr Wirtstiere anwesend und größere Kontaktmöglichkeiten vorhanden sind, während sich die städtischen Hunde eher gelegentlich voneinander, eventuell von Nagetieren, hauptsächlich Ratten, infizieren.

In Nordungarn war das häufige Vorkommen der *L. canicola* in der Tierwelt auf Grund der Untersuchungsergebnisse der menschlichen Leptospirosefälle zu erwarten. In den Jahren 1953—55 war nämlich die überwiegende Mehrzahl der Canicolafieberfälle in diesem Gebiet aufgetreten [12, 13]. In den südlichen und südöstlichen Landesteilen ist jedoch der Canicola-Befall der Tiere gegenwärtig offenbar sehr gering, und dementsprechend wurden menschliche Canicola-Fälle auch nicht beobachtet. Darauf deutet auch das Resultat unserer im Jahre 1955 in einer Ortschaft des Komitats Békés durchgeführten Tieruntersuchung, wobei wir unter 25 Hunden lediglich in 1 Fall Canicola-Positivität feststellten [14]. Der Canicola-Befall der Budapester Hunde ist für städtische Verhältnisse nicht gering. Interessanterweise wurde dennoch bisher in der Hauptstadt, aber auch in den im Norden des Landes gelegenen Städten, nicht ein einziger Fall von menschlichem Canicola-Fieber beobachtet. Sämtliche in den letzten 3 Jahren diagnostizierten menschlichen Erkrankungen stammten aus ländlicher Umgebung. Zur Erklärung dieser Erscheinung kommen drei Möglichkeiten in Frage:

1. In der Provinz wo menschliche Canicola-Fälle vorkommen, ist die Canicolaverseuchung der Hunde wesentlich stärker als in der Hauptstadt.

2. In der Provinz muß man auch mit der canicolaverbreitenden Rolle anderer Tierarten rechnen, was für die Stadt nicht gilt. Einerseits sind also die Expositionsmöglichkeiten auf dem Lande viel größer als in der Stadt, anderseits besteht die Möglichkeit, daß die Bedeutung der letztgenannten Tierarten vom Gesichtspunkt der menschlichen Ansteckung größer ist.

3. In der dörflichen Umgebung und Lebensweise (Barfußgehen, Baden im Freien, usw.) kann es leichter zur Ansteckung kommen.

Auf Grund unserer epidemiologischen Beobachtungen und Tieruntersuchungen müssen diese Umstände offenbar sämtlich in Betracht gezogen werden. Als besonders wichtig erscheint jedoch die canicolaverbreitende Rolle der grö-

ßeren Haustiere. Auf die praktische Bedeutung der Schweine in dieser Hinsicht haben wir bereits früher hingewiesen [11, 12]; unsere neueren Erfahrungen bestätigen diese nicht nur, sondern zeigen auch, daß ebenso wie in anderen Ländern auch in Ungarn die Rinder und Pferde ebenfalls zu den Wirtstieren der *L. canicola* gezählt werden müssen [8, 15—23].

Die *L. pomona-* und *L. sejrő*-Ansteckung kam unter den hauptstädtischen Hunden in verhältnismäßig geringer Zahl vor, doch scheint es, daß es sich bei einem Teil dieser Infektionen um auf dem Lande erworbene Ansteckungen handelt. Von den 3 sejrő-positiven Tieren waren nämlich 2, von den pomona-positiven 1 Tier etwa ½ Jahr vorher aus der Provinz (Berettyóújfalu) in die Hauptstadt gebracht worden. Es sei erwähnt, daß *L. sejrő* in einzelnen Gebieten Ungarns besonders häufig angetroffen und in diesen Gegenden unter den Hunden beträchtliche Verseuchung beobachtet werden kann. Bei unseren vorhin erwähnten Untersuchungen im Komitat Békés [14] kam dieser Typ unter den Hunden am häufigsten vor (40%), und der Sejrő-Befall der Hunde übertraf den sämtlicher an Ort und Stelle vorhandenen Nagetier- und Haustierarten.

Als negatives Resultat sei erwähnt, daß wir icterohaemorrhagiae-positive Tiere nicht fanden, ein Befund, der von den allgemeinen europäischen Erfahrungen abweicht. Dieser Leptospira-Typ ist in Ungarn scheinbar nicht stark verbreitet, was im übrigen mit den Laboratoriumsbefunden bei der Untersuchung der menschlichen Leptospirosen übereinstimmt.

Zusammenfassung

Zur Feststellung des Leptospirenbefalls der Budapester Hunde wurden im Jahre 1955 serologische Reihenuntersuchungen durchgeführt. Mit der Agglutination-Lysis-Reaktion erfolgte die Untersuchung des Blutserums von 104 Hunden, wobei folgende Leptospirentypen zur Anwendung gelangten: *L. grippo-thyphosa*, *L. canicola*, *L. icterohaemorrhagiae*, *L. hebdomadis*, *L. sejrő*, *L. pomona*, *L. mitis*, *L. bataviae*, *L. australis-A*, *L. australis-B*, *L. ballum*. Das Ergebnis war in insgesamt 21 Fällen (21,1%) positiv; am häufigsten war der Canicola-Befall (16), doch kam auch Sejrő- (3) und Pomona-Positivität (2) vor.

Trotz des beträchtlichen Canicola-Befalls der Hunde wurden bisher in der Hauptstadt Fälle von menschlichem Canicola-Fieber nicht beobachtet, obwohl solche in der Provinz nicht selten auftreten. Die charakteristische ländliche Lokalisation der menschlichen Canicola-Infektionen ist in erster Linie darauf zurückzuführen, daß dort auch mit der leptospiraverbreitenden Rolle anderer Haustiere (Schweine, Rinder, Pferde) gerechnet werden muß und die Milieuverhältnisse die Ausbreitung der Infektion begünstigen.

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THE INFLUENCE OF AGE IN SOME INFECTIOUS DISEASES

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The relation between infectious diseases and age has for long been the object of epidemiological investigation. The epidemiological aspects of this problem are not only of theoretical, but also of practical importance.

In the present paper we shall present an analysis of the morbidity by age, as well as of the connection between the age-specific morbidity and the cyclic changes in the epidemic curve in 5 infectious diseases: measles, whooping cough, scarlet fever, diphtheria and poliomyelitis.

Before discussing the results obtained, we wish to make a few general remarks concerning the data used and the methods of calculation employed.

To elucidate the changes occurring in time in morbidity by age we compared the data for the period of from 1949 until 1954 with those for the period 1931—38. For the 1931—38 period we had at our disposal the age incidence data for the country only as well as the number of the population of Hungary as a whole. For the 1949—54 period we had all the necessary details concerning morbidity, but this time, too, the number of population was available only for Hungary as a whole.

Exact studies on the incidence of morbidity according to age can be made by the use of age-specific indices. To make the calculations, we must posses the data showing the cases in the single age groups, as well as the suitable data for the population. The nation-wide population data for 1931—38 have been carefully computed for the population living in the country, relying in the estimation upon the data obtained in subsequent censuses. Such calculations could not be made for the period 1949—54, because no data are available concerning the internal migrations which have taken place since the 1949 census. Therefore we had to compare the morbidity data for the country before World War II with the nation-wide morbidity data for 1949—54. The resulting qualitative difference was naturally taken into consideration when evaluating the results.

In Fig. 1 we present an overall view of the general morbidity of the diseases in question.

In Fig. 1, and in all the other figures, a logarithmic scale was used to demonstrate the morbidity rate. This had to be done for two reasons. 1. great differences existed in the morbidity rate of the single infectious diseases, as well as between the single age groups with each disease. The use of the arithmetic scale would not have made it possible to represent the low values, which however, had to be included. 2. The logarithmic scale has the further advantage of showing the changes of equal proportion by an identical rise or fall in the curve, independent of the absolute numbers. And it was one of the primary aims of this study to examine the ratio of changes.

In Fig. 1 we present the changes in the morbidity of the five diseases, on the basis of the data obtained for the country in 1931—38 and for Hungary as a whole in 1946—54. Of course, the data for the two periods could not be compared without restrictions. The first important difference to be considered was that the data for the post-war period included also those for Budapest.

The other disturbing factor was that the completeness of reporting infectious diseases varies with the disease and with time. In 1945, SOLTH [1] calculated the pertaining data for Budapest and found that for example in 1921—23 only about 40 per cent of the actually occurring cases of measles had bene reported ; the ratio improved by the end of the period. Especially in the period

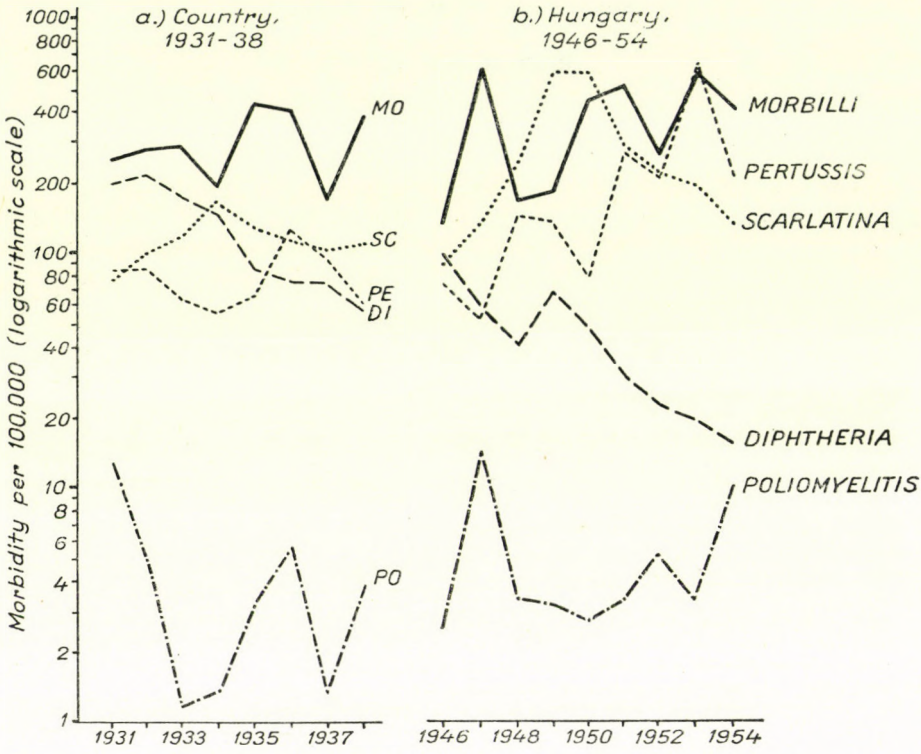

Fig. 1. Morbidity of some infectious diseases per 100 000

1931—1938 the reported cases of measles and whooping cough were probably greatly different from the actual numbers. The ratio of reported to unreported cases is somewhat more favourable with scarlet fever, and appears to be the best in diphtheria. The reported cases of poliomyelitis are almost exclusively those with paralysis. It can be assumed that after World War II the ratio improved and this would explain for example the increase in the morbidity rate of measles and whooping cough.

Fig. 1 shows clearly the remarkably high incidence of measles that is apparently even higher than what is shown in the figure. With both measles and whooping cough even 2 to 3-year periods occurred. The great *scarlet fever* epidemic of 1948—52 is conspicuous by its amplitude, which exceeds several

times that for the 1934 outbreak. Before and after World War II, the epidemic curve for diphtheria showed a definitely decreasing tendency, which continued after the war. The single peak in 1949 was apparently due to the fact that very few immunizations had been carried out in the corresponding period.

As compared to the rate for the other diseases, the poliomyelitis morbidity was quite low. The epidemics of 1931, 1936, 1947 and 1954 are clearly visible

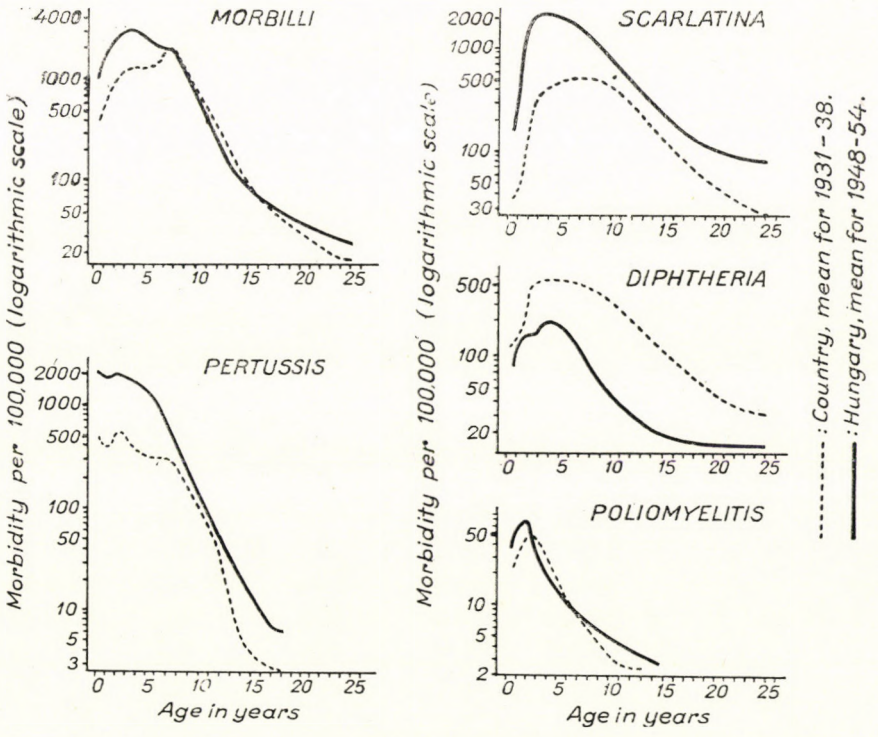

Fig. 2. Morbidity rates according to age

in the graph. It is noteworthy that with poliomyelitis the proportions of the cyclic undulations were usually greater than with the other four diseases.

Fig. 2 drawn in the logarithmic scale, shows the means of the age-specific morbidity rates for the 5 infectious diseases during the two periods under discussion. As already pointed out, as regards absolute values the curves for the two periods are not comparable. Also, it may be assumed that the age curves for the pre-war period would be different, had the data for Budapest been also included. This difference in the shape of the curves cannot, however, be so great as seriously to interfere with a comparison of the pre-war and post-war curves. The figures furnishing the basis to Fig. 2 can be seen in Table I.

Table I

Morbidity per 100 000 of some infectious diseases, according to age groups

A g e	Measles	Whooping cough	Scarlet fever	Diphtheria	Poliomyelitis
a) Rural, mean for 1931—1938					
Under 1 year	36.7	51.6	3.4	12.2	2.2
1 year	66.6	41.8	7.2	23.8	3.5
2 years	106.0	56.5	25.1	51.6	5.0
3 ,, ...	121.2	46.4	39.4	56.8	3.3
4 ,, ...	130.5	36.8	44.4	52.5	1.8
5 ,, ...	131.9	32.8	49.4	56.8	1.3
6 ,, ...	147.6	30.9	48.7	53.0	0.5
7 ,, ...	207.0	31.0	50.7	51.1	
8 ,, ...	166.8	23.2	49.4	47.4	
9 ,, ...	108.6	15.3	44.7	40.0	
10—14 years	34.5	4.5	28.4	14.1	0.2
Above 15 years	1.0	0.1	1.8	22.0	0.0
Total: ...	28.9	7.9	11.4	12.9	0.4
b) Hungary, mean for 1949—1954					
Under 1 year	96.9	211.5	15.1	8.8	3.7
1 year	213.9	190.2	83.3	15.2	6.8
2 years	247.8	202.2	175.3	18.1	4.6
3 ,, ...	280.5	198.6	221.4	22.1	2.8
4 ,, ...	260.4	171.5	212.9	21.6	1.7
5 ,, ...	248.2	131.2	197.4	22.4	1.3
6 ,, ...	207.9	91.5	177.0	15.5	0.7
7 ,, ...	206.4	65.2	155.3	8.9	
8 ,, ...	153.5	38.9	128.5	7.3	
9 ,, ...	94.5	21.5	95.2	5.5	
10—14 years	28.1	6.5	47.7	2.8	0.3
Above 15 years	1.4	0.3	4.8	0.8	0.1
Total: ...	38.1	24.3	32.1	3.3	0.5

In Fig. 2 the curve for measles in the country in the period 1931—38 shows two marked peaks ; the morbidity rate increases steeply after the first year of life until 3 years of age, remaining subsequently at a relatively constant level until school-age. The community life at school strongly enhances exposure, as it is shown by the new rise in the curve, reaching the absolute maximum around 7 years of age. After that the morbidity rate declines steeply.

The post-war curve differs significantly from the pre-war one. The morbidity rate in much higher in the first year of life, the absolute maximum falls to the 3 to 4 years period, after which the curve declines and the school-age is marked by a minor rise only. Subsequently, the shape of the curve is similar to that of the pre-war one.

A comparable shift appears in the age curve for whooping cough. As it has been emphasized, we do not attribute significance to the fact that all parts of the post-war curve are lying higher than the pre-war curve ; this difference might as well be due to a more complete reporting. The difference in the shape of the curves, however, is significant. In both periods the age-curves are two-peaked, there being one peak during the first year of life and another at the age of from 2 to 3 years. The rural curve for 1931—1938 reaches a maximum at two years of age. Later it declines, shows a minor rise at school age, then declines steeply. In the country-wide data for 1949—54, only the first two peaks are present, and the curve declines steeply after it has reached its second peak between 2 and 3 years of age. The beginning of school does not any more alter the shape of the curve. Thus, there is a definite shift in morbidity rate toward the youngest age group. It is also remarkable that in the pre-war period the absolute maximum in the country was at 2 years of age, whereas after the war in the first year.

The whole of the post-war curve for *scarlet fever* runs higher than the pre-war curve. This is the result of the great scarlet fever epidemic of 1948—1952. Here, too, the shape of the curves shows a definite shift toward the younger age groups. Before the war in the rural areas the maximum occurred at about 7 years of age, whereas in the post-war country-wide curve it falls at 3 years of age.

The post-war *diphtheria* morbidity curve runs much lower than the pre-war one. The pre-war curve shows very high values between 2- and 8 years of age, whereas in the post-war period the mean curve falls steeply after 4 to 5 years of age. Immunization undoubtedly plays a significant role in the decrease of diphtheria morbidity rate. This may explain the comparatively small difference between the youngest age groups (i. e. those least protected). The inoculations are obviously responsible also for the flattening of the curve after the first year of life.

The shift toward the youngest age groups is similarly obvious in poliomyelitis morbidity. The shape of the curves is rather similar, but in the pre-war period the maximum is at 2 years of age, whereas in the post-war curve it occurs 1 year earlier.

In Fig. 2 we have presented age curves plotted from the mean values obtained for two periods. For the sake of brevity we do not present the curves for each year, one by one, only their most characteristic values, i. e. those of the absolute maximums of the morbidity rates.

In Fig. 3 are shown the ages at which, the morbidity rate was the highest (the data for pertussis are not included). In general, we attribute greater significance to the changes observable within the single periods than to a comparison of the pre-war and post-war periods. In the pre-war period (1931—38) the maximum morbidity rate for measles almost invariably appeared at around 7½ years of age. In 1931, 1937 and 1938 minor local peaks occurred at around 4½

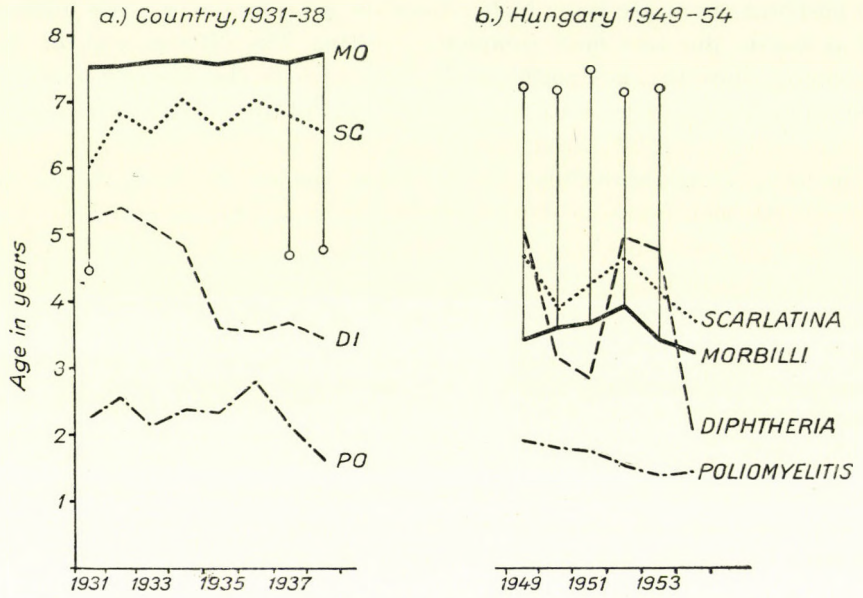

Fig. 3. Maximum morbidity rates according to age

years of age. In the post-war period (1949—54) the absolute maximum fell to 3 years of age in every year, with a slight increasing tendency until 1952, and with a slight decreasing tendency since then. In the period 1949—53 there was also a second, minor peak at 7 years. This second peak did not appear in 1954.

In the pre-war period the age curve for *scarlet fever* showed the maximum around 6 to 7 years of age (with minor variations), without any marked tendency to decrease or increase. In the period 1949—54 the maximum appeared between 4 and 5 years of age, with the last maximum seen below 4 years of age.

The maximum values for diphtheria fall to around 5 years of age during the first four years of the 1931—38 period. In the second half of that period the peaks appear around 3½ years of age, obviously because of the widely employed inoculations. The shifts toward the older age groups in the morbidity rate, as they occurred in 1952 and 1953, are due to a relative fall in the number

of inoculations. In 1954, however, the maximum morbidity rate is found at 2 years of age.

The peaks of *poliomyelitis* morbidity are almost invariably at 2 years of age in the period 1931—36. After that, the maximum has obviously shifted toward younger age. In 1949, the maximum morbidity rate was under 2 years of age ; at present it is around 1½ years.

The maximum morbidity values for *pertussis* have been excluded because we do not possess data grouped according to months of age under 1 year for the period 1931—1938. A study of the corresponding data for the period 1951—54 revealed that the absolute maximum fell invariably to 6 months of age. The second peak at around 3 years of age was lower.

Summing up, in each of the five infectious diseases examined, morbidity has markedly shifted toward the younger, and in some cases toward the youngest, age groups. The shift in the direction of younger age groups is a well-known and much analysed phenomenon in epidemiology. The degree of exposure exerts a fundamental influence upon the shape of the age curves of morbidity. As a rule, infection occurs earlier in the younger age groups of the urbane population, as compared to the rural population. The intensive industrialization started after the war in Hungary increased the number of inhabitants not only in Budapest, but also in the industrial centres in the country, and created a number of new industrial centres as well. It is the mass migration into the towns which presumably is responsible for the described changes in the morbidity rate. Exposure may be increased also by increased travelling and in changes in dwelling. As to the youngest age groups, the development of the network of nurseries may be a factor contributing to the increase of exposure.

What we have elaborated above is by no means considered to be a complete study of the statistical and epidemiological aspects of the problem. We wish to analyse in detail the presumable differences existing between Budapest, the major industrial areas, smaller towns and agricultural colonies, in a later work.

The shift in the morbidity rate in the direction of the youngest age groups is of equally paramount importance in the fight against the infectious diseases under discussion. The importance of the problem is further enhanced by the fact that in the youngest age the diseases are usually more severe in course and have higher case-fatality rates. Correspondingly, this age group requires increased protection. This applies first of all whooping cough. At present, the first inoculation against whooping cough is given during the 6—11 months of life, i. e. at a time when the morbidity rate has already reached its maximum. Knowing the high case-fatality rate of pertussis among infants, it seems imperative to give the first inoculation earlier. Also in the case of poliomyelitis, the inoculations should be so scheduled that sufficient protection be provided for the 1 year old, i. e. for those showing the highest morbidity rate.

Table II/a

Morbidity per 100 000 of some infectious diseases, according to age groups

Rural, mean for 1931—1938

Age group	1931	1932	1933	1934	1935	1936	1937	1938
Measles								
0—2 years ...	64.4	72.6	69.9	38.3	96.5	87.3	33.7	92.3
3—5 ,, ...	102.7	121.5	132.0	80.1	183.0	165.7	71.5	166.5
6—9 ,, ...	123.0	134.8	143.2	103.1	231.6	232.6	95.1	195.4
10—14 ,, ...	31.4	35.5	30.5	20.4	46.8	46.5	19.8	46.0
15— ,, ...	1.0	1.0	0.9	0.7	1.4	1.3	0.6	1.4
Total:	24.9	27.7	28.3	18.4	41.6	39.2	15.9	35.1
Whooping cough								
0—2 years	51.4	54.1	38.2	34.8	41.8	78.1	58.8	42.6
3—5 ,, ...	37.9	40.1	30.1	29.3	34.8	58.7	46.0	32.4
6—9 ,, ...	24.0	24.0	20.4	16.7	18.9	40.1	35.6	20.6
10—14 ,, ...	6.0	4.6	3.8	2.2	2.6	7.8	5.9	3.4
15— ,, ...	0.09	0.08	0.08	0.06	0.04	0.09	0.08	0.09
Total:	8.5	8.6	6.4	5.6	6.5	12.1	9.4	6.2
Scarlet fever								
0—2 years	8.2	10.8	10.3	15.5	12.1	11.8	10.8	14.9
3—4 ,, ...	26.7	36.4	44.9	64.2	53.0	44.2	42.9	43.4
6—9 ,, ...	32.1	39.9	51.1	70.3	55.3	49.6	43.1	50.0
10—14 ,, ...	21.2	26.3	28.8	39.0	30.7	28.6	24.4	25.5
15— ,, ...	1.4	1.6	1.6	2.2	2.0	1.9	1.7	1.9
Total:	7.7	9.9	11.7	16.3	13.0	11.6	10.2	10.6
Diphtheria								
0—2 years	39.2	40.3	37.6	32.6	24.7	21.0	18.6	17.1
3—5 ,, ...	90.3	98.7	80.7	61.4	37.0	31.7	30.7	23.1
6—9 ,, ...	78.5	91.7	65.5	55.7	28.0	24.1	23.1	15.7
10—14 ,, ...	37.4	45.8	29.9	28.2	14.7	13.3	13.7	10.1
15— ,, ...	2.5	2.3	2.1	2.2	1.6	1.6	1.8	1.7
Total:	19.9	22.3	17.3	14.8	8.6	7.5	7.3	5.6
Poliomyelitis								
0—2 years	12.0	3.5	0.8	1.3	2.9	3.6	0.8	3.2
3—5 ,, ...	6.2	2.2	0.6	0.4	1.3	3.7	0.8	1.7
6—9 ,, ...	1.23	0.86	0.17	0.12	0.39	0.87	0.25	0.46
10—14 ,, ...	0.58	0.35	0.10	0.11	0.18	0.23	0.20	0.18
15— ,, ...	0.06	0.05	0.01	0.01	0.05	0.05	0.01	0.03
Total:	1.38	0.51	0.12	0.14	0.32	0.55	0.14	0.35

Table II/b

Morbidity per 100 000 of some infectious diseases, according to age groups
Hungary, mean for 1949—1954

Age group	1949	1950	1951	1952	1953	1954
Measles						
0—2 years ...	86.0	180.1	206.8	117.9	300.9	223.6
3—5 ,, ...	112.3	297.0	334.6	176.5	390.3	237.3
6—9 ,, ...	80.3	217.4	233.3	106.2	232.8	148.9
10—14 ,, ...	12.4	37.4	43.4	18.0	34.1	23.2
15— ,, ...	0.9	1.5	2.3	1.3	1.5	1.0
Total:	17.4	42.7	48.7	25.0	56.7	38.0
Whooping cough						
0—2 years ...	105.7	59.2	206.1	169.5	515.5	155.3
3—5 ,, ...	78.6	49.0	187.9	130.3	408.4	143.9
6—9 ,, ...	40.0	19.4	63.9	48.0	110.9	43.4
10—14 ,, ...	6.4	2.7	8.3	5.5	11.4	4.8
15— ,, ...	0.19	0.12	0.31	0.17	0.64	0.16
Total:	13.0	7.3	26.2	19.9	59.4	20.1
Scarlet fever						
0—2 years ...	136.9	155.9	80.0	57.6	57.0	48.0
3—5 ,, ...	352.5	367.2	197.5	141.5	122.7	82.9
6—9 ,, ...	264.6	233.6	116.3	89.0	80.2	51.2
10—14 ,, ...	105.2	83.3	36.8	25.9	20.8	14.0
15— ,, ...	7.5	9.1	5.0	3.4	2.6	1.4
Total:	56.4	55.5	28.5	20.9	18.5	12.6
Diphtheria						
0—2 years ...	25.1	17.2	15.1	9.8	7.7	8.3
3—5 ,, ...	46.2	37.1	18.5	12.9	10.4	7.4
6—9 ,, ...	18.5	11.3	9.2	6.2	6.3	4.6
10—14 ,, ...	5.9	3.2	2.1	2.2	2.2	1.6
15— ,, ...	1.7	1.2	0.7	0.5	0.4	0.3
Total:	6.7	4.7	3.0	2.2	1.9	1.5
Poliomyelitis						
0—2 years ...	2.7	2.6	2.7	4.8	3.4	14.1
3—5 ,, ...	1.5	1.2	1.6	1.9	1.1	4.0
6—9 ,, ...	0.59	0.55	0.58	0.95	0.44	1.20
10—14 ,, ...	0.31	0.14	0.29	0.57	0.20	0.54
15— ,, ...	0.05	0.03	0.05	0.08	0.04	0.08
Total:	0.32	0.28	0.33	0.53	0.33	1.21

Thus far, we have been discussing the course of the age-curves of the mean of the two periods specified, as well as the maximum morbidity rates. The problem now arises whether there is any correlation between the age-specific morbidity rates and the more or less marked cyclic changes in the epidemic curves. To elucidate the problem, we have determined the specific morbidity rates of five age groups (0—2, 3—5, 6—9, 10—14 years of age and above 15 years of age) for each year of the two periods mentioned. The numerical data can be found in Table IIa and IIb.

Fig. 4 shows the morbidity rates of *diphtheria* for each age group, using the logarithmic scale. The alterations in the shape of the curves caused by inoculation are, of course, slightly different in each age group. This is obvious in the period 1931—38 already. After World War II the order of the single age groups underwent a change in that the post-war values for the age groups above 6 years became significantly lower than the pre-war ones, not only in absolute

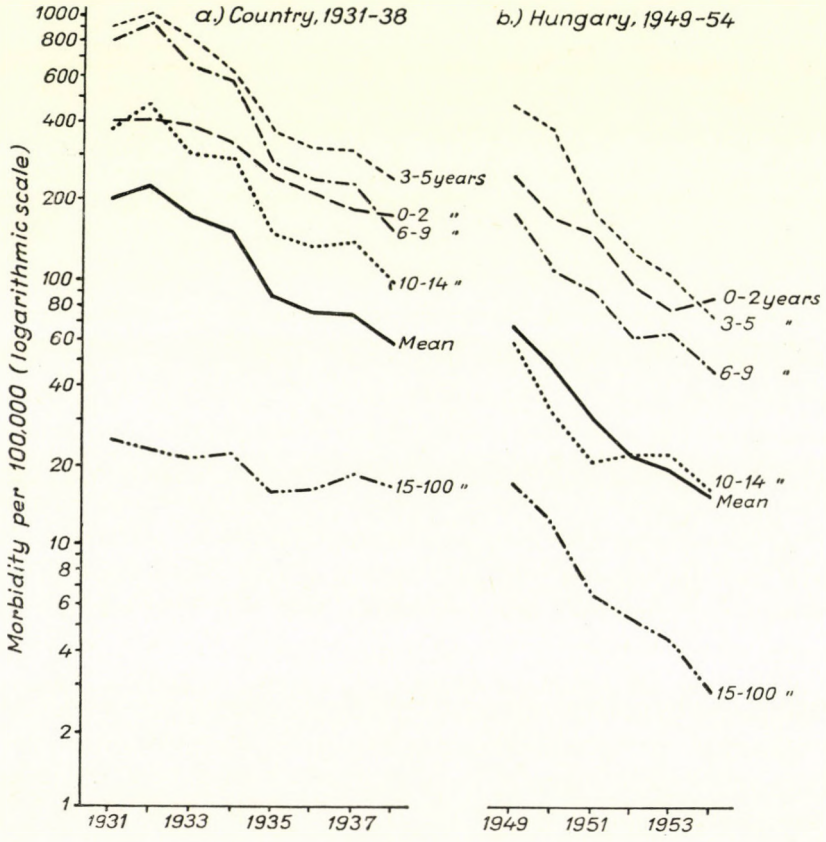

Fig. 4. Morbidity for diphtheria by age groups

values, but also as compared with the age groups 0—2 and 3—5, respectively. In the last year the highest morbidity rate occurred in the 0—2 years age group. The conspicuous feature, however, is the parallel course of the curves for the single age groups.

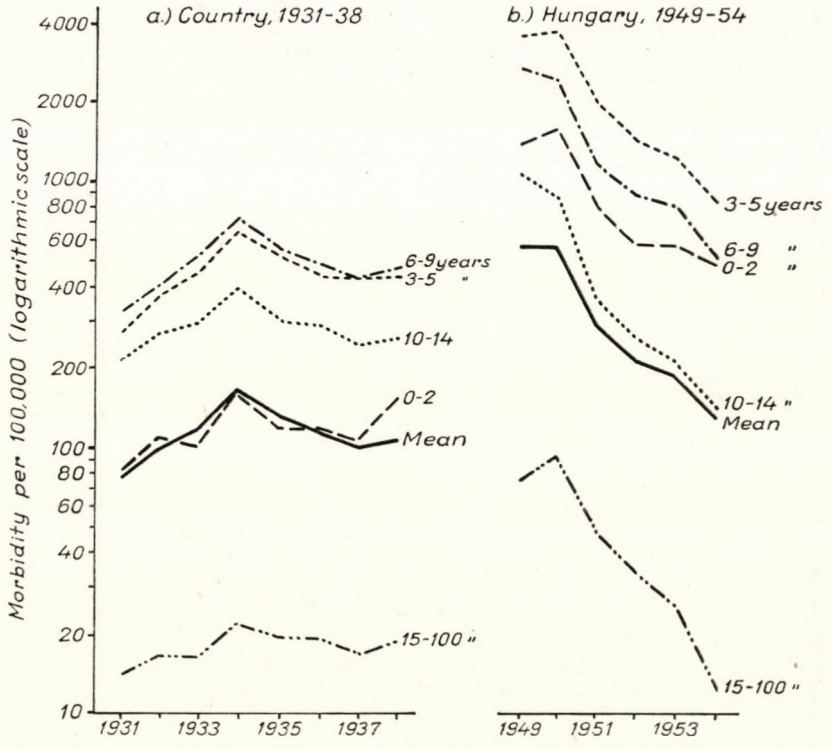

Fig. 5. Morbidity for Scarlet fever by age groups

This phenomenon is even more marked in the case of *scarlet fever* (Fig. 5), in which both the pre-war and post-war curves run parallel with the mean, except for minor variations due presumably to a shift, towards the younger age groups of the morbidity rate. It is most remarkable that the parallelism with the curve for the oldest age group is also almost complete.

Of the five infectious diseases studied, *poliomyelitis* has the lowest morbidity rate (Fig. 6) and, therefore, its expectable deviations from the mean are theoretically greater than those of the four diseases. It is, therefore, even more remarkable that also in this case the parallelism of the curves is almost complete.

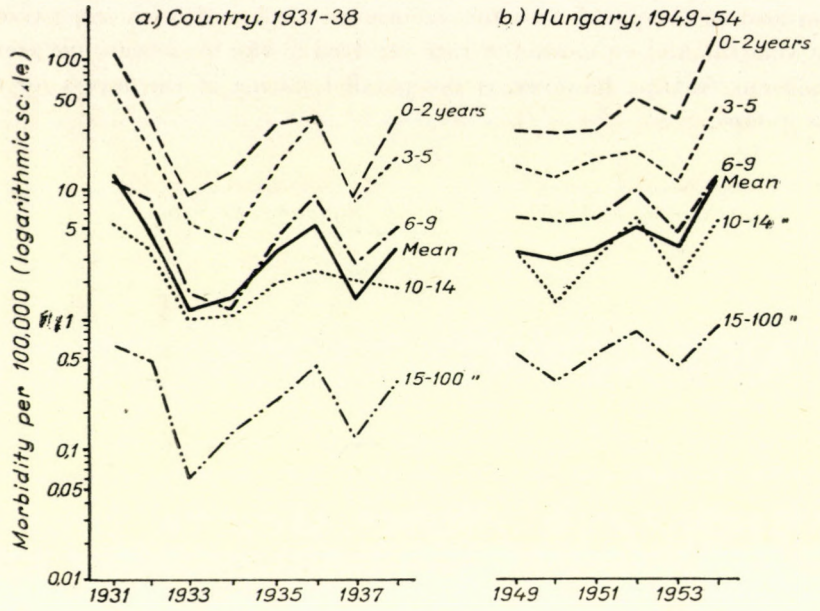

Fig. 6. Morbidity for poliomyelitis by age groups.

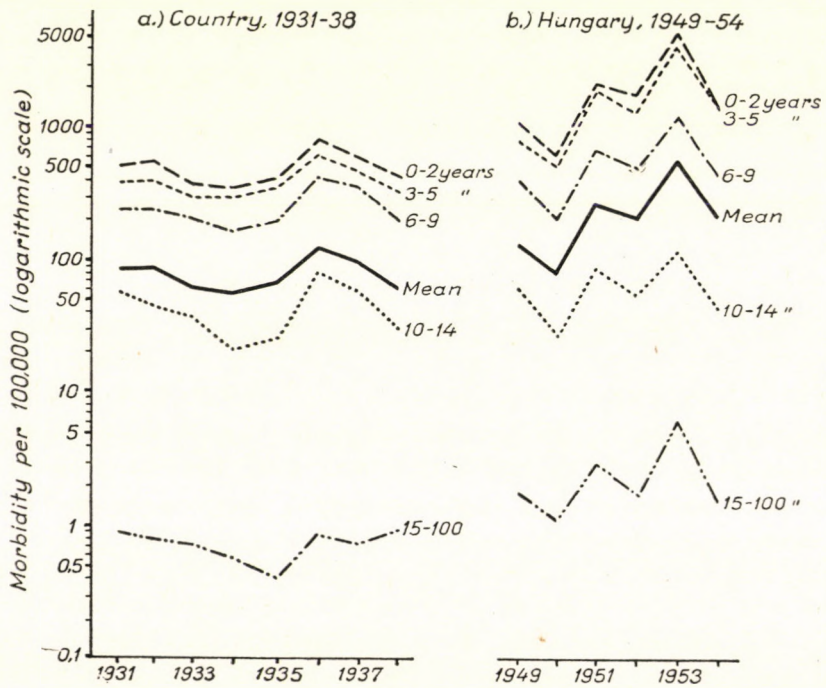

Fig. 7. Morbidity for whooping cough by age groups.

An astonishing parallelism could be detected between the curves for the single age groups also in the case of whooping cough. The parallelism is particularly interesting in the age group above 15 years, because in that age the morbidity rate is extremely low (Fig. 7).

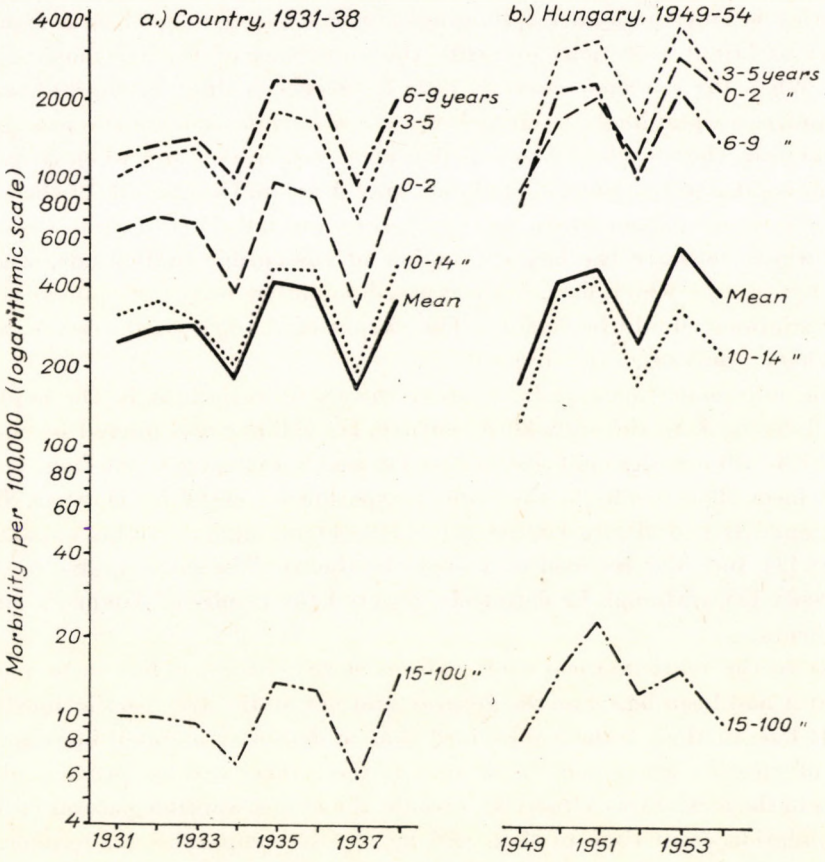

Fig. 8. Morbidity for measles by age groups

Finally, Fig. 8 shows the morbidity rates for measles, according to age groups. Here, too, the curves for the single age groups run completely parallel, with the exception of age group 0—2, in which the morbidity rate increases in relation to the other age groups, independent of the cyclic movement of the epidemic curve.

Discussion

Thus, it was found to be the rule with all five diseases that the cyclic movement of the epidemic curve had no influence on the relation of the morbidity rates of the single groups. In other words, an increase in the epidemic wave

causes a comparable rise in the morbidity of each age group, and a decrease causes a comparable fall. This observation is hardly compatible with the generally accepted theory concerning the causes of the cyclic outbreak and cessation of epidemics of respiratory infectious diseases. According to that theory, at the peak of a cycle the infection of the susceptible population has been completed and the cycle ends because the immunity which has developed as a result of manifest or latent infections prevents the occurrence of further massive outbreaks. When the epidemic curve is near its deepest point, the morbidity rate is low and, correspondingly, there is less chance for infection. As the new generations appear, the number of susceptible persons increases and when it reaches a certain point another wave of epidemic may develop. On grounds of the above theory one would expect to see the cyclic rises and falls first of all in those age groups which embrace the largest number of susceptible individuals, whereas in the age groups which have been exposed to earlier waves of epidemics the cyclic variations should be smaller. The shapes of the age-group curves, as we found them, contradict this hypothesis.

The afore-mentioned explanation of the cyclic variations in the epidemic curves dates back to the early 20th century. Its validity was proved by STOCKS in the 1924—28 measles epidemic in London and SOPER gave it the final mathematical form. The results of the animal experiments made by DANYSZ, BAINBRIDGE and, first of all, by TOPLEY (cit. [2]) all lent support to this view. STALLYBRASS [2], too, was inclined to accept the theory. The same applies to GROMASHEVSKY [3], although he definitely rejected the results of TOPLEY's animal experiments.

As to the mathematical confirmation of the theory, it has to be pointed out that it had been based on the general, and not on the age-specific morbidity rates. It has, further, to be emphasized that in densely populated big cities the cycles of measles are regular and, in addition, there are no sudden, abrupt changes in the birth rates, either. As a result, the new susceptible persons entering the population from time to time will be closely comparable in number. For this reason, any mathematical confirmation, no matter how ingenious, based on general morbidity entails the element of coincidence, beside the causal relationship.

We would not dare to discredit the theory in question solely on the basis of the close comparability of the changes in the morbidity rate in the different age groups. As it has been pointed out by several authors, the cyclic rise in the epidemic curve begins almost invariably at the time when the seasonal variation begins. For this reason, a final opinion cannot be put forward until the seasonal variations are simultaneously analysed. We intend to carry out this work at a later date.

Summary

1. The age-specific morbidity rates have been determined for 5 infectious diseases (measles, whooping cough, scarlet fever, diphtheria and poliomyelitis), for the periods 1931—1938 and 1949—1954. It has been found that with each of the diseases morbidity showed a definite shift towards the young age groups.

2. The age of maximum morbidity has also been determined for the five diseases. The shift of maximum morbidity toward the younger generations is observable not only in the mean values for the two periods, but also in the values for the consecutive years of the post-war period. The shift is the most marked in the cases of whooping cough and poliomyelitis. The absolute maximum of pertussis morbidity appears during the first year of life and therefore it would be advisable to give the first inoculation against pertussis earlier than it is done at present.

3. An analysis of the correlation between age and the cyclic variations in the epidemic curves has revealed that during the cycle the epidemic curves of the single age groups change in closely comparable proportions with each of the five infectious diseases in question. This observation appears to be in contradiction with the generally accepted theory concerning the cyclic variations of respiratory diseases.

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THE OCCURRENCE OF SYSTEMIC MYCOSES IN HUNGARY

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As in most parts of the world, in Hungary the incidence of systemic mycoses has increased in recent years. In agreement with other authors, we see the cause of this increase to lie partly in an improvement of diagnostic methods and partly in the extensive use of antibiotics.

As well known, the antibiotics play a role mainly in the increased incidence of endogenous systemic mycoses, whereas the recent occurrence in Hungary of exogenous mycoses cannot be brought into correlation with the use of antibiotics. In the latter case the obvious explanation seems to be that increased attention has been devoted to these conditions, and that new, improved laboratory methods for the detection of mycoses have extensively been adapted.

During the past 2 years we have examined by laboratory methods 600 patients thought on the basis of clinical symptoms to suffer from systemic mycosis. The diagnosis was confirmed by laboratory methods in 41 of these cases. These 41 cases will be described below.

Well-identified pathogenic fungi were isolated in further 27 cases. However, in these cases the clinical and pathological methods offered no unequivocal proof as to the presence of mycosis. For this reason these cases will not be included in this report.

The methods employed were mostly identical with those described in the literature and will therefore not be discussed in detail. All we wish to point out is that smears made of clinical materials were stained in every case with Schiff's periodic acid leucofuchsin (PAS stain), Grindley's stain, by the Brown-Brenn method and according to Gram. The culture media used were Sabouraud's glucose-agar, blood agar, peptone agar and malt extract; in some cases, when an anaerobic agent was suspected to be present, the thioglycolate medium was also employed. When some exogenous fungus *(Coccidioides immitis, Histoplasma capsulatum, Blastomyces dermatitidis* (GILCHRIST), or *Nocardia asteroides)* had grown, its presence was considered to be pathognomonic after repeated cultivation and identification. When the pathogen present was endogenous *(Candida albicans*, or other species of *Candida, Geotrichum candidum)* or when it was doubtful whether the agent was exogenous or endogenous *(Cryptococcus neoformans, Aspergillus fumigatus)*, only several, unequivocal results have been accepted and this, too, only if the fungus was isolated from a closed body cavity or when the clinical picture was beyond doubt that of a mycosis. The diagnosis of mycosis was rejected when some bacterial pathogen was also present, except when the fungus grew from open fistulae and the associated flora was composed of apathogenic bacteria or of such pathogenic fungi as did not explain the pathological process.

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We do not deal with the systemic mycoses observed in other institutes, because these have been described elsewhere.

*

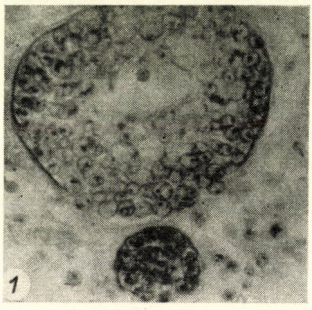
Coccidioidomycosis

(Total number of cases : 1)

1. *Female patient, 22 years of age*

Clinical diagnosis: Suppuration? Lipoma? Angiosarcoma? Chronic villous haemorrhagic synovitis? Mycosis?

Material examined: a) Histological sections made of the synovial membrane of the right knee and left elbow joints. *b)* Fluid aspirated from the right knee joint.



Micrograph 1

Results. a) *Studies of smears and sections.* In the histological sections stained with PAS and Gridley's stain, typical *Coccidioides immitis* spherules were visible. *b)* In *cultures* made of the joint fluid in Sabouraud's agar and blood agar, both at 26° C and 37° C, white colonies of mould, which later turned brown, developed in 7 days.

Identification of the pathogen. a) *Morphology.* Vegetative mycelium composed of several ramifying, septal hyphae. The lateral rami were about twice the diameter of the rami of other hyphae. The arthrospores, about 3 to 4 microns in size, were separated from one another by unstained, light areas. b) *Animal experiment.* Intraperitoneal injection of a suspension of the fungus in physiological saline killed mice in 10 days. Pure cultures of the pathogen grew from the peritoneal fluid. In histological sections of the spleen, typical spherules were present (v. micrograph No. 1) c) *Result of identification, Coccidioides immitis.*

Note: We are indebted to Professor VANBREUSEGHEN, of Antwerpen, for examining the sections and the strain of fungus isolated, and for corfirming our diagnosis. *Publications.* References 1 and 2.

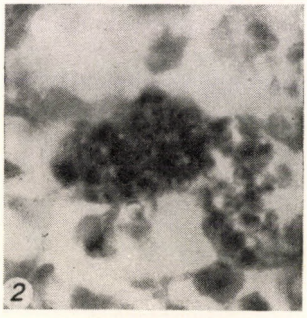
Histoplasmosis

(Total number of cases : 5)

1—5. *Deceased adults of different ages and sex.*

Clinical diagnosis: Hodgkin's disease.
Material examined: Lymph nodes, livers, adrenals from autopsies.

Results of the examinations. a) In cytoplasm of reticuloendothelial cells, Gram positive fungal elements of acute outlines. staining red with PAS and lilac-



Micrograph 2

black with Giemsa were found. The morphological and staining properties, as well as the location suggested *Histoplasma capsulatum.* (v. micrograph No. 2) b) *Result of identification, Histoplasma capsulatum.*

Note: a) By Ziehl-Neelsen's stain no Koch bacillus could be demonstrated in any of the sections. *b)* The histological pattern corresponded to histoplasmosis. *c)* Histoplasmin prepared from a well-defined strain of *Histoplasma capsulatum* by the method of EMMONS *et al* [3] was inoculated intradermally into 395 normal children and children treated for tuberculosis ; the number of positives was 7, i. e. 1. per cent. According to this finding, the incidence of histoplasmosis is relatively high in Hungary. In this country, SINKOVICS was the first to diagnose histoplasmosis in the mouse [5].
Publication. Reference 6.

Nocardiasis

(Total number of cases : 6)

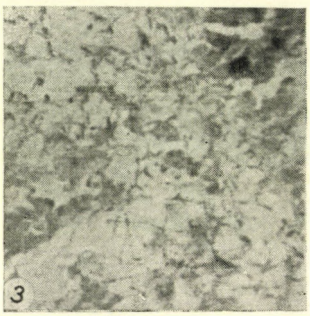
1. *Male patient, 40 years of age.*

Clinical diagnosis: Blastomycosis localized to the gluteus?
Material examined. a) Smears from fistular discharge. *b)* Two samples of discharge from the fistula.

Results. a) In the *smears* stained with Gram's stain, mixed bacterial flora and Gram positive filaments, about 1 micron in diameter, branching and fragmenting into coccoid and bacillary forms were found. b) *Cultivation.* In Sabouraud's agar inoculated with the discharge and incubated for 8 days at 37° C,

colonies with a corrugated, then floury surface, light, then orange in colour and similar in appearance to colonies of *Mycobact. tuberculosis* were grown.

Identification of the cultured pathogen. a) *Morphology.* The branching filaments were around 1 micron in diameter, Gram positive, in part acid-fast (Ziehl—Neelsen's stain, 15″ decoloration); the filaments disintegrated later into bacillarly and coccoid forms. The microorganism did not liquefy gelatine. b) *Animal experiment.* Guinea pigs inoculated intraperitoneally with the fungus



Micrograph 3

suspended in physiological saline died after the 7th day. The pathogen grew in pure culture from the peritoneal fluid. The characteristic form of the pathogen was visible in the sections made of the abdominal wall and of the spleen (v. micrograph No. 3) c) ***Result of identification, Nocardia asteroides***

Note: In the course of the disease, which lasted several years, the fistula closed on sulphadiazine treatment.
Publication. Reference 7.

2. *Male patient, 19 years of age.*

Clinical diagnosis: Localised blastomycosis?
Material examined: Punctate of a rigid infiltration which developed at the site of a penicillin injection.

Results of examinations and identification of cultured pathogen: As in case 1.

Note: Sulphadiazine treatment resulted in a cure within 4 weeks.
Publication. Reference 8.

3. *Male patient, 40 years of age.*

Clinical diagnosis: Pulmonary mycosis?
Material examined: Thoracic punctate, on two occasions.

Results of examinations and identification of cultured pathogen: As in case 1.

Note: The patient has moved to an unknown address and his further fate is unknown.

4. *Female patient*, 10 *years of age.*

Clinical diagnosis: Miliary tuberculosis? Mycosis?
Material examined: Five samples of laryngoscopic, 2 samples of bronchoscopic mucus.

Results of examinations and identification of cultured pathogen: As in case 1.

Note: Serial tests failed to reveal tuberculosis. Sulphadiazine treatment lead to improvement in 3 months.

5. *Male patient*, 56 *years of age.*

Clinical diagnosis: Bronchiectasis?
Material examined: Two samples of bronchoscopic mucus.

Result of examinations: As in case 1.

Identification of cultured pathogen; a) Technical difficulties prevented us from making a detailed morphological study. b) *Animal experiment:* As in case 1. c) *Result of identification, Nocardia sp.*

Note: The patient's condition is unchanged since 8 months.

6. *Female patient*, 30 *years of age.*

Clinical diagnosis: Osteomyelitis.
Material examined: Pus.

Result of examinations. a) In smears and sections Gram positive, branching, not acidfast filaments fragmenting into coccoid forms were found, but no mycelia. b) *Cultivation:* All the three attempts to culture the fungus have failed, presumably because of the strong bacterial contamination.

Identification of the pathogen. As cultivation has failed, the morphological studies did not prove, only made it highly probable the diagnosis, *Nocardia sp.*

Note: The patient's further fate is unknown.

Blastomycosis (Gilchrist)

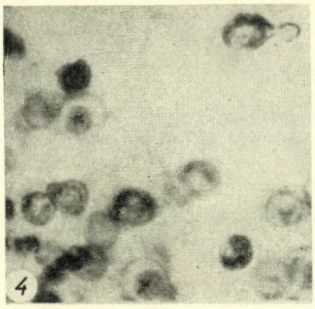
(Total number of cases : 1)

1. *Female patient*, 12 *years of age.*

Clinical diagnosis: Chronic osteomyelitis.
Material examined: Pus and blood from a fistula in the thigh. Mucus obtained by bronchoscopy.

Result of examinations. a) In *smears and sections*, spherical, thick-walled, budding and not budding fungal elements, 8 to 20 microns in diameter and staining red with PAS were present. No fungal elements were demonstrated in the mucus obtained by bronchoscopy. *b) Result of cultivation.* On Sabouraud's agar, at room temperature, white to light brown colonies of mould grew from all the three materials. At 37° C, waxy, yeast-like colonies grew on Sabouraud's agar and on blood agar.

Identification of cultured pathogen. a) *Morphology.* The saprophyte phase culture obtained at room temperature showed branching hyphae. In the parasite phase culture obtained at 37° C thick-walled blastomyces, 8 to 15 microns in diameter, were found. b) *Animal experiment.* The yeast-phase fungus suspended



Micrograph 4

in physiological saline killed the 4 albino mice inoculated intraperitoneally within 14 days. Pure cultures of the pathogen grew from the miliary abscesses in the peritoneum.

The typical form of the pathogen was detectable in sections of the spleen and liver. c) *Result of identification; Blastomyces dermatitides* (v. micrograph No. 4).

Note: The microscopic changes in a biopsy specimen taken from the sequestred left femur of the patient were characteristic of blastomycosis. The fungal elements in this specimen showed the same morphology and staining properties as the fungal elements in the sections made of the organs of experimental animals.
Publication: Reference 9.

Cryptococcosis

(Total number of cases : 8)

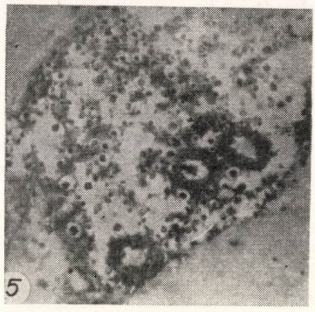
1. *Female patient, 41 years of age*

Clinical diagnosis: Meningoencephalitis.
Material examined: Cerebrospinal fluid, 2 samples.

Result of examinations. a) *Smears and sections.* In the smears and China ink preparations of the sediment, capsulated, budding, spherical fungal elements, 2 to 5 microns in size and staining bright red with PAS were seen. b) *Cultures.* On Sabouraud's agar, as well as on a culture medium composed of equal parts of malt extract and cerebrospinal fluid, both at 37° C and 26° C, a yeast like fungus grew in 6 days from both samples of CSF.

Identification of cultured pathogen. a) *Morphology.* Thick-walled, spherical, budding cells 4 to 15 microns in diameter were seen. The capsule of the cells

varied in diameter, it sometimes amounted to three times the diameter of the cell proper. In some instances the capsule enclosed both the maternal cell and the bud. Pseudomycelia were absent. b) ***Animal experiments.*** Mice inoculated intracerebrally with a suspension of a 48-hour culture in physiological saline



Micrograph 5

died in 8 to 24 days. Pure cultures of the pathogen grew from the mucous area found on the brain of dead animals. PAS stained sections of the brain showed the presence of typical forms of the fungus (v. micrograph No. 5). c) ***Identification; Cryptococcus neoformans (Torula histolytica).***

Note: Postmortem studies, including histopathologic examinations confirmed the diagnosis of cryptococcosis.
Publication: Reference 10.

2. *Male patient, 67 years of age.*

Clinical diagnosis: Relapsing meningitis.
Material examined: Cerebrospinal fluid, 4 samples.

Result of examinations and identification of cultured pathogen: As in case 1.

Note: After some improvement another relapse followed and the condition of the patient remained unchanged for months. Serial tests for Koch bacillus were negative.

3. *Female patient, 40 years of age.*

Clinical diagnosis: Meningoencephalitis? Cryptococcosis?
Material examined: Cerebrospinal fluid, 3 samples.

Result of examinations. a) ***Smears.*** As in case 1. b) ***Cultivation.*** Attempts to culture the pathogen have failed.

Identification of the pathogen. In the absence of cultures, the pathogen was identified tentatively, on grounds of its morphological properties as ***Cryptococcus neoformans.***

Note: Post-mortem histopathologic studies confirmed the diagnosis of cryptococcosis. Cultivation failed probably because of the prolonged treatment with Nystatin.

4. *Male patient*, 1 *year of age.*

Clinical diagnosis: Meningoencephalitis. Sepsis.
Material examined: Blood and 3 samples of cerebrospinal fluid.

Results of examinations. a) *Smears and sections;* No fungal elements were detectable in the blood. In smears made of the 3 samples of CSF, the findings were the same as in case 1. b) *Results of cultivation.* CSF : As in case 1. Blood : No fungus has grown.

Identification of cultured pathogen: As in case 1.

Note: Post-mortem examination confirmed the diagnosis of systemic cryptococcosis. Pure cultures of the pathogen were obtained from the brain, liver, lung and spinal cord. Typical fungi were seen in the histological sections.

5. *Female patient*, 36 *years of age.*

Clinical diagnosis: Mycosis?
Material examined: Discharge and excised specimen from injection abscess.

Results of examination and identification: As in case 1.

Note. The clinical symtoms were those of cryptococcosis. Bacteriological studies revealed no other pathogenic agent. The condition did not respond to any kind of antibiotic.

6. *Male patient* 50 *years of age.*

Clinical diagnosis: Gluteal mycosis?
Material examined: Excised specimen from abscess.

Results of examinations. a) *Smears.* Beside a mixed bacterial flora, fungal elements resembling *Cryptococcus neoformans* were found in China ink preparations, as well as in smears stained with PAS. b) *Culture;* As in case 1. Apathogenic Gram negative bacilli and air cocci grew together with the fungus in question.

Identification: As in case 1.

Note: Post-mortem histopathologic studies cinfirmed the diagnosis of cryptococcosis.

7. *Female patient*, 70 *years of age.*

Clinical diagnosis: Crural ulcer of unknown aetiology.
Material examined: Exudate from ulcer.

Results of examinations. a) *Smears.* As in case 6 b) *Culture.* As in case 1. Accompanying bacterial flora : As in case 6.

Note: Post-mortem examination confirmed the presence of localised cryptococcosis.

8. *Male patient*, 45 *years of age.*

Clinical diagnosis: Mycosis?
Material examined: Sputum.

Results. The direct examinations and culturing were made by DR. VITÉZ, of the *Institute of Public Health, University of Budapest* [11].

Identification: As in case 1.

Note: Patient's further fate unknown.

Aspergillosis

(Total number of cases : 4)

1. *Male patient 26 years of age.*

Clinical diagnosis: Empyema resistant to all antibiotics.
Material examined: Thoracic punctate, 2 samples.



Micrograph 6

Results of examinations. a) *Smears.* Segmented hyphal fragments staining red with PAS and Gridely's stain. In Gram stained smears no bacteria were demonstrable. b) *Cultures.* On Sabouraud's medium at 37° C white colonies of mould, that turned green later, grew from the punctate. The opposite surface of the medium was yellowish.

Identification of cultured pathogen. a) *Morphology:* The conidiophores are short, on the bacilliform vesicle (on the convex side) there is a row of sterigma, on which there are conidia, about 2 microns in size each. There are neither scleorita or perithecia. b) *Animal experiments:* The rats inoculated intravenously with the spore suspension died in 5 to 7 days. Pure cultures of the fungus grew from the miliary abscesses found on the renal cortex. In histologic sections the typical form of the fungus was demonstrable (v. micrograph No. 6). c) ***Result of identification: Aspergillus fumigatus.***

Note: Repeated bacteriological examinations failed to detect the presence of any bacterial pathogen.

2. *Male patient* 45, *years of age.*

Clinical diagnosis: Pulmonary mycosis?
Material examined: Mucus obtained by bronchoscopy, 2 samples.

Results of examinations and identification of cultured pathogen: As in case 1.

Note: Pulmonary tuberculosis and tumour could be excluded on grounds the clinical, bacteriological and radiological studies.

3. *Male patient*, 55 *years of age.*

Clinical diagnosis: Pulmonary mycosis?
Material examined: Bronchial exudate, 1 sample, sputum, 3 samples.

Results of examinations. a) *Smears.* In the bronchial exudate, and the 3 samples of sputum, beside a mixed bacterial flora fragments of hyphae staining red with Gridley's stain and PAS were found. b) *Cultures:* As in case 1.

Identification of cultured pathogen: As in case 1.

Note: After treatment with *Nystatin* for months, the fungus could no longer be demonstrated or cultured.The patients general conditions and radiology showed considerable improvement.

4. *Female patient*, 32 *years of age.*

Clinical diagnosis: Otitis?
Material examined: Discharge from ear, 2 samples.

Result of examinations and identification: As in case 1.

Note: Patient's further fate unknown.

Candidiasis

(Total number of cases : 17)

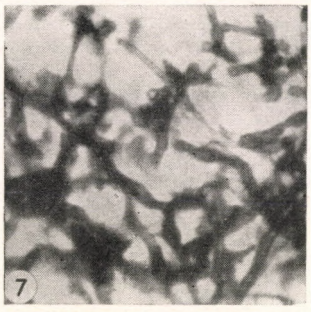
1. *Male patient* 1½ *years of age.*

Clinical diagnosis: Sepsis after prolonged antibiotic treatment.
Material examined: Cerebrospinal fluid, blood (2 samples), urine by catheter (3 samples).

Results. a) *Smears.* In the sediment of the CSF no fungal elements were demonstrable. In the blood smears, as well as in the smear of urinary sediment, budding and non-budding fungal elements, 4 to 6 microns in size, staining red with PAS, were found. b) *Cultures.* On Sabouraud's medium at 37° C in 3 days yeast-like colonies grew, twice from blood, and 3 times from urine.

Identification of cultured pathogen. a) *Morphology.* The culture grown at 37° C revealed under the microscope blastospores, while after prolonged growth on Sabouraud's medium at room temperature, blastospores and pseudomycelia. Growth under semianaerobic conditions on corn meal agar resulted in numerous thick-walled chlamydospores. In liquid medium the fungus tormed a sediment. After one month of cultivation ring formation was observable. b) *Biochemical tests.* The fungus fermented glucose and maltose, as well as galactose (very weakly);

it did not ferment lactose and saccharose. It broke down saccharose without fermentation. No assimilation of nitrate was observable. c) *Animal experiments.* In albino mice the intraperitoneal injection of a suspension in physiological saline of a 48-hour culture of the fungus caused death in 48 hours. Pure cultures of the fungus could be grown from the peritoneal fluid. Large numbers of blasto-



Micrograph 7

spores and pseudomycelia were demonstrable on staining with PAS in sections of the kidney, in the renal cortex (v. micrograph No. 7). d) *Result of identification: Candida albicans.*

Note: Serial tests have failed to demonstrate the presence of any bacterial pathogen in blood or urine. *Paraben* treatment resulted in recovery.

Publications: References 12 és 13.

2—6. *Patients under 2 years of age.*

Clinical diagnosis: Sepsis, preceded by prolonged antibiotic treatment.
Material examined: Blood, urine (by catheter)

Results: a) *Smears.* By the method described with case 1, the fungal elements in question were demonstrated in all samples of urinary sediment. In 4 out of 5 blood smears the fungus was also demonstrated. b) *Culture.* The pathogen in question was grown from all samples of urine and from all samples of blood but one. In the case yielding negative cultures the fungus was demonstrable in smears.

Identification of cultured pathogen: As in case 1.

Note. In 2 of the 5 cases discontinuation of the antibiotic treatment resulted in recovery. In the other 3 cases recovery resulted after the administration of *Paraben.*

7. *Infant, 1 year of age.*

Clinical diagnosis: Sepsis, after prolonged antibiotic treatment.
Material examined: Blood, 4 samples.

Results and identification of cultured pathogen: As in case 1.

Note: Post-mortem examination revealed generalized candidiasis. The changes found in the brain, kidney, pancreas and lung were characteristic of candidiasis. Abundant quantities of the fungus in question were visible in histologic sections.

8—14. *Adult patients, from* 20 *to* 40 *years of age.*

Clinical diagnosis: Pulmonary mycosis? Tuberculosis?
Material examined: Bronchoscopic mucus 2 samples.

Results and identification: As in case 1.

Note: In three of the 6 cases tuberculosis was revealed. In the other three patients *Mycobacterium tuberculosis* was not detected in serial tests. All 6 patients had been given large doses of antibiotics.

15. *Female patient*, 53 *years of age.*

Material examined: Post-mortem specimens of oesophagus, intestinal segments and intra-abdominal exudate.

Results. a) In histologic sections of the materials many typical forms of the fungus in question were visible. b) *Cultures and identifications of cultured pathogen*: As in case 1.

Publication: Reference 14.

16. *Male patient*, 2 *years of age.*

Clinical diagonsis: Meningitis resistant to antibiotics.
Material examined: Cerebrospinal fluid, 3 samples.

Results and identification: As in case 1.

Note: Post-mortem examination showed Candida-meningitis.

17. *Male patient*, 37 *years of age*

Clinical diagnosis: Lobar pneumonia.
Material examined: Sputum, 5 samples.

Results. In the smears made of sputum PAS-positive blastospores appeared in numbers far above the normal. By Gram's stain very few bacteria were revealed, the normal oral flora was nearly absent.

Identification: As in case 1.

Note: The clinical symptoms and the therapeutic results confirmed the diagnosis of secondary candidiasis.
Publication: Reference 15.

Summary

The cases of systemic mycosis diagnosed during the past 2 years at the *State Institute of Hygiene, Budapest* have been briefly described. In each case the clinical diagnosis shown on the material sent in for examination the results of smear studies, cultivation, the morphology and animal pathogenicity of the cultured pathogen and, in some cases, also other observations confirming the presence of mycosis have been reported.

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PROBLEMS CONCERNING THE PHYLOGENESIS OF BACILLUS ANTHRACIS

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Introduction

Although *B. anthracis* is generally known as the first pathogenic bacterium thoroughly studied, difficulties and errors in its identification have been encountered again and again. As to the cause of these failures, we point to the fact that saprophytic organisms resembling to or imitating *B. anthracis* had been described as early as the end of the last century. Hueppe and Wood (1889), Burry (1894) further McFarland (1889) and later also some others gave reports of anthrax-like organisms, under various names as *B. anthracoides*, *B. pseudoanthracis*, *B. anthracis similis*, etc. Of the numerous publications dealing with the diagnostical and pathological importance of these organisms and published during the first three decades of this century we only mention some systematic ones (Wagner, 1920, 1923; Uémura, 1915; Grierson, 1928.). To simplify the matter, all the anthrax-like organisms described in the literature will be referred to in the present paper as "pseudoanthrax bacilli" independently of the names used in any article cited above.

The differentiation of anthrax-like microorganisms from *B. anthracis* very often failed, even on extremely thorough examination. Thus e. g. Uémura (1915) was unable to decide whether the "Hoppe B" strain he studied and found to form capsule in serum was *B. anthracis* of low virulence or a pseudoanthrax bacillus. The introduction of Ascoli's thermoprecipitation method did not diminish the difficulties of anthrax diagnosis; moreover, it often caused failures in differentiation. (See; Poppe, 1913; Sarti, 1921; Schutz and Pfeiler, 1912; Pokschischewsky, 1914.)

Pseudoanthrax bacilli may accidentally cause disease in humans or animals under natural conditions (Wagner, 1920; Senge, 1913; Willamowsky, 1912). Their moderate pathogenicity demonstrable in the laboratory makes it difficult to determine their systemic place. For a long time it was disregarded whether the different names of the pseudoanthrax bacilli mentioned above were of any definite taxonomic significance, and even to-day this aspect is left unconsidered by some workers. Uncertainties in the systematology of mesophilic, aerobic, spore-bearing organisms had not been uncommon until Smith, Gordon and Clark (1946) made their first efforts to settle the problem. The

Bacillus cereus described by FRANKLAND and FRANKLAND in 1887 had failed to attract any particular attention up to the last decade. SMITH *et al.* (1946) regarded *B. cereus* as a prototype of pseudoanthrax bacilli ; it was thus included in their taxology, while the anthrax-like organisms described under various names were deleted as independent species. The correctness of this radical suggestion is now generally accepted.

The similarity of *B. cereus* and *B. anthracis* is so very marked that some authors have claimed the latter to be a pathogenic variant of the former. This idea is not a very recent one ; the possibility that *B. anthracis* were a pathogenic variant of pseudoanthrax bacilli has been suggested by PATOČKA as early as in 1934. SMITH *et al.* (1946) went so far in their radicalism as to be opposed to recognizing even *B. anthracis* as an independent species, and conceived of this organism as a variant of *B. cereus*. The aim of our work was to analyse the above idea. Although we have demonstrate some further relations between the two organisms, we are convinced that *B. anthracis* and *B. cereus* can sharply be differentiated and no failures in diagnosis are encountered if sufficient accuracy is provided for. Before giving an account of our own experimental work, the presentation of the most important literary data concerning these two organisms seems to be necessary.

Present possibilities of differential diagnosis of B. cereus and B. anthracis

When reviewing this problem, attention must also be paid to some early data concerning the characterization of *B. anthracis*, i. e. the differentiation of "true" and "pseudo" anthrax bacilli. Differences in the morphology of the two bacteria are of little diagnostic value except the motile (flagellated) character of *B. cereus* and pseudoanthrax bacilli and the capsule production of *B. anthracis*. Nevertheless, these features alone will hardly suffice for definite differentiation.

Reports on "motile anthrax" strains were had been published from time to time, but none of these observations were confirmed. The observations of BROWN *et al.* (1955) on "motile anthrax" strains obtained by "transduction" seem to be incorrect interpretations of certain methodical errors. The authors themselves later failed in reproducing their own experiments (CHERRY 1956). On the other hand, non-motile pseudoanthrax bacillus and *B. cereus* were described by MCFARLAND and MCFARLAND (1898) and by SMITH *et al.* (1946), respectively. Therefore, motility alone can hardly constitute a basis for differential diagnosis.

Capsule formation in itself should also be weighed cautiously when identifying *B. anthracis*. Apparently some strains of *B. cereus*, whether motile or non-motile, may form capsules. SMITH *et al.* reported on capsulated *B. cereus* though the occurrence of such strains seemed to be exceptional. The "IV Gruppe" strain described by one of us (IVÁNOVICS) in 1937 and lost since that time was

very probably also a *B. cereus* strain. A motile, anthrax-like organism able to produce capsule in animals was recently described by GILLISEN and LUST (1950). NORDBERG also observed a *B. cereus* strain exhibiting capsule formation on dextrose agar medium. These examples are not exceptional, as the capsule production of pseudoanthrax strains was also observed by some other investigators (e. g. UÉMURA, 1915). In the case of "wild" anthrax strains, e. g. those isolated from animal organs, the examination of capsule production is of great practical value, while laboratory strains may soon lose this capacity (CHU, 1952). Under usual cultivation circumstances virulent "wild" strains appear to be R-variants ; in the absence of the necessary physiological conditions they appear as non-capsulated phenotype (IVÁNOVICS, 1938 ; CHU, 1952), for virulent strains produce capsule only under a comparatively high CO_2 tension (IVÁNOVICS, 1937 ; STERNE, 1937). Nothing definite is known concerning the problem whether anthrax bacilli do or do not retain their capsule-producing ability when exposed to natural circumstances, e. g. when they are in the soil for a certain period.

Colonies of anthrax bacilli, of *B. cereus* strains and of pseudoanthrax bacilli exhibit very little differences when cultivated on agar medium (GRIERSON, 1928 ; SMITH *et al.*, 1946). On the base of carbohydrate fermentation, differentiation is hardly possible (NORDBERG, 1951). As to their other enzymatic activities, examination of the haemolytic and lecithine splitting enzymes might be of use in differentiation. As early as in 1913, attention was drown by JÁRMAI to the intensive changes caused by pseudoanthrax bacilli on blood agar medium. A definite haemolytic area (beta-haemolysis) develops around the colonies after 24 hours, with a tendency to further increase by next day. On the other hand, strains of *B. anthracis* do not haemolyse red blood cells, more correctly, there is only a slight haemolysis observable in media containing whole blood. As the hamolytic activity of *B. anthracis* is intimately related to the actual experimental circumstances, no uniform results were achieved by the different authors, while they all agree in regarding this activity as minimal. According to BEKKER (1941), haemolytic activity is very labile even in the same strain, thus it can not be used as a measure of the virulence, as BUZA (1939) had claimed. In the differentiation of *B. anthracis* and *B. cereus*, the examination of haemolysis was regarded as important also by BURDON (1956). Lecithinase production of the strains seems to be of equal value as that of haemolysis (MCGAUGHY and CHU, 1948). Lecithinase is produced very vigorously by *B. cereus* while only moderately and slowly by *B. anthracis*. In both organisms, the enzyme is a phospholipase capable of splitting phosphocholine from the lecithine molecule (CHU, 1949 ; ZWARTOUW and SMITH, 1956). The haemolytic principle and lecithinase are most probably identical (CHU, 1949).

In differential diagnosis, the examination of pathogenicity is in itself only of moderate importance. It is characteristic of "wild" anthrax strains that,

in addition to their intense pathogenicity, the cells appear capsulated in the smears made from organs of animals dying of anthrax. Animal inoculation is of little value in the case of moderately virulent or avirulent strains of *B. anthracis*. Pseudoanthrax strains might similarly cause lethal infection in guinea-pigs and mice. The pathologic events in mice may often resemble a typical anthrax infection, oedematic inflammation of connective tissue may be present, while death ensues usually earlier than in anthrax (GRIERSON, 1928). Well-defined strains of *B. cereus* may occasionally cause disease in mice (BURDON, 1956).

There is a remarkable difference in the penicillin sensitivity of *B. anthracis* and *B. cereus;* POLLOCK (1952) found that the latter is able to produce both adaptive and constitutive penicillinase. JENSEN and KLEEMEYER (1953) observed that strains of *B. anthracis* assumed a teratologic pearl-row-like shape on agar medium containing 0.05 U/ml of penicillin. It has been emphasized also by BURDON (1956) that *B. anthracis*, being sensitive to penicillin, does not grow in the presence of 10 U/ml of that antibiotic.

Application of specific phages appears to be a great assistance in the identification of *B. anthracis*. A phag characterized by its sharp host specificity was isolated by MCCLOY in 1951 from a lysogenic strain of *B. cereus* (designed as W). BROWN and CHERRY (1955) succeeded in further enhancing the specificity of this strain.

On the antigenic structure of B. anthracis and B. cereus

Hot water extractable immune-specific substances of anthrax bacilli attracted great interest in relation to the Ascoli-test. Unfortunately, it turned out very soon that sera prepared for the Ascoli-test might give a precipitation reaction also with extracts of pseudoanthrax bacilli and other aerobic, mesophilic sporebearing bacilli (POKSCHISCHEWSKY, 1914; SCHÜTZ and PFEIFER, 1912; POPPE, 1913; SENGE, 1913; SARTI, 1921; POHL, 1927). It is difficult to evaluate the theoretical bearings of these studies, as no characterization of the sera used has been given by the authors. TOMCSIK and SZONGOTT (1933) were the first to demonstrate the presence of distinct immune-specific antigens in the cells of *B. anthracis* and to prove the immune-biologic difference of "soma" and the capsule. The occurrence of the capsular substance, the D(—)-glutamic acid-polypeptide, of *B. anthracis* (IVÁNOVICS and BRUCKNER, 1937) is very common among members of Genus Bacillus (IVÁNOVICS, 1937). The chemical or serological demonstration of its presence in well-defined strains of *B. cereus* invariably failed (TOMCSIK and GUEX-HOLZER, 1951; IVÁNOVICS, 1953). The encapsulated strains of *B. cereus* cited in the literature were not investigated in this respect. The "soma" antigen of *B. anthracis* obtained in a state of high purity is a polymer of acetyl glucosamine and galactose (IVÁNOVICS, 1940),

to which a peptide moiety consisting of some amino acids is intimately connected (SMITH, STRANGE and ZWARTOUW, 1957).

A part of the non-specific overlapping reactions observed sometimes in connection with the Ascoli-test may be explained by the presence of D(—)-glutamic acid-polypeptide as the common hapten in Genus Bacillus (IVÁNOVICS, 1939). By closed investigation of *B. cereus* new perspectives opened in this field. *B. cereus* strains are apart from a few exceptions, not capsulated, therefore the probable serological relationship of *B. anthracis* and *B. cereus* seems to rest on some other antigenic components.

In 1954 PESTI prepared extracts from *B. cereus* and some pseudoanthrax strains by prolonged shaking with distilled water or in warm dilute hydrochloric acid. Some of these extracts precipitated a commercial horse serum devoid of any capsular antibody. These positive tests indicated a serological relation between the anthrax bacillus and some of the *B. cereus* strains. No attemps were, however, made to determine the antigenic component responsible for the relationship observed ; only the possible role of D(—)-glutamic acid-polypeptide was excluded in PESTI's experiments.

The presence of immune-specific polysaccharide made up of acetyl-glucosamine and galactose in *B. anthracis* has been established by studies on a limited number of strains. It is, therefore, difficult to decide at present whether or not this polysaccharide is a characteristic component. The uncertainity is accentuated by a recent report by JENSEN and KLEEMEYER (1953) on precipitation studies carried out on 50 well-defined *B. anthracis* strains. Of the 50 strains examined by a commercial Ascoli-serum not further characterized, only 25 exhibited a positive reaction, while 13 gave doubtful and 11 entirely negative results.

Considering the bulk of informations referred to above, the immune-biological homogenity of *B. anthracis* strains appears questionable. The nature of the serological relationship between *B. anthracis* and *B. cereus* also seems to need further investigation. The examination of these two problems was the aim of our studies to be presented.

Materials and methods

Media. Two different types of media were used thoroughout. The *YP-medium* consisted of the supernatant of an autoclaved yeast suspension and Witte pepton. As to the details, we refer to the report of IVÁNOVICS and ALFÖLDI (1954). The broth used in our experiments contained horse-meat extract and one per cent pepton. The above media were used also in solid form, after the addition of 1.5 per cent agar-agar.

Phage-material. The strain was kindly supplied by Miss MCCLOY. The phage originated from the lysogenic *B. cereus* W strain. The phage material supplied was designed as : $\frac{\varphi aL}{D}$. The phage was propagated on *B. anthracis* (Davis strain) in liquid YP medium. Seitz filtrates of the culture were used in our experiments.

Immune sera. Anti-anthrax sera both of horses and rabbits were used. The horse serum designated CX was prepared in 1934 by the *Phylaxia Serum Institute* (Budapest). This serum

still contained a considerable amount of anti-polysaccharide antibody, it failed, however, to give precipitation with D(—)-glutamic acid-polypeptide. Rabbit serum has been prepared recently in our laboratory by repeated intravenous injections of washed, formolyzed anthrax bacillus suspensions. Cereus immune serum was made in rabbits. Rabbits were injected several times with a heat killed suspension of strain 116.

Preparation of standard bacterial suspensions. Ten ml of YP-medium were put into a 100 ml Erlenmeyer flask and inoculated. Cultivations was performed at 37° C under aeration by shaking. Growth was estimated by measuring the optical density of the suspension. Measuring was made in a tube 16 mm in diameter, with the aid of an *Oriphot* photometer (IVÁNOVICS and ALFÖLDI, 1954). The suspension of *B. cereus* strains grown in YP broth of an optical density of 0.5 contained usually 10^8 cells per ml. (Cell count was determined in Bürker's haemocytometer.)

Examination of capsule production. Capsule production was examined on cells cultivated in 20 per cent carbon dioxide athmosphere on YP agar medium containing 20 per cent horse serum.

Examination of penicillin sensitivity. Inocula containing 10^3 cells prepared from optically standarized young cultures were spread on the surface of agar plates containing 2.5, 5 and 10 units of penicillin per ml, respectively. Incubation took place at 37° C for 24 hours.

Examination of haemolysis was performed on horse meat broth agar containing 15 per cent whole sheep blood or washed erythrocytes.

Examination of lecithinase. Pepton medium containing egg yolk, as suggested by ZWARTOUW and SMITH (1956), was used. The fluid medium contained 5 per cent egg yolk, 1 per cent Witte pepton and 0.5 per cent sodium chloride. This mixture was sterilized by Seitz filtration, dispensed in test tubes, or, if needed, made up to a solid medium by mixing with equal amounts of a 2.5 per cent solution of agar in water.

Phosphatase test. This was carried out according to BARBER and KUYER (1951). To a meat broth agar medium 0.001 per cent phenolphtalein phosphate was added and the medium was dispensed in Petri-dishes. The inoculated plates were incubated at 37° C for different periods and then a few drops of concentrated ammonium solution were placed on the cover of the Petri-dish.

Examination of the phage effect. Lysates generally used contained 10^{10} plaque-producing units per ml. A tenfold dilution series was prepared from the material. One ml of the bacterial suspension (optical density 0.2) to be examined was mixed with an equal volume of melted YP agar medium and layered onto the agar plates. A drop (0.02 ml) of each phage dilution was put on its surface and incubated.

Phage particle counting was performed by the usual method in a soft agar layer, using the *B. anthracis* (Davis) strain as indicator.

Preparation of autolysates for serological purposes. Cells of a 24 hour agar slant culture were suspended in a mixture of 2 ml saline and 1 ml M/15 Na_2HPO_4 solution. 0.1 ml of an 0.1 per cent trypaflavine solution was added to the suspension which then was incubated for a week.

Performing of precipitation reactions. In a series of tubes different dilutions of the purified polysaccharides were measured in volumes of 0.2 ml each and an equal amount of immune serum diluted 1 : 2 was added to the tubes. The mixtures were kept at 4° C for 48 hours.

To perform quantitative precipitation, 1 ml serum was measured into a cooled centrifuge tube and the optimum amount of hapten was added in 1 ml saline. The tubes were incubated for 3 days in a refrigerator, then centrifuged. The sediment was washed twice, using 5 ml saline each time. The precipitate was digested with sulphuric acid and its N content was estimated by nesslerization. The determinations were made in triplicate.

Isolation of specific polysaccharides. The anthrax polysaccharide was isolated from a virulent strain of *B. anthracis*, according to the method described earlier by one of us (IVÁNOVICS, 1940). For the preparation of immune-specific polysaccharides from *B. cereus* a similar but slightly simplified procedure was applied. Details of the method are presented below.

The organisms were cultivated in Roux flask on YP agar medium for 18 hours. The amount of cells used for a single preparation process was that grown on 0.5 to 0.55 m^2 surface of medium. Cells were washed off with a total volume of 700 ml of saline and washed twice by centrifugation with 700 ml saline each. Bacterial cells thus washed free from agar and medium impurities were resuspended in 400 ml M/50 Na_2HPO_4 solution and 12 mg of trypaflavine was added. This suspension was allowed to stand at 37° C for 7 days in an incubator to let the cells autolyse. This was followed by centrifugation and pH of the supernatant was adjusted to pH 4 with acetic acid. The precipitate thus obtained was removed and the acidity of the clear supernatant was adjusted to pH 6. The volume of the extract was reduced by a warm current of air to 50 ml. During evaporation, temperature never exceeded 60° C. Small amounts of a poorly soluble precipitate appeared during this procedure ; they were centrifuged and discarded. After addi-

tion of 2 g sodium acetate and 1 ml glacial acetic acid the supernatant was mixed with 250 ml alcohol. Next day the precipitate formed was centrifuged and redissolved. The insoluble residue was removed and precipitation repeated by ethanol as described above. This procedure was repeated twice. A third additional precipitation was also made at slightly alkaline reaction (pH 8), in the presence of sodium acetate only. The final sediment dissolved in water was freed from protein by shaking with a mixture of 4 volumes of chloroform and 1 volume amyl alcohol. Finally, the solution was dialyzed. The clear solution thus obtained was evaporated to dryness by a current of warm air. For analytical purposes material dried *in vacuo* at 60° C was used.

Analytical methods. Nitrogen determinations were made by nesslerization according to CLEGHORN and JENDRASSIK (1934). Carbohydrates were determined by the anthron reagent following MOKRASCH's prescription (1954). Extinction values were read at 660 mμ wave-legth. Acetyl determination was made by the method of KUHN and ROTH (1933).

The reducing capacity of samples hydrolysed for 2 hours in *N* hydrochloric acid was measured according to HAGEDORN and JENSEN, and its value was expressed as glucose. For determination of hexoseamines the ELSON and MORGAN reaction was used, as modified by BELCHER *et al* (1954). Values were read by means of a Pulfrich photometer at 530 mμ wavelength.

Paper chromatography was made by the one dimension ascending method. For the determination of amino acids, 20 mg of material were dissolved in 2 ml 6*N* HCl and hydrolysed in a boiling water bath for 9 hours. The solution was deionized by Amberlite IR 120 H+ resine and elution was made by ammonium hydroxide (REDFIELD, 1953). As a solvent for paper chromatography, we used either phenol in ammonia atmosphere or a mixture containing pyridine, ethylacetate, water and acetic acid, 5—5—3—1 volumes, respectively (FISCHER and WEBEL, 1955).

For the identification of carbohydrates, material hydrolysed by *N* HCl for 6 hours was applied. The bulk of hydrochloric acid was evaporated from the hydrolysate *in vacuo*, while its last traces were removed in an exsiccator containing sodium hydroxide. For paper chromatography of carbohydrates, a mixture containing butanol, pyridine and water at a ratio of 3—2—1.5 (HORROCKS and MANNING, 1949) was used. For developing we made use of anilnephthalate.

Results

Strains of B. anthracis and their cultural characteristics. Our study covered a total of 26 *B. anthracis* strains of different origin. Of these 1 (Davis) was kindly supplied by Miss MCCLOY and 3 (Weybridge, Vollum, NPA) by DR. H. SMITH. All the other strains were obtained from the collections of different Hungarian laboratories during the period of 1946 to 1952. Five of these 22 strains were attenuated ones used for vaccine production. As to the other strains no specifications were avaible.

No effort was made to determine the virulence of the strains. In carbon dioxide athmosphere 21 strains exhibited capsule production. Of these, 15 had colonies of mucoid appearance and all the cells were found to be enclosed in well-developed capsules. Colonies of the remaining 6 strains were non-mucoid and the moderate number (1 per cent or less) of capsulated bacteria could be demonstrated only by a thorough microscopic examination. Five typical R-variants were found in the 26 strains studied. Repeated examinations failed to demonstrate capsule production in these 5 strains.

Thus, differences were found in our strains according to their capsule production, while in the biological tests their behaviour was essentially identical. No motile cells could be demonstrated by repeated examination in young cultures. Standard inocula ($2 \cdot 10^6$ cells) of the strains did not grow on media con-

taining 2.5 U/ml of penicillin. On meat extract agar medium containing 15 per cent whole sheep blood haemolysis was absent even after 72 hours of incubation in cultures of all the strains. On the other hand, the same strains exhibited haemolysis on media containing 3 times washed red blood cells instead of whole blood. In this case, a 1 to 2 mm wide haemolytic area was observed around the colonies even after 24 hours. After 48 to 72 hours the haemolysis became more marked and both the diameter and the transparency of the haemolytic area increased. Nevertheless, complete transparency of the haemolytic area could never be achieved.

In our hands the lecithinase test did not prove reliable if performed on solid agar medium. Though after 24 hours around the colonies a certain sort of halo appeared exhibiting sometimes an increasing tendency, a spontaneous clearing up of the medium could also be observed which later confluated with the halos around the colonies. Nevertheless in liquid medium the same test worked accurately. In such tubes minute opalescence or no change whatever appeared after 24 hours. On further incubation the turbidity of the medium increased and by the 3rd or 4th day massive turbidity or even sediment developed. At the end of the experiment (the 120th hour) intensive turbidity was present in every case and a heavy precipitate was mostly formed.

For the differential diagnosis of *B. anthracis* and *B. cereus*, we have attempted to adopt also the phenolphthalein-phosphate test suggested by BARBER and KUYER (1951). This test can be used for the demonstration of phosphatases with the aid of phenolphthalein-phosphoric acid-ester as a substrate. The essence of the reaction is that the intact ester remains colourless on the addition of alkali (in the form of ammonia vapour), while phenolphthalein freed by the enzyme gives its usual colour reaction. Anthrax colonies developed after 24 hours on medium containing phenolphthalein-phosphate failed to exhibit a colour reaction in the presence of ammonia vapour. On further incubation (3 to 4 days), some colonies were found to give a very faint pink colour reaction. This means that the substrate is not decomposed by *B. anthracis*, or, if at all, then very slowly and moderately.

The phage sensitivity of all the 26 *B. anthracis* strains examined was found to be very marked. A confluent plaque formation was present even at 10^{-6} dilution. The confluent, sharply marginated plaque were absolutely clear and translucent.

Antigenic structure of B. anthracis strains. In order to determine whether the presence of specific polysaccharide is species specific and therefore characteristic of *B. anthracis*, attempts were made to identify this cell component in the autolysates of our 26 strains of *B. anthracis*. For the serological identification of this specific substance OUCHTERLONY's (1949) agar diffusion plates were used. In this test, an anti-anthrax horse serum was applied. The serum precipi-

tated anthrax polysaccharide up to a high titre, whereas it was devoid of any anti-polypeptide antibody. The serum was put in a hole in the middle of an agar gel (1 per cent agar in buffered saline) plate while the autolysates of strains were added in holes 12 to 13 mm apart from that containing serum. For identification of precipitation, a plate similarly prepared but containing a 1 to 10 000 dilution of purified polysaccharide instead of the autolysate was used. The results were read after a fortnight's incubation at room temperature. Our observations thus obtained are summarized below.

The autolysates of all the 26 strains were found to give the precipitation reaction characterized by a zone 2.5 to 3 mm in diameter with a sharp margin. The colour of the zone was decreasing in intensity towards the hole containing

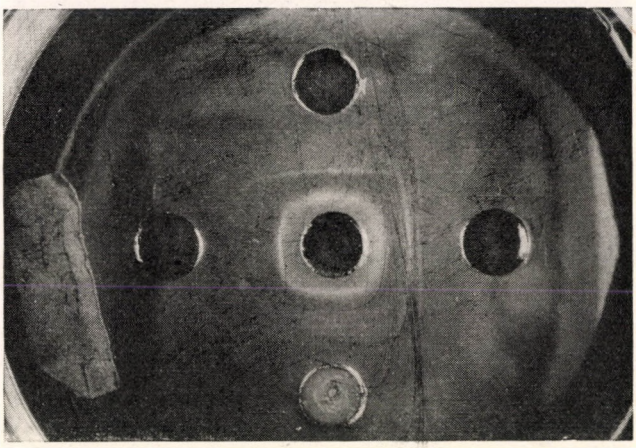

Fig. 1. Agar diffusion precipitation test of autolysates of 4 different *B. anthracis* strains by anti-anthrax horse serum. The central cup contains the serum (Orig. size)

serum. In addition, 1 to 4 fine definite lines also occurred depending on the actual strain and the autolysate. These lines appeared distal from the precipitation zone. One of our typical experiments is presented in Fig. 1. As to the origin of the innermost precipitation zone, we refer to Fig. 2, which presents an experiment performed with purified polysaccharide solution.

Thus we succeeded in demonstrating by serological methods the presence of the well-defined polysaccharide (IVÁNOVICS, 1940) in all the strains examined. In addition to this and the polypeptide type hapten, a minimum of four further different immune specific components seemed to be present in autolysates of *B. anthracis* strains. We did not endeavour to characterize these substances in the present study.

Biological characteristics of B. cereus. A total of 15 strains of different origin was studied in this respect. Of these 9 had been isolated from soil samples, 2 (No. 10 and No. 81) as contaminants from bacteriological samples, while 3, namely NRRC 569, $5/P_3$ and $5/P_{13}$ (parvo-constitutive), were kindly supplied

by dr. POLLOCK from his collection. The fifteenth strain was the lysogenic W strain of Miss MCCLOY.

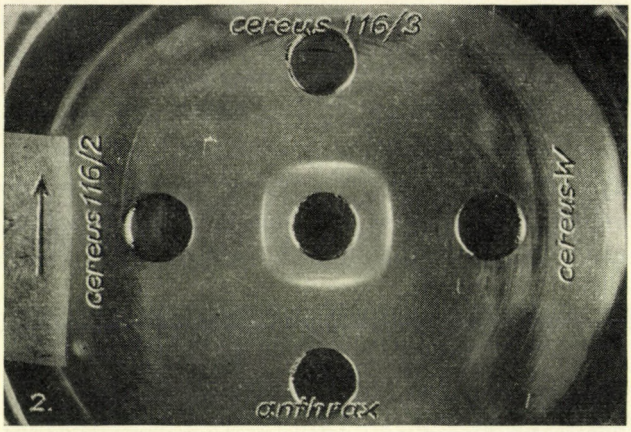

Fig. 2. Agar diffusion precipitation test of solutions of anthrax and cereus polysaccharide (dil. 1 : 10 000) by anti-anthrax horse serum. The central cup contains the serum (Orig. size)

The morphological characteristics of the above strains, agreeing with the criteria given by SMITH *et al.* (1946). Their biological properties are presented in Table I.

As shown in Table I, none of the strains produced capsule. In young broth cultures the single cells or short chains moved vigorously. *B. cereus* 130 strain was an exception forming long filaments consisting of numerous cells. In one of our repeated examinations, the active movement of these filaments could not be stated with certainty.

On agar medium containing 10 U/ml of penicillin the strains grew well if an inoculum containing 10^6 cells from a young broth culture was used. No uniform results could be obtained when using unmeasured inocula prepared from agar slant cultures of 48 or more hours. This is the reason why we consider the penicillin sensitivity test as reliable for the differentiation of *B. anthracis* and *B. cereus* only if standard inocula are used.

A clear-cut differentiation of the strains can be achieved by the haemolysis and lecithinase tests, both on fluid and solid media. The very marked haemolytic activity of *B. cereus* is readily observable as early as after 24 hours of incubation. The lecithinase test is also strongly positive even on the first day of incubation, both in solid and fluid media.

In the differentiation of *B. anthracis* and *B. cereus*, the phenolphthalein-phosphate splitting test was also used with success. Colonies of *B. cereus* developed an intensive red colour in ammonia vapour in 24 hour old cultures. It seems very probable that for the splitting of this substrate the same phophatase might

Table I

Biochemical characters, virulence and reaction with anti-anthrax polysaccharide antibody of B. cereus strains

Strain	(1) Capsule production	Motility	(2) Penicillin resistance	Haemolysis	Lecithinase	Phosphatase	(3) Phage sensitivity	(4) Precipitation	(5) Virulence 10^6	(5) Virulence 10^7
No 2	—	+	+	+	+	+	—	—	0/3	2/3
No 4	—	+	+	+	+	+	—	—	0/3	0/3
No 5	—	+	+	+	+	+	—	—	0/3	2/3
No 6	—	+	+	+	+	+	—	—	0/3	2/3
No 7	—	+	+	+	+	+	—	—	1/3	3/3
No 8	—	+	+	+	+	+	—	—	0/3	3/3
No 10	—	+	+	+	+	+	—	+	3/3	3/3
No 81	—	+	+	+	+	+	—	+	1/3	
No 114	—	+	+	+	+	+	—	+	0/3	3/3
No 116	—	+	+	+	+	+	—	+	3/3	
No 130	—	+	+	+	+	+	—	—	1/3	1/3
No $5/P_3$	—	+	+	+	+	+	—	—	0/3	0/3
No $5/P_{13}$	—	+	+	+	+	+	—	—	0/3	0/3
NNRC 569	—	+	+	+	+	+	—	—	2/3	3/3
W	—	+	+	+	+	+	—	+	2/3	3/3

Remarks: (1) Capsule production on serum agar in 20 per cent CO_2 atmosphere.
(2) Growth on agar medium containing 10 U/ml penicillin.
(3) — No lysis by concentrated phage preparate.
(4) Tested by agar diffusion method.
Only a reaction specific to anthrax polysaccharide taken as positive.
(5) Animals infected with 10^6 or 10^7 bacilli.
The numerator represents the number of dead, the denominator that of the inoculated mice.

be responsible which was described by CHU in 1949 in connection with the lecithinase effect. It is rather peculiar that the splitting of the substrate occurred only in the colonies themselves and never in the medium even just around them. Nevertheless, the presence of enzyme activity was established in the supernatant of centrifuged *B. cereus* broth cultures ; this points to the extracellular character of the enzyme.

As stated by MCCLOY (1951), further by BROWN and CHERRY (1955), strains of *B. cereus* invariably exhibit a complete resistance against anthrax-phage. No lysis could be observed on *B. cereus* inoculated agar plates on the addition of a drop of lysate containing about 10^{10}/ml phage particles. In addition, we examined also the phage-adsorbing ability of some of our strains. The results are presented in Table II.

Table II

Adsorption of phage by different strains of B. cereus

To 4 ml of YP broth culture (optical density, 0.5) of individual strain 1 ml of lysate diluted to 1 : 100 ($2 \cdot 10^9$ plaque forming units) was added. The mixture was incubated at 37° C for 20 minutes and for 1 hour in a refrigerator. After centrifugation the plaque forming unit content of the supernatant was determined on *B. anthracis* Davis strain

Bacterial suspension added to the lysates	Plaque forming units in the supernatant ($\times 10^6$)	Per cent of adsorbed phage
B. cereus 116	940	53
B. cereus 130	1570	21
B. cereus 114	1100	45
B. cereus W	1600	20
B. anthracis (Davis)	5	99.8

As Table II reveals, the amount of phage adsorbed by *B. cereus* strains was negligible, thus specificity seemed fairly questionable. The anthrax strain used as a control adsorbed, on the other hand, 99.8 per cent of the phage added.

As to another problem in differential diagnosis, we refer to a further observation. The No. 114 strain of *B. cereus* gave at an occasional inoculation to YP medium 2 different types of colonies, *viz.* type *a)*, slightly translucent colonies of about 4 mm in diameter, exhibiting a homogeneously rough granulation when transilluminated ; type *b)* colonies somewhat larger than type *a)*, with a slightly opaque central area and a translucent, roughly granulated margin. The characteristics of the variants were retained in subcultures. The biological characteristics of the variants were essentially similar, except their motility. Variant *a)* moved vigorously, while variant *b)* formed motionless filaments in broth. No motile cells were found by repeated examination in cultures of variant *b)*, thus this can be regarded as a non-motile variant of *B. cereus*, supporting the view that motility is not a definite criterion of the difference between *B. cereus* and *B. anthracis*.

The lysogenic character of *B. cereus* strain W was described by McCLOY (1951). No data are available on the so-called "induction" of this strain. In our experiments young cultures of this strain in YP broth exhibited progressive lysis on UV irradiation and further incubation. In the lysates, 50×10^6/ml phage particles were found. Thus this strain appeared to belong to the group of inducible lysogenic bacteria (LWOFF, 1953).

Antigenic structure of B. cereus strains. As mentioned above, different anthrax and anthrax-like strains may very often give false reactions in the Ascoli test. The probable identity of these strains with *B. cereus* suggests the presence of related antigenic components in this species and *B. anthracis*. Recently, CHU (1950) has pointed out that there are certain serologically related components in the spores of these two species (Personal communication to

McCLOY). According to PESTI, dilute hydrochloric acid extracts of *B. cereus* are precipitated by anti-anthrax sera. BROWN and TREECE (1956) in some preliminary experiments have also found related antigenic components in the cells of *B. anthracis* and *B. cereus*.

Nevertheless, none of these studies gave definite information on the serological relations of the two species. As the above studies revealed, in the extracts of *B. anthracis* there are several specific components in addition to the D(—)-glutamic acid-polypeptide (IVÁNOVICS and BRUCKNER, 1937). That is why detailed studies could only solve the problem of the serological relationship of these two species.

Autolysates of our *B. cereus* strains, when tested against anti-anthrax sera by the agar-gel method, exhibited different precipitation patterns. Extracts of some of our strains [10, 81, 114, 116, and W] gave a definite precipitation zone beginning at the serum containing hole as a diffuse area about 3 mm wide, with a border. More distally one or two, occasionally three, very fine but definite lines could also be observed, their number depending on the actual strain and the experiment. Thus, the precipitation pattern observed exhibited certain similarities to those referred to in connection with *B. anthracis* autolysates.

The rest of our *B. cereus* strains did not exhibit serological relations of such an extent with these strains the characteristic zone was not present, only some thin lines at about the middle of the distance between the holes containing the serum and extract, respectively.

To establish the details of these serological relationships, the specific substance was isolated from the lysates of some of our strains. As to the chemical characteristics of the substances isolated we refer to Table III.

Table III

Chemical characterization of substances isolated from B. anthracis and from different strains of B. cereus

Strain	(1) Yield g	Per cent N	Hexosamine %	Anthron value (2) Gluc. %	Anthron value (2) Galact. %	(3) Reduction %	(4) Acetyl %
B. anthracis		3.20	36.2		38.2	64.5	9.9
B. cereus 116	0.272	3.29	29.8		36.2	66.4	13.7
	0.282	3.25	29.5		34.5	63.4	12.4
	0.314	3.67	31.0		36.8	67.0	
B. cereus W	0.288	3.76	35.00		39.8	67.3	10.7
B. cereus 6	0.363	5.69	53.0	13.5		67.0	11.5
B. cereus 8	0.330	5.51	52.2	14.0		67.1	
B. cereus 569	0.267	5.36	50.6	13.7		64.8	

(1) Yield from an amount of bacteria grown on medium about 0.5 square meters surface.
(2) Anthron value is expressed in glucose or galactose.
(3) Expressed in glucose.
(4) Mean of two determinations.

As Table III reveals, the substances isolated from the different *B. cereus* strains from two groups, according to the characteristics of the polysaccharide present. One group consisted of preparates with a comparatively low (3.25 to 3.75 per cent) N content and a high anthron value were amino sugars. Such preparates were obtained from *B. cereus* strains 116 and W, and were found to be very similar to those obtained from virulent *B. anthracis*. The other group was obtained from *B. cereus* strains 6, 8, and 569. These had a high N content and only about ¼ of their reducing substances reacted with anthron.

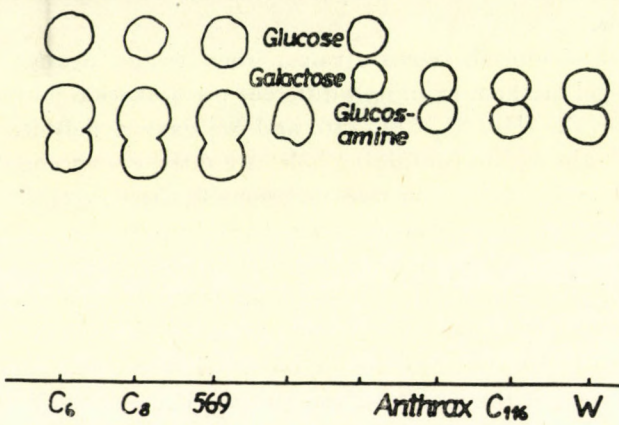

Fig. 3. Chromatogram of hydrolysates of polysaccharides isolated from different *B. cereus* strains and from *B. anthracis*. Developed by anilinephthalate

In hydrolysates of these polysaccharides we succeeded in demonstrating the presence of monosaccharides and certain amino acids. A paper chromatogram run in butanol pyridine water solvent and developed by aniline-hydrophthalate is presented in Fig. 3.

In hydrolysates of the anthrax and 116 strains galactose and glucosamine were present. A spot corresponding to glucosamine was found on the chromatograms of hydrolysates of *B. cereus* 6, 8 and 569 strains. In addition, spots characteristic of glucose and of another substance with a somewhat lower R_f value than glucosamine, were also obtained. The latter spot gave a positive reaction with ninhydrine and Elson—Morgan's reagent.

Substances hydrolyzed with 6*N* HCl for 9 hours and chromatographed in pyridine, ethyl acetate, water and acetic acid solvent, when developed by ninhydrine revealed the pattern presented in Fig. 4. In the hydrolysates of all the substances tested the following amino acids were demonstrated: *a*-*ε*-diaminopimelic acid, aspartic acid, glutamic acid and alanine. The spot representing aspartic acid was very faint in the case of anthrax polysaccharide. The spot characteristic of glucosamine was present in every preparates. On chromato-

grams of polysaccharides isolated from strains 6, 8 and 569, an additional ninhydrine positive spot was also present, giving a colour reaction with Elson—Morgan's reagent.

There was no possibility to identify the hexosamine mentioned. Two types of amino sugar might have been taken in consideration, *viz.* chondrosamine and the not yet definitely identified hexosamine described by STRANGE and

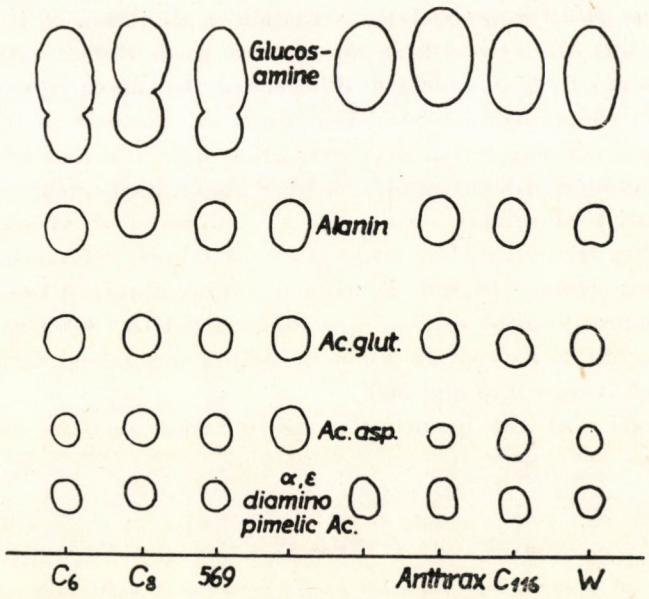

Fig. 4. Chromatogram of hydrolysates of polysaccharides isolated from different *B. cereus* strains and *B. anthracis*. Developed by ninhydrin

POWELL (1954) in extracts of *B. cereus* and related bacteria. The latter was also found by SMITH, STRANGE and ZWARTOUW (1956) in hydrolysates of *B. anthracis* and of the cell walls of different bacteria (Lactobacillus, Gram positive cocci, etc.) by CUMMINS and HARRIS (1956). These latter authors differentiated the hexosamine in question from glucosamine and chondrosamine by means of two-dimensional chromatography in phenol and lutidine. Unfortunately, we could obtain neither lutidine nor authentic samples of chondrosamine and the unidentified hexosamine. In one-dimension chromatograms run in phenol and developed by ninhydrine the unknown hexosamine could not be identified because of the interfering effect of alanine. According to FISCHER and WEBEL (1955), the migration of chondrosamine in pyridine, ethyl acetate, water and acetic acid solvent is slower than that of glucosamine. Therefore, we could not exclude the possibility that our second amino sugar spot also represented chondrosamine.

The results of our serological examinations carried out with the above preparates might be summarized as follows. Substances isolated from *B. cereus* strains 6, 8 and 569 were absolutely inactive when tested against anti-anthrax sera. Polysaccharides obtained from strains 116 and W exhibited characteristics essentially similar to those obtained from *B. anthracis*. Fig. 2 presents the reaction patterns of these substances in the agar gel precipitation test.

Polysaccharides isolated from anthrax bacilli and *B. cereus* W and 116 strains gave a positive precipitation reaction in dilutions of 1 to 400 000 to 1 to 800 000 with the anti-anthrax horse serum used. Rabbit anti-anthrax sera were precipitated by 1 to 1 600 000 dilutions of the above polysaccharide preparations. On the contrary no reaction could be obtained in the presence of any of the anthrax sera tested with extracts strains 6, 8 and 569 of *B. cereus*.

In accordance with the results outlined above, anti-cereus serum obtained by immunization of rabbits with strain 116 exhibited an identical serological specificity when tested with bacterium polysaccharides. Polysaccharides isolated from *B. cereus* strains 116 and W, as well as that obtained from *B. anthracis* gave positive precipitation tests up to a dilution of 1 to 1 600 000. On the other hand, no precipitation occurred when the serum was tested with cereus polysaccharides of strains 6, 8 and 569.

The results of the quantitative precipitation test are summarized in Table IV.

Table IV

Maximum amount of precipitate (expressed as N) formed at a slight excess of polysaccharides

Polysaccharide	Amount of N precipitated μg/ml		
	Anthrax sera		Cereus 116 serum
	Horse(1)	Rabbit(2)	Rabbit(3)
Anthrax	166 ± 4.1	369 ± 4.0	1012 ± 9.0
B. cereus 116 (lst. prep.)	156 ± 2.7	368 ± 5.4	992 ± 8.0
(2nd. prep)	163 ± 7.7		
(3rd. prep.)	158 ± 1.7		
B. cereus W	166 ± 3.0	368 ± 5.4	1044 ± 9.0

To 1 ml serum was added 1. 40 μg ; 2. 120 μg and 3. 300 μg of hapten

It is seen that both anthrax and cereus polysaccharides precipitate similar amounts of antibody from either anthrax or cereus 116 sera. It seems worth mentioning that sera adsorbed by heterologous polysaccharides gave no reaction with homologous hapten.

Discussion

In the monograph of SMITH, GORDON and CLARK (1952), the authors have put the following question: "When a strain of *B. anthracis* loses its pathogenicity is it still the anthrax bacillus or is it *B. cereus*? From the incomplete work of the writers and from the natural relationships of *B. cereus* it would seem that the latter represents the true status of the non-virulent strains and that from the academic viewpoint *B. anthracis* is taxonomically a pathogenic variety of *B. cereus*."

We believe that the authors cited had formed their opinion on the basis of certain onesided points of view and by taking into account some not yet corroborated results. This concern especially the problem of the so-called "motile anthrax" strains. We recall here to our doubts presented also in the introduction.

According to our opinion, motility and virulence cannot be regarded as main features in the differentiation of *B. anthracis* and *B. cereus*. Even capsule production seems to be of minor importance, there being more characteristic properties to be made use of in differentiation.

On the basis of our own examinations and of the pertaines literature, we think a definite differentiation of *B. anthracis* and *B. cereus* to be possible by the following three features : 1. phosphatase production ; 2. presence or absence of anthrax phage receptors ; 3. examination of penicillin sensitivity.

The vigorous and extensive haemolytic capacity of *B. cereus* is well-known ; the same enzyme seems to be responsible also for the lecithin decomposing activity as well as for phenolphtaleinphospate splitting ability of the organism. As to the haemolytic activity of *B. anthracis* strains, the data available are rather controversial, probably because the phosphatase activity of these strains is not only weak, but, in addition, it seems to be inhibited by the presence of blood serum. Accordingly, the two bacteria can easily be differentiated on grounds of their lecithin decomposing and haemolysing activities. The difference of the two species can readily be demonstrated also by the phenolphthalein-phosphate test. Making use of this test the difference in cultures incubated for 24 hours is as marked as in the case of lactose fermenting and non-fermenting enteric bacteria on Endo medium.

The most correct differentiation of these two species is made by testing them with properly chosen mutants of the phage strain isolated from a lysogenic *B. cereus* strain by MCCLOY (1951). The absence of the phage receptor in *B. cereus*, and its presence in *B. anthracis*, strains seems to be a very characteristic feature. This phage receptor of *B. anthracis* appears to be different from both the specific polysaccharide and the D(—)glutamic acid-polypeptide present in the organism. These substances when isolated in purified form failed to inacti-

vate the phage. On the other hand, the phage was inactivated by extracts obtained under appropiate conditions from anthrax bacillus (IVÁNOVICS and LANTOS, to be published).

The sensitivity to penicillin is another decisive feature in the differentiation of these two related bacteria. We again emphasize that in performing this test, certain technical criteria must always be kept in mind. The penicillin sensitivity of *B. anthracis* can be measured either directly on agar plates or by the examination of cell wall production inhibition (LEDERBERG, 1956), by the method suggested by JENSEN and KLEEMEYER (1953).

In some respects our studies have added further data to knowledge about the antigenic structure of *B. anthracis*. We could identify by serological methods the presence of the specific glucosamine-galactose polymer in all the 26 anthrax strains studied. The use of the agar-gel diffusion method has made it possible to identify this substance by separating it from the other antigenic components. Thus we are inclined to suppose that the fact that Ascoli's reaction was positive with only 50 per cent of the strains studied by JENSEN and KLEEMEYER (1953), might have been due to technical error. The apparent serological inhomogeneity of the anthrax strains might have resulted from possible failures either in the extracts prepared or in the immune sera used, or probably in both.

In an earlier study one of us (IVÁNOVICS, 1940) had pointed to the fact that polysaccharides isolated from autolysates of *B. anthracis* might contain certain peptide parts. This supposition was later confirmed by the studies of STRANGE and BELTON (1954) on *in vitro* cultivated, and by those of SMITH and ZWARTOUW (1956) on *in vivo* obtained anthrax bacilli. These authors have found in hydrolysates of their bacterial polysaccharide preparates in addition to the monosaccharides also alanine, aspartic acid, glutamic acid and glycine. Furthermore, SMITH, STRANGE and ZWARTOUW (1956) reported the presence of α-ε-diamino pimelic acid, and STRANGE anf POWELL (1954) described an additional hexosamine not definitely identified.

In our own anthrax polysaccharide preparates we succeeded in demonstrating all the amino acids described above but glycine. No special efforts were made at demonstrating the presence of the unidentified hexosamine.

As to the cell-wall origin of the polysaccharide isolated from filtrates of anthrax cultures, we agree with SMITH, STRANGE and ZWARTOUW (1956). We had stated in an earlier study (IVÁNOVICS, 1940) and were also able to demonstrate in the present work that this hapten was readily precipitable both by horse and rabbit anti-anthrax sera. The differences occasionally encountered may have resulted from either the differences in antibody content of the actual sera or from the peculiarities of the reaction mechanisms characteristic of the two kinds of sera (IVÁNOVICS, 1940). Naturally, none of these differences are specific. Therefore the description of a further polysaccharide in addition to the above galactose-glucosamine polymer by CAVE—BROWN—CAVE, FREY,

EL KHADAM and RYDON (1945) appears to be of certain interest. According to their claims, the second polysaccharide of *B. anthracis* has a mannan character giving a remarkable precipitation reaction with rabbit but none with horse anti-anthrax sera. Nevertheless, it is rather strange that this mannan-type polysaccharide should have been isolated only from unwashed suspensions of bacteria cultivated on media containing yeast extract. Accordingly, contamination of the anthrax extracts by polysaccharides from the medium might have occurred. This is the more probable if we remember that mannan-type polysaccharides are well-known components of the yeast cells. To separate mannan from anthrax cell extracts the above authors made use of baryta. This reagent readily precipitates also the mannan isolated from yeast cells (IVÁNOVICS, unpublished observation). Thus it seem easily explainable that commercial anti-anthrax horse sera failed to precipitate the mannan type polysaccharide isolated by the above authors. On the contrary the rabbit sera prepared in their laboratory might have reacted with mannan because the vaccines used for immunisation of the animals had probably been also produced on media containing yeast extract.

Strains of *B. cereus* are inhomogeneous in respect of their antigenic structure. From some of the strains a substance essentially similar in both chemical and serological respect to the anthrax polysaccharide could be obtained, while from others we isolated an entirely different substance of polysaccharide character. Accordingly, at least two different types of *B. cereus* may exist. No remarkable differences were observed in the virulence of the two types of *B. cereus*. Naturally, as our studies covered a moderate number of strains, further types might also occur.

The similarity of the polysaccharide of a certain type of *B. cereus* to that of anthrax bacillus points to an intimate relation between the two organisms. Nevertheless, in spite of the presence of the same polysaccharide, we must assume important differences in the structure of the cell surface of the two species, even if non-motile *B. cereus* and uncapsulated *B. anthracis* are dealt with. It is known that a species specific phage receptor substance is present on the surface of anthrax bacilli. This is absent or more probably substituted by a receptor of different specificity in *B. cereus*.

As to the phylogenetic relation of the two bacteria, their possible origin from a common ancestor cannot be excluded. This suppositions seems to be valid in spite of the immunological heterogeneity of *B. cereus*. There is already an example on the mutation of the cell-wall in the group of Genus Bacilli namely in connection with *B. megaterium* (IVÁNOVICS, 1955). One may suppose that a similar change might have taken place during the period of evolution from the hypothetical common ancestor. Another possibility of a relation of the two bacillus species might be supposed through a certain type of *B. cereus*. As to the definite way of development, one is still confined to speculation. This is

why we think that all the differences correctly demonstrable in the two organisms must be taken into account. These differences appear to be so characteristic — that according to our actual knowledge on systematology — they should suffice to make the two bacteria accepted as a distinct species each.

Summary

Strains of *B. anthracis*, *B. cereus* and even their variants can be differentiated with certainty, though in the latter case the cell morphology,colony formation and pathogenicity are not satisfactory grounds for such differentiation. The well-developed phosphatase system (lecithin and phenolphthalein-phosphate splitting enzyme and haemolytic activity), together with the penicillinase activity of *B. cereus* make differential diagnosis — under appropriate experimental conditions — fairly accurate. Differences in phage sensitivity of the two bacteria offer an additional possibility for definite differentiation. Regarding these characteristics, the two bacteria are to be looked upon as belonging to two different species and there is no reason to design *B. anthracis* as a variant of *B. cereus*.

The presence of a specific glucosamine-galactose polymer described earlier as a constituent of *B. anthracis* could be established in all our anthrax strains [26] studied. In this respect this species appears to be homogeneous. The same specific substance was found to be present also in some strains of *B. cereus*, i. e. a certain type of this bacterium has a polysaccharide component similar to that of *B. anthracis*. This type of *B. cereus* can be differentiated sharply from those characterized by a polysaccharide of essentially different chemical and serological nature. The latter polysaccharide consists roughly of 1 mol. glucose and 3 mol. of aminosugar. The amino sugars present are glucosamine and probably the "unidentified hexosamine". Also acetic acid (acetyl) and a peptide part contribute to the polysaccharide molecule. In the peptide part the presence of α-ε-diaminopimelic acid, alanine, glutamic acid and aspartic acid could be demonstrated.

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Addendum: A recent statement by M. STERNE and H. PROOM (J. Bact., *74*, 541, 1957 October) is in accordance with the authors' opinion in every respect.

STUDIES ON THE INTERACTION BETWEEN COXSACKIE AND POLIOMYELITIS VIRUSES

II. LATE RESISTANCE TO POLIOMYELITIS OF YOUNG MICE PREVIOUSLY INFECTED WITH COXSACKIE B1 VIRUS

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Interference between the Group Coxsackie B and the poliomyelitis virus under appropriate circumstances has been demonstrated by several authors [1—6]. In an earlier paper [7] we reported our findings on the interference of a Coxsackie B1 strain (C B1) with the Lansing strain of poliomyelitis virus and emphasized that the age of the mice used in the experiments was an important factor. In nine-day-old mice the development of the poliomyelitis infection was inhibited by simultaneous infection with C B1 virus. The survivors of this experiment were challenged with poliomyelitis virus on the 36th day following the first inoculation. Those groups which had been previously infected with C B1 virus or with both viruses, showed a surprisingly greater resistance than the appropriate control groups of normal mice and mice pre-infected with poliomyelitis virus only.

The investigations to be reported below were designed to provide further information on the phenomenon of late resistance ; the aims were (a) to present more quantitative data on late resistance ; (b) to examine the role of the route of inoculation ; (c) to determine the duration of diminished susceptibility ; (d) to test the specificity of late resistance, with special regard to other viruses capable of growing in the CNS of the mouse ; (e) to investigate the role of humoral antibodies.

Materials and methods

Viruses. Details of our C B1 strain and those of the mouse adapted Lansing strain have been given in a previous paper [7]. The tick encephalitis virus strain KEm_1 was isolated by FORNOSI and MOLNÁR [8] in this laboratory. The encephalo-myocarditis virus strain *EMC* was obtained by the courtesy of Professor H. PETTE (Hamburg), while the *WE* strain of LCM virus was kindly supplied by Professor G. IVÁNOVICS (Szeged). The three latter strains have been maintained by intracerebral passage in mice in this laboratory.

Standard viral suspensions. A 20 per cent suspension prepared from torsos of mice infected on the first day of life was used as C B1 standard suspension. Details of its preparation have been published elsewhere [9]. In order to prepare Lansing, KEm_1, LCM and EMC standard suspensions, mice of 14 to 16 g body weight were infected intracerebrally and killed after the characteristic symptoms had developed. The pooled CNS was ground with quartz sand and diluted 1 in 10 with 0.9 per cent saline containing 1000 units of penicillin and 2 mg of streptomycin per ml. The viral suspensions were kept sealed in ampoules in CO_2 ice and titrated before use. The method of titration of C B1 viral suspensions has been described in detail [7]. In the present

paper the term LD_{50} refers to the dose which killed 50 per cent of one-day-old mice when inoculated intracerebrally in 0.02 ml. Lansing, LCM, KEm_1 and EMC viruses were titrated in the same manner as described earlier for the Lansing virus [7].

C Bl immune serum was prepared in mice [9]. In a neutralisation test, serum in a final dilution of 1 in 3000 protected 50 per cent of the one-day-old mice against 100 LD_{50} of C Bl virus. The mice were inoculated subcutaneously with 0.02 ml of the serum-virus mixture.

Mice. Albino mice from the polygamic breeding stock of this Institute were used. The litters were kept under observations from the time of delivery. Only healthy and well-developed 9-day-old sucklings were used in the experiments. Litter-mate animals were equally distributed in the experimental and control groups.

General characterization of the experiments. 9-day-old mice were divided into groups, called T (Test) and S (Standard). Mice in group T were inoculated with 10^5 LD_{50} of C Bl virus in 0.02 ml. Mice in group S were inoculated with 0.02 ml of a suspension prepared from uninfected torsos. Inoculation was intracerebral except in *Experiment (b)*, where it was subcutaneous. Only some of the infected animals showed symptoms (see also [7]) and 17 per cent (150 out of 900) died with symptoms of encephalitis characteristic of C Bl infections. Thirtysix or 60 days later the survivors were challenged. 0.02 ml of the challenge virus was inoculated intracerebrally into the animals of both the T and S groups, in tenfold dilutions. Groups T and S were divided into several subgroups according to the number of different dilutions. The period of observation was different, depending on the challenge virus.

The experiments may be considered as parallel titrations in differently pre-treated animals. However, all groups could not be inoculated on the same day because of shortage of mice of the same age. Nevertheless, the same dilution was always offered on the same day and inoculation was completed within 48 hours. In the intervals the virus was kept in dry ice.

Observation and evaluation. During the period of observation, each mouse was examined every day. Death or any symptom was recorded. Comparison of the groups T and S was based on the following data : death rate ; average incubation period ; average duration of illness ; average survival time ; the relative frequency of the different symptoms. Deaths caused by the trauma of inoculation or by some intercurrent disease were subtracted from the total number of animals. The experimental results were submitted to statistical analysis [10]. Instead of the survival time we calculated with its reciprocal $\left(\frac{1}{t_i}\right)$ as recommended by GARD [11]. The mean of the $\frac{1}{t_i}$ values was calculated according to the formula $\frac{\Sigma 1/t_i}{N}$, where N is the number of animals.

Results

(a) *Degree of late resistance afforded by intracerebral injection of C Bl virus.* Subgroups of groups T and S (see above) were challenged with four different

dilutions of poliomyelitis virus on the 36th day following the first inoculation. Both C B1 and Lansing viruses were given intracerebrally. Period of observation, 30 days.

Table I

Results of Experiment (a)

A) Poliomyelitis mortality

Designation of groups	Log. dil. of Lansing virus	No. of mice	Poliomyelitis deaths		Relative potency	Fiducial limits
			No.	per cent		
S	—4	25	15	60	100	
	—3	24	19	79		
	—2	21	21	100		
	—1	23	23	100		
T	—4	26	4	15	5.0	0.7—18.5
	—3	23	12	52		
	—2	21	16	76		
	—1	20	17	85		

B) Survival time

Designation of groups	Log. dil. of Lansing virus	No. of mice	Mean survival (days)	Reciprocal of survival times (days)			Relative potency	Fiducial limits
				Sum ($\Sigma\, 1/t_i = y_i$)	Sum of quadrates ($\Sigma\, y^2$)	Mean ($\bar{y}_i$)		
S	—4	25	14.9	1.1043	0.08905	0.0442	100	
	—3	24	12.0	1.7154	0.17208	0.0714		
	—2	21	9.5	2.3983	0.30378	0.1142		
	—1	23	7.8	3 1211	0.44425	0.1357		
T	—4	26	20.0	0.2036	0.01053	0.0078	3.7	0.9—11.0
	—3	23	19.1	0.8806	0.10445	0.0382		
	—2	21	14.1	1.2920	0.11785	0.0615		
	—1	20	11.2	1.7632	0.21287	0.0882		

The results are given in Table I. Each dilution of the challenge virus induced remarkably lower death rates and longer survival times in group T than in the appropriate control subgroup. Moreover, the death rate was slightly lower and the average survival time was slightly longer in each subgroup of group T than in the subgroup of the control mice which had received tentimes more diluted challenge virus.

The dose-response curves calculated either from the death rates by the probit analysis, or from the reciprocals of the survival times by regression analysis,

proved to be linear (Fig. 1). Both kinds of analysis revealed significant differences between the responses of groups T and S. The lethal effect of Lansing virus in group T has been calculated as 5 per cent of that in group S, while in terms of the reciprocals of the survival times the percentage was 3.7.

Prolongation of the survival time can almost completely be explained by the longer incubation period. The average duration of illness was only a few

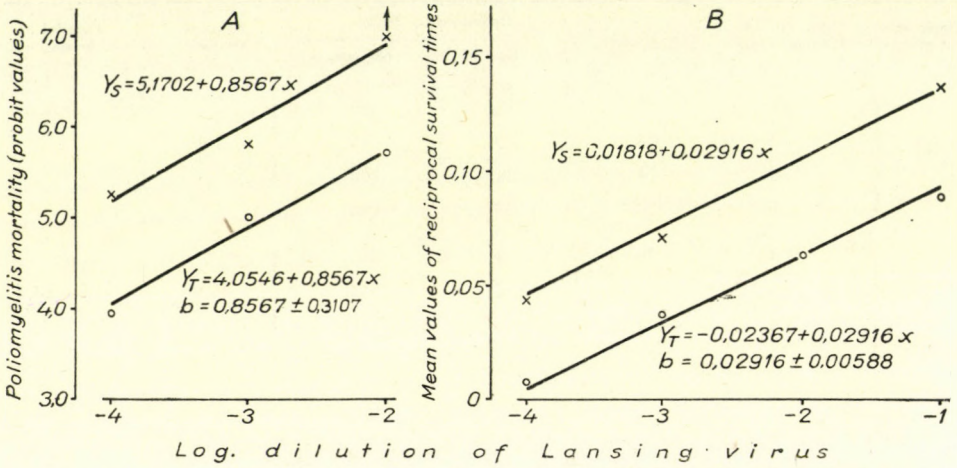

Fig. 1. Evaluation of Experiment (*a*). *A :* Regression-lines according to poliomyelitic mortality. *B :* Regresson-lines according to reciprocal survival times

Explanations : + Values of S groups
○ Values of T groups

hours longer in group T than in the corresponding subgroups of group S. In both groups the majority of the ill animals died within 48 hours following the first appearance of symptoms. In group T, however, death occurred exceptionally as late as on the 5th or 6th day of illness. The incidence of different symptoms was about the same in both groups.

In conclusion, it may be stated that mice which had been infected with C B1 virus by the intracerebral route at 9-days of age, 36 days later proved to be about 20 times more resistant to the Lansing strain of poliomyelitis virus than the appropriate controls.

(b) *Late resistance following subcutaneous injection of C B1 virus.* C B1 virus was administered subcutaneously and only 3 dilutions of poliomyelitis virus were tested. Otherwise, this experiment was the same as *Experiment* (*a*).

The results obtained and presented in Table II and Fig. 2 are very similar to those of the intracerebral experiment. Both the linearity of the relations and the significance of the differences were clear when calculated in the same manner. In group T the relative potency of Lansing infection was 0.7 and 0.9 per cent of that in group S, according to death rates and reciprocals of the survival

Table II

Results of Experiment (b)

A) Poliomyelitis mortality

Designation of groups	Log. dil. of Lansing virus	No. of mice	Poliomyelitis deaths		Relative potency	Fiducial limits
			No.	per cent		
S	—4	30	19	63	100	
	—3	37	30	81		
	—2	46	46	100		
T	—4	17	2	12	0.7	0.04—3.7
	—3	27	13	48		
	—2	38	20	53		

B) Survival time

Designation of groups	Log. dil. of Lansing virus	No. of mice	Mean survival (days)	Reciprocal of survival times (days)			Relative potency	Fiducial limits
				Sum ($\Sigma 1/t_i = y_i$)	Sum of quadrates (Σy_i^2)	Mean ($\bar{y}_i$)		
S	—4	30	17.2	1.3188	0.11088	0.0434	100	
	—3	37	12.3	2.7344	0.28363	0.0739		
	—2	46	10.0	4.9388	0.56600	0.1074		
T	—4	17	29.5	0.0690	0.00238	0.0040	0.9	0.2—3.1
	—3	27	19.8	0.6975	0.03816	0.0255		
	—2	38	14.0	1.5811	0.13609	0.0416		

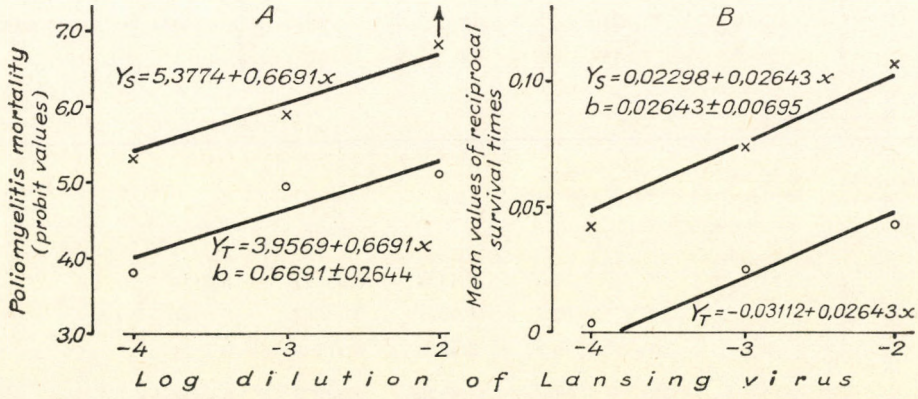

Fig. 2. Evaluation of Experiment (*b*). *A*: Regression-lines according to poliomyelitic mortality. *B*: Regression-lines according to reciprocal survival times

Explanations: × Values of S groups
○ Values of T groups

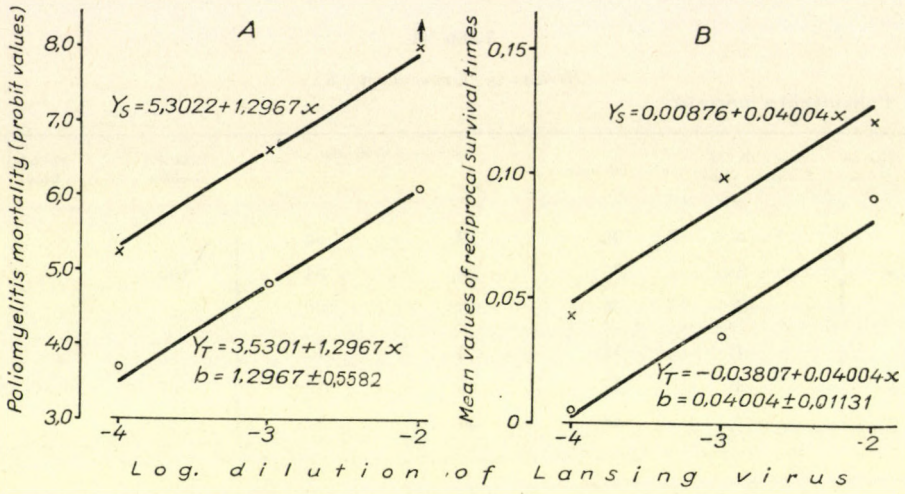

Fig. 3. Evaluation of Experiment (c). *A :* Regression-lines according to poliomyelitic mortality
B : Regression-lines according to reciprocal survival times
Explanations : × Values of S groups
○ Values of T groups

Table III

Results of Experiment (c)

A) Poliomyelitis mortality

Designation of groups	Log. dil. of Lansing virus	No. of mice	Poliomyelitis deaths		Relative potency	Fiducial limits
			No.	per cent		
S	—4	10	6	60	100	
	—3	20	19	95		
	—2	20	20	100		
T	—4	11	1	9	4.3	0.5—15.3
	—3	24	10	42		
	—2	15	13	87		

B) Survival time

Designation of groups	Log. dil. of Lansing virus	No. of mice	Mean survival (days)	Reciprocal of survival times (days)			Relative potency	Fiducial limits
				Sum ($\Sigma\, 1/t_i = y_i$)	Sum of quadrates ($\Sigma\, y_i^2$)	Mean ($\bar{y}_i$)		
S	—4	10	14.2	0.4380	0.03321	0.0438	100	
	—3	20	10.7	1.9815	0.23504	0.0991		
	—2	20	8.9	2.4223	0.31768	0.1211		
T	—4	11	19.0	0.0526	0.00277	0.0048	6.8	1.3—20.2
	—3	24	13.1	0.8413	0.07880	0.0351		
	—2	15	10.6	1.3668	0.15840	0.0911		

times, respectively. Thus, protection against poliomyelitis virus was 4 to 7 times greater after subcutaneous infection than after intracerebral. However, the difference is not statistically significant.

(c) *Duration of the resistance.* In these experiments mice were challenged with poliomyelitis virus on the 60th day following the intracerebral inoculation with C B1 virus or with control material. Period of observation, 30 days.

Two, similar experiments were performed but less animals were used in Experiment I than in Experiment II. Table III and Fig. 3 show the results of Experiment I.

The difference between the resistances of group T and S was significant and nearly equalled those in 36 day experiments. The relative potency of Lansing infection in the group T was 4.3 and 6.8 per cent, respectively.

The results of Experiment II were very similar to those of Experiment I. Nevertheless, the data of Experiment II were not suitable for statistical analysis. The two mortality curves do not run parallel and the survival times were not unequivocally longer in the subgroups of group T than in the corresponding control groups. It should be mentioned that on the fifth day following infection with Lansing virus the ventilation of the animals was stopped for 12 hours because of a technical defect. The warm and damp air killed a great number of mice, especially in the group which had received the 10^{-3} dilution of the Lansing virus. Later the irregularity in the survival times in the same group was most striking. Some reports [12] suggest that sudden bad conditions could be responsible for the altered susceptibility of the survivors to the poliomyelitis virus.

The results of Experiment II support the conclusion which we could be drawn from the results of Experiment I alone, that the increased resistance to poliomyelitis virus would persist at least until the 60th day following the infection by C B1 virus. Moreover, according to the results of Experiment I this resistance does not seem to diminish between the 36th and the 60th days.

(d) *Late resistance to other viruses capable of growing in the CNS of the mouse.* Group T and S of mice were challenged with tenfold dilutions of tick encephalitis, LCM and EMC viruses by the intracerebral route following the first inoculation. Animals inoculated with LCM or tick encephalitis virus were kept under observation for 20 days, those with EMC virus only for 10 days. Each mouse was examined individually twice a day.

Mice in group T did not show diminished sensitivity to tick encephalitis virus. On the contrary, the death rates were slightly higher than in the appropiate control groups. The average incubation periods were approximately the same (Table IV).

Some difference between the responses of the two groups to EMC infection is apparent in Table V. Death rates are lower and average survival times are longer in group T. Although our experimental data are not sufficient to demonstrate the statistical significance, the very regular differences in the survival times may indicate a slight late protection against EMC virus by a previous C B1 infection. This slight protection is much less than that observed in the above experiments against poliomyelitis virus.

Table IV

Results of the challenge with KEm_1 strain of tick encephalitis virus

Designation of groups	Log. dil. of KEm_1 virus	No. of mice	Specific deaths		Mean survival (days)
			No.	per cent	
S	—5	28	1	4	9.0
	—4	12	4	33	6.5
T	—5	20	2	10	9.0
	—4	13	10	77	7.1

Table V

Results of the challenge with EMC virus strain

Designation of groups	Log. dil. of EMC virus	No. of mice	Specific deaths		Mean survival (hours)
			No.	per cent	
S	—7	52	26	50	94.6
	—6	21	21	100	70,8
	—5	30	30	100	58.0
T	—7	58	17	29	110.0
	—6	25	24	96	88.0
	—5	34	34	100	73.8

According to the data in Table VI, challenge of groups T and S with LCM virus resulted only in moderate differences both in death rates and average survival times. In a later experiment, therefore, we challenged about 80 mice in each of groups T and S with a 10^{-4} dilution of the virus. In this experiment

Table VI

Results of the challenge with "WE" strain of LCM virus

Designation of groups	Log. dil. of "WE" strain	No. of mice	Specific deaths		Mean survival (days)
			No.	per cent	
S	—5	22	1	5	—
	—4	14	8	57	9.9
	—3	12	12	100	8.4
T	—5	14	0	0	—
	—4	11	3	27	10.0
	—3	15	15	100	9.7
S	—4	79	33	42	8.6
T	—4	83	9	11	8 0

we were able to demonstrate a significant difference between the death rates ($P < 0.1$ per cent) in the group T and group S. Thus, infection of mice at 9 days of age with C B1 virus resulted in a late resistance to LCM virus, too. However, this resistance is significantly weaker than that to the poliomyelitis virus.

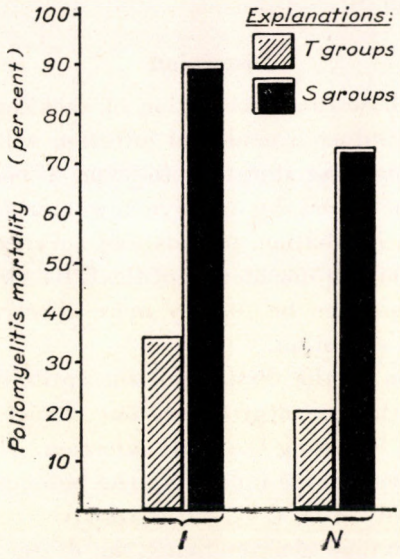

Fig. 4. Results of Experiment (e)
I: Groups treated with C Bl immune serum.
N: Groups treated with normal serum

(e) *Experiment on the role of specific humoral immunity in the development of the late resistance.* A hundred mice of nine days of age were equally distributed in 4 groups. Groups T_i and T_N were inoculated with C B1 virus, groups S_i and S_N with normal torso suspension, by the intracerebral route. Thirtysix days later a Lansing virus suspension containing 1000 LD_{50} per ml was mixed with the same volume of pooled C B1 hyperimmune serum (v. Materials and methods) (Mixture I). Another sample of the same virus suspension was mixed similarly with a pool of normal mouse serum (Mixture N). Both mixtures were kept at $+4°$ C for an hour. Then groups T_i and S_i were inoculated with Mixture I, groups T_N and S_N with Mixture N, intracerebrally. The individual dose was 0.02 ml which contained 10 LD_{50} of virus. Moreover, on the same day and on the following two days groups T_i and S_i received daily individual doses of 0.25 ml of a 1 : 10 dilution of hyperimmune serum intraperitoneally. Groups S_N and T_N were given the same doses of pooled normal mouse serum by the same route. The mice were kept under observation for 30 days.

As shown in Fig. 4, the death rates in the groups T_i and S_i were even slightly higher than in the control groups. Thus neither virus neutralization nor passive immunity was produced with a serum containing high level C B1

antibodies ; *i.e.* neither specific nor non-specific humoral immunity is responsible for late resistance.

The usual difference between the responses of groups T and S was apparent in this experiment also. Its value was approximately the same in the relation $S_i - T_i$ as in the relation $S_N - T_N$.

Discussion

Our experiments show that inoculation of suckling mice with a dose of C B1 virus sufficient to induce a nonlethal infection will produce an enhanced resistance of considerable long duration to type 2 poliomyelitis virus. The greater resistance, characterized by relative low morbidity and death rates, as well as by prolonged incubation periods and survival times, was regularly observed whatever the route of inoculation of the C B1 virus. In our experiments the peripheral route seemed to be slightly more effective than the direct injection of the virus into the brain.

The whole duration of the diminished susceptibility is so far unknown. However, it is obvious that its degree does not change significantly between the 36th and 60th days following the C B1 infection. The extent of resistance may be expressed as a percentage indicating the potency of the Lansing virus in mice infected previously with C B1 virus relative to its potency in control mice. The average percentage characterizing the degree of late resistance obtained in *Experiments (a), (b)* and *(c)* was 3.6. The similar average value obtained from earlier experiments, when the two viruses had been given simultaneously, was 12 per cent [7] ; *i.e.* the early resistance which was clearly explanable by the interference phenomenon was about three times weaker than the late resistance we have described in the present paper.

Is the late resistance an interference phenomenon? Our experimental data are sufficient to exclude the role of humoral immunity as well as that of a non-specific defence mechanism acting generally against neurotropic viruses. Although some late resistance to EMC and LCM viruses could be observed, no decrease but a statistically non-significant increase of susceptibility was found in mice which had been challenged with the Hungarian tick encephalitis virus on the 36th day following the infection by C B1 virus. The late resistance might be supposed, therefore, to have some kind of "specificity". Other workers [13—24] have shown that under appropriate circumstances LCM and EMC viruses could give rise to interference with poliomyelitis virus and *vice versa*. We were not able to find similar data in connection with the tick encephalitis virus. It is reasonable, therefore, to suppose some parallelism between the specificity of the interference phenomenon and that of the late resistance. This supposition might be supported by our finding that we could regularly observe both interference and late resistance to poliomyelitis virus in mice which had been inoculated with C B1 virus at 9 days of age, while inoculation of 21-day-old

mice was never followed by either phenomenon. The explanation of the late resistance as interference would be clear if the persistence of the C B1 virus for 60 days could be demonstrated. Long persistence of active virus, as demonstrated with a few viruses [25—34], is in this case very improbable because all our efforts to recover the virus from the CNS or other organs of the mice 36 or 60 days following the infection have been unsuccessful. As a further argument against the role of active virus, there was no decrease in the resistance of those mice which had been injected both intraperitoneally and intracerebrally with anti-C B1 serum by the time of the development of the poliomyelitis infection. We suppose that in presence of active C B1 virus *in vivo* neutralization would be occurred and some modification of the late resistance ought to have been observed.

More recent reports [35—39] have drawn our attention to the possible role of masked virus. Although provirus corresponding to prophage has not been proved to exist [40], there is no reason to exclude the intracellular persistence of the C B1 virus in our experimental animals.

As a further possible explanation of the late resistance, the infection with C B1 virus might be supposed to induce some persistent changes in the cell wall or in the metabolism of the susceptible cells. These changes unrelated to the presence of the virus might prevent infection with related viruses. The role of local virus inhibitors [41—43] produced on initiation by C B1 virus may neither be excluded.

Similar late resistance to some neurotropic viruses has been observed by several authors. THEILER [25, 44] was able to demonstrate a late cross-resistance of undetermined degree between the mouse encephalitis virus and the mouse adapted poliomyelitis virus. RHODES and CHAPMAN[24] revealed greater resistance to the strain *MM* (Group EMC) of hamsters which had been infected with LCM virus 30 days previously. BAUTISTA, JUNGEBLUT and KODZA [45] demonstrated a late resistance in hamsters between the strain *Col SK* of the Group EMC and the strain MEF_1 of poliomyelitis virus. The earlier observation by DALLDORF and WHITNEY [46] of the late resistance to the strain Lansing, of mice previously infected with the strain *MM* was considered by the authors to be an immune protection based on the hypothetical antigenic relation between the two viruses.

The mechanism of the late resistance we have shown obviously requires further investigations. More accurate analysis of this phenomenon may provide information even about host-virus relationship.

Summary

1. An enhanced resistance of long duration to the Lansing strain of poliomyelitis virus has been demonstrated in mice which had been infected at 9 days of age with Coxsackie B1 virus.

2. Mice previously infected with Coxsackie B1 virus were about 28 times less sensitive to poliomyelitis virus 36 to 60 days after infection than control mice. Sensitivity was judged by death rates due to poliomyelitis virus and by survival times.

3. The route of infection with Coxsackie B1 virus has little or no influence on the development of the late resistance.

4. Humoral antibodies play no part in the late resistance. Possible explanation have been discussed on the basis of interference and other possibilities.

Acknowledgements. The author is greatly indebted to DR. G. BARSY for his advices in the statistical evaluation and to DR. E. FARKAS for his encouragements and help.

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STUDY ON THE CYCLIC MULTIPLICATION OF INFLUENZA AND ND VIRUSES IN TISSUE CULTURES

By

S. HORVÁTH

*Institute of Microbiology, University Medical School, Szeged.**

(Received May 23, 1957)

The cyclic multiplication of influenza and related viruses has been extensively studied [1—13]. Thus far, embryonated and de-embryonated eggs were used in the investigations, and also ultraviolet irradiated heterologous virus, this being the only possible means to observe one step growth.

In the present work we deviated from the common practice and followed up the growth of virus in minced chorioallantoic membranes, using a titration method of sufficient accuracy developed by us. In studies on the growth of the influenza virus the tissue culture offers better conditions than the embryonated or de-embryonated egg. For this reason, we attempted to confirm the step growth of influenza virus without the use of U. V. irradiated heterologous virus.

Materials and methods

1. Viral infectivity has been determined by a method developed earlier, involving the use of rolling drums, which has been slightly modified recently [14, 15]. The nutrient fluid used in the titrations and in the experiments was a glycosol solution containing 0.2 per cent egg white [14]. In the tests for infectivity titre there were 5 parallel tubes for each dilution.

2. The haemagglutination titrations (HA titrations) were carried out essentially by the method of TAKÁTSY [17, 18], as modified by us [19].

3. The influenza virus strains PR8 and Weiss were used. Strain PR8 was passaged not only in eggs, but also in minced chorioallantoic membranes. The tissue culture passages were made in minced chorioallantoic membranes in 2 ml nutrient fluid, using 0.1 ml 10^{-3}-diluted viral suspension as the inoculum.

The ND virus was the strain designated ND_{26}, isolated in Hungary. The virus was maintained by passaging in eggs.

The virus-bearing fluids obtained in the course of the egg and tissue culture passages were used in the experiment immediately after harvesting and pooling in every case.

Preliminary experiments

In studies on the adsorptive and elutive properties of viruses the HA titration procedures were mostly used. We, however, employed the virus infectivity test in similar studies, as in this way even minute amounts of virus could be demonstrated. Before setting up the experiments on the growth of virus we examined the conditions of inactivation and tested the viruses for adsorption and elution.

1. *Inactivation of PR8 virus passaged in ovo, at 37° C, and at 4° C.* These temperatures were selected because these occurred most frequently in subsequent experiments. At 37° C we made the virus-growth experiments, whereas at + 4° C we stored the virus-bearing

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fluids before use. The experiments were made in a physiological NaCl solution containing 0.2 per cent egg white, as well as in physiological NaCl (a solution not containing egg white). Inactivation resulting under different conditions is shown in Fig. 1. The 0.2 per cent egg white in the nutrient fluid had a very marked protective effect on the virus.

2. *Adsorption-elution studies on the basis of the measurement of viral infectivity.* From the results of the adsorption and elution studies made on erythrocytes and chorioallantoic membranes by means of the HA titration procedure [6, 21] we drew the conclusion that a receptor of a certain unit surface absorbs from different amounts of virus within a given time equal percentage of virus. Of course, with the change in the quantity of the receptor the percentage amount of absorbed virus, too, will change in the same sense.

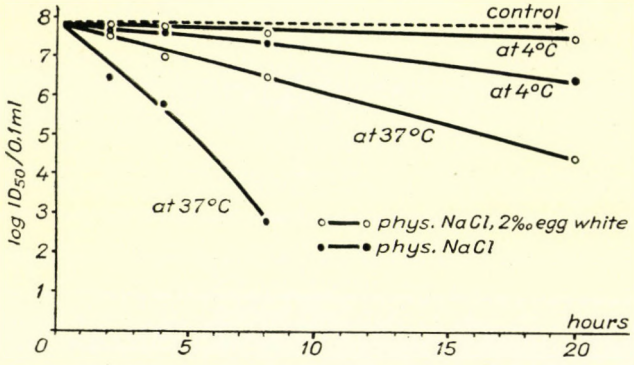

Fig. 1. Inactivation of influenza PR8 virus under different conditions

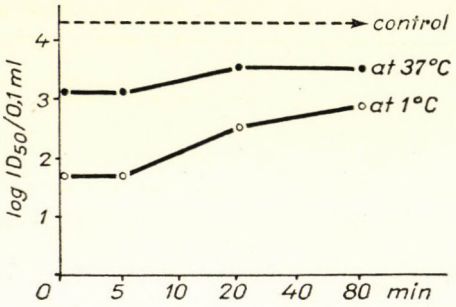

Fig. 2. Elution at different temperatures of influenza PR8 virus adsorbed onto chorioallantoic membrane

In the preliminary experiments we investigated the adsorption to, and the elution from, the chorioallantoic membrane by determining the infective titre of the virus.

PR8 virus was alloved to adsorb onto chorioallantoic membranes from 13-day embryonated eggs, then the membranes were washed 5 times in succession with physiological saline, using 8 ml with each washing. One group of the membranes was then placed into 2 ml 37° C, and the other group into 2 ml 1° C, glycosol solution containing egg-white, testing from time to time the supernatant for infectivity. The results are presented in Fig. 2.

It is remarkable that after washing 5 times a certain amount of virus was instantly eluted from the membranes, even at 1° C and that at both temperatures elution progressed to an equilibrium at about the same rate. It would be difficult to establish the measure of fermentative, in contradistinction to non-fermentative, elution in the experiment.

Next we investigated how we could remove by washing the viruses adsorbed onto the surface of the membranes. The membranes were placed for 2 minutes into a fluid containing $10^{6.97}$ $ID_{50}/0$ 1 ml of PR8 virus passaged *in ovo*. This was followed by washing 15 times in succession, partly with 37° C, partly with 5° C physiological saline containing 0.2 per cent egg

white, in volumes of 5 ml. The washing fluids contained virus in decreasing amounts. In the experiment made at 37° C the second washing fluid contained $10^{3.60}ID_{50}/0.1$ ml of virus, and the fifteenth $10^{2.50}ID_{50}/0.1$ ml. Similar results were obtained at 5° C, except that at that temperature the virus titres were by an average of 0.4 to 0.6 log. values lower. It thus seems that virus adsorbed onto the surface of membranes cannot be removed therefrom by washing.

Keeping the chorioallantoic membranes under other experimental conditions for prolonged periods of time, in fluid containing virus caused no substancial difference either in the changes in the infectivity of the supernatant, or in the amounts of virus found in the membrane extracts. We could not recover from the membrane extracts the amount of virus that these had presumably taken up in the course of adsorption [20].

The observations reported above demonstrate clearly how complex the process of adsorption and elution is, particularly when the experiments are made on living tissues and the quantitative changes of virus are determined on the basis of infectivity and not by means of the HA test.

Experimental

Making use of the results obtained in the above-described preliminary experiments, attempts were made to register the growth of virus in the chorioallantoic membrane. Only the infective and HA properties have been taken into consideration, although agglutination was rarely observable in the course of the experiments owing to the smallness of inocula and the short duration of the experiments.

1. *Changes in the quantity of virus in the membrane and in the culture fluid.* Finely minced chorioallantoic membranes were obtained from 13-day embryonated eggs and a suspension was prepared in the manner described in the case of infectivity titrations on influenza virus [14]. After washing off the excess PR8 virus from the membranes, the membrane suspension was continued to

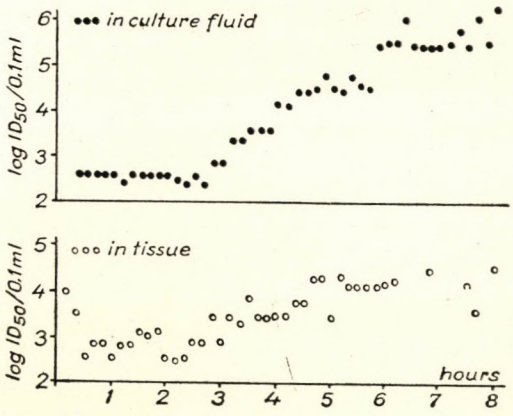

Fig. 3. Quantitative change of the infectivity of PR8 virus passaged *in ovo* in the supernatant and in the membrane extract in the course of cultivation

be cultured in separate sealed dishes, in an apparatus shaking at a rate of 100/minute in a water bath, 37 ± 0.2° C in temperature. The tests for virus content made from time to time on the supernatant and membrane extracts yielded the following results (Fig. 3).

It is conspicuous in Fig. 3 that the titration results for the supernatant show some regularity, whereas this cannot be stated concerning the membrane extracts.

2. *Study on the growth of PR8 virus, passaged in ovo and in tissue cultures, respectively.* As experiments carried out as described above, yielded no reliable information as to the growth of virus in the cells, in the subsequent series we measured only the amount of virus in the nutrient fluid. To facilitate demonstration of the changes occurring in the course of viral growth, the nutrient fluid was titrated for virus in the course of viral growth in the same dish. At first, only PR8 virus adapted to chorioallantoic membrane was studied, as it was thought that in this way we might obtain more precise growth curves.

The experiments of this type, which then yielded reliable results, were made as follows. From the sediment of the washed, minced and centrifuged membrane 0.5 ml was measured into a 50 ml Erlenmeyer flask, then 4 ml of nutrient fluid and diluted viral suspension in 0.5 ml nutrient fluid were added. After 2 minutes of adsorption the excess fluid was decanted, the membranes were washed 5 times with 25 ml physiological NaCl and finally 4.5 ml of nutrient fluid was added. Adsorption and washing required about 5 to 6 minutes, because with the washing no centrifuging was necessary, owing to the fast rate of settling of the membranes. After stoppering the flask, the tissue-virus suspension was placed into the shaker on the 37° C water bath. At intervals, the suspension was sampled by means of a 0.05 ml spiral loop, the samples were diluted tenfold in 0.45 ml of nutrient fluid without delay and kept at 4° C until titrated. The results obtained by this procedure were superior to those obtained by the former method.

In one case we carried out an experiment with the 72th passage of PR8 virus adapted to chorioallantoic membrane. Of a tenfold dilution of a viral suspension with a titre of $10^{6.62}$ ID_{50}/0.1 ml were added 0.5 ml to 4.5 ml of membrane suspension and the experiment was made according to the above principles. The results are shown in Fig. 4.

Fig. 4 shows that the virus began to grow 3 hours after inoculation, the viral titre increased for about 90 minutes, followed by a constant period, also about 90 minutes in duration. After about 6 hours another phase of multiplication began. During the first 3 hours no substantial change occurred in the virus content of the nutrient fluid and after growth one step was found. We have reproduced the experiment 4 times, with identical results.

Under the same experimental conditions we examined also the growth of PR8 virus passaged *in ovo*. 0.5 ml of a 100-folds dilution of allantoic fluid (titre, $10^{7.62}$ ID_{50}/0.1 ml) was added to 4.5 ml of membrane suspension. The results are shown in Fig. 5.

The result roughly corresponds to the value in Fig. 4. The constant period after the phase of growth was somewhat shorter in duration than with tissue-passaged PR8 influenza virus.

In the subsequent experiments we used tissue-passaged virus as the inoculum and it was examined in what way the viral titre changed in the nutrient fluid following inoculation of different amounts of virus. In these experiments 0.5 ml volumes of viral suspensions with titres of $10^{3.83}$ ID_{50}/0.1 ml and $10^{6.62}$

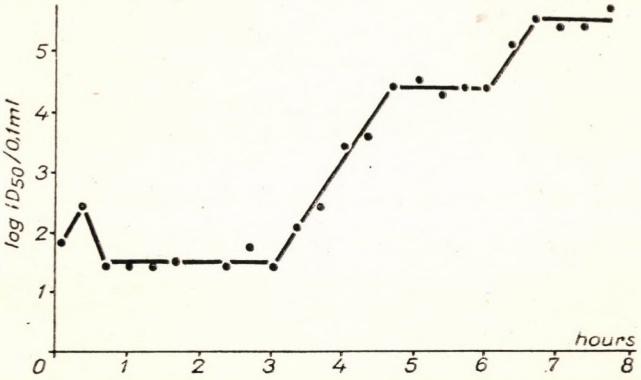

Fig. 4. Growth curve of influenza PR8 virus from the 72th tissue culture passage in tissue culture

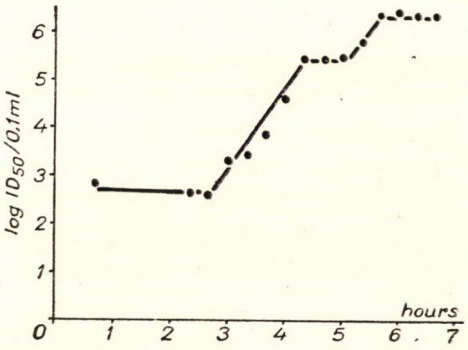

Fig. 5. Growth curve of egg-passaged influenza PR8 virus in tissue culture

ID_{50}/0.1 ml, respectively, were added to 4.5 ml nutrient fluid containing membranes. In this case the excess virus was not washed off from the membranes, but the growth of virus was started immediately by placing the mixtures into the shaker in the 37° C water bath. The results are presented in Fig. 6, in which the inocula show the quantities of virus contained in 0.5 ml.

The two growth curves shown in Fig. 6 do not run parallel and their deviations cannot be ascribed to eventual errors. In the cultures inoculated with greater amounts of virus growth began much sooner and the curve was less steep than in the case of smaller inocula. In both cases the lag phase was followed by a second cycle, occurring at about the same time.

3. *Growth of the influenza virus strain Weiss.* From a 100-fold dilution of the allantoic fluid with a titre of $10^{6.22}$ ID_{50}/0.1 ml, was added 0.5 ml to 4.5 ml

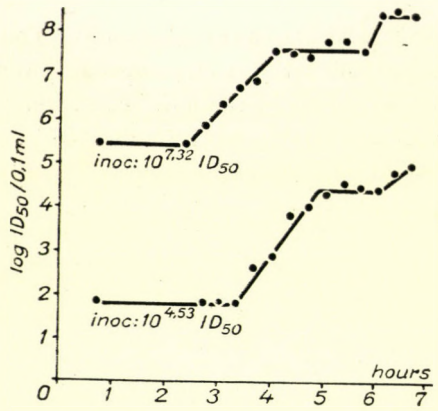

Fig. 6. Growth curves in tissue culture of influenza PR8 virus from the 90th tissue culture passage. (After inoculation the membranes were not washed with physiological saline)

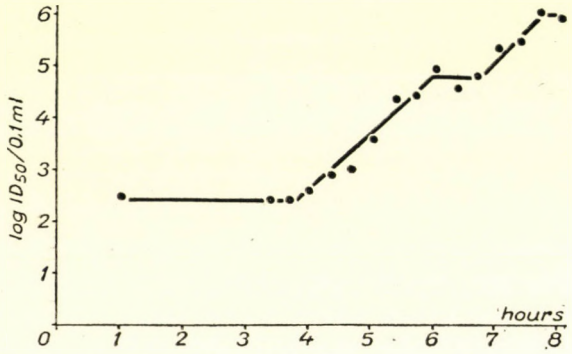

Fig. 7. Growth curve of influenza Weiss strain in tissue culture

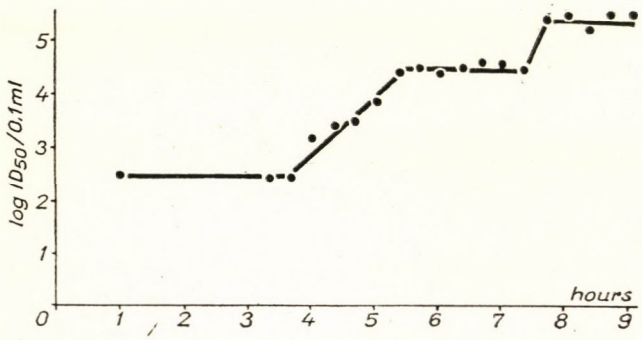

Fig. 8. Growth curve of strain ND_{26} in tissue culture

of membrane suspension. The results obtained after the usual washing and incubation are shown in Fig. 7.

According to Fig. 7, the virus begins to be released from the membrane in somewhat less than 4 hours. After an increase in titre for about 90 minutes

a constant period about 2 hours in duration begins. The second cycle starts after about 7½ hours to be soon followed by another constant period.

4. *Growth of ND virus in chorioallantoic membrane.* The virus used was the strain designated ND_{26}. 0.5 ml of a 10-fold dilution prepared from allantoic fluid with a titre of $10^{7 \cdot 68}$ ID_{50}/0.1 ml was added to 4.5 ml of membrane suspension. After the usual post-adsorption washing we obtained the growth curve shown in Fig. 8.

In somewhat less than 4 hours the virus escapes from the cells. The titre of the nutrient fluid then increases for about 2 hours. The short constant period that follows continues in the second growth cycle, beginning at about the 7th hour.

Discussion

The growth curves presented above may be considered the resultants of complex processes of which it would be extremely difficult to determine the single details. We do not know precisely the details of adsorption and elution, the inactivation of virus caused by heat and other agents, the elution of virus from the cells, etc.

The method employed for determining the infectivity titre of the virus has made it possible to carry out large numbers of titrations simultaneously, against suitable parallels, in a comparatively simple way. Thus, the amounts of virus contained in the nutrient fluid could be controlled at frequent intervals. In the early phase of the experiments we examined the growth of virus at 5 to 10 minute intervals, but later it became obvious that sampling every 15—20 minutes was sufficient. In the literature [1, 2, 9, 10, 11] intervals as long as 1, 2 or 4 hours are mentioned ; this must have been the reason that no step-like growth could be demonstrated without the use of U. V. irradiated heterologous virus. A further explanation for the difference between our results and those reported by other investigators is that we have used tissue cultures, whereas others used embryonated or de-embryonated eggs in the experiments. In the first, so-called lag phase of growth the titre of the supernatant showed no substantial changes within the limits of error, as it has been reported also by other investigators [1, 2, 10, 13]. According to HORSFALL [12], however, the infective titre of the supernatant decreases during the lag phase and in the absence of cells 42 per cent of the virus is inactivated in 2 hours and 60 per cent in 4 hours. In the light of these results it is difficult to find a satisfactory explanation for the closely comparable titration values we have obtained during the lag phase, although these do not yet contradict HORSFALL's statements.

Opinions vary as to the duration of the lag phase of the influenza virus PR8. It has been reported to last 4 to 6 hours [1, 8, 11, 12, 13] ; in our experiments it was about 3 hours. The differences of the methods employed might explain the different results. This is why in our experiments the second cycle

began much earlier and lasted for about 6 to 8 hours according to the virus tested.

The second cycle has been reported to be shorter [9] than the first one. We can confirm this view. However, we have found no such great differences as have been found by FAZEKAS DE ST. GROTH and CAIRNS [9] (cycle 1, 8½ hours ; cycle 2, 4½ to 7 hours).

According to HENLE *et al.* [1], the duration of the lag phase is not at all influenced by the size of the inoculum. On the other hand, we found the duration of the lag phase to be the function of the inoculum. For example, between the two inocula shown in Fig. 6 there is an about 600-fold difference, the lag phases differ by about 1 hour in duration and the growth curves are not parallel. The differences are readily explained by considering the heterogeneity of the particles contained in a viral suspension. Thus, even when greater amounts of virus are inoculated, extreme deviations may occur. In the case of big inocula particles greatly differing in the duration of the cycle may reach the cell surface, making the cyclic nature of growth slightly indistinct.

We have computed on the basis of the experiment shown in Fig. 6 (calculating according to W. HENLE *et al* [1]) the number of viral particles that may be formed from one viral particle in the case of the two inocula used. We have chosen this experiment, because in it the virus was not washed out of the membranes after inoculation. Not only the values shown in Fig. 6 were used in the computations, but we calculated also with the potential limits of error (± 0.2 $\log_{10}$). Computing in the case of the smaller inoculum with the values shown in Fig. 6 we have found a 51-fold growth after the first cycle. Taking into account the extreme possibilities, growth was computed to vary from 19 to 153-fold. In the case of the bigger inoculum a 240-fold growth was computed, with the extreme values ranging from 50 to 600-fold.

Such computations apparently yield more reliable values when small amounts of virus are inoculated and marked deviations occur with the use of massive inocula.

For the time being, such calculations are difficult to make, since the circumstances of adsorption, elution, etc., are too little known to allow computation by a more accurate method the number of viral particles formed from one particle during a single growth cycle.

Summary

Two influenza strains and one strain of ND virus have been studied for adsorption and elution. In model experiments we registered the cyclic growth of viruses without the use of U.V. irradiated heterologous virus, in tissue cultures. Growth of the viruses tested began 3 to 4 hours after the lag phase. After the first cycle the logarithmic multiplication of virus lasted 1½ or 2 hours. This was followed by a shorter constant period. Then began the second cycle, which was somewhat shorter in duration than the first one.

Acknowledgement: Author is indebted to DR. E. NAGY for passaging the PR8 virus in tissue culture.

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ISOSEROLOGICAL STUDY OF AN ANTI-0 AGGLUTININ CAUSING HAEMOLYTIC COMPLICATION IN TRANSFUSION

By

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The anti-0 agglutinins present in the blood of recipients belonging to groups A_1 and A_1B usually do not cause grave haemolytic complication even when subgroup-incompatible or 0-group blood is transfused. All they may cause is an occassional mild reaction.

This behaviour can be explained by the low temperature (0 to 4° C) at which these agglutinins are active ; at body temperature they are inactive.

As to their origin, they can be considered to be "natural agglutinins", because in the individuals in whom they are present no antigenic stimulus starting an immunisation process is usually demonstrable. Neither have these antibodies serological properties indicative of an immune type. At a given temperature the agglutinins are not more active in media containing albumin (their titre is not higher), the heat amplitude is narrow and is restricted to low temperatures, their titres are usually low, they have no haemolytic activity and their indirect *Coombs* test is negative.

As far as their specificity is concerned, the natural agglutinins react most intensely with 0-group cells, but act on the A_2-group cells as well, whereas their reactions with the A_2B, A_1, B and A_1B cells are very weak or totally absent.

MORGAN and WATKINS [1] distinguished between two types of anti-0 sera. The anti-0 sera inhibitable with saliva from secretors belonging to any group are designated sera of anti-H specificity, and those not showing this inhibition have been termed sera of anti-0 specificity.

SANGER [2] studied human anti-H and anti-0 sera and found a close correlation between these two types and the Lewis blood group of the donors supplying them. All the donors supplying anti-H sera were Le(a+), whereas those supplying anti-0 were, with one exception, Le(a—). The correlation between anti-H and Le(a+) showed a very high statistical significance.

The reactions of anti-H, either human or animal, are not in harmony with the AB0 genotype, whereas the anti-0, in the strict sense of the word, that has been found in human sera only, shows a certain relationship to it. BOORMAN, DODD and GILBEY [3] require from the latter group to react exclusively with erythrocytes containing either 0, or A_2, or both antigens. However, no such

anti-0 serum has been found and thus it seems that the reactions of the anti-0 and anti-H facilitate quantitative, rather than qualitative distinctions to be made, as it has been suggested by HIRSZFELD and AMZEL [4].

In the following, we shall discuss studies made on an anti-0 agglutinin which caused a serious haemolytic reaction in the course of a blood transfusion carried out because of haemorrhage in a case of abortion.

Methods

a) The qualitative tests were made at +4, +22 and +37° C, simultaneously with the tube and the test plate technique. In both cases equal amounts of a 2 per cent suspension of the test cells in saline and of the serum to be tested were used, 0.2 ml single volumes in the case of the tube technique and 0.05 ml with the test plate method. The test tube results were read after 2 hours, the test plate ones after 20 minutes. The adequate temperatures were provided by a refrigerator, a water bath and an incubator, respectively. To avoid drying with the test plate method, the plates were incubated in a wet chamber previously cooled or heated to the adequate temperature.

b) The quantitative studies were made simultaneously in media containing salts and in media containing albumin. The test temperatures were +4, +22 and +37° C. The serial twofold serum dilution tube technique was used. For diluting the serum and for suspending the test cells, irregular agglutinin-free AB serum containing 1/3 part human albumin was used as the albumin medium. The results were read after 2 hours of incubation.

c) In the absorption tests 1/3 part of the twice washed sediment of the test cells was added to the serum at +4° C. Absorption lasted 1 hour ; with shaking the serum — cell mixture every 1/4 hours. After each absorption the serum was tested for residual antibody.

d) The enzyme modification tests (trypsin, papaine) on 1 : 2 serial serum dilutions were made according to MORTON and PICKLES [5], and KUHNS and BAILEY [6] respectively.

e) The indirect Coombs tests [7, 8, 9] were made with the lowest dilution of serum not agglutinating the 0-group cells at 37° C.

f) To detect the presence of haemolysin, 0.2 ml of a 1 per cent suspension was added to 0.2 ml of active serum not older than 4 hours. The results were read after incubation for 2 hours in a water bath of 37° C, then again after centrifugation of the gently shaken content of the tube.

g) The inhibition test to determine the antibody type (anti-H or anti-0) of the subject supplying the anti-0 serum was performed with the saliva from an 0 and from an A_1B secretor. Fresh saliva was boiled for 20 minutes, cooled, centrifuged and 0.3 ml of the supernatant was mixed to the serum in question. After incubation for 30 minutes, the mixture was serially diluted in 1 : 2 steps with physiological saline. To each dilution was added 0.3 ml of a 2 per cent suspension made of the twice-washed sediment of 0-group cells. The results were read after 2 hours of incubation at room temperature. Unneutralized serum from the subject in question, an anti-H serum neutralized with the above-mentioned salivas, as well as an unneutralized anti-H serum were used as controls.

Brief clinical description of the case

Mrs. J. L., 27 years old, was admitted to the Obstetrics Department of Marcal Hospital on December 29, 1953, with the diagnosis of incomplete abortion and haemorrhagic shock.

There was no evidence of previous pregnancy, abortion or transfusion in the history.

In view of the strong haemorrhage, severe anaemia and shock, curettage was carried out without delay and a blood transfusion was given beside drugs to improve heart action and circulation.

The only blood available at the moment was 0-group, Rh positive. 300 ml of this was transfused into the patient whose blood group was AB, Rh positive.

Four hours after transfusion the patient developed the typical symptoms of transfusion haemolysis. Treatment of the haemolytic complications was begun without delay and blood samples from the patient and from the donor were sent to the Central Blood Group Serological Laboratory, National Blood Transfusion Service, to investigate the case.

The patient improved gradually and recovered without showing any symptom (anuria) of a more serious renal lesion.

Results of analysis

The recipient belonged to the A_1B, CcDe-group ; the donor to 0, CcDe. The serum of the recipient contained an irregular agglutinin which intensely agglutinated the erythrocytes of the donor in both a saline and an albumin medium, at +4, +22 and +37° C alike.

The observation that except for a few A_1, B and A_1B samples of the 580 blood samples used in the test (0 300, A_1 100, A_2 20, B 100, A_1B 50, A_2B 10) the antibody reacted with each sample at +4° C, giving the strongest reactions with the 0 and A_2-group cells, called attention to the possibility of anti-0 specificity.

Table I

Titres of the antibody found in the recipient's serum against different ABO-group cells selected at random, in saline medium, at +4° C

Blood group	Serum dilutions									
	T	1/2	1/4	1/8	1/16	1/32	1/64	1/128	1/256	1/512
0	++++	++++	++++	++++	++++	+++	++	+	—	—
A_2	++++	++++	+++	++	+	—	—	—	—	—
A_2B	+++	++	+	—	—	—	—	—	—	—
B	++	+	—	—	—	—	—	—	—	—
A_1	+	±	—	—	—	—	—	—	—	—
A_1B	+	—	—	—	—	—	—	—	—	—

Subsequently, it was examined whether the antibody would give a constant titre, and if so, with blood of which group. It has been found that it reacted with constant intensity with the 0-group cells.

To exclude the possibility of the serum of the recipient containing several kinds of antibody, the serum was subjected to absorption tests against test cells of different blood group combinations. The results showed clearly that the serum of the recipient contained only one kind of antibody, which was independent of the Rh—Hr, MNSs, P systems and belonged to the anti-0 group. The results of the absorption tests showed namely that, independently of the various combinations of the blood group antigens of the absorbing blood, the antibody was completely absorbed from the serum, with the only difference that in the course of absorption with the originally weakly reacting B and with the very weakly reacting A_1 and A_1B bloods, serial absorption had to be made, whereas only one single absorption was required with the 0-group and one or two with the A_2-group bloods, under the same conditions.

To determine the anti-0 type, the serum was examined by the agglutination-inhibition test described by MORGAN and WATKINS. Saliva from an 0-group and from an A_1B group secretor was used in parallel tests. No inhibition resulted and thus the serum was typed as anti-0.

To confirm this finding in the light of the evidence published by SANGER, attempts were made to determine the Lewis blood group type of the donor of the anti-0 serum. We had no anti-Le^a or anti-Le^b test sera ; knowing, however, the close correlation between the secretor, non-secretor and Lewis blood group characteristics, we tested the donor for secretor and non-secretor properties. The donor was found to be a strong secretor of A and B groups substances, i. e. Le(a-) in agreement with the results published by SANGER.

Further studies revealed that the antibody did not cause haemolysis *in vitro* and gave a negative indirect Coombs test, suggesting that it was complete, not of the immune type.

The results of the titrations made at +4, +22 and +37° C against 0-group cells are presented in Table II.

Table II

Medium and temperature		Serum dilutions									
		T	1/2	1/4	1/8	1/16	1/32	1/64	1/828	1/256	1/512
Saline	+ 4° C	++++	++++	++++	++++	++++	+++	++	+	—	—
	+22° C	++++	++++	++++	+++	++	±	—	—	—	—
	+37° C	+++	++	±	—	—	—	—	—	—	—
Albumin	+ 4° C	++++	++++	++++	++++	++++	+++	+++	++	+	—
	+22° C	++++	++++	++++	++++	+++	++	+	—	—	—
	+37° C	++++	++++	+++	+	—	—	—	—	—	—

It can be seen that the anti-0 agglutinin found in the serum of the recipient had a remarkably high titre and, in accordance with KETTELS' rule [10], it had an extremely wide heat amplitude, being highly active even at body temperature. Although not convincingly, it reacted more strongly in the albumin medium.

Enzymatisation (trypsin, papine) of the cells used in the titrations did not substantialy influence the results of titration.

Discussion

In some properties (titre, heat amplitude, activity in albumin medium) the anti-0 agglutinin found in the serum of the recipient differed substantially from the usual properties of the so-called "cold-type", "natural" anti-0 agglutinins.

These properties indicated that even if it was not an immune antibody, the anti-0 agglutinin present in the recipient's serum was of a form as may have caused haemolysis *in vivo* at transfusion of incompatible blood.

The other potential causes of haemolysis, such as haemolytic or infected preserved blood, "dangerousness" of the 0-group donor, were excluded : the sample from the preserved blood used for transfusion proved haemolysis free and sterile ; the donor's blood showed a low isohaemagglutinin titre and contained neither isohaemolysin, nor incomplete anti-A and anti-B. The development of the haemolytic complication had, therefore, to be ascribed to the peculiar form of the anti-0 antibody in the serum of the recipient. This form may be a transient one toward the immune type.

The antibody titre in the recipient's serum was followed up in the period of from December 20, 1953, to September 5, 1956. The time of the first test (December 22, 1953) coincided with the second month of her pregnancy, with the time of spontaneous abortion and of the development of the haemolytic complication. The antibody titre gradually decreased in 1954 and on October 27 only the 1 : 4 dilution reacted with 0-group cells in albumin medium at +4° C. In 1955, a few days before another spontaneous abortion, the titre was found again to be as high as 1 : 128. From that time on the titre of the antibody decreased gradually and no antibody was detected in the sample taken on September 15, 1956, when the patient was in the fifth month of her third pregnancy. It is noteworthy that from this pregnancy she gave birth to a normal child, at full term.

Although we have had no occasion thus far to test the blood of the husband and that of the newborn, on the basis of the close correlation between the pregnancies ending in abortion, the consequent appearance of the antibody and the rises in the titre, we feel compelled to suggest that a correlation existed between the abortions and the presence of antibody, confirming thereby the observations made by HENRY [11].

The case seemed to merit interest since to our best knowledge only one single case has been reported in which haemolytic complication was caused by an undoubtedly anti-0 antibody in the course of transfusion [12].

Summary

Serological studies made on an anti-0 antibody causing a haemolytic complication have been described. The antibody studied showed some serological properties essentially different from the usual ones ; this was interpreted as suggesting that the antibody may have been in the process of conversion into the immune type. Apparently, in support of this assumption were a close correlation between the appearance and rise in the titre of the antibody, and pregnancies ending in abortion, as potential antigenic stimuli.

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A SHORT-TERM GLASSHOUSE TEST FOR ESTIMATING PIRICULARIA RESISTANCE OF DIFFERENT RICE VARIETIES

By

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The resistance to *Piricularia* of 12 rice varieties and of 20 selected lines from a further one was studied under glasshouse conditions. The purpose was to develop a rapid test on young plants, yielding results comparable to those of field trials and to facilitate any further breeding and qualifying programme.

The actual resistance of different varieties may be readily determined by statistical evaluation of field damage caused by *Piricularia*, even if nothing is known about the factors responsible for the resistance. Since, however, in field trials the irregularity of the yearly *Piricularia* incidence might spoil the experiment, artificial infection seemed desirable [7]. This is readily performed on young plants bred in glasshouse and permits to examine a great number of varieties in a short time, thus rendering the selection of resistant varieties unquestionably more effective.

Naturally, results obtained on glasshouse bred young plants can be applied to *Piricularia* epidemics in the field only if the development of resistance of the actual variety is known in detail. The pathological change of damaged plants of any age is characterized by wel-defined areas of tissue necrosis, each caused by a separate infection with *Piricularia*. No systemic toxic manifestations are ever encountered. Thus, of the possible mechanisms resulting in resistance to disease described by GÄUMANN [2], only two main types might be distinguished as likely to occur in rice — *Piricularia* relations, *viz.* (i) resistance to the local penetration of the agent; (ii) a certain essential discrepancy in the metabolism of the parasite and host, resulting in the former's impeded vegetative and/or generative growth. The possibility of determining the relative degree of resistance brought about by either mechanism is given in tests involving the use of artificially infected glasshouse plants, but the evaluation must conform with the mechanism resulting in resistance of the host.

Ad (i). Supposing the resistance to be of penetrational character infection with a standard inoculum must cause the less lesions on unit leaf surface, the more resistant the plant. This theory had been accepted by Japanese workers (especially SUZUKI and co-workers) in the twenties and thirties, and

though SHIMADA's [12] microscopic studies did not support it, SAKAMOTO [9] developed laboratory resistance test based on the above suggestion. Ad (ii). Resistance brought about by discrepancies in the metabolism of the symbiotic organisms, on the other hand, must be characterized by the morphology of the lesions. This has been accepted by most workers [1, 3, 11] who, accordingly, choose the different types of leaf spots to differentiate between sensitive and resistant varieties.

Materials and methods

Pure seeds harvested in 1955 were obtained from different Hungarian experimental farms. The *Experimental Rice Breeding Division of the Collective Farm, Szarvas*, kindly supplied the varieties Dunghan Shali (DS); Uzross—17 (U17); Uzross—72 (U72); Linia—45 (L45); Agostano (Ag) and Precoce Allorio (PA); further, selected strains of the Kendzo-population (BTK—45, —91, —104, —110, — 177, —204, —302, —254, —364, —423, —464, —490, —522, —560, —561, —582, —584, —589, —595 and —603).

From the *Experimental Farm of Örki, Kopáncs*, we obtained samples of the strains Hokkaido Early (HE); Ooba (OB); and Santahezszky (Sz). The resistance of the three further varieties Dubovsky—129 (D-129), Vross—213 (V213) and Vross—3716 (V3716) imported from the Soviet Union in the spring of 1956 was also examined.

Rice varieties used for the experimental infections were bred in glasshouse boxes at a temperature varying between 22 to 28°C. Seeds of different varieties were sown in separate pots 12 cm in diameter each. These were placed in concrete tubs containing water at a level 3 to 5 cm lower than the soil surface in the pots. By this method irrigation was avoided. Thirty to forty grains of seed were sown on the surface of the muddy soil of each pot. After germination had begun about 15 young plants exhibiting uniform and vigorous development were kept for further examinations.

Infection was performed with a watery suspension of conidia containing 0.2 per cent gelatine and 0.05 per cent sodium oleate. These adjuvants enhance the moistening capacity of the watery suspension without exerting any untoward effect on the germination of conidia [1, 7]. The conidia were obtained either from cultures grown on different media, such as porridge agar, or sterilized rice grains in Blake flasks, or from infected plants. Suspensions containing 10 to 50 000 conidia per ml were found to be the most suitable for the infection of plants. The conidium count was determined in Buerker chamber.

Infection was performed at the time when the first leaf had reached its full size but no unfolding of the second leaf blade had yet occurred. Under the above experimental conditions this stage of development was attained on the ninth to twelfth day after sowing. The conidium suspension was carried onto the surface of the first leaf by a brush, as this method has been found to yield the most reproducible results.

Infected young plants were kept in a moist atmosphere for 24 hours following the infection. To achieve the appropriate moistness, the plants were covered by a filter-paper "tent" stretched over a wire frame, allowing the lower edge of the paper to dip into the water surrounding the plant pot. After 24 hours the tents were removed. Further breeding of the plants was made under usual glasshouse conditions, as described above.

The results were read on the 6th to 8th day following infection. By that time all the spots were clearly visible while no confluence of spots or drying off of the leaf had yet occurred. Four main types of spots were differentiated.

Type 1, necrosed spots of needle point size (Fig. 1).

Type 2, minor spots 1.5 to 2.0 mm in diameter, with a marked necrosed margin (Fig. 2).

Type 3, greater spots with a thin necrosed margin (Fig. 3).

Type 4, extensive spots without necrosis (Fig. 4).

The development of the four spot-types was found to follow each other in a certain chronological order. Spots of type 1 appeared 2 to 3 days after infection, then the spots of the 2nd and 3rd type, and last (6 to 8 days after infection) the extensive lesions without a necrosed margin.

To facilitate comparison of the degree of resistance of different varieties, a tentative grouping of the morphological changes according to sensitivity was made. Thus, leaves exhibiting only type 1 and/or type 2 lesions were designated as resistant, "R"; those carrying type 3 and/or type 4 lesions, sensitive, "S". Diseased leaves with pathological changes belonging to



Fig. 1. Type 1 lesions on first leaves of the variety Uzross—17



Fig. 2. Type 2 lesions on first leaves of the variety Agostano



Fig. 3. Type 3 lesions on first leaves of the variety Precoce Allorio



Fig. 4. Type 4 lesions on first leaves of the variety Dunghan Shali

none of the above two groups, while exhibiting a variety of different spot types, were designated as intermediary, "I". Characterization of the varieties was made by the incidence of the above R-I-S resistance types in the individuals of the variety under investigation.

Experimental

The results were not well reproducible in preliminary experiments. This failure could not be ascribed to differences in the source of inoculum, therefore studies were carried out on the possible role of the actual state of development of leaves and the age of the plant.

Results of such studies are presented in Table I. The experiment was carried out on the most sensitive and the most resistant varieties available (DS and U17, respectively). Data of Table I reveal that the severity of symptoms on variety DS decreased with age. This gradual mitigation of symptoms finally lead to their total absence, to macroscopic immunity. The essentially similar results obtained with U17 are not presented in Table I.

Table I

Changes in the sensitivity of the variety DS to Piricularia in relation to the age of its first leaf

Age of the first leaf in days	Incidence of different leaf types in %*			
	Negative	R	I	S
9	0	0	7	*93*
10	0	0	0	*100*
11	4	12	*28*	*56*
12	23	*23*	*48*	8
13	*69*	0	13	18
14	*70*	22	8	0

* Plants were infected by carrying conidia on the first leaf by a brush. For each of the experiments 23 to 27 plants were used.

In the subsequent experiments due attention was paid to the age factor, and very young plants were subjected to infection. Temperature and light effects were also standardized. The variety DS was included in every experiment, as reference standard. Experiments in which variety DS exhibited a decreased sensitivity were not evaluated. Data in the further tables represent the results of those experiments, selected from 3—4 parallel runs, in which maximum sensitivity was exhibited by the variety in question.

The results concerning the resistance to *Piricularia* of 12 varieties are summarized in Table II. The degree of sensitivity or resistance was estimated on basis of the pathological changes observable on the leaves, as described above.

Under conditions optimal for the multiplication of *Piricularia*, on the leaves of V213, OB, HE and PA, all the spot types were simultaneously pre-

Table II

Piricularia resistance of 12 rice varieties

Variety	Number of plants examined	Incidence of different leaf types in %			Qualification
		R	I	S	
Dunghan Shali	57	5	14	*85*	sensitive
Vross-3716	69	5	20	*75*	
Vross-213	46	15	35	50	intermediary
Ooba	26	16	46	38	
Hokkaido Early	36	23	34	43	
Precoce Allorio	67	12	64	24	
Uzross-17	46	*96*	2	2	resistant
Uzross-72	50	*98*	2	0	
Dubovski-129	45	*78*	20	2	
Santakhessky	36	*75*	19	6	
Agostano	29	*72*	28	0	
Linia-45	27	*96*	4	0	

sent, thus the incidence of R-I-S leaves was mostly equal. Further studies are required to decide whether this phenomenon resulted from the intermediary character of the variety's resistance or from the inhomogeneity of the population.

In the sensitive group, the DS variety was characterized by the presence of type 4 lesions only, while V3716 simultaneously exhibited both type 3 and 4 lesions.

Resistance was most marked in the two Uzross varieties and in L45. These developed only needle-point size spots of type 1. Of varieties D-129, Sz and Ag, the mixed occurrence of type 1 and 2 lesions was characteristic, while type 3 spots were exceptional. Type 2 spots of Ag were especially characteristic, having a regular spherical shape with a thick necrosed margin.

The resistance of 20 lines selected from the variety Kendzo was also studied. In the preliminary experiments this commercial variety exhibited typical intermediary characteristics similar to PA. It was expected to obtain resistant Kendzo lines by stock-selection at the *Experimental Division of the Collective Farm* (*Szarvas*). The results summarized in Table III reveal the differences obtained and show that, in spite of the appearance of a wide variety of transitory forms, no extreme variants similar to those of DS or U-16 occurred.

The pathological changes observed in the different groups were as follows. In the group qualified as sensitive, the 91, 490 and 584 lines displayed

Table III

Piricularia resistance of 20 lines obtained by stock-selection from the commercial variety Kendzo by the Experimental Rice Breeding Division of the Collective Farm, Szarvas

Variety	Number of plants examined	Incidence of different leaf types in %			Qualification
		R	I	S	
BTK- 91	45	18	43	40	
490	19	16	63	29	
582	34	15	29	56	sensitive
584	47	17	42	41	
595	33	18	12	70	
BTK- 45	38	47	26	27	
110	27	30	22	48	
177	29	48	17	38	
364	31	23	29	48	
464	43	42	21	37	intermediary
522	66	29	29	42	
560	50	46	34	20	
589	48	31	42	27	
609	31	55	16	29	
BTK-104	27	52	33	14	
204	42	64	29	7	
302	26	73	15	22	resistant
354	25	75	7	18	
423	44	52	23	25	
581	29	66	13	21	

extensive irregular spots with a thin necrosed marginal area. Some additional minute necrosed spots were also frequent. The type 2 and 3 lesions observed on the leaves of 582 differed only in size, both being approximately circular with thin margins. Type 1 lesions were rare. The lesions found on the leaves of line 595 were unique in their appearance, exhibiting extensive brownish, broadly marginated type 3 lesions, spreading along the leaf fibres.

Lines 104, 354, 423 and 581, considered resistant, exhibited lesions similar to those of Ag, *i. e.* round lesions 1 to 4 mm in diameter with broad necrosed margins. Type 1 lesions were exceptional. Varieties 204 and 302 displayed mostly type 1 and 2 lesions.

The eight lines qualified as belonging to the intermediary group exhibited a multiformity of pathological changes covering all the types described in the two extreme groups.

Discussion

The sensitivity to *Piricularia* of leaves of young rice plants was found to decrease gradually with age. The phenomenon was invariably observed in all the varieties tested. The results of ANDERSEN *et al.* [1], HASHIOKA [3], SALMON [10], PADMANABHAN *et al.* [5] and TANAKA *et al.* [13] might be interpreted partly in the light of this phenomenon.

Certain biological changes in the rice plants in relation to their development are also known to influence resistance to *Piricularia*. *Piricularia* sensitivity is enhanced (a) in vigorously metabolizing young leaves ; (b) in all the leaves during blossoming, when they contain a high concentration of nutritive substances, as a result of degradation of their "mobilizable store materials" [4, 13] ; (c) after the period of blossoming in the basis of the leaf blades, in nodi and in almost any parts of the head, and it is felt that these plant parts very probably also excel with their greater metabolic activity during the latest developmental stage of the rice plant.

These developmental changes in sensitivity were found to be characteristic of any variety of rice, not only in the experiments described above but also in field trials [1, 5, 7]. Thus the resistance of a certain variety must involve also some additional property limiting the multiplication of the parasite even in the most sensitive host tissues. This type of resistance must be genetically stable and, accordingly, essentially differing from the resistance connected with certain developmental phases. It may, therefore, be rightfully expected that a test method involving the use of young glasshouse-grown plants will yield results greatly advancing the practical purpose of resistance breeding.

There having been fair agreement between the results of the glasshouse test method on young plants and the behaviour of fully developed plants in the field, the method described will always allow to differentiate between clearly resistant and sensitive varieties. Field trials made with such varieties support this statement. (Unpublished observations independently made by K. KÁLLAY, R. SZABÓ and J. PODHRADSZKY, respectively.)

On the other hand, no definite conclusions could be drawn in the case of intermediary varieties, though most of them proved fairly resistant in field trials. Precoce Allorio *e. g.* can be regarded as practically free from *Piricularia* damage, as we have reported earlier [7]. The remarkable resistance of HE in field trials may be due to its extraordinarily short breeding period. Further studies are required to obtain more information about the field resistance of the varieties V 213 and OB.

A decreasing order could be established in the sensitivity of the 20 lines obtained by stock-selection from the commercial variety Kendzo, on the basis of the well-producible differences observed. However, differences in sensitivity

were so gradual between the successive members of the series, that their classification into resistant, intermediary and sensitive groups seemed arbitrary. Field trials are therefore required to determine the degree of resistance sufficient for avoiding *Piricularia* damage in the field.

Summary

A new, well-reproducible and rapid glasshouse test has been described to determine the sensitivity to *Piricularia* of rice varieties.

Examination of 12 varieties by this method revealed its applicability for sorting out the markedly resistant and sensitive varieties.

No definite results were obtained when examining varieties of intermediate resistance, though some regularly observable differences made it possible to establish a decreasing order of sensitivity.

The supposition that there is an essential difference between the developmental changes in sensitivity of any variety and the genetically determined differences characteristic of varieties, has been discussed in detail.

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A RAPID BIOLOGICAL TEST FOR DETERMINING OXYTETRACYCLINE AND CHLORTETRACYCLINE ACTIVITY

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The comparatively long time taken by the usual oxytetracycline and chlortetracycline activity tests render them impractical for continuous control of industrial fermentation processes. It seemed desirable to find a new method permitting simple, rapid and exact determination of the above antibiotics.

There are certain methods available to render biological antibiotic tests less time consuming. Thus, incubation of media seeded with microorganisms before addition of the antibiotic [1] and the use of a special colour reagent [2] might be useful in testing certain antibiotics (*e. g.* penicillin).

None of these methods could be applied for testing tetracyclines.

The method presented below makes use of the spore germination- and growth-stimulating activity of certain organic compounds.

Materials and methods

Medium. The basal medium contained the following ingredients, agar-agar 45 g, peptone 7.5 g, horse-meat broth 1500 g. pH before sterilization 7.0 to 7.1. Sterilization at 1.4 atmospheres for 30 minutes.

Test organism. Strain FDA 6633 of *B. subtilis*, kindly supplied by the *Research Institute of the Pharmacological Industry*.

Standard spore suspension. The microorganism was cultivated on the medium described above, in Blake flasks, at 37°C, for a week. This was followed by keeping the cultures in a refrigerator for 3 hours. Organisms were suspended in 100 ml cold sterile distilled water and collected in glass-corked vials. Vegetative cells were killed by keeping the suspension at 65°C for 30 minutes. This was followed by washing the cells 3 times by centrifugation with sterile distilled water.

Spore concentration was determined by the light adsorption method, using a Lange Colorimeter (Model IV) provided with an orange filter. Distilled water served as a control. Only suspensions of 80 to 90 per cent light absorbing capacity were used.

Next, the spore suspension was reincubated at 65°C for 30 minutes. Suspensions thus prepared could be stored over several months at 0°C in glass-corked vials.

Standard spore suspensions were appropriately diluted before used.

Addition of substances stimulating spore germination and growth. A separate buffer solution was used with each antibiotic. The concentration of each stimulating substance stock solution was chosen so as to permit its use as diluent for the spore-suspension preparation. Thus, 2 ml of a suspension of a preliminarily determined spore count was usually added to 98 ml of the appropriately diluted stimulating substance solution. Depending on the actual antibiotic to be tested, two different types of buffer solution were used. For oxytetracyline a 1/3 molar KH_2PO_4 buffer of pH 5 was used. Chlortetracyline was diluted in a 1/3.4 molar NaH_2PO_4 buffer

of pH 4.5. The determination of the pH of the buffer solution was performed by means of an electric pH-meter.

Preparation of agar plates. The basal medium was melted by autoclaving for 30 minutes under 1 atmosphere pressure and then cooled to 50°C in a water bath. Inoculation of the cooled medium was made by adding 5.5 ml of an appropriately diluted spore-suspension to each 100 ml of the medium, and after due mixing it was poured into Petri dishes or other suitable flat glass containers with this type of test only a single agar layer was prepared, containing both the test organism and the stimulating substance. Independently of the actual type of container used, the 3 mm thickness of the agar plate was found to be essential.

Agar plates prepared as described above, can be stored for about 2 days at +4°C. It is advisable to keep refrigerator stored plates at room temperature for 30 minutes before use.

Experimental

Depending on the actual antibiotic to be tested, the substance was either put in the cups of the plate or carried by paper discs onto the agar prepared as described in the previous section. Incubation was performed at 43°C for 3 to 4 hours, as it was found that in the presence of stimulating substances this time was sufficient for the development of visible bacterial growth. We studied the effects of the stimulating agents both on the growth of the test organism and on the inhibitory effect of the actual antibiotic. As a control, the same experiments were carried out on media not containing the stimulating factors.

The results are presented in Table I.

Table I

Compound	Concentration* %	Effect on the spores	Effect on 24 hour cultures	Effect on the antibiotic	Effect on the inhibition zone
Nicotinamide	0.0002—0.0005	ø	ø	ø	ø
Aneurin.....	0.0005	inhibitory			
Cobalamine	0.2 μg/ml	ø	ø	ø	ø
dl-aspartic acid	0.0002—0.0014	stimulatory	ø	ø	ø
Glutamic acid	0.0002	stimulatory	stimulatory	ø	ø
Folic acid	0.00002—0.002	stimulatory	stimulatory	ø	ø
Indol acetic acid	0.00002—0.0014	stimulatory	stimulatory	ø	ø
Biotin	0.00002	ø	ø	ø	ø
Glucose	0.08—0.008	ø	ø	ø	ø
Lactose	0.02	stimulatory	stimulatory	ø	ø

* The concentrations given are those finally present in the agar.

The best results were obtained by using folic acid or indol acetic acid as a stimulant. The inhibition zones were found to be uninfluenced by the presence of either of these substances.

In our experiments *B. subtilis* exhibited a more rapid growth in media of pH 6.9 to 7.0 then in those commonly used and with a pH of 6.0 to 6.5. The pH of our media did not interfere with the action of chlortetracyline and oxytetracyline if the antibiotics had been dissolved and diluted in a phosphate buffer of pH 4.5 to 5.0.

The fact that in our experiments the growth-stimulating effect of dl-aspartic acid was exerted also in the absence of non-caramelized sugars, appeared to be in contrast with the results of HACHISUKA *et al.* [3]. Nevertheless, the possible presence of caramelized polysaccharide components of agar-agar, produced by autoclaving, might have been sufficient to elicit the effect. Simultaneous addition of certain active growth-stimulating substances does not seem to enhance their effect, while it causes the appearance of a concentric wide bacteriostatic zone with only a narrow sterile area of total inhibition. As an explanation of the phenomenon one might suppose that microorganisms stimulated to vigorous development by the stimulators present are in a special physiological condition rendering them more resistant to the action of the poorly diffusing oxytetracycline and chlortetracycline.

Discussion

There are certain criteria to be observed at selecting a development-stimulating substance for short-term biological antibiotic tests, *viz.* (i) it must enhance spore germination of the organism ; (ii) it must stimulate growth and multiplication in the lag and the early logarithmic phases ; (iii) it must not interfere with the action *in vitro* of the actual antibiotic on the test organism ; (iv) it must ensure the development of a clear inhibition zone with clear-cut margins.

Rapid biological antibiotic tests may be developed by using such substances stimulating either the growth of a single microorganism or having a general stimulating effect. This type of test usually does not require a special technique or new type standard curves.

The mechanism of action on *B. subtilis* of the stimulating factors examined was not studied. The different stimulating factors probably have a different mechanism of action on the spores and the vegetative cells of the microorganism.

Our observation that after 3 to 4 hours of incubation at 43°C a more opulent development of the organisms was present than after a similar incubation at 37°C, suggested the former temperature to be superior for the spore germination of *B. subtilis*.

Summary

A short-term biological activity test has been developed for oxytetracycline and chlortetracycline. A simple horse-broth agar with 0.0014 per cent of growth-stimulating factor (folic acid or indol acetic acid) permitted testing of the above two antibiotics within 3 to 4 hours, using *B. subtilis* FDA 6633 strain as test organism.

The application of stimulating factors shortening the time necessary for a certain biological test appears to be a method practicable also in other biological test systems.

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CHANGES IN THE ANTIBIOTIC SENSITIVITY OF PATHOGENIC BACTERIA IN THE PERIOD 1953—1956

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Shortly after the discovery and introduction into therapy of antibiotics, certain pathogens began to show an increasing resistance to these agents. Since then, the resistance to antibiotics has become a highly important problem of theoretical and practical significance. Laboratory diagnosis to-day must include beside the identification of the pathogen, also data concerning its sensitivity to the different antibiotics.

The Department of Bacteriology of our Institute has been studying the sensitivity to antibiotics of the isolated pathogens since 1953, by the blood agar method of FŰRÉSZ and KUBINYI [1], involving the use of 24-hour broth cultures of isolated bacteria. The sensitivity tests made against penicillin, streptomycin and chloramphenicol were supplemented in 1956 by the introduction of tests for sensitivity to terramycin.

During the past 4 years a very considerable number of results has been accumulated, requiring statistical analysis and evaluation. In the following the results of the statistical analysis will be described. We shall not deal with several important theoretical and practical aspects of the problem of resistance, and we do not intend to give a survey of the pertaining literature as we feel that within the scope of such a report these problems could not be discussed in sufficient detail.

The following points were investigated:

1. The incidence of different pathogens in the different specimens tested.

2. Eventual changes in the antibiotic sensitivity of isolated pathogens in the period 1953—1956.

3. Eventual correlations in the response of certain types of pathogen to different antibiotics.

In Table I are comprised the data for the pathogens isolated from different specimens.

During the past 4 years a total of 6765 specimens was tested, from which a total of 8908 pathogens were isolated. Horizontally, the most important test materials and vertically the pathogens more frequently encountered in

Table I

Number of pathogens isolated from different test materials in the period 1953—1956

	Total	Test materials						
		Urine	Bile	Blood	Aural discharge	Naso-pharyngeal discharge	Pus, punctate	Other
Number of test materials.....	6765	1880	468	157	1040	369	1286	1565
Pathogens isolated								
Staphylococcus	2481	116	101	74	445	221	891	633
Streptococcus	431	30	9	12	122	40	90	128
Pneumococcus	249	5	—	3	58	28	32	123
Enterococcus	485	206	27	42	38	2	102	68
E. coli.....	2384	1325	323	18	61	18	199	440
Klebsiella	446	135	60	—	51	109	6	85
Ps. pyocyanea	985	143	14	8	531	7	111	171
Proteus	1217	462	55	1	349	37	115	198
Other.....	230	36	13	12	38	19	33	79
Total	8908	2458	602	170	1693	481	1579	1925

the tests are shown. Under the heading "others" are included the less often encountered test specimens and pathogens, partly that they should not interfere with evaluation and partly because they are not sufficient in number to allow for general conclusions. The commonest test materials were urine, pus, punctates and aural discharge and the most frequently isolated pathogens were *Staphylococcus pyogenes aureus*, *Escherichia coli*, *Bacillus proteus*, and *Pseudomonas pyocyanea*.

Table II

Percentage incidence of microorganisms in the commonest test materials, 1953—1956

Pathogen	Urine	Bile	Blood	Aural discharge	Naso-pharyngeal discharge	Pus, punctate
Staphylococcus	6	22	47	43	60	69
Streptococcus	2	2	8	12	11	7
Pneumococcus	0	—	2	6	8	2
Enterococcus	11	6	26	4	1	8
E. coli.....	70	69	11	6	5	15
Klebsiella	7	13	—	5	30	0
Ps. pyocyanea	8	3	5	51	2	9
Proteus	25	12	1	34	10	9

The absolute numbers presented in Table I being difficult to evaluate. in Table II are shown the percentage incidences of the single pathogens in the different test materials.

The sum of the percentage values presented in Table II exceeds 100, because often more than one pathogen was isolated from a given test specimen. The commonest pathogens were *Escherichia coli* (in urine and bile), *Staphylococcus pyogenes aureus haemolyticus* (in pus, naso-pharingeal discharge and blood), and *Pseudomonas pyocyanea* (in aural discharge).

Except for some minor variations, the annual incidence ratios of the different pathogens were roughly identical. This does not apply to Klebsiellae, the incidence of which is presented in Table III.

Table III

Incidence of Klebsiella strains in some specimens

Specimens	1953—1956 total	1953	1954	1955	1956
a) Number of specimens					
Urine	1880	304	397	674	505
Bile	468	83	104	131	150
Aural discharge	1040	19	252	537	232
Naso-pharyngeal discharge	369	11	40	212	106
b) Klebsiella positivity; number of					
Urine	135	—	14	52	69
Bile	60	—	11	16	33
Aural discharge	51	—	10	27	14
Naso-pharyngeal discharge	109	—	10	52	47
c) Klebsiella positivity; percentage of					
Urine	5	—	4	8	14
Bile	10	—	11	12	22
Aural discharge	3	—	4	5	6
Naso-pharyngeal discharge	23	—	25	25	44

In column 1 are shown the overall results for the 4-year period, followed by the data for the single consecutive years. It is noteworthy that in 1953 Klebsiellae were not isolated from any of the test materials, whereas after 1954 they grew in increasing numbers from the specimens, with the aural discharge as the probable exception, in which their incidence remained relatively low until 1956. In the 3 other kinds of test material, however, their incidence rose in a statistically significant measure. It is most characteristic that for example in 1956 Klebsiellae were isolated from 44 per cent of naso-pharyngeal discharges. This increasing incidence of Klebsiellae was by itself most remarkable. In Hungary STEINER [2], as well as STEINER and PUTNOKY [3], have observed in recent years an increasing incidence of Klebsiella pneumonia among premature infants and newborns. In other countries OBRINSKY *et al.*

[4], as well as WEISS *et al.* [5] have made similar observations. The increase in the frequency of Klebsiella pneumonia is ascribed to the wide-spread use of antibiotics, especially of penicillin. What we deal with is probably a sequel to a shift in flora, resulting from the wide-spread use of antibiotics

As to the changes in antibiotic resistance, we studied them also by examining the different specimens separately. It was found that, except for chance variations, the pathogens occurring in the different specimens did not differ from one another in antibiotic sensitivity, and for this reason the sensitivity of bacteria to antibiotics can be discussed independently of their origin.

In Table IV are presented the antibiograms of the most often encountered Gram-positive and Gram-negative bacteria, expressed in percentage of the 4-year totals.

Table IV

Antibiotic sensitivity of frequently isolated microorganisms, in per cent, 1953—1956

Antibiotic	Grade of sensitivity	Stapyhlococcus	Streptococcus	Pneumococcus	Enterococcus	E. coli	Klebsiella	Ps. pyocyanea	Proteus
Penicillin	S	22	82	88	3	0	1	0	1
	M	12	8	7	9	0	1	1	1
	R	65	10	5	88	100	98	99	98
Streptomycin	S	68	20	21	4	53	57	7	24
	M	12	44	45	5	9	9	31	20
	R	20	36	34	91	38	34	62	56
Cloramphenicol	S	85	97	99	77	80	77	13	61
	M	11	3	1	21	7	9	42	26
	R	4	0	0	2	13	14	45	13
Terramycin*	S	91	96	100	84	79	78	10	15
	M	1	3	—	1	8	7	43	4
	R	8	1	—	15	13	15	47	81
	Total:	100	100	100	100	100	100	100	100

* For 1956 only.

S = sensitive,
M = moderately sensitive,
R = resistant.

It can be seen that about 65 per cent of Staphylococci were resistant to penicillin. Most of the Streptococci and Pneumococci were susceptible to penicillin and to the broad-spectrum antibiotics. The Enterococci, E. coli and Klebsiellae were sensitive first of all to the broad-spectrum antibiotics. In E. coli and Klebsiella infections streptomycin was also effective.

The B. proteus, but even more so the Ps. pyocyanea strains were highly resistant to the four kinds of antibiotic tested, and nearly 50 per cent of the infections caused by them could not be influenced by the use of the antibiotics in question.

Combined antibiotic therapy, or the new, more effective antibiotics (neomycin, polymyxin) may be decisive in the treatment of infections caused by the above pathogens.

The changes in the antibiotic sensitivity of the pathogens under discussion, as they occurred during the 4-year test period, were studied by careful analysis.

In Tables V to VIII are shown the antibiotic sensitivity data for the pathogens showing some change in this respect during the test period. These microorganisms were : *Staphylococcus pyogenes aureus haemolyticus*, *Pneumococcus*, *E. coli* and *Ps. pyocyanea*.

Table V

Percentage distribution of Staphylococcus strains according to antibiotic sensitivity. 1953—1956

Antibiotic	Grade of sensitivity	1953	1954	1955	1956
Penicillin	S	25	23	22	22
	M	31	17	9	7
	R	44	60	69	71
Streptomycin	S	70	67	68	68
	M	8	12	13	11
	R	22	21	19	21
Chloramphenicol	S	82	88	86	82
	M	13	9	12	10
	R	5	3	2	8
	Total :	100	100	100	100
Number of strains tested :		263	541	1059	618

In Table V are found the data for the antibiotic sensitivity of Staphylococci for the single calendar years. The number of the strains tested in each year shows clearly that the changes that had taken place were in fact highly significant. The data for terramycin have not been included, as tests with this antibiotic were carried out only in 1956. The sensitivity of Staphylococci to penicillin underwent a substantial change during the test period. In 1953, only 44 per cent of the strains isolated were resistant, whereas in 1956, 71 per cent showed resistance to penicillin. It is also remarkable that the percentage of sensitive strains remained almost unchanged during the test period, whereas

that of the moderately sensitive ones decreased gradually, in favour of the resistant strains, until the former had disappeared almost completely.

The fact that the resistance of Staphylococci to penicillin is increasing is known throughout the world and has been emphasized in numerous reports, both abroad and in this country [1, 6—12].

In Table VI are presented the corresponding data for Pneumococci.

Table VI

Percentage distribution of Pneumococcus strains according to antibiotic sensitivity. 1953—1956

Antibiotic	Grade of sensitivity	1953	1954	1955	1956
Penicillin	S	92	84	89	88
	M	8	10	4	8
	R	—	6	7	4
Streptomycin	S	54	19	27	11
	M	38	52	46	40
	R	8	29	27	49
Chloramphenicol	S	100	97	100	99
	M	—	3	—	—
	R	—	—	—	1
Total :		100	100	100	100
Number of strains tested :		13	63	89	84

As Table VI shows, the incidence of streptomycin-resistant Pneumococci rose from 8 per cent in 1953 to 49 per cent in 1956. Meanwhile, the incidence of sensitive strains fell from 51 per cent to 11 per cent. The percentage of moderately sensitive strains remained roughly the same. Thus, at present, nearly 50 per cent of the strains isolated are resistant to streptomycin.

Table VII contains the data illustrating the changes in the antibiotic-sensitivity of E. coli strains. There was a remarkable increase in the resistance to chloramphenicol. SZABÓ [13], in 1954, found that 100 per cent of the E. coli strains grown from cases of biliary tract infection were sensitive to chloramphenicol. In the same year, KUBINYI and HAMAR [14] found only 2 among the 300 strains of E. coli isolated from various discharges and body fluids to be chloramphenicol-resistant. In foreign countries where chloramphenicol had been introduced earlier, the incidence of resistant strains was reported to be higher than in Hungary [15]. In 1953, we found 5 per cent of the E. coli strains to be chloramphenicol-resistant, as compared to 26 per cent in 1956.

Table VII

Percentage distribution of E. coli strains according to antibiotic sensitivity. 1953—1956

Antibiotic	Grade of sensitivity	1953	1954	1955	1956
Penicillin	S	—	—	—	—
	M	—	—	—	—
	R	100	100	100	100
Streptomycin	S	52	50	57	51
	M	6	8	8	11
	R	42	42	35	38
Chloramphenicol	S	81	87	86	70
	M	14	7	5	4
	R	5	6	9	26
	Total:	100	100	100	100
Number of strains tested:		371	451	850	712

As it is revealed in Table VIII, Ps. pyocyanea strains underwent similar changes.

Table VIII

Percentage distribution of Ps. pyocyanea strains according to antibiotic sensitivity. 1953—1956

Antibiotic	Grade of sensitivity	1953	1954	1955	1956
Penicillin	S	1	—	—	1
	M	2	0	0	—
	R	97	100	100	99
Streptomycin	S	8	3	9	7
	M	24	24	35	33
	R	68	73	56	60
Chloramphenicol	S	18	12	13	11
	M	61	43	37	39
	R	21	45	50	50
	Total:	100	100	100	100
Number of strains tested:		106	257	462	160

The increase in the chloramphenicol resistance of Ps. pyocyanea strains is a most grave fact, this microorganism being almost totally unresponsive to the other antibiotics.

It is remarkable that the increased resistance of the four pathogens had developed against different antibiotics. The cause of this is believed to the following. Staphylococci and Pneumococci usually occur in the naso-pharynx.

E. coli is the dominant bacterium of the intestinal flora. Thus, any antibiotic administered against any infection will be in touch with these microorganisms and if it affects them, it will alter their metabolism. The response to the antibiotic depends on the metabolism of bacteria. For example, in the metabolism of Staphylococci any action on the penicillin-binding component must affect the formation of the enzyme penicillinase. Likewise, it is only natural that Pneumococci, in whose metabolism the carbohydrate play an important role (capsule), should develop resistance to streptomycin, which is active just along these lines. The high lipid content of the cellular wall of E. coli and Ps. pyocyanea strains indicates that these bacteria have an intensive lipid metabolism ; thus they might adapt themselves to the broad-spectrum antibiotics by changes in their lipid metabolism.

Accordingly, the bacteria in question may have increased their resistance either by a frequent selection of mutants of different properties or as a result of an enhanced adaptive capacity.

The data presented indicate that in Hungary, too, certain microorganisms are developing an increased resistance to certain antibiotics. Beside the known increase in the penicillin-resistance of Staphylococci, our findings lend a new significance to the streptomycin-resistance of Pneumococci and to the remarkable increase in the resistance to chloramphenicol of E. coli strains. This calls for further observations to determine the trend of evolution and also to work out plans for effective therapy.

Finally, we have investigated the eventual correlation in the sensitivity of pathogens to several antibiotics. To elucidate this point, the sensitivity of the single microorganisms to the different antibiotics has been analysed by a combinative method. As this requires very meticulous work, the studies have been restricted to the commonest pathogens, such as Staphylococcus, E. coli, Klebsiellae, Ps. pyocyanea and B. proteus. Penicillin being inactive on all microorganisms but the first, in these cases the results for streptomycin- and chloramphenicol-sensitivity were compared. Some remarkable features were observed, which are presented in Tables IX—XII.

In Table IX are comprised the data for the combined antibiotic sensitivity of staphylococcal strains. Of the 2481 strains, 551 were sensitive to penicillin, 316 were moderately sensitive and the others were resistant. Of the penicillin-resistant strains 978 were sensitive to this antibiotic and 196 were moderately sensitive. The remaining strains were resistant to streptomycin. Of the strains resistant to both penicillin and streptomycin, 199 were sensitive and 155 were moderately sensitive to chloramphenicol, and 86 of the strains were resistant to all three antibiotics. To facilitate evaluation, in column 2 of Table IX we present the percentage values.

The above data reveal that during the test period only 22 per cent of the cases of staphylococcal infection would have responded favourably to the

Table IX

Combined antibiotic sensitivity of the Staphylococcus strains tested. 1953—1956

Antibiotic sensitivity			Number	Per cent
Penicillin	Streptomycin	Chloramphenicol		
S			551	22
M			316	13
R	S		978	40
R	M		196	8
R	R	S	199	8
R	R	M	155	6
R	R	R	86	3
		Total:	2481	100

Table X

Comparative analysis of the penicillin- and streptomycin-sensitivities of the Staphylococcus strains tested

Sensitivity to penicillin	Sensitivity to streptomycin			Total
	S	M	R	
a) Number of strains				
S	457	73	21	551
M	244	25	47	316
R	978	196	440	1614
Total	1679	294	508	2481
b) Penicillin-sensitivity per cent				
S	27	25	4	22
M	15	8	9	13
R	58	67	87	65
Total	100	100	100	100
c) Streptomycin-sensitivity, per cent				
S	83	13	4	100
M	77	8	15	100
R	61	12	27	100
Total	68	12	20	100
d) Theoretically expectable number of strains, provided no causal relationship exists				
S	372.9	65.3	112.8	551.0
M	213.9	37.4	64.7	316.0
R	1092.2	191.3	330.5	1614.0
Total	1679.0	294.0	508.0	2481.0

usual doses of penicillin. In a further 13 per cent increased doses of penicillin might have been effective. After penicillin, the usual and increased doses of streptomycin could have been anticipated to act favourably in about 50 per cent of the pathogens. However, in 14 per cent of the cases only chloramphenicol would have been effective, and 3 per cent could not have been influenced at all.

The most important purpose of this combined analysis was to determine whether there existed a correlation in the behaviour of the single types of pathogens to the different antibiotics, and, if so, what was the nature of the correlation.

In the first part of Table X are included the numerical values for the sensitivity of some Staphylococcus strains to the two antibiotics. Of the 551 strains sensitive to penicillin 457 were sensitive also to streptomycin. 73 were moderately sensitive to streptomycin and 21 were resistant to it. The other columns and rows can be interpreted in the same way.

In the second part of Table X are presented the percentage values for the sensitivity of Staphylococci to penicillin. The column "Total" shows the penicillin-sensitivity of all strains tested and it is seen that only 22 per cent of the strains were sensitive to penicillin. It is remarkable that among the strains sensitive to steptomycin 27 per cent were sensitive also to penicillin, whereas among the streptomycin-resistant strains only 4 per cent were penicillin-sensitive. 65 per cent of the strains were resistant to penicillin. However, only 58 per cent of the streptomycin-sensitive, and as many as 87 per cent of the streptomycin-resistant strains were resistant to penicillin. This shift in the percentage values suggests that the sensitivities to the two antibiotics are not independent of each other. Were they so, the percentage incidence of penicillin-sensitive or -resistant strains were equal in the streptomycin-sensitive or -resistant groups. The same can be seen in the third part of Table X which shows the percentage values for streptomycin-sensitive strains. Of all the strains 68 per cent, but of the penicillin-sensitive strains 83 per cent, were sensitive to streptomycin. Twenty per cent of all the strains, but only 4 per cent of the penicillin-sensitive strains, were resistant to streptomycin. Again, the identical properties tend to occur together and the opposite ones eliminate or neutralize one another to a certain degree.

What would be the percentage incidence of the strains if the two properties, notably sensitivity to penicillin and sensitivity to streptomycin, were independent of each other? The data pertaining to this point are shown in the fourth part of Table X. The incidence of penicillin-sensitivity was roughly the same in the groups showing sensitivity, moderate sensitivity and resistance to streptomycin, as well as in the group including all the strains tested. The single categories of streptomycin- and penicillin-sensitivity naturally contain the actually encountered strains. A comparison of these theoretically

expectable values with the real ones will supply us with relevant information as to the correlation between the sensitivity to penicillin and that to streptomycin. The comparison can be seen in Table XI.

Table XI

Staphylococcus: Correlation between sensitivity to penicillin and sensitivity to streptomycin

Sensitivity to		Number of strains		x — m		$\frac{(x-m)^2}{m}$
penicillin	streptomycin	found (x)	calculated (m)	+	—	
S	S	457	372.9	84.1		19.0
	M	73	65.3	7.7		0.9
	R	21	112.8		91.8	74.7
M	S	244	213.9	30.1		4.2
	M	25	37.4		12.4	4.1
	R	47	64.7		17.7	4.8
R	S	978	1092.2		114.2	11.9
	M	196	191.3	4.7		0.1
	R	440	330.5	109.5		36.3
	Total :	2481	2481.0		$\chi^2 = 156.0$	

From left to right the columns of Table XI show the degree of sensitivity to the two antibiotics ; the values found ; the values calculated ; and, finally, the difference between the two. It is remarkable that great positive differences were found in the case of identical properties, *i. e.* in the case of strains resistant or sensitive to both antibiotics. On the other hand, great negative differences occurred with the antagonistic properties, *i. e.* in the case of strains sensitive to one antibiotic and resistant to the other. Little were the differences when sensitivity to one antibiotic was moderate. Thus, there obviously exists a positive correlation as regards the sensitivity to the two antibiotics. The last column of Table XI contains the results obtained by dividing the square of difference by the calculated value, the sum of these ratios giving the χ^2 value. Its significance ($n = 7$) shows that a causal relationship exists between the two properties.

Table XII shows our results for the sensitivity of Staphylococcus strains to penicillin and chloramphenicol, as well as to streptomycin and chloramphenicol, as determined by the method outlined above. In both cases differences closely comparable to those mentioned above were obtained and again the significance of the χ^2 values confirms the existence of a correlation in the sensitivity to the different antibiotics. It is remarkable that the least close correlation occurred in the case of penicillin *vs.* chloramphenicol. Likewise, a correlation was established between the streptomycin and chloramphenicol

Table XII

Staphylococcus : Comparative analysis of sensitivity to penicillin and chloramphenicol, resp. streptomycin and chloramphenicol

Sensitivity to		Number of strains		Sensitivity to		Number of strains	
penicillin	chloramphe-nicol	found	calculated	streptomycin	chlor-amphenicol	found	calculated
	S	533	464.8		S	1606	1427.3
S	M	17	58.8	S	M	64	179.3
	R	1	23.8		R	9	72.4
	S	278	268.6		S	260	249.9
M	M	29	33.8	M	M	29	31.4
	R	9	13.6		R	5	12.7
	S	1298	1372.0		S	243	431.8
R	M	219	172.4	R	M	172	54.3
	R	97	69.6		R	93	21.9
Total :		2481	2481.0	Total :		2481	2481.0
		$\chi^2 = 90.4$				$\chi^2 = 725.8$	

sensitivities of E. coli, Klebsiellae, Ps. pyocyanea and B. proteus. These data are shown in Table XIII.

Table XIII

Gram-negative bacteria : Comparative analysis of sensitivity to streptomycin and chloramphenicol

Sensitivity to		E. coli		Klebsiella		Ps. pyocyanea		Proteus	
strepto-mycin	chlor-amphenicol	f	c	f	c	f	c	f	c
	S	1217	1017.2	240	194.7	28	8.9	250	181.8
S	M	34	107.4	6	22.0	18	28.9	29	71.2
	R	16	142.4	5	34.3	23	31.2	14	40.0
	S	170	167.0	28	32.6	39	39.4	169	153.2
M	M	23	17.6	12	3.6	150	126.7	57	60.1
	R	15	23.4	2	5.8	114	136.9	21	33.7
	S	527	729.8	78	118.7	61	79.7	336	420.0
R	M	145	77.0	21	13.4	244	256.4	210	164.7
	R	237	102.2	54	20.9	308	276.9	131	92.3
Total :		2384	2384.0	446	446,0	985	985.0	1217	1217.0
	χ^2	500.5		* 121.0		63.9		119.6	

f = number of strains, found,
c = number of strains, calculated

* = Ignoring M M = 3.6.

The behaviour of the pathogens on exposure to the different antibiotics therefore indicates that these properties are not independent of one another and, consequently, some common factor must play a role also in their development. This is in apparent contradiction with the earlier view that during the test period the resistance of Staphylococci to penicillin had increased, whereas their resistance to streptomycin and chloramphenicol had not changed. The resistance of Pneumococci to streptomycin showed a similar increase and it did not change against penicillin and chloramphenicol. Finally, the resistance to chloramphenicol of E. coli and Ps. pyocyanea increased, but no change occurred in their sensitivity to streptomycin. If the same factor were responsible for the sensitivity to the different antibiotics, it would justifiably be expected that the increase in the resistance to one antibiotic would be associated with an increase against the other.

It has to be emphasized that from a purely statistical point of view there may exist a positive correlation between the sensitivities to two or more antibiotics not necessarily involving a simultaneous change in the resistance to these antibiotics. The only possible interpretation of this phenomenon appears to be that the sensitivity of the pathogen to the antibiotic is determined not by one, but by several factors. Microorganisms have the fundamental property of being able to develop resistance to antibiotics in general but there exist also specific factors that make it possible that resistance to a given antibiotic should develop. This specific resistance depends on the metabolic type of the bacterium and once it has developed it affords protection against the given antibiotic.

Summary

The results of antibiotic sensitivity tests involving 8908 pathogens isolated from 6765 test specimens during the period 1953—1956 have been analysed statistically. It has been found that

(i) in the above period the incidence of Klebsiellae in the different test materials has considerably increased ;

(ii) the resistance of Staphylococcus to penicillin, that of Pneumococcus to streptomycin, and that of E. coli and Ps. pyocyanea to chloramphenicol greatly increased during the period mentioned ;

(iii) a causal correlation exists in the sensitivity of the single pathogens to the different antibiotics. This statement is in apparent contradiction with an earlier one, according to which resistance changes against one antibiotic only, whereas resistance to the other remain unchanged. This phenomenon appears to have the sole explanation that for the development of resistance not one, but several factors are responsible, some of which are inherent in the general and common properties of bacteria, whereas others affect such characteristics of the bacteria which are connected to one certain antibiotic.

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ÜBER DIE ANWENDBARKEIT DER MIDDLEBROOKSCHEN HÄMOLYSE-REAKTION ZUR FESTSTELLUNG DER MENSCHLICHEN UND TIERISCHEN TUBERKULOSE

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Im Rahmen unserer Untersuchungen über die Beziehungen zwischen menschlicher und tierischer Tuberkulose stellten wir u. a. auch das Vorkommen und den Grad der Infektion der Einwohner und der Rinderbestände einer Ortschaft fest. Hierbei nahmen wir neben einer allergischen Probe auch ein serodiagnostisches Verfahren in Anspruch, um uns von dessen Brauchbarkeit zu überzeugen.

Seit den 1948 veröffentlichten Untersuchungsergebnissen von MIDDLEBROOK und DUBOS [33] haben sich erneut weitere Kreise für die serologische Feststellung der Tuberkulose interessiert. Es stellte sich nämlich heraus, dass die verschiedenen roten Blutkörperchen mit den auf ihre Oberfläche geschichteten Antigenen spezifisch gemacht werden können und daher zum Nachweis der entsprechenden Antikörper geeignet sind. Inzwischen haben zahlreiche Autoren ihre Erfahrungen bei Anwendung der Hämagglutinations- (Hg-) und hämolyse- (Hl-) Reaktionen bzw. bei deren gemeinsamer Anwendung zur Feststellung der Tuberkulose und zu ihrer Prognose mitgeteilt. Zahlreiche Versuche wurden mit menschlichen und tierischen Blutsera zur Bestimmung sowohl der verschiedenen Krankheitsformen der Erwachsenen- und Kindertuberkulose, als auch der Tiertuberkulose durchgeführt. Die Meinungen der Autoren gehen jedoch stark auseinander; manche äusserten sich günstig, während andere die Methode als wenig wertvoll beurteilten. Noch unsicherer und widersprechender erscheinen ihre Auffassungen, wenn man die Resultate der verschiedenartigen Untersuchungen vergleicht.

Die Frage konnte auch durch die in Ungarn vorgenommenen einschlägigen Untersuchungen nicht geklärt werden. Über derartige Versuche berichteten ALMÁSSY [1], FÖLDES und KÖRÖSI [16], KERTAY und SZABÓ-SZÜCS [29], BUKOWINSZKY, FÖLDES, PIUKOVICH und CSERNOK [8], FLESCH, HALÁSZ und BAINTNER [15], TÖRÖK und VASTAGH [40].

Im Hinblick auf die Wichtigkeit des serologischen Nachweises der Tuberkulose schien es angezeigt, die Frage an der nicht selektierten Einwohnerschaft und am Tierbestand einer Ortschaft zu untersuchen. Wir sind der Ansicht, dass die unter natürlichen Verhältnissen gewonnenen Ergebnisse charak-

teristischer sind, als wenn die Untersuchungen am ausgewählten Krankenmaterial einzelner Heilanstalten bzw. an einzelnen Tiergruppen oder womöglich an künstlich infizierten Versuchstieren durchgeführt werden.

Laut LAGERCRANTZ [30], POPP [35], KERTAY und SZABÓ-SZÜCS [28], BERLIK [4] sowie WEICHSEL [41] ist die Hl-Probe empfindlicher und zuverlässiger als die Hg-Probe, weshalb wir bei unserer Arbeit die erstere anwandten.

Untersuchungsmethoden

Die Feststellung des Infektionsgrades der Einwohner erfolgte mit Hilfe der Mantouxschen Probe. Vom Kochschen Tuberkulin in der Verdünnung 1 : 10 000 wurde 0,1 ml intrakutan injiziert und das Ergebnis der allergischen Probe nach 48—72 Stunden abgelesen. Als positiv betrachteten wir nur 10 mm grosse oder grössere Reaktionen, wobei auch das Ausmass der Infiltration und Hyperämie berücksichtigt wurde. Zur Auswahl der Kranken wurden neben der physikalischen Untersuchung und Röntgendurchleuchtung auch bekteriologische Verfahren (Züchtung, Typus usw.) in Anspruch genommen.

Den Tuberkulosebefall bzw. die Erkrankung der Rinder stellten wir durch klinische Untersuchung und mit der intradermalen Tuberkulinprobe fest. Zur Impfung wurde 50%iges Koch-Tuberkulin benutzt. Die klinische Untersuchung der Tiere und die Beurteilung der Tuberkulinproben erfolgte jeweils durch zwei Tierärzte. Als positiv wurde die Reaktion angesehen, wenn die an der Einspritzungsstelle des Tuberkulins — an der Halsseite — entstandende Schwellung mindestens des Anderthalbfache der ursprünglichen Dicke der Hautfalte erreichte. In den Fällen, wo die Tuberkulinprobe negativ, die Blutuntersuchung jedoch positiv ausfiel, wurde die Tuberkulinprobe nach 2 Monaten wiederholt. Die klinisch als tuberkulös oder tbc-verdächtig befundenen Tiere wurden von einer aus vier Mitgliedern bestehenden Kommission beurteilt.

Zu gleicher Zeit mit den vorstehend angeführten Untersuchungen wurden sowohl von Menschen wie Tieren Blutproben entnommen, um die mit der allergischen Probe und den anderen erwähnten Untersuchungen gewonnenen Resultate mit den Ergebnissen der Hämolyse-Reaktion vergleichen zu können. Die Ausführung der MIDDLEBROOKschen Hämolysenprobe [29] mit den Blutsera der Menschen und Tiere geschah folgendermassen: Die auf übliche Weise gewonnenen Testsera wurden nach Inaktivierung (30 Minuten bei 60° C) mit der TAKÁTSYschen Öse zu 0,1 ml an der MANDULAschen Platte in physiologischer Kochsalzlösung in doppelt erhöhter Serie (1 : 2 : 4 : 8 usw.) verdünnt. Die in *Alsever*-Lösung aufbewahrten und vor Gebrauch dreimal gewaschenen Schaferythrozyten wurden mit Tuberkulin (2,5%) sensibilisiert und erneut dreimal gewaschen. Zu jeder einzelnen Serumverdünnung gaben wir 0,1 ml der 1%igen sensibilisierten Erythrozythensuspension. Auf Grund des Titrierungsergebnisses wurden den Testserenverdünnungen und Erythrozythengemischen aus dem verdünnten und mit normalen roten Schafblutkörperchen abgesättigten Komplement 0,05 ml zugegeben und die Röhrchen nach Schütteln bei 37° C in den Thermostat gestellt. Die Reaktionen beurteilten wir nach 2 Stunden auf Grund der mit den Kontrollen verglichenen Hämolyse. Als positiv wurde eine Hämolyse in der Serumverdünnung 1 : 8 oder höher gewertet.

Untersuchungsergebnisse

Die Mantoux-Probe in der Verdünnung 1 : 10 000 wurde an 1113 Einwohnern der Ortschaft vorgenommen. Die Reaktion fiel in 594 Fällen positiv aus, d. h. 53,4% der untersuchten Personen waren tuberkuloseinfiziert. Die Einzelergebnisse sind nach Altersgruppen auf Tabelle I zusammengefasst. Die Resultate der serologischen Untersuchung der Einwohner sind in Tabelle II wiedergegeben.

Das Blutserum von 965 der wahllos untersuchten 1322 Personen enthielt keine hämolysierenden Antikörper und erwies sich demnach als negativ (73%), während in 357 Fällen = 27% ein positives Resultat zustande kam.

Tabelle I

Ergebnis der Mantoux-Probe in der Verdünnung 1 : 10 000

Anzahl der Einwohner nach Altersgruppen		Mantoux		
		negativ	positiv	
			Anzahl	%
0—6	171	127	44	25,7
7—14	327	141	186	59,6
15—19	84	52	32	38,1
20—34	215	74	141	65,6
über 35	316	125	191	60,5
Insgesamt	1113	519	594	53,4

Tabelle II

Serologische Untersuchung der Einwohner auf Tbc

	Anzahl	Hämolysetiter						Insgesamt	
		1 : 8	1 : 16	1 : 32	1 : 64	1 : 128	1 : 256	positiv	negativ
Insgesamt untersucht	1322	131	114	72	33	6	1	357	965
		36,6%	31,9%	20%	9,2%	1,7%	0,2%	27%	73%
Mantoux-positiv	546	84	45	23	17	3	1	173	373
		48,5%	26%	13,2%	9,8%	1,7%	0,5%	31,6%	68,4%
Kranke mit aktiver Tbc	48	6	3	4	2	1	—	16	32
								33,3%	66,7%
Mantoux-negativ	479	40	52	35	12	—	—	139	340
		28,7%	37,4%	25,1%	8,6%			29%	71%

Bei der serologischen Untersuchung erwies sich demnach nur die Hälfte der Personen als tuberkuloseinfiziert, die bei der mit der Verdünnung 1 : 10 000 durchgeführten einmaligen Tuberkulinprobe als tuberkulös nachgewiesen werden konnten. Noch unzuverlässiger war das Resultat, wenn wir die von einzelnen Autoren empfohlene Serumverdünnung 1 : 16 als Grenztiter nahmen. In diesem Fall waren nur 17% der Untersuchten positiv. Unter den 546 mantouxpositiven Personen enthielt nur das Blutserum von 173 hämolysierende Antikörper, d. h. nur 31,6% waren positiv, und lediglich 33,3% der an Tuberkulose erkrankten Menschen erwiesen sich auch serologisch als tuberkulös. Von den mantouxnegativen 479 Personen waren hingegen 71% auch serologisch negativ, während 29% positive Hl-Reaktion gaben.

Bei der Untersuchung der Tiere betrug der Tuberkulosebefall der Rinder in der Ortschaft auf Grund der Tuberkulinprobe 26,7%. Bei der klinischen Untersuchung fanden wir 0,3% sämtlicher untersuchten Tiere und 1,2% der tuberkulinpositiven tuberkulös. Die serologischen Untersuchungsergebnisse der Tiere sind in Tabelle III enthalten.

Tabelle III

Serologische Untersuchung der Rinder auf Tbc

	Anzahl	Serumverdünnungen 1:						Insgesamt	
		8	16	32	64	128	256	positiv	negativ
Insgesamt untersucht	427	88	44	17	15	30	7	201	226
		20,6%	10,3%	3,8%	3,5%	7,0%	1,9%	47,1%	52,9%
Tuberkulin-positiv	122	17	10	5	2	9	2	45	77
		14%	8,3%	4,1%	1,6%	7,3%	1,6%	36,9%	63,1%
Tuberkulin-negativ	305	71	34	12	13	21	5	156	149
		23,2%	11,2%	4%	4,2%	7,0%	1,6%	51,2%	48,8%

Laut Tabelle III gab das Blutserum von 201 der wahllos untersuchten 427 Tiere (47,1%) in der Verdünnung 1 : 8 bzw. in höherer Verdünnung positive Hl-Reaktion. Wenn wir jedoch den von einigen Autoren empfohlenen Grenzwert von 1 : 16 oder noch höhere Serumverdünnungen als massgebend betrachten, so kommt die 26%ige serologische Positivität dem 26,7%igen Wert der Tuberkulin-Positivität bereits sehr nahe. Die Ergebnisse der beiden immundiagnostischen Proben stimmen aber nur scheinbar überein. Betrachten wir nämlich die mit dem Blutserum der tuberkulinpositiven und -negativen Tiere gewonnenen Resultate auch in diesem Falle gesondert, so sehen wir bereits eine wesentliche Abweichung. Die serologische Probe fiel nämlich nur bei 36,9% der tuberkulinpositiven Tiere, hingegen bei 51,2% der tuberkulinnegativen Tiere, positiv aus. Die der Verdünnung 1 : 16 und den grösseren Serumverdünnungen entsprechenden prozentualen Werte sind auf Tabelle III angeführt.

Die Blutsera der klinisch tuberkulosekranken bzw. krankheitsverdächtigen Tiere enthielten ebenso wie die der aktiv tuberkulösen Menschen nur in 1/3 der Fälle hämolysierende Antikörper.

Besprechung

Die zum Nachweis der tuberkulösen Infektion dienenden und seit Jahrzehnten nach verschiedenen Methoden und mit vielerlei Antigenen durchgeführten serologischen Untersuchungen hatten keine zufriedenstellenden Resultate gezeitigt. Es war daher verständlich, dass die erwähnte Mitteilung von

MIDDLEBROK und DUBOS [33] grosses Interesse erweckte. Seither wurde aber auch dieses Hg-Verfahren vielfach modifiziert, offenbar deshalb, weil es sich nicht so bewährte, wie man erwartet hatte. Die Mehrzahl der Forscher ist allerdings der Ansicht, dass die Hg- und Hl-Probe, hauptsächlich aber letztere, alle bisherigen serologischen Verfahren in bezug auf Empfindlichkeit und Spezifität übertrifft. BRODHAGE [6,7] hält die Hg-Reaktion zur serologischen Ermittlung der Tuberkulose am geeignetsten, denn wenn sie auch nicht immer ein vollgültiges Resultat ergibt, so kann sie doch die Ergebnisse anderweitiger Untersuchungen ergänzen.

CREMER[11] misst der Hg-Reaktion bei der Beurteilung der Pathogenese chronischer Augenkrankheiten Bedeutung bei. Nach FÖLDES und KŐRÖSI [16] gibt die Hg-Reaktion Auskunft über die immunbiologischen Verhältnisse des Organismus. Bei 13,4% der Bevölkerung war das Ergebnis positiv, während die Hg-Positivität der Tuberkulosenkranken 65,9% übertraf.

Nach BUKOWINSZKY und Mitarbeitern [8] bietet die Hg-Reaktion eine gute Hilfe zur klinischen Bewertung des Zustandes und der Aktivität der Erkrankung. Auch ALMÁSSY [1] hält die Hg-Probe zur Feststellung der Tuberkuloseinfektion von Menschen und Haustieren für brauchbar. Laut BURGER [9] eignen sich die Hg- und Hl-Reaktionen zum Nachweis der fortgeschrittenen Tuberkulose von Rindern. In der europäischen Literatur äusserten sich ferner POPP [35], BOVET und Mitarbeiter [5], BERBLINGER [3], EINHUBER [12], HEIN und SROKA [24] sowie WIDMER [43] günstig über den diagnostischen Wert der Hg- und Hl-Reaktion. Andere Autoren, so HEIN [21], TAKEDA [39], KERTAY und SZABÓ-SZÜCS [29], beobachteten Titererhöhung der Hg- und Hl-Reaktion entsprechend der Progression des Krankheitsprozesses bei der Tuberkulose von Meerschweinchen und Rindern, SPECK und DEDIE [38] bei der von Fleischfressern. Nach FEILS [13] vermögen sich die allergischen und serologischen Proben gegenseitig zu ergänzen. HOLLENDER und Mitarbeiter [26] äussern sich zurückhaltender.

Im Gegensatz zu vorstehenden Autoren stehen in letzter Zeit immer mehr Forscher (MEISSNER und ORLOWSKY [32], POTHMANN und WILMS [36], FLESCH und Mitarbeiter [15] u. a.) dem diagnostischen Wert der Hg- und Hl-Proben erheblich kritischer gegenüber. KERTAY und SZABÓ-SZÜCS [29] erhielten bei der experimentellen Tuberkulose von Meerschweinchen in so hohem Prozentsatz [92%] positive Reaktion, dass das Ergebnis dem der Tuberkulinprobe beinahe gleichwertig war. Die mit Rinderblutproben im vorliegenden Versuch gewonnenen abweichenden Resultate sind offenbar auf den andersartigen Verlauf der Meerschweinchentuberkulose zurückzuführen.

Nach den in den Tabellen angeführten Einzelangaben sind zwischen den Ergebnissen der Tuberkulin- und Hl-Probe sowohl bei gesunden, wie bei tuberkulösen Menschen als auch bei Tieren wesentliche Unterschiede zu beobachten.

Auffallend ist das serologische Verhalten der Blutsera der tuberkulinnegativen Menschen und Tiere. Das Serum der tuberkulinnegativen Menschen ergab in 29% der Fälle, das der gesunden Tiere zu 51,2% positives Resultat. Ähnliches wurde auch von anderen Autoren beobachtet. In seiner zusammenfassenden Mitteilung stellte BRODHAGE [7] fest, dass das Blutserum von 15% der nichttuberkulösen Menschen positive Hg-Reaktion gab. FLEMING und Mitarbeiter [14] sahen ebenfalls bei 40% der Nichttuberkulösen ein positives serologisches Ergebnis. TÖRÖK [40] gewann bei 27,4% der tuberkulinnegativen 10—14jährigen Kinder mit der Verdünnung 1 : 16 und höheren Verdünnungen positive Hg-Reaktion, während die Hg-Probe bei 50,3% der Tuberkulösen negativ ausfiel. Laut FLESCH und Mitarbeitern [45] war das Blut tuberkulinpositiver Kinder bei Anwendung der Hg-Probe zu 54,3% negativ, das Serum der gesunden zu 12,9% positiv (Titergrenze 1 : 16). Bei den Untersuchungen von ADCOCK und Mitarbeitern [45] fiel die Hg-Probe bei 54,4% der aktiv tuberkulösen Menschen positiv aus, während 49,7% der auf Tuberkulin positiv reagierenden serologisch negativ waren. Dagegen gaben 13,4% der tuberkulinnegativen Personen positive Reaktion (Titergrenze 1 : 8). In den Untersuchungen von ARNOLD [2] waren bei Anwendung der Hg-Probe 38,4% der Lungentuberkulösen positiv, während laut YAGI [44] 20% der Tuberkulinpositiven auf die Hg-Probe positiv reagierten. Nach MEISSNER und ORLOWSKY [32] sowie HONKAPOHJA [22] reagiert ein hoher Prozentsatz der Lungentuberkulösen serologisch negativ ; der Hg- sowie Hl-Probe sei kein diagnostischer Wert beizumessen.

Die Erfahrungen ausländischer Autoren bei den mit Rindersera vorgenommenen Untersuchungen stimmen im wesentlichen mit den Feststellungen der ungarischen Verfasser überein.

In den durch Sektion nachgeprüften Fällen von SCHRÖDER [37] gab die Hl-Probe bei 80,9% der nicht tuberkulösen Schlachttiere ein positives Ergebnis, während sich 45,9% der tuberkulösen Rinder als negativ erwiesen. FEILS [13] beobachtete unter ähnlichen Verhältnissen bei der Perlsucht der Rinder (Tbc der serösen Häute) 96%ige, bei der Erkrankung mehrerer Organe 34%-ige und bei der Tuberkulose eines Organs 16%ige serologische Positivität. HARTWIGH und WEINHOLD [23] sahen bei 50% der tuberkulinnegativen bzw. zweifelhaft reagierenden Tiere mit der Hg- und Hl-Probe positive Resultate. BERLIK [4] vermochte im Blutserum von 85% der Kühe mit Eutertuberkulose hämolysierende Antikörper nachzuweisen. Nach GOURDON und HENRY [20] geben gesunde Tiere in hohem Prozentsatz positive, dagegen tuberkulöse oft negative Hg- bzw. Hl-Reaktion.

Nach unseren Untersuchungsergebnissen sowie den vorstehend angeführten Literaturangaben schliesst die Negativität der Hl- oder Hg-Probe bei der gegenwärtig gebräuchlichen Technik die Anwesenheit der tuberkulösen Infektion, ja auch eine Erkrankung nicht aus. Der Hl-Titer des Blutserums tuber-

kulinpositiver, ja auch an Tuberkulose erkrankter Menschen und Tiere erreicht jedoch in einem verhältnismässig hohen Prozentsatz der Fälle nicht den Grenztiter 1 : 8, d. h. sie sind serologisch als negativ zu bezeichnen. Zwischen der Tuberkulinpositivität und dem Hämolysetiter besteht kein Zusammenhang. Die allergischen Proben und die serologischen Reaktionen funktionieren offenbar nach unterschiedlichen Mechanismen. Mit den serologischen Verfahren werden bekanntlich die im Blut zirkulierenden humoralen, durch die intrakutane Tuberkulinprobe die an Zellen gebundenen sessilen Antikörper nachgewiesen. Bei den sehr mannigfaltigen Krankheitsformen (Schwankungen, Remissionen, Rezidiven) kann die Menge der im Blut kreisenden Antikörper im Laufe des abwechslungsreichen tuberkulösen Krankheitsgeschehen, je nach den damit zusammenhängenden und sich verändernden allergischen Zuständen hochgradig schwanken, was bei den stärker an die Mesenchymzellen gebundenen Antikörpern nicht der Fall ist.

Die Bedeutung der Funktionen der humoralen Antikörper im Tuberkuloseverlauf ist noch nicht geklärt. Die bis zur Negativität gehende Schwankung der Titerwerte, die bei den serologischen Untersuchungen in verschiedenen Stadien der Krankheit bzw. der Allergie gewonnen wurden, liess sich bisher nicht auf zufriedenstellende Weise erklären.

Die bei tuberkulinnegativen, d. h. mit Tuberkulose nicht infizierten Menschen und Tieren, in einem nicht zu vernachlässigenden hohen Prozentsatz in Erscheinung tretende serologische Positivität gibt dagegen Anlass zu Bedenken in bezug auf die Spezifität der Probe. LAGERCRANTZ [30, 31] hält die MIDDLEBROKschen Reaktionen vom Gesichtspunkt der tuberkulösen Infektion nicht für spezifisch ; bei seinen Versuchen reagierten mit säurefesten saprophytischen Mykobakterien beimpfte Meerschweinchen nicht auf Tuberkulin, während ihr Blutserum serologisch positiv war. ALMÁSSY sah in einem Fall von pseudotuberkulöser Darmentzündung (Enteritis paratuberculosa) eines Rindes positive Hg-Reaktion von hohem Titer. Andere Autoren, wie JOSIUKAS und Mitarbeiter [27], MIDDLEBROOK und DUBOS [34], vermochten im Blutserum von Versuchstieren, die mit anderen säurefesten Bakterien behandelt worden waren, mit der Hg-Probe Agglutinine nachzuweisen. FORSCHBACH und Mitarbeiter [17] sowie unter den ungarischen Autoren FLESCH und Mitarbeiter [15] ziehen bei der Erklärung der an tuberkulinnegativen Kindern beobachteten positiven Hg-Reaktionen auch die Rolle heterophiler Antigenen in Betracht.

Aus vorstehenden Ausführungen geht hervor, dass die serologischen Proben, d. h. genauer die Hl-Probe, — zumindest in ihrer heutigen Form — auf Grund immunbiologischer Erwägungen zur Feststellung der Tuberkulose als ungeeignet bezeichnet werden müssen. Zum selben Ergebnis gelangt man, wenn man die Frage technisch betrachtet. Es sei in diesem Zusammenhang lediglich auf die grossen Unterschiede in der chemischen Natur und Zusammen-

setzung der bisher verwendeten Antigene hingewiesen. Hinzu kommt noch, dass die chemische Zusammensetzung der sensibilisierenden Substanz noch umstritten ist (Polysaccharid? Lipopolysaccharidprotein?). Die Lösung dieser Fragen erfordert weitere Untersuchungen. Hiernach wird man erst ein serodiagnostisches Verfahren ausarbeiten können, das den allergischen Proben gleichwertig ist.

Zusammenfassung

Im Zusammenhang mit Studien über die Beziehungen zwischen menschlicher und tierischer Tuberkulose wurden die Einwohner und Rinderbestände einer Ortschaft auf Tuberkulosebefall untersucht. Um den praktischen Wert der serologischen Probe zu ermitteln, wurden die mit diesem Verfahren gewonnenen Ergebnisse mit den Resultaten der allergischen Probe verglichen. Neben der intradermalen Tuberkulinprobe gelangte die MIDDLEBROOKsche Hämolysereaktion zur Anwendung. Von den untersuchten Einwohnern erwiesen sich bei Benutzung von Alt-Tuberkulin in der Verdünnung 1 : 10000 53,4% mantouxpositiv, dagegen nach der Hämolysereaktion nur 27% als serologisch positiv.

Von den Tieren waren nach der Intradermalprobe 26,7% tuberkulinpositiv; nach dem Hämolyseverfahren dagegen 47,1%. Nur $^1/_3$ der Blutsera der an aktiver Tuberkulose leidenden Menschen und Tiere enthielt hämolysierende Antikörper. Von den mantouxpositiven Menschen waren 68,4%, von den tuberkulinpositiven Rindern 63,1% serologisch negativ, während sich 29% der mantouxnegativen Einwohner und 51,2% der tuberkulinnegativen Tiere bei der Hämolyseprobe als positiv erwiesen. Als positiv wurden die mit der Verdünnung 1 : 8 und höheren Serumverdünnungen gewonnenen Reaktionen gewertet.

Die Versuche ergaben, dass die MIDDLEBROOKsche serologische Methode in ihrer gegenwärtigen Form die allergischen Proben nicht zu ersetzen vermag und zum Nachweis der tuberkulösen Infektion von Menschen und Tieren nicht geeignet ist. In ihrer Spezifität und Zuverlässigkeit bleibt sie hinter der intradermalen Tuberkulinprobe zurück, sie entspricht nicht den praktischen Anforderungen, ja auch ihre Spezifität erscheint fraglich.

*

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SUGAR DECOMPOSING ACTIVITY OF SARCINA FLAVA IN CERTAIN PHASES OF ITS LIFE CYCLE

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Several observations have suggested the occurrence of certain differences in the biological characteristics of microorganisms regenerating from their filterable form, as compared to those of the original strain [2, 3, 9]. Thus, differences were found in Gram staining, in the lability of acid fastness of acid-fast bacteria [1, 6], in the colony morphology of the secondary colonies, in pigment production, in serological characteristics, etc. [6, 7, 14]. Differences in sugar fermentation are also frequent, consisting of enhanced fermentative activity as well as changes in the gas production [9].

In the early phases of regeneration, the sugar fermenting activity is often lacking. It usually reappears only stepwise, in the secondary colonies [7, 8]. Rather an exception than a rule is the phenomenon mentioned by KALINA [5], that an organism usually not decomposing sugar should exhibit a variety of sugar decomposing activities in the early phases of its regeneration from the filterable form. Parallel to the completion of regeneration, fermentative activities gradually disappeared. The present report deals with similar observations in connection with *Sarcina flava*.

Materials and methods

To obtain filterable forms, we used the method of ageing the cultures [10, 11]. A strain of *Sarcina flava* was inoculated in bouillon medium and incubated for some days at 37°C. This was followed by an additional incubation for 150 days at room temperature. The culture was filtered through a collodium membrane of 310 (± 10) mμ pore diameter. For regeneration experiments filtrates were dispensed to 20 serum bouillon and 20 chocolate agar media each. Regeneration was accomplished by the so-called protracted incubation method [9].

To avoid possible contamination by the ubiquitous natural *Sarcina flava* strains, all possible precautions, preliminary and control experiments were made. As to the details of these techniques and methods we refer to our earlier publications [9—13].

Experimental

Bacterium-free filtrates of *Sarcina flava* were inoculated into serum bouillon media. In one of the tubes the formation of a fine granular sediment was observed on the 5th day of incubation. Under the phase-contrast microscope the sediment appeared as irregularly grouped amorphous granules.

The organism thus isolated was transferred to chocolate agar medium where poor, small (0.5 to 1.0 mm), white colonies formed after 24 hours of incubation. No haemopepsis had occurred. Microscopic examination revealed small (0.5 μ) Gram-negative cocci and cocco-bacilli, some of them in pairs. The organism was designated as "substrain 1". Of the 20 sugars examined it decomposed glucose, saccharose, maltose, trehalose and d-mannose.

Substrain 1 was serially subcultured on the different media used in our earlier experiments [9, 10]. After some passage the appearance of a few transparent 0.5 to 1.0 mm colonies of marked haemopeptic activity was observed in certain subcultures. Microscopic examination of these colonies revealed polymorphous, aggregated cocci 0.5 to 2.0 μ in diameter [9, 10]. These organisms were designated as "substrain 2". They had a sugar fermenting activity essentially similar to that of "substrain 1". The only difference observable was the 14 days' delay in the decomposition of trehalose.

When serially subculturing pure lines of substrains 1 and 2 on chocolate agar medium, they underwent certain further changes.

From the unchanged colonies of substrain 1 the small cocci and cocco-bacilli gradually disappeared and were replaced by irregular groups of 4 to 8 somewhat bigger "sarcinoid" cocci. These organisms designated as "substrain 3" exhibited a markedly decreased sugar fermentation, as far as they decomposed only maltose and trehalose, the latter with a delay of 14 days. Substrain 3, when carried through further passages, gradually assumed an appearance characteristic of typical *Sarcina flava*, both as regards colony and cell morphology ("substrain 5"). Parallel to these changes the fermentative activity was entirely lost.

In cultures of substrain 2 the appearence of big, yellow colonies of typical Sarcines took place. These organisms ("substrain 4") have lost their abi-

Table I

Strains	Dextrose	Lactose	Saccharose	Maltose	Mannit	Arabinose	Xilose	Trehalose	Inosite	Raffinose	D-galactose	D-tartarate	D-sorbose	Adonite	Inuline	Salicine	L-sorbose	Dulcit	L-rhamnose	D-mannose
Original	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Substrain 1	+	—	+	+	—	—	—	+	—	—	—	—	—	—	—	—	—	—	—	+
Substrain 2	+	—	+	+	—	—	—	+14 days	—	—	—	—	—	—	—	—	—	—	—	+
Substrain 3	—	—	—	+	—	—	—	+14 days	—	—	—	—	—	—	—	—	—	—	—	—
Substrain 4	—	—	+14 days	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Substrain 5	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—

lity to decompose any sugar but saccharose, and even that only after 14 days. This activity was also lost on further passages.

A summary of the results described above is presented in Table I.

Table I reveals that the filterable form of *Sarcina flava*, while becoming a fully developed cell, loses its ability to decompose sugars. This phenomenon develops gradually and is intimely connected with the actual phase of regeneration. In certain cases the final loss of sugar decomposing activity is preceded by delayed fermentation.

Similar observations are very scarce in the literature. Further experiments are required to detect more details concerning the phenomenon.

Summary

Sarcina flava not known to decompose any sugar in its normal vegetative form was found to ferment certain sugars while regenerating from its filterable form. With the completion of regeneration, sugar fermentation was found to disappear gradually.

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AETIOTROPIC TREATMENT OF EXPERIMENTAL SHIGELLOSIS

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The use of modern aetiotropic preparations has fulfilled every hope in the treatment of infectious diseases, including dysentery. The more or less serious symptoms are usually rapidly relieved and the patient feels himself cured within a few days. In most cases the faeces soon become bacteriologically negative and remain so permanently. Notwithstanding the excellent results, the problem of dysentery cannot be considered solved. Instead of going into details we refer to two data in the literature. BODA *et al.* [1] state that chloramphenicol, the antibiotic most effective against dysentery, cures the disease in 55 to 65 per cent only and 5 to 10 per cent of the patients do not make a recovery. TROITSKY *et al.* [10] recommend combined immunochemotherapy not only in chronic, but also in acute dysentery. This problem, so important in clinical medicine and public health, cannot be solved before the causes responsible for the partial failure of aetiotrop measures have been elucidated.

Conjunctival infection and the keratoconjunctivitis shigellosa that can be elicited by it appeared suitable for such studies [7, 8, 17]. In the following we shall give an account of 300 treatments made in connection with 500 cases of keratoconjunctivitis shigellosa in 250 guinea pigs. The agents tested were chloramphenicol, oxytetracyclin, streptomycin, sulphamethythiazole and sulphaguanadine.

Methods

Treatment began at various point of time after conjunctival infection, usually when all the symptoms were doubtlessly present. In most cases antibiotics were administered over two days, as recommended by BODA *et al.* [1], but in some instances for 5 to 6 days. The preparations were applied mostly locally, into the palpebral fissure, but oral and intramuscular administration was also performed. In the overwhelming majority of cases the doses were computed from the scheme used in human therapy. For example, the daily dose of the broad-spectrum antibiotics was 50 mg/kg into each eye.

The experimental animals were subjected daily to clinical and laboratory examinations; the latter consisted in culturing conjunctival discharge in agar and DC medium. Animals simultaneously infected by the same method but not subjected to antibiotic treatment served as controls. Since permanent lesions may interfere with the exact determination of the time of full recovery from keratoconjunctivitis shigellosa, instead of complete reparation the subsidence

of the acute process was accepted as the criterion of clinical recovery. The end of the acute phase is marked by the cessation of discharge, relief of chemosis and a flattening of the swollen cornea.

The results of the experimental therapy were controlled by comparing the mean clinical and bacteriological sanation times of the different groups. As the same therapeutic measure may produce excellent results in some cases and moderate or poor results in others, the standard deviation of the mean recovery times showed considerable scattering in some cases (Table I). For this reason we have used also a further method of evaluation. Aetiotropic treatment can be considered successful when the pathogenic agent disappears rapidly from the conjunctival discharge in response to treatment. In the cases in which the ocular discharge turned negative 3 days after the discontinuance of treatment and remained permanently negative, the clinical symptoms also subsided soon and full recovery ensued shortly. Such cases have been termed "directly cured". On the other hand, the term "not directly cured" has been used to denote the cases of keratoconjunctivitis shigellosa in which the pathogen could be grown from the conjunctival discharge for 3 days or longer after treatment had been discontinued.

It is common practice to administer the daily dose of the antibiotics at equal intervals within 24, but not less than 16 hours [11]. For technical reasons we had to prescribe a less regular mode of administration, giving the daily dose evenly distributed within 7 to 8 hours. It was therefore necessary to compare the results attained by our method to those by the usual therapy. The pertaining results have been grouped according to recovery times in Table I. Of the

Table I

Results of continuous and discontinuous chloramphenicol therapy of keratoconjunctivitis shigellosa

Cf: No. 275 *S. flexneri* 2a, 1000 million germs
Duration of treatment: 2 days

Duration of daily treatment, hours	Mean recovery time, days after onset of disease		Standard deviation of recovery times	
	Clinical	Bacteriological	Clinical	Bacteriological
Experiment 1			*Onset of treatment:* 1st day of keratoconjunctivitis shigellosa. *Number of cases:* 20 in each group.	
24	12.1	10.5	7.86	9.60
7 to 8	11.0	9.2	3.24	5.01
Experiment 2			*Onset of treatment:* 3rd day of disease *Number of cases:* 10 in each group	
16	12.1	5.1	2.19	1.18
7 to 8	11.5	5.2	1.56	1.08
Untreated	20.8	18.0	1.40	2.79

30 cases of keratoconjunctivitis shigellosa, 20 were "directly cured" and 10 were "not cured directly" in the regularly treated group, as compared to our irregular treatment which resulted in the two figures 18 and 12, respectively. Thus, there was no significant difference in effect between the two modes of administration, as it has been shown also by GOTTSEGEN and PÁNCÉL [5] for the penicillin treatment of pneumonia.

The sensitivity of Shigella strains *in vitro* was studied by a method described earlier [9], except that the media were not plated out, but slanted (in 5 ml volumes) and were inoculated with a normal loopful of a one-day bouillon culture of the strain to be tested. In the case of antibiotics the basic medium was the usual broth-pepton-agar.

Results

Keratoconjunctivitis shigellosa will respond excellently to a properly chosen aetiotropic treatment and, if treatment is begun immediately after conjunctival infection, the disease may not develop at all. As a result of starting the treatment immediately after the development of ocular symptoms, the grave acute changes are rapidly relieved and the volume of discharge is soon reduced, sometimes to nil. The time required for ocular restitution is also shortened, but it has to be emphasized again that tissue reparation may not be complete for days after the subsidence of clinical symptoms. The bacteriological response to aetiotropic therapy is very rapid and sensitive. When treatment is adequate, a significant decrease in the Shigella count of the conjunctival discharge will be evident one day after the onset of treatment and the cultures soon turn negative. It can be stated that administration of the aetiotropic agent markedly reduces the time required for the clinical and bacteriological cure of keratoconjunctivitis shigellosa (Table I).

This result was attained by the use of any of the aetiotropic agent listed above. It has to be noted that the antibiotics in general, and oxytetracyclin in particular, proved to be local irritants, but with the exception of tetracyclin they caused no serious lesions. It was observed that guinea pigs are nearly as sensitive to oxytetracyclin as to penicillin. Keratoconjunctivitis shigellosa, however, responded well to the former drug, too.

The above-outlined effect of aetiotropic therapy is, however, not always so consequent. As illustrated by the example of the 236 cases of keratoconjunctivitis shigellosa treated locally with chloramphenicol, only about $^2/_3$ of the cases recovered within one week after onset of treatment and in the other $^1/_3$ the process was prolonged or turned chronic. In 3 cases recurrences were noted, whereas no recurrence occurred among the many hundreds of untreated cases. Fig. 1 also shows that in 12.5 per cent of the cases treated with chloramphenicol the pathogen could still be grown from the conjunctival discharge one week, or even several weeks, after the onset of treatment.

The observations made in connection with the aetiotropic treatment of keratoconjunctivitis shigellosa may be summarized as follows. *Like human dysentery, the disease responds well to aetiotropic agents, but treatment will fail in numerous cases.*

The causes of failure

Studies *in vitro* and clinical observations suggest that the failure of aetiotropic therapy may be due to several factors. Of these, we are going to deal with the following.

(i) *Resistance of the pathogenic agent.* In contrast with the view of some authors [2], it has been almost generally accepted that *in vivo* aetiotropic

agents are ineffective against the microorganisms resistant *in vitro*. As far as we know, in the case of dysentery this statement has not been confirmed by animal experiments. We have therefore investigated the effects of different aetiotropic agents on such cases of keratoconjunctivitis shigellosa as had been elicited by Shigella strains resistant to the given preparation *in vitro* (Table II and III). Of the 8 cases in which keratoconjunctivitis shigellosa had been

Table II

Results of sulphamethylthiazole treatment of keratoconjunctivitis shigellosa caused by sensitive and resistant pathogens

Cf: No. 272 (sensitive) and No. 53,538 (resistant) *S. flexneri*, 1000 million germs of each.

Onset of treatment: 4th day of disease
Number of cases: 8 in each group
Duration of treatment: 6 days
Mode of administration: local.

In vitro sensitivity of the pathogen	Mean recovery time after onset of disease, days		Standard deviation of recovery times	
	Clinical	Bacteriological	Clinical	Bacteriological
Sensitive	10.0	5.8	1.00	0.92
Resistant	15.0	13.6	2.45	2.35

Table III

Results of chloramphenicol treatment of keratoconjunctivitis shigellosa due to a resistant pathogen

Cf.: No. 787 (resistant to 100 micrograms/ml of chloramphenicol *in vitro*) *S. flexneri*, 1000 million germs.

Onset of treatment: 1st day of disease.
Duration of treatment: 3 days.
Number of cases: 10 in each group.

Groups of experimental animals	Mean recovery time after onset of disease, days		Standard deviation of recovery times	
	Clinical	Bacteriological	Clinical	Bacteriological
Treated	16.0	17.8	2.34	4.29
Untreated	15.6	14.6	3.03	2.26

caused by microorganisms sensitive to sulphamethylthiazole, all 8 were "directly cured", whereas in the case of agents resistant to that drug none of the cases was "directly cured" by the same treatment. Likewise, the 10 cases of keratoconjunctivitis shigellosa caused by chloramphenicol-resistant micro-

organisms and treated with chloramphenicol had to be classified as "not cured directly". Thus, aetiotropic therapy will fail whenever the pathogenic agent responsible for the disease is resistant to the drug used.

(ii) *Quantity of the pathogen.* Studies *in vitro* have shown that the bacterial count affects aetiotropic treatment and beyond a certain bacterial count there is no antibacterial effect. A similar correlation can be demonstrated also *in vivo*, if treatment by aetiotropic agents is begun during the latency period. Twenty guinea pigs were divided into two groups. The agent used for infecting the conjunctiva was the same strain of Sh. flexneri in both groups, but the infective dose was 50 million in one group and 1000 million in the other. Administration of chloramphenicol was in both groups started during the latency period. In the group infected with the smaller dose only a single eye developed the disease, as compared to the 10 eyes in the other group. Thus, in this relation there is an unquestionable correlation between the bacterial count of the infective dose and the effect of aetiotropic treatment.

Next, the effect of treatment begun after the latency period was studied. Using the same method and the same doses as above, two groups of 14 guinea pigs each were infected with S. flexneri and treatment with chloramphenicol was begun after the development of the symptoms. The control animals were infected but not treated. Of the 28 eyes infected with the smaller dose 17 were "directly cured". In 11 both bacterial and clinical recovery was delayed. In the group infected with the higher dose the results were better. 22 cases were "directly cured" and the disease ran a protracted course in 6 case only.

The significance of the actual microbial count developing by the time treatment is begun is apparently easy to demonstrate. In general, therapy lead to rapid success if treatment had been started at a time when only few Shigella colonies grew from the ocular discharge. It seemed therefore only natural to conclude that it is the actual microbial count which determines the success of the failure of causal therapy. Nevertheless, it is also unquestionable that aetiotropic therapy may lead to full success also when the ocular discharge contains a great number of viable Shigellae at the time treatment is begun. To decide the issue, 50 guinea pigs were infected conjunctivally and a normal loopful of ocular discharge was inoculated every day onto agar plates and the Shigella colonies that had grown were counted. On the third day following completion of chloramphenicol treatment the experimental animals were divided into two groups. Into the first group were included the animals in which treatment had been effective, *i. e.* in which no pathogens could be cultured from the ocular discharge. The second group contained the failures. Subsequently, we determined from the records the average colony count from one loopful of ocular discharge immediately before onset of treatment in the two groups. In the first group the average colony count was 360 ; in the second group 430 . The two values are not significantly different. This means that

factors other than the actual microbial count also influence the effect of therapy.

(iii) *The daily dose of the aetiotropic agent and the duration of administration.* Aetiotropic treatment will obviously fail to produce the desired results if the concentration of the antibacterial agent does not reach the focus and does not maintain there for the time required the concentration necessary to destroy the pathogens. We have found that failures are more frequent if

(a) the dose of the aetiotropic agent is too low ;

(b) treatment is discontinued too early, for example after 1 day ;

(c) the mode of administration is such that the required concentration cannot be attained at the site of the lesion. For instance, by mouth daily doses of 200 mg/kg of chloramphenicol were required to produce the desired effect, whereas on local application 50 mg/kg daily sufficed. The effect of the locally administered aetiotropic agent is clearly demonstrated if we infect both eyes but treat only one of them after the symptoms have developed. In such cases the properly treated eye will improve rapidly and heals 1 or 2 weeks earlier that the other.

Prolonged treatment (lasting for 5 to 6 days) does not ensure better results, just as it is the case in human dysentery [1]. Of the 27 cases of keratoconjunctivitis shigellosa treated with chloramphenicol for 5 to 6 days 22 were "directly cured" and 5 were "not cured directly".

Of the 210 cases treated with chloramphenicol for 2 days 154 were "directly cured" and 56 were "not cured directly".

Repeated treatment markedly improves the chances of treatment. In 23 of the above 56 cases "not cured directly" treatment was repeated. The 2-day course of chloramphenicol was repeated in 15 animals and streptomycin was administered to 8 after an interval of 2 to 3 days. After the second cycle only 2 animals of those treated with chloramphenicol were "not cured directly". These also recovered completely when a further short course was given after an interval of 2 to 3 days.

(iv) *The organism's immunological state.* Keratoconjunctivitis shigellosa leads to immunity [8], as it is reflected by the rise in the mouse-protecting antibody titre of serum and, on reinfection, by the increase in protection. Immunity is, however, only relative, as larger doses may induce keratoconjunctivitis shigellosa even in recovered animals. The effect of acquired immunity manifests itself also in recurrent disease, for example in the more favourable result of aetiotropic therapy as compared to its effectiveness on the first occasion. It is seen in Fig. 1 that in cases of keratoconjunctivitis shigellosa developing after reinfection the bacteriological findings turn finally negative within a few (at the most 4) days after the beginning of aetiotropic therapy and at the same time the clinical symptoms also subside.

Frequently, but not always, recurrent keratoconjunctivitis shigellosa heals faster, even without treatment, than the first disease [8], this means that exposure to shigellosis enhances the antebacterial powers of the organism. However, even during treatment of the first disease signs of a systemic antibacterial activity are often apparent. Cultures of the ocular discharge frequently remain positive after administration of the aetiotropic agent has been discontinued, and turn negative only after more or less time. In these cases the bactericidal power of the aetiotropic agent can be ruled out and the de-

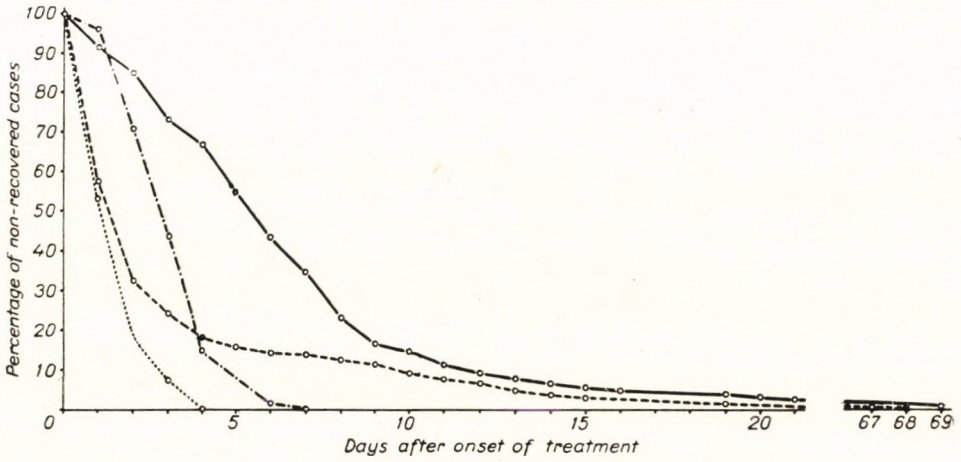

Fig. 1. Recovery from keratoconjunctivitis shigellosa on chloramphenicol treatment.

Explanations:

First disease (236 cases)
———— clinical recovery
— — — — bacteriological recovery
Recurrent disease (64 cases)
—·—·—·— clinical recovery
. . . . . bacteriological recovery

struction of the pathogen is due exclusively to the action of the antibodies of the host. In one series both eyes of 25 guinea pigs were infected with a *S. flexneri* culture and the 50 eyes were treated with chloramphenicol. FOSSIN *et al.* showed that *in vitro* chloramphenicol is bactericidal against *S. flexneri.* As late as three days after the discontinuance of treatment Shigellae could be cultured from 22 eyes in 1 to 9 occasions, all in all in 53 instances.

(v) *Time of treatment.* According to the universally accepted view, recovery from infectious diseases, including shigelloses, will be the faster the earlier treatment is begun. The failure of an otherwise effective therapeutic measure should be ascribed to the delay in beginning treatment. To investigate the significance of the time factor, both eyes of 14 guinea pigs were infected with 50 million, and both eyes of further 14 animals with 1000 mil-

lion, microorganisms from the same culture of *S. flexneri*. We started to treat with chloramphenicol both eyes of two animals from each group on the 1st, 2nd, 3rd, 4th, 6th, 8th and 10th day, respectively, leaving two animals in each group as controls. The results are presented in Table IV. According to the data

Table IV

Effect of the time factor on the results of aetiotropic treatment in keratoconjunctivitis shigellosa

Onset of treatment (day of disease)	Size of dose used for conjunctival infection			
	50 million S. flexneri		1000 million	
	Number of cases			
	"Directly		"Directly	
	cured"	not cured"	cured"	not cured"
1	3	1	2	2
2	4	—	2	2
3	4	—	2	2
4	1	3	4	—
6	2	2	4	—
8	—	4	4	—
10	3	1	4	—

in Table IV, the disease that had developed after infection with the smaller dose was usually cured more effectively by early treatment and responded poorly to aetiotropic therapy instituted at a later point of time. On the other hand, on infection induced by the larger infective dose the time factor had an opposite effect. These were not the only data to show that the time at which therapy is begun does not determine its effect. If we examine the role of the time factor independently of the size of the infective dose, it will be found that for example of the 70 cases of keratoconjunctivitis shigellosa in which treatment had begun on the first day of the disease 37 (53 per cent) were "directly cured", whereas of the 120 cases in which aetiotropic therapy had begun on the 6th day of disease 74 (57 per cent) were "directly cured".

While investigating why the time factor had a demonstrable influence in one group and not in the other, our attention has been directed to the changes in the parasite count with in the focus. If in the course of spontaneously healing keratoconjunctivitis shigellosa equal volumes (one normal loopful) of the ocular discharge are identically plated out and the Shigella colonies which have grown after 24 hours of incubation at 37°C are counted and the colony counts are shown on the ordinata, the curves obtained will show 4 types (Fig. 2). High peaks are formed when the parasite count is persistently high, when the balance between pathogen and systemic defense has shifted in fa-

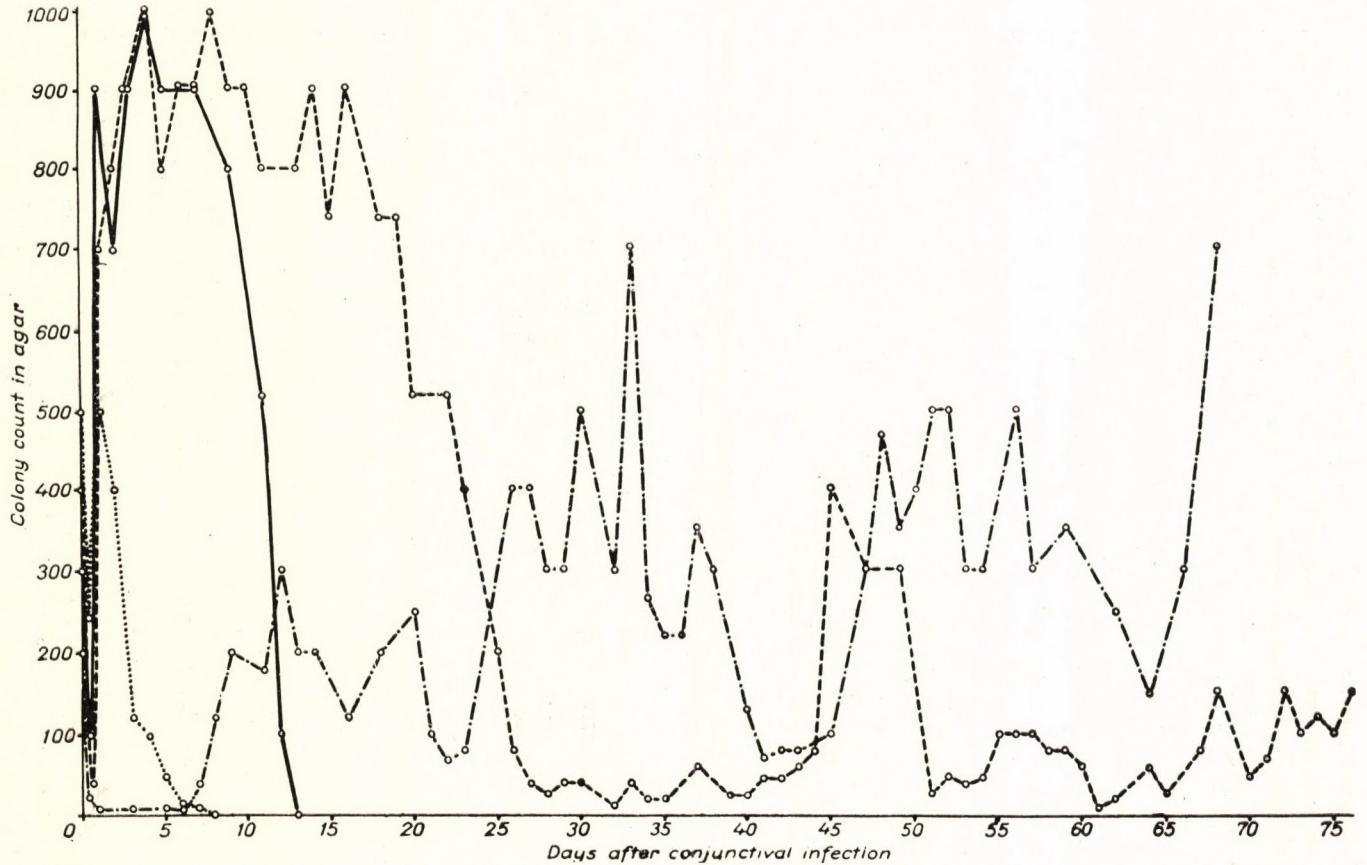

Fig. 2. Changes in the number of Shigella colonies grown from different cases of keratoconjunctivitis shigellosa

Explanations :

a.) ———— acute disease ; cf.: 1000 million *Sh. flexneri* germs.
b.) — — — — chronic disease ; cf.: 1000 million *Sh. flexneri* germs.
c.) — . — . — . — chronic disease, repeated infection ; cf.: 10 million *Sh. flexneri* germs.
d.) ..... acute disease, repeated infection ; cf.: 1000 million germs.

vour of the pathogen, whereas the deep and long falls in the curves represent the period of effective systemic defense. According to the curves, the aetiotropic agents will be most effective during the periods indicated by the falls. Thus, the time factor will play a role in those cases in which the parasite count is persistently low during the first days disease (curve *c*) or when at this time it undergoes a steep fall (curve *d*).

Discussion

It is believed that to explain our results we must devote attention not only to the parasite, but also to the host when analysing the mode of action of aetiotropic agents [12]. It has been shown for typhoid fever and brucellosis [3] that *in vivo* the aetiotropic agent has a bacteriostatic effect only and the pathogen is destroyed by the interaction between the aetiotropic agent and the systemic defense. The experiments of FOLEY revealed that *in vivo* the effect of tetracyclin requires an active systemic defense effort. On the basis of our results it seems justified to extend the above statements to the shigelloses. The role of the mouse-protecting antibodies in these diseases is well-known, but our investigations [17] suggest that beside humoral immunity also cellular defense plays a role. The significance of cellular immunity in shigelloses is still obscure, though the presence of antibodies in plasma cells has already been demonstrated [13, 14].

On the basis of the above, the effect of aetiotropic agents in shigellosis is explained as follows. During the disease the Shigellae show a tendency to unrestricted growth. This is checked by the aetiotropic agent, which makes it thereby possible for the systemic defense to kill off the pathogens. This explains the difference of the antibiotic effect *in vitro* and *in vivo* pointed out by MOSONYI [6]. Thus, the full success of aetiotropic therapy depends on the following criteria. The pathogen should be sensitive to the aetiotropic agent used. The aetiotropic agent should reach a concentration sufficient in comparison with the microbial count in the focus. The antibacterial forces of the host organism should be intensive. If any of these criteria are missing, therapy will not lead to success.

If the pathogen is resistant to the aetiotropic agent used, this will be unable to check growth and the disease will be cured only after the antibacterial antibodies have killed off the large numbers of Shigella in the infected focus. Under such circumstances the aetiotropic agent has no aetiotropic effect. This lends a certain interest to the sensitivity data of the strains of *S. flexneri* endemic in Hungary. (Table V and VI).

We have to emphasize that strains resistant to any of the aetiotropic agents used nowadays occur. The percentage of *S. flexneri* strains resistant to sulphonamides is especially high.

Table V

Grades of sensitivity to different antibacterial preparations

(According to WELCH)

Antibacterial agent	Inhibitory concentration			
	Highly sensitive	Sensitive	Moderately resistant	Resistant
	strains		strains	
Chloramphenicol	0.1—5	6—15 microgram/ml	16— 50	51—
Streptomycin		0—5 microgram/ml	6— 20	21—
Oxytetracyclin	0.01—1	2—5 microgram/ml	6— 20	21—
Sulphamethylthiazole		3—6 mg per 100 ml	7—100	101—
Sulphaguanidine		3—6 mg per 100 ml	7—100	101—

Table VI

Sensitivity of S. flexneri strains in vitro

Preparation	Number of		Number of		Total
	highly sensitive	sensitive	moderately resistant	resistant	
	strains		strains		
Chloramphenicol	93	—	—	7	100
Streptomycin	—	31	45	24	100
Oxytetracyclin	66	30	2	2	100
Sulphamethylthiazole	—	15	4	81	100
Sulphaguanidine	—	9	12	79	100

An other prerequisite of successful aetiotropic therapy is that a sufficient amount of the aetiotropic agent be administered over a sufficiently long period. To continue dosage for 5 to 6 days is aimless, and not only because of the well-known risks. It is also unreasonable because excessive doses, even when administered for a prolonged period of time, will also fail if systemic defense cannot annihilate the pathogen during the period of treatment. Repeated short courses promise a better effect and can be continued without any serious risk as long as bacteriological and clinical data make it necessary. A cyclic course allows time for the systemic defense to develop.

The most important factor in the success of aetiotropic therapy is the intensity of the systemic defense mechanism. If carried out properly, modern

aetiotropic therapy will achieve in every case that the number of pathogens in a focus should fall suddenly and steeply, but if the organism fails to kill off the surviving parasites, no recovery ensues, the Shigellae begin to multiply again and instead of recovery deterioration will follow. Thus the failure of modern aetiotropic therapy should be ascribed to a weakness of systemic defense.

In our opinion the time factor has little role in the therapeutic effect. Nevertheless, this must not be interpreted as meaning that it makes no difference at what point of time treatment is started. Therapy should be started as early as possible. We do not intend to go into the details of this question (to bring relief as soon as possible; to eliminate the source of infection as fast as possible, etc.). It should be borne in mind, too, that during the first few days of illness systemic defense may be very active. We have investigated the direct curative effect of the aetiotropic agent and not the quality of recovery. Nonetheless, our observations indicate that a treatment begun in the earliest possible phase of the disease will ensure recovery with the least permanent tissue lesions. By saying that the role of the time factor is insignificant we mean practically that a proper procedure will produce results even in the late stages of the disease. CSELEY and ÁRKOSY [16] have shown that chloramphenicol has a beneficial effect in any stage of typhoid fever.

Thus, aetiotropic therapy of shigelloses must be started as early as possible and an eventual failure should be ascribed not to the time factor, but, provided that the procedure employed meets modern standards, to a weakness of systemic defense. In such cases cyclic courses may still be effective, because this method allows time for the systemic defense to gain strength. To achieve complete success, however, the development of such immuno-chemotherapeutical procedures [10] may be required as can enhance the bactericidal powers of the organism.

Summary

The results of 300 treatments in 500 cases of keratoconjunctivitis shigellosa have been discussed. The agent used were chloramphenicol, oxytetracyclin, streptomycin, sulphamethylthiazole and sulphaguanidine. It has been shown that, like human dysentery, experimental shigellosis responds well to aetiotropic treatment, but in a considerable percentage of the cases the desired result cannot be attained. The failure of therapy may be due to the resistance of the pathogen ; an insufficient concentration of the therapeutic agent in the focus ; a too short duration of its presence in the concentration required ; the weakness of antibacterial systemic forces. Our results indicate that *in vivo* the aetiotropic agents act merely bacteriostatically and it is assumed that the destruction of the pathogen is the task of systemic bactericidal forces. A weakness of systemic defense must be blamed for the failure of aetiotropic therapy carried out by up-to-date methods. Cyclic courses of treatment and the introduction of immuno-chemotherapeutical methods are recommended.

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EVALUATION OF A NEW TYPE OF MUMPS VACCINE IN HUMANS

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The improvement of diagnostical procedures in mumps lead to the revision of the earlier opinion about the clinical importance of this disease. The increasing frequency of greater or smaller epidemics, the incidence of certain central nervous system complications, the sexual gland injuries, etc., rendered prevention a problem of increasing importance. The preparation of a specific prophylactic vaccine was endeavoured by several authors [1—9].

To determine the actual immune state of the subject to be vaccinated against mumps, several methods are known, such as skin tests and certain serological reactions [10, 11].

The present report will deal with immunization experiments carried out on military personnel with a new type of mumps vaccine.

Materials and methods

Vaccine. The vaccine was prepared by the *Virus Department* of the "*Human*" *State Vaccine Institute, Budapest*, according to the prescription of KOCH [9]. The essential steps of the preparation method were as follows. Pools of mumps virus were harvested from eggs infected with the *Enders* strain. After clarifying with low speed centrifugation, the virus was adsorbed to $Al(OH)_3$ gel *in statu nascendi*. After sedimentation by centrifugation with 3000 r. p. m. for 20 minutes, the sediment was resuspended in buffered saline, $^1/_{10}$ of the original pool volume. The gel adsorbed virus was then inactivated by the addition of 0.4 per cent formaldehyde. The final product contained 5×10^4 haemagglutinating units of virus and 0.8 mg of gel per ml.

Skin test. This test was performed by the intracutaneous injection of 0.1 ml each of the "Mumps Cutaneous Diagnostic" and of the "Mumps Control" materials produced by the "Human" Institute. Both of the materials contained 0.01 per cent final concentration of formaldehyde, so that intensive aspecific local erythemata were observed on the first day following the injection. Nevertheless, the differences between the diameters of the reactions caused by the control and the test material permitted a reliable evaluation of the results. Differences less than 10 mm were regarded as negative, while those exceeding that value as positive.

Serological tests. Haemagglutination inhibition (HI) and complement fixation (CF) reactions (the latter both with S [13] and V antigens) were carried out by the micromethod of TAKÁTSY [12]. Each reaction was regarded as positive if the titres exceeded 1 : 16.

Experimental

A group of 296 military personnel was examined by the skin test. Of them 157 (53.1 per cent) proved to be positive and 139 (46,9 per cent) negative.

These results, together with those obtained by examining the blood samples, are presented in Table I.

Table I

Mumps immunity in 296 military personnel, as revealed by different tests

Per cent	Reaction			
	HI	CF(V)	CF(S)	skin
Positive	31.6	1.6	7.6	53.1
Negative	68.4	98.4	92.4	46.9

The data presented in Table I agreed well with the history of the subjects, as none of them was known to have suffered from mumps recently.

Immunization experiments were performed in two groups, in part of the skin test negative individuals (Group I, 91 persons ; Group II, 13 persons).

Immunization of Group I was made by injecting 6 weeks apart two 0.5 ml doses of vaccine into the upper arm muscles. Blood samples were taken at the time of the first injection, on the 22th day following the first injection, at the time of the second injection (6th week) and 3 months after the first injection. At that time the skin reaction of the immunized persons was also reexamined. The results are presented in Table II.

Table II

Immune responses in Group I

Immunization	Blood sample	Per cent of positive reactions in			
		HI	CF(V)	CF(S)	skin
1st	0 week	20.4	0	5.2	0
	3 weeks	83.8	49.9	11.9	—
2nd	6 weeks	84.9	62.3	2.1	—
	3 month	97.8	96.7	77.3	79.2

Table III

Immune responses in Group II

Immunization	Blood samples, taken after	Per cent of positive reactions in			
		HI	CF(V)	CF(S)	skin
Single dose	0 week	30.7	0	0	0
	3 weeks	69.2	38.4	7.6	—
	3 months	100.0	84.6	46.1	91.7

Immunization and blood sampling in Group II was made as in Group I, with the only difference that Group II was given only a single 0.5 ml dose of vaccine. The results are presented in Table III.

Both Table II and Table III reveal the development of a remarkable immunity, as determined by different serological tests and by the skin test.

Discussion

When examining sero-immune responses to the injection of a gel adsorbed, concentrated, formaldehyde inactivated mumps virus vaccine, it was thought important to use most reliable serological reactions. It was therefore concluded to perform all the three reactions in parallel. The results appeared to confirm those of HENLE *et al.* [8] concerning the seroimmune responses to an appropriate amount of antigen.

An advantage of the vaccine used was that a small amount (0.5 ml) of it sufficed to provoke an immunity not inferior to that obtained by HENLE. To support the above statement the presentation of some of our earlier data [14] seems to be relevant.

We have found that gel adsorbed mumps vaccine, if given in a single 0.2 ml dose (10 000 haemagglutinating units of formalized virus) at the onset of an epidemic afforded 50 per cent protection as calculated from the morbidity rate of vaccinated (244 persons) and control (1044 persons) groups. Skin tests were positive in 79 per cent of the vaccinated individuals, while the mean positive antibody titre incidence, as measured by the three serological reactions, was only 35 per cent. These results are in agreement with those of HENLE *et al.* [8], PETTINEN *et al.* [15], and also of some other authors who used large amounts of differently prepared, concentrated unadsorbed vaccine.

In the present study no epidemiological evaluation of the effect of vaccination was made. The serological and skin reactions suggest the larger amount of antigen (0.5 ml single or double dose) to be superior in initiating immunity to the smaller (0.2 ml) one used earlier. With one 0.5 ml dose of gel adsorbed, inactivated vaccine a rapidly developing and long lasting immunity seems to be attainable. As the vaccine is easy to prepare, it appears to present several advantages over earlier preparations.

Summary

The immunogeneity of a gel adsorbed, concentrated, formaldehyde inactivated mumps vaccine has been tested in humans.

In Group I, 91 skin test negative individuals were inoculated with two 0.5 ml doses of the vaccine, six weeks apart. In Group II, 13 persons were vaccinated with a single dose of 0.5 ml.

Immune responses were determined by testing the HI, CF(S), CF(V) antibody content of blood samples taken at the beginning of immunization, in the third and sixth weeks and after 3 months, when the skin test was repeated.

In both Group I and II rapid and lasting immunity developed in 3 weeks without remarkable loss of titre up to the end of the third month. The incidence of skin positivity markedly increased following immunization.

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STUDIES ON THE LIPIDS OF INTESTINAL BACTERIA*

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Very little is known about the physiological (biological) role of bacterial lipids. The pertaining data in the literature are few and often contradictory, and have been obtained by divergent methods. Nonetheless, we attribute importance to these lipids for two reasons. On the one hand, there exists a certain correlation between the lipid content and the resistance of some bacteria, such as *Mycobacterium tuberculosis*. On the other hand, the lipids, being basic components of the cell wall or the cytoplasmic membrane, presumably influence the regulation of cell permeability. Also, certain bacterial lipids, for example the TB. phosphatides, contribute substantially to the effect of bacteria on tissues.

ASSELINEAU and LEDERER [1] found that the lipids of bacteria are more complex than those of higher animals. Characteristically, they contain relatively little glycerol and no sterols at all. Besides neutral fatty acids, they are rich in free fatty acids of high carbon number (32, 50, 80). They often contain beta-oxy-acids. Their phosphatides are often choline-free. The lipids combine with the different polysaccharides, inosito-glycero-diphosphoric acid, etc., by complex linkage. According to WESTPHAL [2], the complexes form giant molecules with molecular weights around 1 000 000.

The investigations concerned with bacterial lipids often lead to divergent results, partly owing to differences in the method of culturing, as well as in the methods of determination. ECKSTEIN [3] extracted lipids from *E. coli* with alcohol, ether, then with chloroform and analysed the pooled extracts. In contrast with this, BLOOR [4] extracted with warm alcohol, then with ether and analysed the pool of the two extracts. WESTPHAL [5] extracted the lipoprotein and lipopolysaccharides of Gram-negative intestinal bacteria by treatment with phenol water. The lipids of *Salmonella* were studied in some detail by BOIVIN [6] and CMELIK [7], after warm extraction with alcohol, alcohol-ether and acetone, respectively. They found that the Gram-negative bacteria

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contain significantly more lipids (5 to 10 per cent) than the Gram-positive microorganisms (1 to 2 per cent).

In a previous study [8] we showed that of the Gram-negative intestinal bacteria those resistant to chloramphenicol contain more of the ether-soluble lipids than the sensitive variants. In resistant bacteria the rise in lipid content was proportionate to the degree of resistance, *i. e.* the ether-soluble lipid content of resistant cells increased with the increase in the resistance to antibiotics. In view of this change it seemed interesting to investigate the other soluble lipids of bacterial pairs.

Materials and methods

The chloramphenicol-resistant and-sensitive *E. coli* O : 111 and *S. paratyphi B* bacteria were grown in Kolle flasks in an agar culture medium. The 24-hour cultures were washed off with physiological saline and centrifuged. Washing with physiological saline, sterile distilled water and centrifugation were followed by drying at 102°C for 24 hours. Ten to 12 g of the bacterium (dried to constant weight) were finely pulverized and extracted with hot solvent fumes in a modified Soxhlet apparatus. Before the experiment, the empty extracting shell had been extracted for one hour with each of the solvents used. The bacteria were extracted first with petrol-ether (boiling point 35° to 37°C) for 8 hours, then with ether for 8 hours and finally with chloroform for 16 hours. After extraction was complete, the extract was poured into previously weighed dishes, rinsing the flasks 4 times with 5 ml of solvent. The solvent was then evaporated and, after constant weight had been attained in an exsiccator, the dish was weighed on analytical scales.

The extraction with three different solvents was intended to achieve separation of the lipids into different fractions. Petrol-ether extracted the most accessible lipids. By extraction with ether new, more strongly bound lipid fractions were obtained. Chloroform, which has at higher boiling point and is more polar, dissolved the greatest amount mainly of lipids rich in phosphatide and in complex linkages.

We also investigated the quality and quantity of the extract obtained by treatment with chloroform alone. It has been found that there was no difference in quantity and composition between the chloroformic extracts and the pooled extracts obtained by the above fractionation from the same bacterial culture. For this reason, extraction with chloroform was carried out when pooled extracts were studied.

Phosphatides were determined by acetonic precipitation. MACLEAN [9] in 1927 employed precipitation with $CdCl_2$ from alcoholic solutions (complex metallic salts). This method has been abandoned, since phosphatides are degraded by it. BLOOR [10], BULL [11], BORGSTRÖM [12] and CMELIK [13] estimated the phosphatides in bacterial lipids by acetonic precipitation in the presence of $MgCl_2$. Originally, this method had been developed for the estimation of lecithin, but was used also for determination of the more complex phosphatides in bacteria. From the total phosphatides those of the cephalin type were determined according to LEVENE and ROLF [14], as well as PARNAS [15] and BULL [16], by dissolving the phosphatides in 2 ml of petrol-ether and precipitating the cephalin-type phosphatides with 20 ml of absolute alcohol, standing overnight in a refrigerator, centrifugation, washing with absolute alcohol, drying and gravimetric determination.

Nitrogen and phosphorus were also determined in the lipid fractions. Nitrogen was estimated by the Kjeldahl method, in a Wagner—Parnas micro-apparatus. After incineration phosphorus was determined with ammonium molybdate in the presence of ascorbic acid, using a Beckmann spectrophotometer.

Results

The results for the lipid fractions in the chloramphenicol-sensitive (2 micrograms/ml) and *in vitro* chloramphenicol-resistant (200 micrograms/ml) variants of *S. paratyphi B* are presented in Table I.

Table I

Lipid content of chloramphenicol-sensitive and -resistant S. paratyphi B strains, in percentage of dry-weight

Lipid	Sensitivity to chloramphenicol	SOLVENT			Total
		Petrol-ether	Ether	Chloroform	
Total lipid	S	1.92	0.87	1.63	4.42
	R	2.25	0,98	2.22	5.45
P content	S	0.001	0	0.027	0.028
	R	0.003	0.001	0.053	0.057
Phosphatide	S	0.099	0.070	0.915	1.084
	R	0.140	0.108	1.422	1.670
Cephalin	S	0.017	0.001	0.266	0.284
	R	0.037	0.003	0.538	0.578

S = sensitive
R = resistant

The quantity of soluble lipids was higher in every fraction in the resistant bacteria. The total lipid content of the susceptible cell was 4.24 per cent, that of the resistant cell 5.45 per cent. This represents a difference of 23 per cent. This is not much and, in spite of the fact that the means of repeated tests did not vary by more than 5 per cent and that the shift was unequivocal in all of them, that difference alone did not reveal more than what had already been known from earlier work. There was, however, a much more characteristic difference in the phosphorus content and the composition of the lipids extracted from the two kinds of bacteria. The lipids of resistant bacteria contained twice as much phosphorus as those of the susceptible microorganisms. This indicate that, apart from a slight quantitative change, the lipids of the bacteria which had become resistant underwent a more characteristic qualitative alteration. The amount of phosphorus-containing lipids, the so-called phospholipids had apparently increased in the resistant cells. These data are shown in the third row of Table I. It is obvious that resistant cells contained significantly higher amounts of phosphatides than sensitive ones. The difference was even more marked in the case of the cephalin-type phosphatides, the amount of which was doubled in resistant cells.

Table II contains the results for the chloramphenicol-resistant and chloramphenicol-sensitive *E. coli* O : 111 strains. Here the difference was much more pronounced. Beside an insignificant increase in the total lipid content, the phosphorus content was trebled in the resistant microorganism; correspondingly, the amount of total phosphatide was nearly trebled and the quantity

Table II

Lipid content of chloramphenicol-sensitive and resistant E. coli O : 111 strains, in percentage of dry weight

Lipid	Sensitivity to chloramphenicol	SOLVENT			Total
		petrol-ether	ether	chloroform	
Total lipid	S	1.46	0.90	1.55	3.91
	R	1.28	1.03	1.91	4.22
P content	S	0	0	0.016	0.016
	R	0	0	0.048	0.048
Phosphatide, total,	S	0.013	0	0.458	0.471
	R	0.040	0.036	1.112	1.188
of this Cephalin	S	0	0	0.035	0.035
	R	0	0	0.378	0.378

S = sensitive
R = resistant

of cephalin-type phosphatide was 10 times more than in the sensitive bacteria. Thus, the bacteria showing resistance *in vitro* differed from the sensitive ones in their higher phosphatide content and, within that, in the significantly higher cephalin-type phosphatide content.

Table III

Lipid content of chloramphenicol-sensitive and -resistant microorganisms

Microorganism	Lipid	Phosphatide	Cephalin
a) Lipid content, g/100 g dry weight of material			
E. coli O : 111 strains			
Strain No. 5, sensitive	3,23	1,050	0,082
Strain No. 102, resistant *in vivo*	4,43	2,351	0,898
S. paratyphi B.			
Strain No. 2, sensitive	4,38	0,954	0,266
The *in vivo* resistant variant of strain No. 2.	5,25	1,829	0,538
b) Lipid content of resistant strains in percentage of the lipid content of sensitive strains			
E. coli O : 111 strains			
Strain No. 102/5	137	224	1092
S. paratyphi B	120	192	202

In Table III are shown the data of lipid analysis for a chloramphenicol-sensitive *E. coli* O : 111 strain freshly isolated from a patient and for a strain *in vivo* resistant to chloramphenicol and to the other broad-spectrum antibiotics. To facilitate comparison, we present the lipid-composition data for a sensitive and for an *in vitro* chloramphenicol-resistant strain of *S. paratyphi B.* The remarkable feature in this case, too, was that in the *in vivo* resistant *E. coli* O:111 strain more than 50 per cent of the lipids were phospholipids, of which 38 per cent cephalin-type phosphatide. At the same time, only 32 per cent of the lipids of the sensitive strain were phospholipids, of which only 8 per cent were cephalin-type. In the *S. paratyphi B* bacteria a similar, though less marked, change occurred.

The studies were repeated on cultures of sensitive and resistant *E. coli* strains grown in liquid medium in fermentors of 15 litres capacity each. In this case, too, it was the rule that the phosphatides, and within them the cephalin-type phosphatides, increased in the resistant bacteria. The only difference from the results obtained by growth in solid medium was that the quantity of the first (petrol-ether) fraction was somewhat greater in the sensitive and the resistant bacteria alike. It seems that the higher lipid content in the liquid culture medium may be ascribed to a more intense flagellum formation.

It is known that *Streptococcus haemolyticus* is extremely sensitive to antibiotics, and to all broad-spectrum ones. Resistance to antibiotics cannot be induced *in vivo*, and only quite exceptionally *in vitro*, by a most difficult adaptation process. It seemed, therefore, interesting to investigate the quantity and composition of lipids in such a bacterium, in the light of the evidence obtained for the surface lipids of Gram-negative bacteria. A strain of *Streptococcus haemolyticus* "*A*" was grown in a liquid medium in a 15-litre fermentor. After the above-described treatment had been carried out, the soluble lipid, phosphatide and cephalin contents were determined. The total lipid content was 1 per cent. In this quantity the cephalin-type phosphatides did not amount to more than 2 per cent. It seems that these bacteria fail to develop resistance to antibiotics because their metabolic responses to environmental effects are dominated not by the formation of lipids, but by a process forming a hyaluronic acid capsule, as it takes place *in vivo*.

We have determined the nitrogen and phosphorus content in the fractions from sensitive and resistant *E. coli* O:111 strains, as well as in the phosphatides. The results are shown in Table IV.

It can be seen that phosphatides of the resistant cells are richer in both phosphorus and nitrogen than the sensitive cells.

The N content of the single lipid fractions increased, *i. e.* it was always higher in the chloroformic fraction than in the petrol-ether extract and it was invariably higher in the resistant variant than in the sensitive one. In

Table IV

Percentage N and P content of the phosphatides of chloramphenicol-sensitive and -resistant E. coli O: 111 strains

	Sensitive	Resistant
N content	2.06	3.26
P content	2.10	3.81

the course of extraction, especially in the case of the etheric extract, a substance insoluble in petrol-ether, but soluble in water separated. Also, on redissolving the precipitated phosphatides it was found that a smaller amount of insoluble material had separated from them. Chromatography after hydrochloric acid hydrolysis showed the separated materials to contain a great amount of amino acids. Assumedly, in such cases peptides loosely linked to lipids had split off and these substances combined more firmly with the lipids of resistant bacteria.

Discussion

Capsule formation in noxious environment is a well-known feature of bacterial aggressivity and self-protection. In the human or animal organism the Anthrax bacillus forms this capsule from amino acids (glutamic acid), the Pneumococcus from carbohydrate, the Streptococcus from polysaccharide, etc. Earlier [8] and the present results suggest that the Gram-negative intestinal bacteria develop resistance to antibiotics on the basis of similar biological changes and processes. The noxious agent, the antibiotic, contacts the cell at the surface ; accordingly, it is only logical that the changes serving self-preservation should take place mainly on the surface. The rearrangement will affect the surface lipids, because it is the high lipid content of the surface and the much more intense lipid metabolism that distinguish this group of bacteria from the Gram-positive microorganisms. The quantitative and qualitative increase in cellular lipids results not only in an increase of the overall bacterial resistance, but affects also the changes in specific resistance, for example that to antibiotics. The role of lipids in the ability of the single antibiotics to invade the cells is suggested by the observation made by COGHILL *et al.* [17], and ABRAHAM *et al.* [18] that a close parallelism exists between the activity and lipid-solubility of the different antibiotics, *e. g.* the different penicillins. According to FEW and SCHULMAN [19], it is the lecithin in the cell wall which forms the layer permitting the passage of penicillin into the cell. When the phospholipid ratio in the cell wall changes at the expense of lecithin or of the lecithin-type phosphatides, it becomes difficult for the anti-

biotic to penetrate the cell, or, in other words, the resistance of the cell increases. Quantitative and qualitative changes in the surface lipids thus serve to enhance the protection of bacteria against antibiotics, which substances are hindered in reaching the vulnerable cellular component through the altered cellular barrier.

Summary

(i) The composition of lipids extractable with petrol-ether, ether and chloroform from chloramphenicol-sensitive and -resistant strains of *S. paratyphi B* and *E. coli* O : 111 has been studied.

(ii) The chloramphenicol-resistant variants of both species of bacteria contained more lipid than the sensitive ones.

(iii) The lipids of the -resistant bacteria were found to differ from those of the sensitive ones. The lipids of resistant bacteria contained significantly more phosphorus and phosphatides. Among the phosphatides the amount of the cephalin-type phosphatides was greatly increased.

(iv) A similar change of lipid composition in favour of the phosphatides and cephalin occurred in *E. coli O : 111* strain resistant *in vivo* to antibiotics.

(v) The phosphatides of resistant bacteria contained about 1½ times as much nitrogen and phosphorus as those of the sensitive microorganisms.

(vi) As compared to the former strains, the strain of *Streptococcus haemolyticus "A"* studied contained very little lipid, phosphatide and cephalin.

(vii) It is suggested that the change in the composition of surface lipids is responsible for the increased resistance of bacteria to antibiotics.

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A QUICK METHOD FOR TESTING ANTIBIOTIC SENSITIVITY USING PAPER STRIPS IMPREGNATED WITH TRIPHENYL TETRAZOLIUM CHLORIDE

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Triphenyl tetrazolium chloride (TTC) has been widely used for investigating oxidation-reduction changes in metabolic processes. For testing sensitivity to antibiotics, TTC was recommended by RAJAM and ADCOCK [1], later by NEAL and CALBERT [2]. The former method, carried out by using 24-hour cultures and fluid medium, is time consuming for routine laboratory examinations. In Hungary, VÁCZI *et al.* used TTC for investigating the mode of action of bacterial enzymes [3]. SCHÖNBERG [4—7], SCHÖNBERG and KRAUS [8, 9], KRAUS [10] and LIENHOP [11] worked with media containing TTC for examining pollution of foods.

In the present experiments antibiotic sensitivity was determined prior to the actual isolation of the pathogenic organisms.

Materials and methods

Pathological materials (sputum, pus, urine, etc.) were streaked directly on agar plates. The inoculated media were dried in an incubator at 37°C for 10 to 15 minutes, then paper discs impregnated with antibiotics were placed on the surface of the plates. The discs contained the drugs at the following concentration:

Penicillin G,	5 units
Streptomycin,	50 μg
Aureomycin,	50 μg
Terramycin,	50 μg
Chloromycetin,	30 μg .....[12]
Polymyxin,	5, 10 or 30 units
Sulfathiazole,	100, 300 or 500 μg
Sulfamethylthiazole,	100, 300 or 500 μg
Tetracyn,	10, 30 or 50 μg..... [13, 14]

Subsequently, the plates with the discs were incubated at 37°C for 4 hours (first incubation). Then paper strips impregnated with TTC were placed over every antibiotic disc. Readings were taken after incubating the plates for another hour at 37°C (second incubation).

These strips, 25 × 3 cm in size, were made of Macherey-Nagel 214 filtre paper and were impregnated with the solution described by RAJAM and ADCOCK [1]: TTC, 0.5 g ; sodium succinate, 2 g ; sodium chloride 0.85%, 100 ml. After impregnation they were dried hanging at room temperature for 6 hours. Then the paper was cut into small strips 3 × 0.6 to 0.7 cm in size. The strips prepared in this way could be stored in the refrigerator for three months.

Experimental

TTC is reduced by bacteria into red formazone. If bacteria sensitive to the corresponding antibiotic are present, they will give rise to a red colour only outside the zone of inhibition. Resistant bacteria produce redding at various distances from the central part of the paper, according to the degree of resistance. The strips are coloured at a very early stage of growth, when colonies are still unvisible to the naked eye. The red colour of the strips appears earlier on the surface facing the medium. Prolongation of the second incubation period not only produces a red colour in the strip, but also the appearance of a red line beside it. According to the distance between the redding and the central part of the paper strip, various degrees of antibiotic sensitivity can be distinguished, as follows:

(i) very sensitive, 15 mm ;
(ii) sensitive, 10 to 15 mm ;
(iii) moderately sensitive, 10 mm ;
(iv) slightly sensitive, 5 mm ;
(v) resistant, with the colour developing in the central part of strip.

These incubation times were established in preliminary experiments by determining the incubation period yielding the most sensitive results. The experiments were carried out on 210 materials, each streaked on three plates. One of the plates served for testing sensitivity by the quick method under discussion. As a control, on the remaining two plates, only antibiotic discs and TTC strips, respectively, were placed. These control discs showed (1) whether without the TTC strip the result of the examination was the same after 24 hours ; and (2), whether multiplication of bacteria was inhibited by TTC.

As to the latter circumstance, it was found that placing TTC strips on the plate immediately after inoculation, an area of inhibition appeared which corresponded to the shape of the strip.

TTC was bacteriostatic to the organisms most frequently isolated from routine materials (*Staphylococcus aureus*, *Staphylococcus albus*, *E. coli*, *B. proteus*, *Ps. pyocyanea*, *Streptococcus*, *Pneumococcus*).

The longer the period between inoculation and placing the TTC strips, the smaller the area of inhibition. After a minimum period of 3 hours the reagent did not influence the multiplication of bacteria. The first incubation period of the quick method had therefore to last not less than 3 hours. By incubating the plates for 4 hours greater accuracy was obtained, and the growth of organisms was sufficient to reduce TTC.

Of 210 materials sent to this laboratory, antibiotic sensitivity was determined in 164. In 30 cases no bacteria were cultured. In 16 instances bacteria were isolated from the material, but no reaction developed.

For testing organisms not growing on simple agar the method can be performed also on other media, such as blood agar. Agar plates are, however, more advantageous in that they can be read ½ hour sooner than blood agar plates, by inspecting the inner surface of the strip, the one in contact with the medium. With blood agar, which is not transparent, the result must be read from the upper surface of the TTC strip. Thus testing will take 5½ hours.

Discussion

By using solid media for testing sensitivity to antibiotics the possibility of certain errors has to be considered. A lack of colour on the TTC strip may be due to the following.

(ia) Bacteria cannot be cultured from the material ;
(ib) the material to be examined contains anaerobic bacteria.
(ii) Growth of bacteria occurs but no red colour appears when
(a) the number of viable cells is very low ;
(b) the diffusion of bacterial products into the medium is not adequate ;
(c) some other chemical reaction takes place in the reagent ;
(d) the lag phase of bacteria is protracted ;
(e) the biochemical activity of the organisms is insufficient.

To (iia), it has to be noted that when examining materials from which only a few colonies can be cultured the testing of sensitivity must be made by the usual methods.

Using the present method the physician could be informed of the antibiotic sensitivity of organisms 5 hours after the pathological material had been received in the laboratory. The isolation of pathogenic bacteria and testing their sensitivity to antibiotics in the usual manner were carried out parallel with the quick sensitivity testing method. The two methods of testing yielded the same result in most of the cases. Of discrepant results the physician should be immediately informed.

Summary

By the use of paper strips impregnated with triphenyl tetrazolium chloride a method has been worked out for testing sensitivity of bacteria to antibiotics from the original pathological material. Placing on inoculated agar plates the strips over discs impregnated with antibiotics the red colour of reduced tryphenyl tetrazolium chloride shows the zone of inhibition within 5 hours of incubation.

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BEITRÄGE ZUR SYMPTOMATOLOGIE DES SHWARTZMAN-PHÄNOMENS

Von

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In früheren Versuchen [1] hatten wir festgestellt, dass bei der Entwicklung des Shwartzman-Phänomens konsequenterweise Leukopenie auftritt, während es in der Erythrozytenzahl, im Gesamteiweissgehalt des Serums und in den einzelnen Serumeiweissfraktionen zu keiner bewertbaren Veränderung kommt. Nach unseren Ergebnissen beeinflussen demnach die das Shwartzman-Phänomen herbeiführenden wiederholten Endotoxinmengen den Eiweisshaushalt entweder überhaupt nicht oder nur sehr geringfügig, und auch auf den Wasserumsatz sind sie ohne wesentliche Wirkung.

Nachdem aus den Untersuchungen von DELAUNAY, BOQUET und Mitarbeitern [2, 3] bekannt geworden ist, dass die Endotoxine tiefwirkende sympathomimetische Reaktionen hervorrufen, schien es von Interesse, unsere eine Ergänzung der Symptomatologie des Shwartzman-Phänomens bezweckende Versuchsserie auf die Untersuchung des Verhaltens des Salz- und Zuckerstoffwechsels auszudehnen.

Methodik

Zur genauen Auswertung der Intensität der an verschiedenen Tieren herbeigeführten Shwartzman-Phänomene wandten wir die unsererseits ausgearbeitete quantitative Shwartzmansche Reaktion an. Ebenso wie bei der Titrierung des Jensenschen Diphtherie-Toxins wurde die depilierte Rückenhaut des Kaninchens in 6 etwa 15—20 cm^2 grosse Flächen eingeteilt und dann als vorbereitende Impfung in die einzelnen Flächen jeweils 0,1 ml verschiedener Verdünnungen (konzentriert — 1 : 32) des sterilen $\overline{aa}$-Filtratgemisches der 48stündigen Bouillonkultur von Dyspepsia coli-Stämmen mit der Antigenstruktur O : 111 : B_4, O : 55 : B_5 und O : 26 : B_6 intrakutan eingespritzt. Nach 24 Stunden erfolgte die Auslösung der Reaktion durch die intravenöse Zweitinjektion von 2 ml/kg Körpergewicht des konzentrierten Filtrats. Die Reaktion wurde 4 Stunden nach der provozierenden Impfung bestimmt. Das hervorgerufene Shwartzman-Phänomen ist in Abb. 1 zu sehen.

Auf Abbildung 1. sind die der Stelle der vorbereitenden Injektionen mit der konzentrierten Lösung und den Verdünnungen 1 : 2, 1 : 4, 1 : 8, 1 : 16 und 1 : 32 ensprechenden nekrotischen Flecke deutlich erkennbar; die die Nekrosen umgebenden hyperämischen Gebiete treten weniger scharf in Erscheinung. Sowohl die hyperämischen wie die nekrotischen Gebiete wurden auf Cellophanpapier kopiert, mit Hilfe eines Planimeters ihre Flächen bestimmt und sodann die Grösse der bei den einzelnen Tieren festgestellten nekrotischen bzw. hyperämischen Gebiete summiert. Gleichzeitig wurde auch bei sämtlichen Versuchstieren der sog. Titer des Coli-Filtrates, d. h. diejenige grösste Verdünnung festgestellt, bei deren Anwendung als vorbereitende Impfung an der Einspritzungsstelle noch Nekrose aufgetreten war.

Es gelang uns, das Shwartzman-Phänomen auf diese Weise bei 19 von 20 mit Standarddiät gefütterten, 2,5—4 kg schweren normalen männlichen Kaninchen auszulösen. Nach unseren Resultaten steht die Grösse der nekrotischen bzw. hyperämischen Gebiete, in cm^2 ausgedrückt, bei den verschiedenen Kaninchen im umgekehrten Verhältnis zum Titer, d. h. je grösser das nekrotische und hyperämische Gebiet, um so stärker verdünnt war das vorbereitend gespritzte

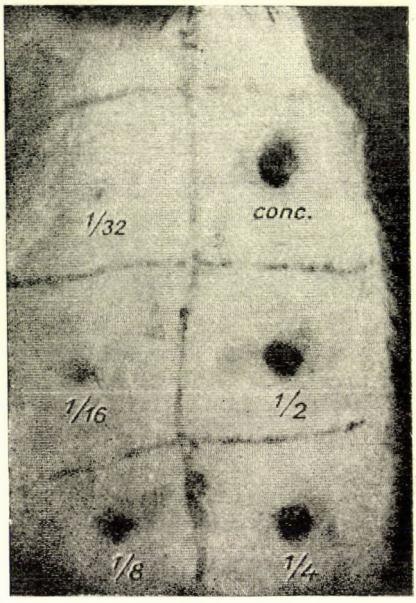

Abb. 1. Quantitatives Shwartzman-Phänomen

Endotoxin, an dessen Injektionsstelle Nekrose auftrat. Auf Grund dieses gesetzmässigen Zusammenhanges erwies sich der Titerwert zur Charakterisierung der Intensität des Phänomens als ausreichend.

Sämtlichen Tieren wurde vor der vorbereitenden Impfung, dann 24 Stunden später unmittelbar vor der Auslösung und 4 Stunden danach, beim Ablesen der Reaktion, Blut entnommen und der K-, Na- und Ca-Gehalt der Sera mit dem Flammenphotometer, die Cl-Menge nach der RUSZNYÁK—VAN SLYKEschen Methode bestimmt.

Experimenteller Teil

Das Verhalten der Na-, K-, Ca- und Cl-Konzentrationen im Serum unserer 19 normalen Versuchstiere während der Entwicklung des Shwartzman-Phänomens ist im Diagramm der Abb. 2 zusammengefasst. Die waagerechten Linien in den Säulen des Diagramms entsprechen den Mittelwerten, die Höhe der Säulen ergibt das Ausmass der Streuungen, indem alle Säulen von den extremsten analytischen Werten begrenzt sind. Sämtliche Resultate wurden auch statistisch ausgewertet.

Wie das Diagramm deutlich zeigt, führten die vorbereitenden und auslösenden Endotoxinmengen in der Na-Konzentration des Serums keine be-

wertbare Veränderung herbei. Ähnlich verhielt sich der Serum-Cl-Wert. 24 Stunden nach der Vorbereitung sanken zwar die Na- und Cl-Mittelwerte um 5--8%, gleichzeitig waren aber auch grössere Streuungen vorhanden, so dass diese Abweichung nach der statistischen Bewertung nicht signifikant ist. Dieses Verhalten des Na und Cl stimmt mit unseren bereits erwähnten Versuchsergebnissen überein, da wir auf Grund der Unveränderlichkeit des Serumgesamteiweisswertes und der Erythrozytenzahl während der Entwicklung des Shwartzman-Phänomens bereits früher den normalen Zustand des Wasserhaushalts, dessen natürliche Vorbedingung der unveränderte NaCl-Gehalt des

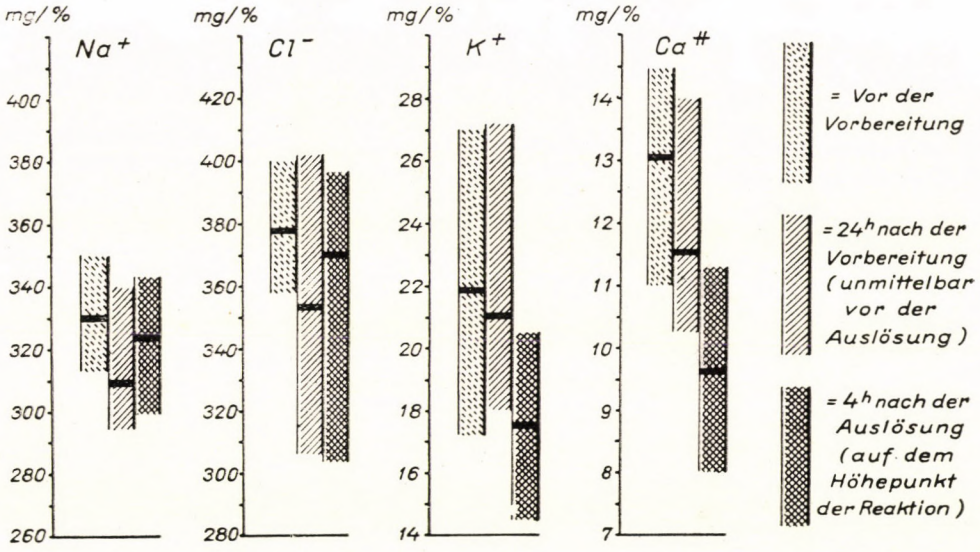

Abb. 2. Veränderung der anorganischen Ionen während des Shwartzman-Phänomens im Blutserum der Kontrollkaninchen (19 Tiere)

Serums ist, vorausgesetzt hatten. Der K-Gehalt des Serums ist nach der Vorbereitung ebenfalls normal, sinkt aber während der Entwicklung des Shwartzman-Phänomens im Mittelwert um etwa 20%. Eine ungefähr gleiche Senkung zeigt auch der Serum-Ca-Spiegel. Statistisch sind beide Senkungen signifikant. Anhand unserer experimentellen Ergebnisse kann also festgestellt werden, dass die zur Vorbereitung intrakutan verabreichten Endotoxinmengen im Mineralsalzgehalt des Serums keine bedeutenden Veränderungen oder höchstens vorübergehende, sich rasch ausgleichende Verschiebungen verursachen, während die auslösende i. v. Endotoxindosis erhebliche K- und Ca-Senkung herbeiführt. Unter Berücksichtigung der vorhandenen Zusammenhänge zwischen K- und Ca-Gleichgewicht sowie dem Funktionszustand des vegetativen Nervensystems bieten unsere Resultate eine Stütze für die Auffassung DELAU-

NAYS, der auf Grund der Gefässreaktionen den neuroendokrinen Angriffspunkt der Endotoxine hervorhob.

Teils im Hinblick auf diese Beobachtung, teils auf die konsequente Senkung der Serum-K-Konzentration schien es wahrscheinlich, dass während des Shwartzman-Phänomens auch im Kohlenhydratstoffwechsel Störungen bestehen. Wir nahmen daher an 10 Tieren in den ersten 4 Stunden der Vorbereitung und Auslösung Blutzuckerbestimmungen nach HAGEDORN—JENSEN vor. Die Ergebnisse sind auf Abb. 3 veranschaulicht.

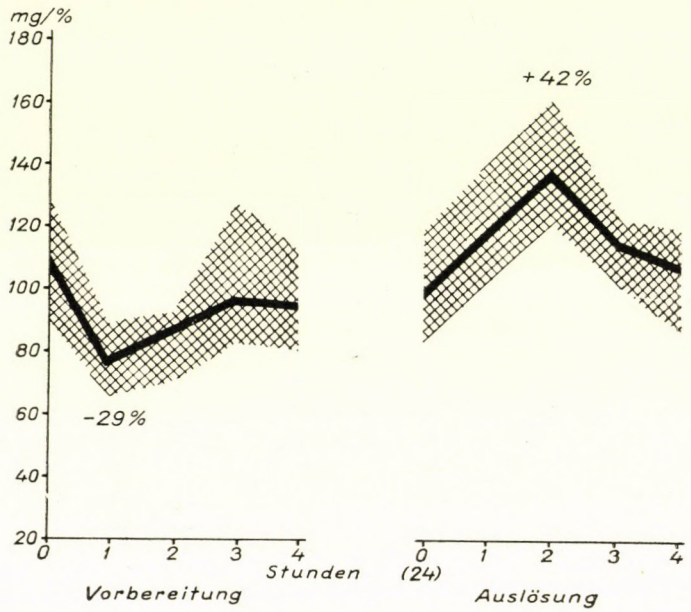

Abb. 3. Blutzuckerspiegel während der Shwartzmanschen Reaktion (10 Tiere)

Wie aus Abb. 3 ersichtlich, sinkt der Blutzuckerwert 1—2 Stunden nach der Vorbereitung im Mittelwert um 29%; diese schwache Hypoglykämie normalisiert sich schnell. Die auslösende Endotoxindosis hingegen führt zu ausgeprägter Hyperglykämie (im Mittelwert 42%), die sich ebenfalls nach 3—4 Stunden normalisiert. Eine ähnliche Hyperglykämie wurde von BURROWS [4] nachgewiesen, doch folgt seiner Ansicht nach die Hypoglykämie der Hyperglykämie nur nach grossen Endotoxingaben im Terminalstadium, während bei unseren Versuchen auch die minimale, intrakutan verabreichte vorbereitende Endotoxindosis schwache Hypoglykämie verursacht hatte. Die Abweichung dürfte sich am einfachsten damit erklären lassen, dass in der Entwicklung der Symptome neben der Endotoxinmenge auch die Anwendungsstelle eine Rolle spielt. Die bei der Auslösung auftretende Hyperglykämie lässt sich mit der K-Senkung schwer in Einklang bringen, da ja K-Senkung im allge-

meinen bei Glykogenbildung beobachtet wird [5]. KUN und MILLER hatten jedoch nachgewiesen, dass bei der Entwicklung der hyperglykämischen Endotoxinwirkung das Leberglykogen erheblich reduziert wird [6].

Zwecks eingehendener Untersuchung des Kohlenhydratstoffwechsels bestimmten wir auch die Phosphohexosisomerase-Aktivität in den von 8 Kanin-

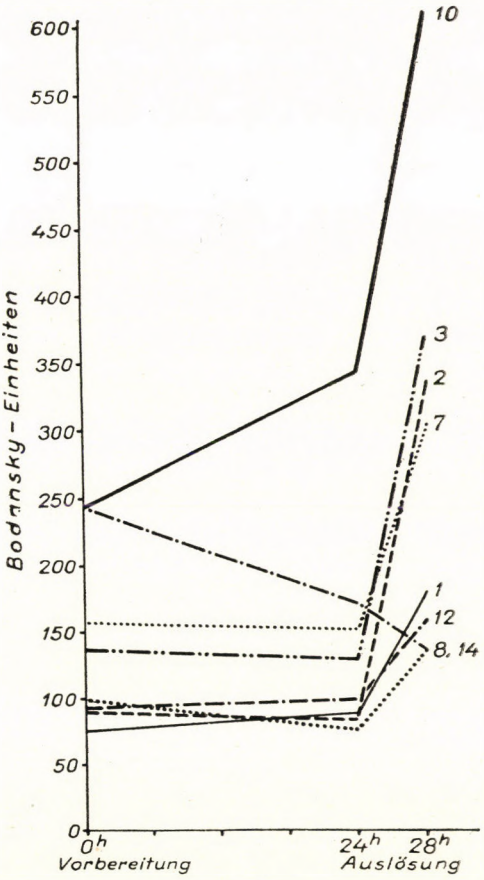

Abb. 4. Phosphohexosisomerase-Aktivität des Serums während des Shwartzman-Phänomens (8 Tiere)

chen vor der Vorbereitung, vor und 4 Stunden nach der Auslösung entnommenen Seren. Von Isomerase wird das beim Glykogenabbau oder bei der Zuckerresorption gebildete Glukose-6-phosphat in einer reversiblen Reaktion zu Fruktose-6-phosphat umgestaltet, was die weiteren Dissimilations- bzw. Assimilationsstufen ermöglicht. Das Enzym kommt in grossen Mengen in der Leber und in den Muskeln vor, ebenso im Blut, wo es in den roten Blutkörperchen in 160mal grösserer Menge enthalten ist als im Serum. Seine Bestimmung wurde nach BODANSKY [7] durchgeführt.

Wie Abb. 4 zeigt, nimmt die Isomerase-Aktivität des Serums (von einem Fall abgesehen) um 200—300% zu. Diese Steigerung lässt sich nicht auf eine Schädigung der roten Blutkörperchen zurückführen, weil einerseits die Erythrozytenzahl während der Entwicklung des Shwartzman-Phänomens unverändert bleibt, anderseits die K-Senkung im Serum eine mit Permeabilitätssteigerung einhergehende Schädigung geradezu ausschliesst. Die Isomerasevermehrung kann man auch nicht mit einer Leberschädigung erklären, da wir die Isomerase-Aktivität an einigen Tieren in vor der Auslösung und nach der Entwicklung des Phänomens herausgeschnittenen Leberstückchen bestimmt hatten und weder bei dieser noch bei der im intermediären Kohlenhydratstoffwechsel eine wichtige Rolle spielenden Glukose-6-phosphatase bzw. Phosphorylase-Aktivität keine bewertbare Abweichung fanden. Die während des Shwartzman-Phänomens eintretende Erhöhung der Serumisomerase-Aktivität lässt sich daher nur mit Muskelschädigung erklären, wobei vorausgesetzt wird, dass das Enzym aus der geschädigten Muskulatur in das Serum gelangt. Auf Grund unserer Befunde sind wir der Ansicht, dass sich die beim Shwartzman-Phänomen beobachtete Kohlenhydratstoffwechselstörung vor allem auf die Muskulatur lokalisiert und die Leber sich lediglich sekundär und später in den Pathomechanismus einschaltet. Diese Annahme wird durch die wohlbekannten, aber in pathophysiologischer Beziehung noch kaum analysierten Vergiftungssymptome der Endotoxine gestützt, nämlich den Muskeltremor, auf den später Paraplegie, allgemeine Muskelschwäche usw. folgen. Auf Grund dieser Arbeitshypothese wollen wir unsere Versuche fortsetzen.

Im letzten Teil unserer Versuche wollten wir das Versäumnis nachholen, das auch THOMAS [8] in seiner 1954 erschienenen Monographie betonte, dass nämlich die Wirkung der Sympathikolytika auf den Verlauf der Shwartzman-Reaktion bisher nicht untersucht wurde. Unter Berücksichtigung des sog. Titers erscheint die unserseits beschriebene Methode zur Durchführung derartiger vergleichender Untersuchungen geeignet.

Die Titer der 20 Kontrollkaninchen sind in Abb. 5 wiedergegeben. Mit Ergotamin behandelten wir 11 Tiere (vor der Auslösung wurden i. p. 0,8 mg/kg Ergotamin verabfolgt). Wie aus dem mittleren Diagramm ersichtlich, gingen 3 Tiere an generalisiertem Shwartzman-Phänomen zugrunde, wodurch sich das ganze Diagramm zu Gunsten der höheren Titer und zu Lasten der niedrigeren nach links verschiebt. Die Ergebnisse zeigen eindeutig, dass die Entwicklung des Shwartzman-Phänomens durch die Ergotaminvorbehandlung nicht gehemmt, sondern seine Auslösbarkeit gesteigert wird. Der Salzhaushalt verhielt sich nach Ergotaminvorbehandlung ebenso wie bei den Kontrollen, statt Hyperglykämie trat jedoch nach der Auslösung extreme Hypoglykämie auf. 12 Tieren gaben wir vor der Auslösung s. c. 10 mg/kg Chlorpromazin. An generalisiertem Shwartzman-Phänomen verendeten 3 Tiere, ebenso stieg der Titer bei der Mehrzahl der Tiere. Die Linksverschiebung der Titerwerte ist indes-

sen weniger eindeutig als nach Ergotamin ; bei 2 Tieren blieb das Phänomen nach Chlorpromazinvorbereitung aus, weshalb wir die Resultate in dem Sinne

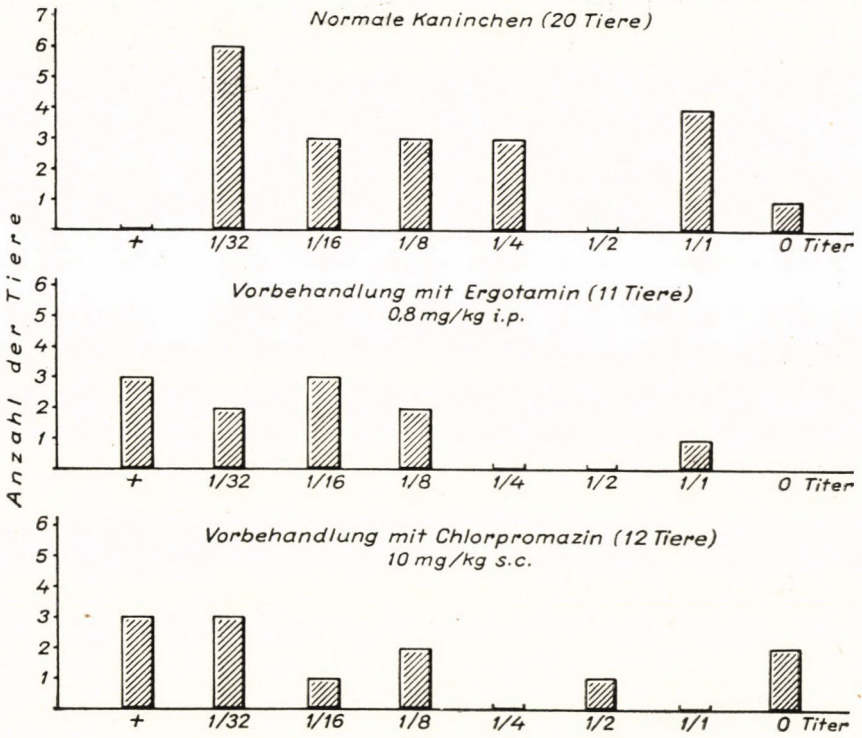

Abb. 5. Quantitatives Shwartzman-Phänomen
\+ = an generalisiertem Shwartzman-Phänomen eingegangen
O = reaktionsfrei

bewerten, dass zwar die Auslösbarkeit des Shwartzman-Phänomens durch Chlorpromazinvorbereitung im allgemeinen erhöht wird, diese Wirkung aber wesentlich schwächer ist als die des Ergotamins. Nach i. v. Vorbehandlung mit 4 mg/kg Dibenamin bzw. 2 mg/kg Malonsäuredinitril sahen wir ähnliche Ergebnisse wie nach Chlorpromazin. Diese Untersuchungen können wir also mit der Feststellung abschliessen, dass die Sympathikolytika die Auslösbarkeit des Shwartzman-Phänomens im allgemeinen fördern und in der Mehrzahl der Fälle nicht imstande sind, seine Auslösung zu verhindern. Infolgendessen halten wir es für unwahrscheinlich, dass die adrenerge Wirkung der Endotoxine in der Entwicklung des Shwartzman-Phänomens als primärer kausaler Faktor mitwirkt. Allerdings muss man in Betracht ziehen, dass es keine leichte Aufgabe ist, den diesbezüglichen Einfluss der Sympathikolytika an Kaninchen zu untersuchen' da diese Spezies laut HARVEY und NICKERSON [9] den adrenergblockierenden Substanzen gegenüber hochgradige Resistenz zeigt.

Zusammenfassung

Es wurde festgestellt, dass während der Entwicklung des Shwartzman-Phänomens unter den anorganischen Ionen des Serums die K- und Ca-Konzentration erheblich sinkt und auf Wirkung des vorbereitenden, intrakutan verabreichten Endotoxins Hypoglykämie auftritt, während nach der Auslösung der Blutzuckerwert steigt. Zu gleicher Zeit mit der Hyperglykämie nimmt die Phosphohexosisomerase-Aktivität des Serums extrem zu. Diese Steigerung wird vermutlich durch Muskelschädigung verursacht, so dass an der Störung des Kohlenhydratstoffwechsels vor allem die Muskulatur beteiligt ist. Durch Sympathikolytika (insbesondere Ergotamin) wird die Auslösbarkeit des Shwartzman-Phänomens gesteigert, weshalb es unwahrscheinlich sein dürfte, dass die sympathikomimetischen Effekte der Endotoxine an der Entwicklung des Phänomens als primäre kausale Faktoren beteiligt sind.

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CARBON METABOLISM IN BOTRYOTINIA FUCKELIANA AND ITS BEARINGS ON SWEET ROT IN GRAPES*

I. ORGANIC ACIDS, THE ONLY CARBON SOURCES OF THE MOULD

By

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(Received May 28, 1957)

Botryotinia fuckeliana WETZ., formerly called *Botrytis cinerea* PERS., is the mould responsible for both the appreciated sweet rot and the harmful gray rot in grapes.

Attacking the berries while still clustered, sweet rot raises their relative sugar content, for the mould consumes the organic acids in them, and by thinning down their skin, intensifies evaporation. By this it imparts to the grape a characteristically pleasant flavour, yet causes the berries to shrink. From such berries is "aszu" or raisin wine produced.

It is striking that apart from certain wine-growing districts, such as Tokajhegyalja in Hungary, the country around Arad in Roumania and Bordeaux in France, *Botryotinia fuckeliana* everywhere in the world gives rise not to the afore-described sweet rot, but the damaging gray rot.

In gray rot it is the relative acid content of the berries which increases, for the mould consumes the sugar in the grape juice and raises the organic acid content. In addition, it imparts a disagreeably bitter taste to the grape and causes entire clusters to rot away completely.

Obviously, to throw light upon either of these processes it is imperative to study the carbon metabolism of *Botryotinia fuckeliana* in its relation to organic acids.

Materials and methods

A strain of *Botryotinia fuckeliana* WETZ., derived from a Tokajhegyalja collection in monoculture, was used in the experiments ; it was labelled 26/e. Taxonomically, the fungus genus has been described earlier in detail by one of us [V.-Felkai ; 1]. The characteristics of the studied strain are presented in Table I. The usual zymographic and auxanographic methods were applied in making determinations [2].

The fungal stock culture was maintained in CZAPEK—DOX nutrient medium containing 3 per cent saccharose [3]. The material required for the investigations was obtained by surface-

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culturing in test tubes on 5 ml CZAPEK—DOX medium containing 3 per cent saccharose. The cultures were incubated at 27° C.

Table I

Characteristics of strain 26/e of Botryotinia fuckeliana

Decomposition of sugar		Production of acid		N and S source	
glucose	+	glucose	+	NH_4Cl	+
arabinose	+	arabinose	±	$NaNO_2$	+
lactose	—	lactose	—	$NaNO_3$	+
saccharose	+	saccharose	+	asparagine	±
maltose	+	maltose	+	peptone	+
d-mannose	+	— — — — —		$MgSO_4$	+
glycerin	±	glycerin	—	$KHSO_3$	+
mannite	—	mannite	—	$Na_2S_2O_3$	+
starch	+	starch	+	NH_4SCN	±

Spore measures 9,9 μ × 10 μ.

In the individual experiments the appropriate substances enumerated in the experimental part of this paper were added to CZAPEK—DOX basic medium containing no source of carbon. The nutrient media were sterilized in the autoclave at 1.5 atm. for 20 minutes, or by filtering them through a sterile Seitz EK plate.

Having determined the feasible dilution limits in a number of preliminary experiments, 5, 3, 1, 0.5, and 0.25 per cent dilutions were prepared with CZAPEK—DOX nutrient medium containing no carbon source. To certain stronger acids, for which the buffer action of the CZAPEK—DOX medium was found to be insufficient, the sodium salt of the respective acid was added simultaneously. CZAPEK—DOX medium devoid of a carbon source, served as control. Readings were taken every other day, using arbitrary gradations for germination, growth and development.

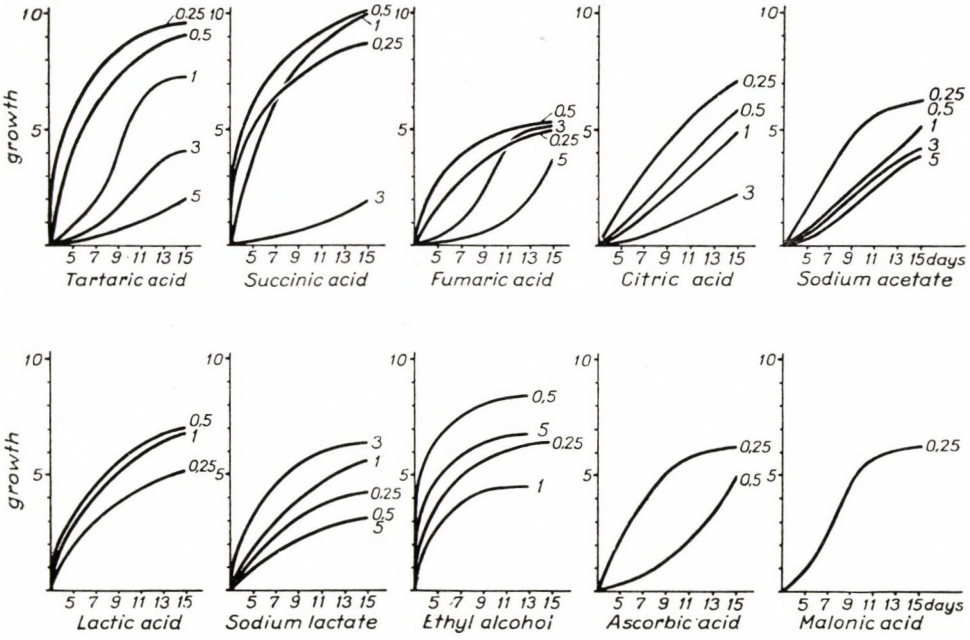
The following were the carbon sources used : acetic acid, sodium acetate, lactic acid, sodium lactate, ascorbic acid, tartaric acid, succinic acid, fumaric acid, citric acid, sodium citrate, formic acid, propionic acid, oxalic acid, sodium oxalate, malonic acid and ethyl alcohol.

Experimental

The graphs in Fig. 1/b show that the growth rate of the mould was the highest in tartaric and succinic acid; slightly less in citric acid, lactic acid, sodium acetate, and ethyl alcohol; while sodium lactate and ascorbic and malonic acid proved to be inferior sources of carbon.* In acetic, oxalic, formic, and propionic acids, as well as in sodium oxalate, there was no germination at all. The failure to germinate may have been due to the low level

* At this point it needs to be mentioned that WOLF, BRYDEN and MCLAREN [Mycologia 42, 233, 1950], in addition to 30 other carbon sources, cultured the mould *Monosporium apiospermum (Allescheria boydii)* also on malonic acid. We cannot, however, consider here their results, since they have failed to publish details, e. g. on the method used in sterilizing the unstable malonic acid.

(a) Growth as a function of concentration and time



(b) Growth in 15 days

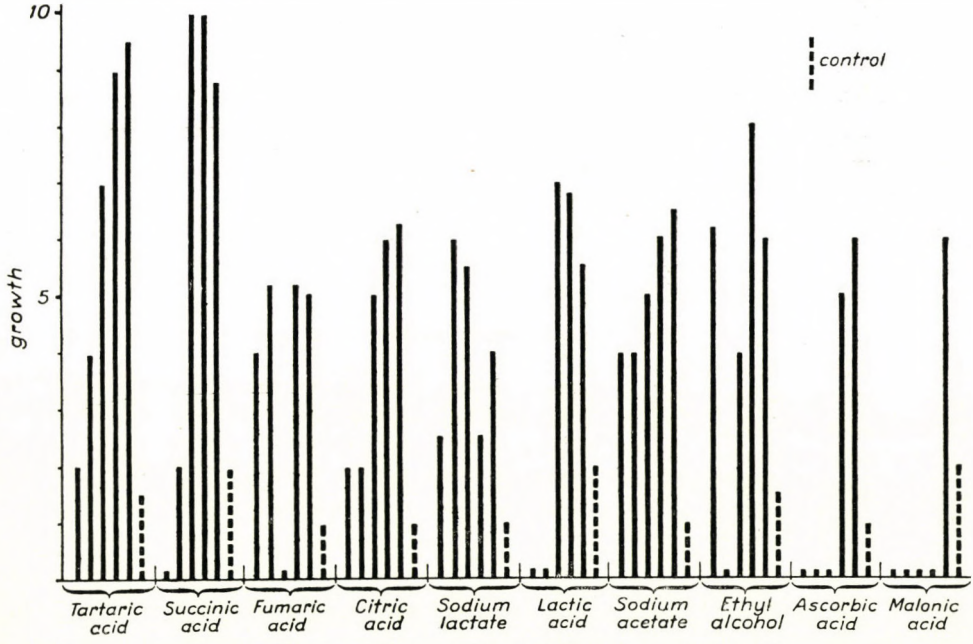


Fig. 1. Growth of *Botryotinia fuckeliana* in organic acids

of energy in these substances. As regards sodium oxalate, WEHMER's [4] claim is probably valid : during the metabolic process oxalic acid lends itself readily to oxidation in an acid medium, while in an alkaline one the salts of oxalic acid are stable.

Discussion

The steep time curves in Fig. 1/a and the growth rates attained in 15 days, as shown in the graphs in Fig. 1/b, equally testify that tartaric acid, succinic acid, and ethyl alcohol are the substances which in the metabolic process the mould utilizes with considerable ease (steep curves), at a high rate (15-days values), and to great advantage. The flatness of the curves for the other acids indicate that the fungus had so to say, to adapt itself, to the respective acid as its only source of carbon and energy. Accordingly, it could utilize these substances in a lesser degree only.

Efforts were made to reconcile our findings with the transition processes of acids described in the literature. For moulds *(Aspergillus, Penicillium, Botrytis)* the comprehensive work of FOSTER [5] entitled "Chemical Activities of Fungi", was considered in the first line. NORD and WERKMANN's [6] "Advances in Enzymology" and PORTER's [7] "Bacteriological Chemistry and Physiology" were also taken into account.

The metabolic processes known from the data in the literature to occur in moulds, and mostly comprising the usual acid cycles, seem to offer a satisfactory explanation for the utilization of tartatic, succinic, acetic, fumaric, citric and lactic acid. No data motivating the utilization of ascorbic acid by the mould could be traced.

The mould's utilizing malonic acid, is a new observation ; this acid has always been known as an agent inhibitory to metabolism ; its transformation cannot proceed *via* any route involving succinic acid, since it is an inhibitor of just the succinic acid dehydrogenase.

These data, too, show that several parallel metabolic routes are possible, and that the one most favoured by the prevailing conditions will operate more effectively than the others.

On the evidence obtained we ought to postulate the operation of a SZENT-GYÖRGYI—KREBS tricarboxylic acid cycle, though in view of our findings with malonic acid, not without some hesitation. In favour of a tricarboxylic acid cycle speaks the mould's rapid and powerful growth in citric, succinic, tartaric acid (which, though not a member, might easily transform to any one member of the cycle), fumaric acid, and acetate. One might say that the mould has, as it were, adapted itself to work up these substances and is already in possession of the enzymic systems required to that end. However, this is not possible unless the same intermediates and the same

enzymic systems converting them, are active in the carbohydrate metabolism as well.

We wish to mention the findings of GENTILE [8], who studied *Botrytis cinerea* PERS. *(Botryotinia fuckeliana* WETZ.) giving rise to oxalic acid from various carbon sources, e. g., from glucose and organic acids. He showed that the acids of the Krebs cycle intensified, while malonic acid inhibited respiration. On this evidence, he regards the operation of the Krebs cycle as confirmed. In addition, he demonstrated the direct oxidation of glucose *via* gluconic acid — glucuronic acid — (with chain reaction) oxalic acid + erythrose — tartaric acid, leading finally to oxalic acid. It should be borne in mind that in this chain of reactions there appears no succinic-acid dehydrogenase, which means that in this case the inhibitory action of malonic acid is unable to assert itself.

Summary

A strain of the mould *Botryotinia fuckeliana* WETZ. has been found to utilise the following ten out of sixteen tested organic compounds, viz.: lactic acid, sodium lactate, citric acid, tartaric acid, succinic acid, sodium acetate, fumaric acid, malonic acid, ascorbic acid, and ethyl alcohol.

Formic acid, propionic acid, oxalic acid, sodium oxalate, and acetic acid yielded negative results.

While the experimental results, in agreement with data from the literature, support the idea of the operation of a postulated Krebs cycle, the fact that the mould utilises malonic acid, speaks against it. The situation probably is that of several possible metabolic routes, external conditions (e. g. the addition of malonic acid) favour the action of a single one. This question is the subject of a simultaneously published paper.

Acknowledgement : The authors wish to express their thanks to Professor J. BÁNHEGYI for helpful advices.

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CARBON METABOLISM IN BOTRYOTINIA FUCKELIANA AND ITS BEARINGS ON SWEET ROT IN GRAPES*

II. UTILIZATION OF MALONIC ACID AND ITS EFFECT ON THE METABOLISM OF THE MOULD

By

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(Received May 29, 1957)

The aim of the present work was to clarify, in the light of previous experiments, the behaviour of the mould *Botryotinia fuckeliana* Wetz. in the presence of malonic acid.

Until recently, malonic acid has been regarded as of no biochemical significance except for its role as an inhibitor of terminal respiration ; little attention has been paid to its conversion in the metabolic process. Although den Dooren de Jong [2] showed more than 30 years ago that certain bacteria were capable of utilizing malonic acid as the sole carbon source, and although beet has been known for a long time to contain much potassium malonate [3], it was not until 1952 that Lara [4] and Hayaishi and Kornberg [5] pointed out some bacteria that degrade pyrimidines to malonic acid. A malonate-decomposing enzyme was found to be present in the skeletal muscle of fish [6] and in sap expressed from runners of certain Leguminosae [7]. Using C^{14} isotopes, Lee and Lifson [8] found that in rats fed malonic acid the instable carbon is built into succinic acid. Some short time ago the enzyme system which decomposes the acetoacetic acid in heart muscle, was shown to decompose malonic acid equally well [9].

Some research workers [10, 11] believed that the first step in the subsequent transformation of malonate was decarboxylation, while others [12, 13] thought it was oxidation. In 1953, Hayaishi [14] showed that coenzyme A (CoA) and adenosine triphosphate (ATP) were involved in malonate decarboxylation. The results of Hayaishi [14] were confirmed in 1954 by Wolfe, Ivler, and Rittenberg [15], and by Wolfe and Rittenberg [16], who by demonstrating the presence of acetyl CoA, clarified the process of decarboxylation by *Pseudomonas fluorescens*.

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Materials and methods

Culture procedure. Strain 26/e of *Botryotinia fuckeliana* WETZ. [1] was grown at 27° C on a CZAPEK—DOX agar slant containing 3 per cent saccharose.

For the *respiratory tests*, precultivation was carried out in test tubes on 5 ml of CZAPEK—DOX nutrient medium, to which the carbon source required as per the experimental part of this paper was added.

The one-week cultures undergoing initial sporulation were either used directly after a flush of distilled water, or as much of them as a test tube holds was homogenized in 10 ml of 1/15 M phosphate buffer, pH 7.2, respectively in 5 ml of CZAPEK—DOX medium containing 0.05 per cent malonic acid.

Conventional manometric procedures were used for the determination of oxygen uptake (QO_2) and respiratory quotient (R_Q) [17, 18]; the nitrogen content was determined by the Kjeldahl technique. The manometric determinations were carried out in 2 ml of CZAPEK—DOX medium at pH 7.5 and 20° C, in an air atmosphere with 140 shakes per minute.

Dehydrogenase activity was determined from the phosphate homogenates with triphenyltetrazolium chloride (TTC).

To demonstrate the presence of *hydroxamic acids*, the phosphate-buffered homogenates were either used crude, or they were centrifuged at 3500 r. p. m. for 10 minutes, if after centrifugation up to 5 per cent CCl_3COOH were added to them and then they were centrifuged for another 10 minutes.

To demonstrate the presence of activated acid radicals, the hydroxamate method of LIPMANN and TUTTLE [21, 22] and STADTMAN [23, 24], were applied.

Paper chromatography was carried out using GIRI and RAO's [25] method with certain modifications [26, 27, 28, 29, 30] and, in addition, the rapid procedure developed by ourselves [31].

Identity and purity of the *malonic acid* was checked by its inhibiting the succino dehydrogenase of heart muscle and by paper-chromatographic analysis, respectively.

Experimental

Effect of malonate on the utilization of saccharose. In view of the finding that the mould utilizes malonate as the sole carbon source [1], the next step was to study the effect malonate exerts on the utilization of other carbon sources, e. g., saccharose.

In our experiments, 0.05, 0.10 and 0.15-per cent malonic acid was found to double cultural growth in Czapek-Dox medium containing saccharose.

With a view to demonstrating the differences in growth, stab macrocultures were produced on agar plates (Fig. 1).

It was found that malonic acid in no way inhibited saccharose utilization: the mould grew quicker and the culture developed more powerfully than the control.

Effect of malonate on oxygen uptake. Wishing to throw light upon the mechanism of the action accelerating and intensifying growth, oxygen uptake was determined in the presence of 0.05-per cent (about 0.005 M) malonic acid (Fig. 2).

Fig. 2 shows substantially higher values for the oxygen-uptake quotients in cultures that do, than in those that do not, contain malonic acid. The differences are manifolds of the average 10 to 20 per cent scatters over the parallels in the Warburg experiments and the nitrogen determinations.

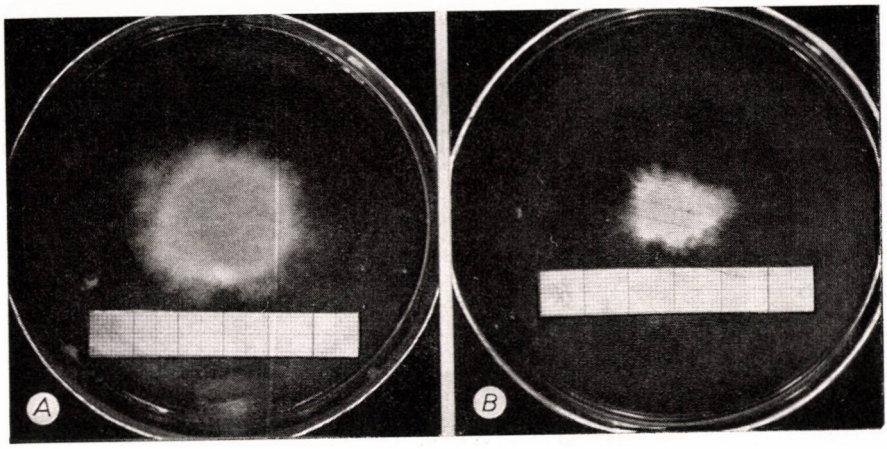


Fig. 1. The effect of malonate on the growth of *Botryotinia fuckeliana*
One-week old stab cultures. A = Czapek—Dox agar containing 3% saccharose and 0,05% malonate. B = Czapek—Dox agar containing 3% saccharose ; control

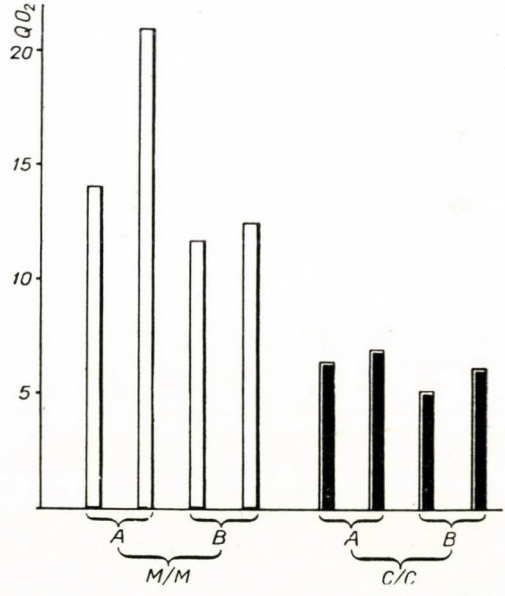


Fig. 2. The effect of malonate on the oxygen consumption of *Botryotinia fuckeliana*

Explanations :

M/M = Grown and Warburg measurements made in Czapek—Dox medium containing 3% saccharose and 0.05% malonate. C/C = Grown and Warburg measurements made in Czapek—Dox medium containing 3% saccharose.
A = One-week cultures. B = Two-week cultures

The effect achieved with quite low concentrations appears to point to malonate as some kind of a stimulant acting on the metabolism of the mould. Fig. 2 also shows that the effect is weaker in the older cultures, for while the one-week (A) and the two-week (B) cultures were started at the same time, the Warburg measurements in the latter were made one week later. The settled organism, probably possesses less adaptive capacity.

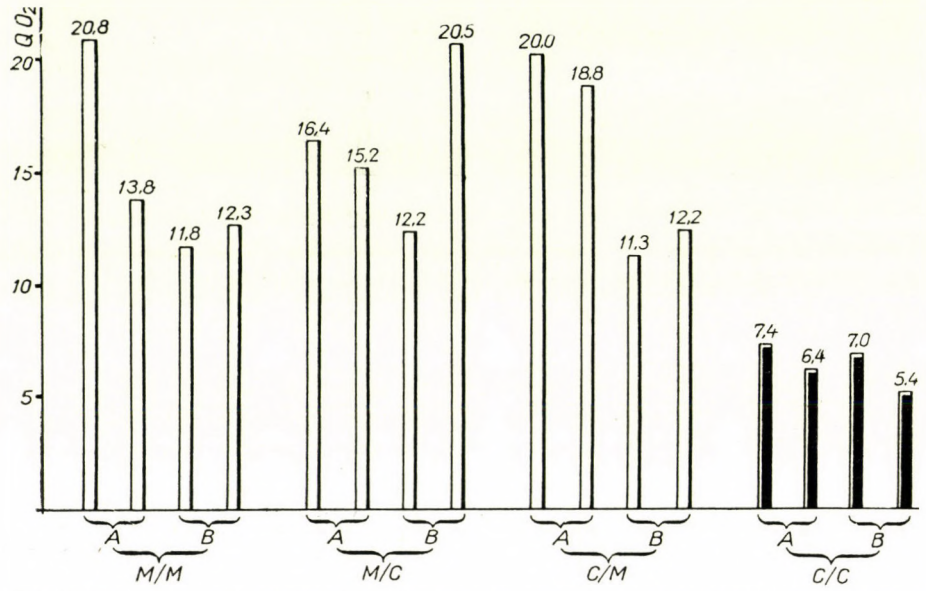


Fig. 3. The effect of malonate addition on the oxygen consumption of mycelia grown in different carbon sources

Explanations :

M/M = Grown and Warburg measurements made in Czapek—Dox medium containing 3% saccharose and 0.05% malonate. M/C = Grown in Czapek—Dox medium containing 3% saccharose and 0.05% malonate, but Warburg measurements made in Czapek—Dox medium containing 3% saccharose. C/M = Grown in Czapek—Dox medium containing 3% saccharose, but Warburg measurements made in Czapek—Dox medium containing 3% saccharose and 0.05% malonate. C/C = Grown and Warburg measurements made in Czapek—Dox medium containing 3% saccharose.
A = One-week cultures. B = Two-week cultures

To study the said stimulating effect in greater detail, and to set more exact limits to the amount of malonate required, two-phase experiments were carried out, in which malonic acid was added alternatingly in the culture phase and in that of the Warburg measurements (Fig. 3).

In the M/C alternative it can be seen that although to the cultures no malonate has been added in the Warburg measurements, they nevertheless displayed a QO_2 equal to that shown by the cultures provided with malonic acid in both phases of the experiment. This indicates that the amount of malon-

ate originally present in the cells of the mycelium ensured the higher respiratory level for the full 3 hours of the experiment.

Considering that the wet weight of the cultures used in the experiments did not exceed 200 mg, corresponding to about 0.25 to 1.1 mg of nitrogen, the mycelium must have contained a minimum amount of malonate only, and even this could but decrease during the life of a week's duration.

These contemplations still further substantiate the stimulating effect directly indicated by the experimental results and set a considerably reduced upper limit to the amount of substance supposed to be necessary to elicit that effect.

Very significant is the experiment carried out with the C/M group, because the cultures in it were provided with malonate during the Warburg

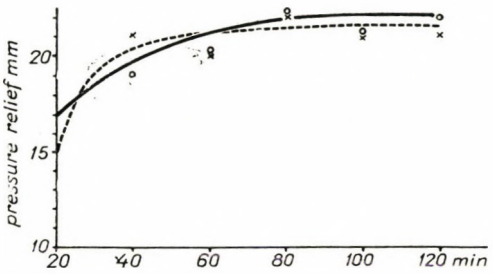


Fig. 4. Effect of malonate on changes in the oxygen consumption of a saccharose culture. Changes, in dependence on time, in the intensity of respiration in the C/M group. Experimental conditions are the same as for the C/M group in Fig. 3

measurements only. What we can see here is that initially the respiration of the cultures was feeble, but in 30 to 35 minutes became intensified, and thereafter stayed on the same level. This respiratory intensity corresponded roughly to that of the other groups provided with malonic acid. In this manner, by the end of the 3-hour experiment, every culture which had ever been in contact with malonate, yielded more or less the same QO_2.

The graph in Fig. 4 clearly shows that 50 to 60 minutes were needed for respiration to reach its peak intensity. Including the time from the preliminaries to the reading taken at 0 hour, about 70 minutes were required to allow a sufficiently large amount of low-concentration malonate to diffuse into the mycelial cells and there to produce the appropriate effect. This finding constitutes further support for the stimulating action mentioned above.

Paper chromatography was used for the approximate determination of the amount of malonic acid diffusing into the mycelium in 3 hours. In analogy with the Warburg experiments, a culture each was placed for 0, 15, 30, 60, 120 and 240 minutes into test tubes holding 2 ml of a 0.05 per cent malonate solution. After the lapse of the appropriate time, the individual cultures

were washed, dipped into liquid air, and fixed. Following pulverisation and thawing, the fixed cultures were chromatographed. The presence neither of malonate nor of any of its derivatives, — into which it may transform after entry, and of which so many moles arise as malonate moles are consumed, — was demonstrable with an indicator of about 100 μg sensitivity (0.04-per cent alcoholic bromocresol green), not even after incubation for as much as 4 hours.

Thus, through this experiment it has become possible to characterize the limit value of the stimulating amount of malonate by a more readily definable value : by one we now know lies below the amount that can be chromatographed without condensation.

Dehydrogenase activity in malonate cultures. It was deemed important to clarify the effect malonic acid, as a succinodehydrogenase inhibitor exerted on the formation and activity of that enzyme, under a variety of culturing conditions.

Table I shows that cultures grown on sugar displayed feeble dehydrogenase activity without the addition of a substrate ; a more intense one on the addition of succinic or malonic acid ; and none at all on the combined addition of these two acids. Accordingly, saccharose cultures are capable of "dehydrogenating" not only succinic acid, but malonic acid as well.

Table I

Effect of growth in malonate on dehydrogenase activity

The test tubes contained 0.5 ml of the appropriate homogenate, 0.5 ml of 5% succinate, and 0.5 ml of 5% malonate as indicated. Total volume : 5 ml ; 1/15 M phosphate buffer; incubation at 27° C for 16 hours

Homogenate plus additions	Saccharose culture	Malonate culture	Malonate saccharose
Nil	+	—	—
Succinate	++	—	—
Malonate	++	—	++
Succinate plus malonate	—	—	—

Cultures grown on malonate gave no dehydrogenase reaction with either of the substrates in the dilutions applied, not even after the lapse of 3 days.

Saccharose cultures containing 0.05 per cent malonate showed dehydrogenase activity on the addition of malonic acid only.

The results obtained with malonate in sugar cultures made us postulate that the homogenate first decarboxylates the malonate and that dehydrogenation affects solely to the arising acetate. This postulate found support not only in a study of the structure of malonate and acetate, made from the point of view of dehydrogenation, but also in certain data in the literature

[14, 15, 16] that had been obtained with bacteria and prove the decarboxylation of the malonate and the oxidation of the acetyl CoA formed.

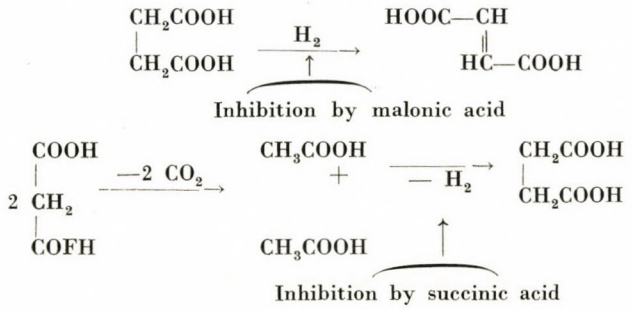
The pathway of the dehydrogenation of the acetate we believed to have found in the Thunberg—Wieland reaction on the strength of the following considerations, viz.:

(i) According to the Thunberg—Wieland reaction, by giving up one molecule of hydrogen two molecules of acetate transform to succinic acid.

(ii) On this basis, it must in all probability be possible to inhibit the Thunberg—Wieland reaction with succinic acid, as a product of the reaction, because of the chemical equilibrium.

(iii) Succinic acid inhibited the dehydrogenation of the malonate, for both of the two separate dehydrogenations which it had been possible to demonstrate, ceased on the simultaneous addition of succinic and malonic acid. Thus, malonic acid was not the only agent to inhibit the dehydrogenation of the succinic acid ; the action is a two-way action and can be deduced only as above.

Since, to explain this two-way action, a step that can be inhibited with succinic acid — namely the Thunberg — Wieland reaction —, had to be postulated to take place in the metabolism of the malonate, the processes ensuring the negative result can be summarized as follows.



Obviously, an inhibition of the second process too must have occurred, or "dehydrogenation" in itself would have ensured a positive TTC reaction. On the other hand, in the conversion of any other acetate where succinic acid could exert its inhibitory action but later, the other dehydrogenations taking place in the meantime would yield a positive TTC reaction. At the same time, the second process provides a working hypothesis for the metabolism of the malonate.

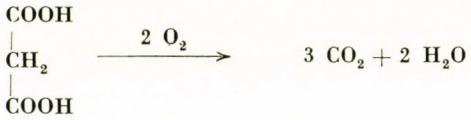
Because growth on malonate-containing saccharose inhibited succinodehydrogenase, "dehydrogenation" of the malonate only could be demonstrated in such cultures, and even this it was found possible to inhibit with succinic acid.

Transformation of malonate. With a view to verifying the decarboxylation postulated in the transformation of malonate on the strength of our investigations with TTC, we determined the respiratory quotient (R_Q) of both the live and the homogenized mould, following growth on malonate.

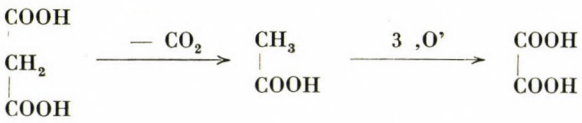
In the presence of 0.05 per cent malonic acid the respiratory quotient of the live cultures was less than 1 ($R_Q < 1$), while that of the homogenized cultures exceeded 1 ($R_Q > 1$).

The explanation for the respiratory quotient being less than 1 in live cultures the fact is that decomposition proceeds as far as oxalic acid, as according to GENTILE [32], the mould oxidises the respirational acids to oxalic acid. In the present case, oxalic acid would originate from the acetic or succinic acid arising from the malonate. The capacity of the strain used in these experiments to produce oxalic acid has recently been demonstrated [33], while this capacity of the species has been known for long [34, 35, 36].

Comparing the R_Q values for the total and partial (oxalic acid) oxidation of malonate, $R_Q < 1$ is in favour of oxalic acid formation, as



$$R_Q = \frac{CO_2}{O_2} = \frac{3}{2} = 1.5$$



$$R_Q = \frac{CO_2}{O_2} = \frac{1}{1.5} = 0.66.$$

In the homogenized cultures there was marked decarboxylase activity ($R_Q > 1$). This shows that decarboxylation is the primary feature in the transformation of the malonate, since during homogenization it is mainly the structure-bound respirational enzymes that are paralysed. Would decarboxylation affect solely some oxidation product, it could not occur in homogenized cultures, because these are not active with the oxygen in the air, only with some other hydrogen acceptor, e. g. with TTC, and, consequently, the substrate of the decarboxylase enzyme could not arise either. Thus, in our case the CO_2 production of the homogenized cultures affords proof that decarboxylation is the primary process, which the oxidation processes follow.

Activated acid radicals. In mycelium grown on either sugar or malonate an activated acid fraction, hydroxamic acid, can be demonstrated by means of hydroxylamine.

Judged by its behaviour when centrifuged during purification with a protein precipitator and precipitates of adsorptive capacity, the activated acid fraction is a substance of non-protein nature and great molecular weight. It is probably an acyl derivative of CoA.

Chromatography shows that the hydroxamate which arises on the action of hydroxylamine is a homogeneous product ; however, for technical reasons we have so far failed in identifying it.

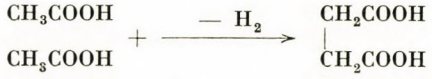
Discussion

Evidence has been brought forth that *Botryotinia fuckeliana* can utilize malonate as the sole carbon and energy source. By the determination of the dehydrogenase activity and the respirational quotient, light has been thrown upon the first two steps of the utilization process and the nature of its end product. In keeping with the data in the literature relative to other carbon sources, our experiments show that through several intermediate steps, malonate finally transforms into oxalic acid. On the strength of our findings with homogenized cultures, the first step is decarboxylation ; on the evidence of our experiments with TTC, the arising acetate is dehydrogenated to succinic acid ; the succinic acid, in the manner clarified by GENTILE [32], is converted to oxalic acid.

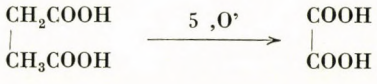
Accordingly, the mechanism of the conversion of malonate is as follows.



(I)



(II)



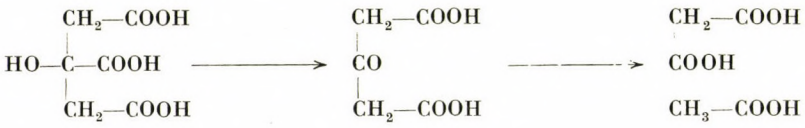
(III)

In addition to serving as the energy source, malonic acid also serves as the carbon source. In our culture tests the mould showed no gross changes

consequent upon its inability to obtain carbon from a source other than the malonate.

The further transformation of succinic acid very probably cannot proceed in the usual way ; the malonic acid present would inhibit this, unless a strong malonic acid decarboxylase activity afforded protection to the succinic acid dehydrogenase. The first assumption is favoured by the observation that in a saccharose medium the presence of as little as 0.05 per cent malonate sufficed so completely to inactivate the succinic-acid dehydrogenase in the cultures that it was no longer demonstrable with TTC, at a time when with malonic acid as the substrate dehydrogenase activity was still present.

As regards the effect of malonate on the utilization of sugar, the point to be emphasized is that by inhibiting succinodehydrogenase, malonic acid shunts the metabolic process to an until then feeble pathway, in which it can participate and thereby stimulate the process. This role of a stimulant is not conceivable unless, in addition to the pre-existence of a possible metabolic pathway, we also postulate the presence of malonic acid in the mould's normal metabolism. That this is so is clear from the intensifying of respiration which presents itself in about 20 or 30 minutes after the addition of malonic acid, and which a metabolic product foreign to the organism would be unable to produce. In view of the shortness of time, the idea of adaptation can probably be discarded. Another point in favour of the presence of malonate is one of the degradation processes of citric acid in moulds, which is accompanied by malonate formation [34].



Upon the action of malonic acid a direct oxidation of the sugars, associated with an uninterrupted splitting off of oxalic acid, might come into the foreground a phenomenon, the occurrence of which was demonstrated by GENTILE [32] for the mould in question. While it could not inhibit this route, malonic acid might, directly or *via* its derivatives, join the process and this would present an explanation for the stimulating effect.

Our study of the malonate-saccharose cultures has indeed confirmed that the presence of malonate brings succinodehydrogenase activity to a stop.

Malonic acid need not necessarily exert its stimulating action directly, it may act also through its derivatives. For instance, in one route by which the stimulating effect exerts itself, the acetyl CoA possibly present in the metabolism of the malonate, comes probably into play, since it is the acetyl CoA which supplies the key to the trans-acetylase and CoA-transferase systems.

The capacity of malonate to stimulate the degradation of sugars has been demonstrated in bacteria, isolated animal tissue samples, and organs, by several authors in the years 1953—1956 [40, 41, 42]. Stimulative action on respiration has been observed in objects of plant, animal, and bacterial origin [43, 44, 45].

These conclusions might be controverted on the ground that increased oxygen consumption was not a sign of intensified metabolism or sugar uptake ; on the contrary, it arose because by the malonate the metabolic processes had been shifted to a pathway of poor efficiency, and it was this pathway that had consumed so much oxygen ; oxygen consumption might be but a process to detoxicate malonic acid.

Such arguments can be refuted by reference to the fact that in our experiments the presence of malonate in the cultures was found to hasten and intensify growth : a phenomenon impossible without enhanced and intensified previous liberation of energy.

Based upon our own findings and the relative data in the literature, we present in the following a hypothetical explanation of the processes taking place in sweet rot.

According to present knowledge, in these processes the malonate uptake of *Botryotinia fuckeliana* is of no significance ; yet, since it was our experiments carried out with malonic acid that allowed us a gradually deepening insight into the carbon metabolism of the mould and yielded a growing number of data on its general metabolism and enzymic systems, these experiments, indirectly, were certainly conducive to our elaboration of a hypothesis consistently including all essential data known to date. It is satisfactory to note that our hypothesis had enabled us to foreconclude certain facts of pedology which, as will be seen in the following, were later substantiated.

Botryotinia fuckeliana can give rise to either of two processes : to sweet rot, in certain regions and under favourable climatic conditions ; to gray rot, in all other parts of the world, and under adverse climatic conditions, in the first-mentioned regions too. Simplified, the essential feature of sweet rot is a drop, and that of gray rot, a rise in acid contents.

Having dealt with them in a previous report [1], we need not go into the details of the sweet rot process. Let it be suffice to point out its dependence on certain geographical and meteorological factors [36, 37], and to mention that it is the metabolic process, in which this dependence is given concrete and objective existence.

The sporadic incidence of sweet rot in a few places of the earth remote from one another suggests individual biochemical convergency elicited, or at least promoted, by some "local factor". That this factor is a localised one, follows clearly from the experience that a transference of either the mould, or the grape-vine, or both, to other places has never yet resulted in sweet rot, but invariably in gray rot. Accordingly, soil and exposure are the essentials to be considered. Identity and major role of climatic conditions are ruled out by the divergent geographical situation of the few sweet-rot regions.

The dependence on the meteorological conditions reveals itself in that a humid warm atmosphere favours a decrease, while cold weather rich in precipitation favours an increase, in acid production.

The acid producing capacity of *Botryotinia fuckeliana in vitro* has long been known [32, 34, 36, 38]. It has also been shown that acid accumulation is not inevitable ; the acids may be oxidized even to carbon dioxide [34, 36]. VÖRÖS-FELKAI [33] demonstrated that in the first week of growth in a Czapek—Dox nutrient medium oxalic, citric, glycuronic, and ascorbic acids make their appearance, with the pH dropping to 2 or 3, and that in the second week these acids disappear, with the pH regaining its former value of 6 to 7. In a previous communication [1] we reported the capacity of *Botryotinia fuckeliana* to utilize any one of several organic acids as the sole carbon source.

As regards the cause behind the meteorological dependence, this is to be looked for in the changes that take place in the nutritive conditions. In warm and humid autumn weather both the relative and absolute sugar contents of the berries increase, rendering their juice hypertonic in relation to the fungal cells. Now, to counteract the osmotic force, the mould is compelled to take up osmotically active substances from the berries. But sugars diffuse through the cell membrane about a hundred times slower than do charged ions, and so it is mainly the organic acids of the berry that participate in osmoregulation. From all this it evidently follows that energy production by the mould shifts in the direction of the oxidation of the organic acids ; this the more as they are readily assimilated by the mould [1]. In sweet rot, therefore, *Botryotinia fuckeliana* oxidises not only the acids it has earlier produced from glucose, but also those it takes up from the berry.

In cold autumn weather with much rainfall the berry juice becomes diluted owing to a substantial water uptake, and is therefore hypotonic in relation to the fungal cells. When this is the case the mould is forced to give off osmotically active substances and thereby relieve the pressure increasing in consequence of the water intake. It is again the faster diffusing organic acids that take part in osmoregulation, *i. e.*, the mould gives off to the berry the organic acids it has produced earlier. This process is fed by a glycolysis always intensified in this case, and as the organic acids produced from sugar leave before their further transformation, it also is associated with an enhanced sugar consumption. Eventually, there will be an accumulation of acids which leads to gray rot and ruins the berries.

On the basis of the foregoing, both the sweet-rot and the gray-rot processes may be explained by a chain of intensified oxidative and glycolytic reactions, respectively, brought forth by some trophic action. A chain like that clarifies not only the question of meteorological dependence but also the interrelation of the two different processes, provided they derive from a preponderance of the normal processes present in the metabolism of the mould. For the normal and joint presence of glycolytic (acid producing) and oxidative (acid consuming) enzymic systems, evidence has been furnished by several authors including, for example, WEHMER [36, 38], GENTILE [32] and VÖRÖS-FELKAI [33].

As an explanation for the temperature dependence of the two processes, one might directly invoke the difference in the temperature optimum of the enzymic activities ; this would mean glycolysis $<$ oxidation in warm, and glycolysis $>$ oxidation in cold weather, but the probability of this assumption is slight ; it has never yet been observed in a living organism.

The role of the local factor is to give rise to that potential capacity which in dependence on the weather will or will not manifest itself. Since in our case the "local factor" is ultimately to provide a basis for an oxidation of substantial activity (loss of acid, sweet rot), the soil is to be taken into account in the first line, for the soils might really agree in the geographically different places. Now from the soils, the heavy metals might be the activators of enhanced oxidation. It has been shown by FOSTER [34] that to develop, moulds require a minimum quantity of Mn, Cu, Fe and Co ions. In addition to the heavy metals, they also definitely need Mg, Ca and Zn. The Zn supply is particularly important, for in its absence incomplete carbohydrate metabolism is bound to lead to an accumulation of acids. The intensified loss of acid induced by weather conditions may of course inhibit activated oxidation from asserting itself, and this is where the two part processes synthesize to a uniform whole, which is dependent on geographical and meteorological factors.

It follows from the foregoing that certain similarities must prevail in the pedologic conditions of the above-enumerated sweet-rot regions. And, indeed, it is an essential common feature, for example, of the regions around Tokajhegyalja, Aradhegyalja, and Bordeaux that the soil is underlain by the same riolite-tufa parent rock under a loess cover. It does not lie within our scope to discuss in detail the significance of this common origin of soil. However, it is most probably not accidental that riolite is a silicate rock of high side-ion content.

Further support is afforded for the trophic theory by the finding that if unripe grapevine is infected with the mould gray rot results, while infection of the ripe fruit leads to sweet rot [36]. For this phenomenon, too, the osmotic theory may furnish the explanation, since the juice of the ripe berry may be hypertonic, and that of the unripe fruit hypotonic in relation to the mould.

An explanation attempted on the basis of the biological balance enables us to account not only for the two extreme stocks, but also for the various grades existing between them, *e. g.*, for the "aszu", or raisin, or sweet rot grape of higher acid content. In this, acid oxidation is diminished, but evaporation increases the relative sugar content. Accordingly, changes in the weather conditions induce changes in the metabolism.

The capacity of the loess cover to reflect light and heat cannot explain changes in the acid content ; it can, in the utmost, account for increased evaporation, but this is not enough.

Summary

In studying the utilization of malonic acid by the mould *Botryotinia fuckeliana* it has been found that malonate decarboxylizes to acetate and oxidizes *via* succinic acid to oxalic acid. In the liberation of energy by the mould grown on malonate and in the mould's biological synthetizing processes, a major role is probably played by the acid fraction appearing as hydroxamate. The same acid fraction is encountered in the mould grown on sugar.

In a concentration of 0.005 M, and even less, malonic acid stimulates oxygen consumption and growth of the mould in a saccharose medium.

The action of malonate manifests itself not only in stimulation, but also in changing the metabolism which liberates energy. There is no succinodehydrogenase activity in malonate-saccharose cultures, wherefore direct oxidation of the sugars is the more probable pathway of degradation.

Data from the literature and from our own experiments has made it possible to reconcile the metabolic processes of the mould with the processes taking place in sweet rot and gray rot, and to formulate their interrelations in a hypothesis. This rests on the nutritional conditions influenced by osmoregulation and elucidates the dependence of sweet rot on geographical and meteorological factors.

Acknowledgements: We wish to express our thanks to Professor J. BÁNHEGYI, who made it possible to carry out this series of experiments and gave us valuable advice. Thanks are also due to Professor I. SOÓS for helpful criticism of this paper from the ampelological point of view.

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THE ROLE OF SERINE IN THE METABOLISM OF ESCHERICHIA COLI STRAINS

By

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Since KAUFFMANN's investigations, typing of *E. coli* has been performed almost exclusively by serological means. Biochemical examination serves generally for determining the genus of bacteria. In the typing of strains within the genera, serological examination is given preference over biochemical methods. The serological method has been used in recent extensive investigations concerning the classification of *E. coli* strains [1, 18, 19].

Instead of the generally applied method based on the antigenic structure, some authors still use biochemical examinations for typing *E. coli* strains. In addition to examining fermentation of carbohydrates and alcohols, strains can be differentiated by determining their antibiotic sensitivity [8, 16], and their amino acid requirements. Investigations into the amino acid metabolism of *E. coli* have been carried out chiefly in genetic studies. In examining X-ray and ultra-violet light induced mutations, the determination of the amino acid requirement of bacteria can be regarded as the method of choice [2, 6, 9, 10, 12, 15, 20—23].

The amino acid metabolism of *E. coli* strains occurring in nature has been investigated in less detail. SCHREIER and RUTHE examined the amino acid metabolism of *E. coli* strains by determining amino acids in bacterial hydrolysates and in media in which growth had taken place, and by observing the amino acid requirements [17]. They found no difference between pathogenic and non-pathogenic strains of *E. coli*.

KELNER and HELLMUTH, using 19 amino acids, found that some of them inhibited and others stimulated the growth of bacteria [11]. DAGLEY *et al.* determined the amino acid production of *E. coli* strains [4]. DAWES showed that in the presence of glucose *E. coli* strains could utilize only glutamic acid, aspartic acid and serine as a source of nitrogen [5]. DAWES found no difference among various *E. coli* strains as regards utilization of these amino acids.

The purpose of the present investigations was to study eventual differences in the amino acid utilization of the *E. coli* strains used in this study.

Materials and methods

In the experiments 220 *E. coli* strains were used. All the strains produced indole, were Voges-Proskauer negative, methyl red positive and neither of them grew in Koser's citrate medium. Twenty of the strains were isolated from the faeces of calves with or without enteritis by Dr. P. Áldásy, who determined their morphology, their biochemical behaviour and classified them by O antigens. One hundred of the strains were isolated by Dr. L. Pesti from the faeces of normal pigs and from the faeces of pigs with enteritis. The remaining 100 strains were cultured by the authors from the faeces and organs of calves and lambs with enteritis. The strains were preserved on peptone agar slants.

In the first part of the investigations, *E. coli* strains were cultured in synthetic media, each containing only one amino acid as a sole source of nitrogen and carbon. Growth in these media indicated the utilization of the corresponding amino acid. The basal synthetic medium consisted of NaCl, 0.75% ; KH_2PO_4, 0.1% ; Na_2HPO_4, 0.05% ; $MgSO_4$, 0.01% ; $Na_2S_2O_3$, 0.01% ; in distilled water. To the basal medium the following amino acids were added at concentrations of 5 mM. Glycine, dl-alpha-alanine, dl-phenylalanine, dl-valine, l-serine, dl-lysine, l-leucine, dl-methionine, l-cystine, l-cysteine HCl, dl-tryptophan, l-tyrosine, dl-threonine, l-proline, l-arginine, dl-isoleucine, dl-norvaline, l-asparagine, l-aspartic acid, l-glutamic acid and dl-histidine. The medium was adjusted to pH 7.1 with NaOH, autoclaved at 110° C for 20 minutes and finally tubed in 5 ml amounts. Inoculations were made with 10^6 organisms grown on agar slants. Prior to inoculation the bacteria were washed several times in saline. The cultures were incubated at 37° C for 7 days. Growth was examined daily by means of a Pulfrich nephelometer. The results were generally expressive in the 72nd hour ; no significant changes occurred on further incubation.

Decomposition of amino acids by *E. coli* was shown as follows. In a 0.02 M phosphate buffer of pH 7.5, amino acids were dissolved at concentrations of 20 mM. The solutions contained pyruvic acid at a concentration of 40 mM as a nitrogen acceptor, for showing possible transamination. Fermentation was performed in a final volume of 1.5 ml. To the 1.5 ml samples bacteria corresponding to 10 mg dried organisms were added. Cultures were grown on peptone-agar slants for 16 hours and before addition the bacteria were washed several times.

Tubes were incubated for 16 hours at 37° C under aerobic conditions. After incubation the suspension was centrifuged and changes in the amino acid content of the supernatant were studied by paper chromatography.

For paper chromatography, Schleicher—Schüll's filter paper was used. With a platinum loop 0.02 ml of the supernatant was deposited on the paper. As a solvent, butanol-acetic acid saturated with water was used. After an upward development for 18 hours the paper sheets were sprayed with ninhydrin solution (0.2 per cent in butanol), then heated 20 min. at 90° C. Thus, the amino acids appeared as coloured spots at various distances beyond the point of original application of the supernatant, according to the R_F values.

Detection of pyruvic acid in the fermentation tubes was performed as described by Friedmann and Haugen [7].

Results

The growth of 30 *E. coli* strains was examined in synthetic media for the utilization of 21 various amino acids. The results of the repeated experiments are summarized as follows.

Of the amino acids listed above, all the 30 strains could utilize glutamic acid, aspartic acid, asparagine and alpha-alanine as a sole source of nitrogen and carbon. There was no difference between these amino acids as to the rate of multiplication and the number of organisms after 72 hours of incubation. In media containing tryptophan or cysteine no growth of some strains occurred. Other strains, however, showed a degree of multiplication never reaching that of strains cultivated in media containing glutamic acid or aspartic acid.

A variable degree of utilization of tryptophan and cysteine was observed in various *E. coli* strains. In the metabolism of *E. coli* the clearest results were yielded by serine. In a synthetic medium containing serine as a sole source of nitrogen and carbon, some strains showed abundant growth, while others completely failed to grow in it. In the utilization of serine no such various degrees of growth were demonstrable as in the case of tryptophan or cysteine. Neither of the strains multiplicated in media containing any of the remaining amino acids.

Two hundred and twenty *E. coli* strains of various sources were examined as to the ability of utilizing serine. Of the strains, 97 failed to grow in the synthetic medium containing serine, while the growth of 123 strains was supported by this amino acid. However, all the 220 strains grew when to the medium pyruvic acid or glucose was added at concentrations of 1 mM and 0.2 per cent, respectively.

Using adequate controls, the fermentation of 21 amino acids was investigated by strains growing in the presence of serine and by strains unable to utilize this substance. Of the 21 amino acids, asparagine and aspartic acid were fermented by all the strains while serine was fermented only by part of the cultures. Neither fermentation of other amino acids nor transamination of pyruvic acid into alanine was observed.

E. coli strains utilizing serine perfectly decomposed it in the fermentation tubes, while those not growing in serine medium did not attack this amino acid. Decomposition of serine was not due to transamination, as in the presence of pyruvic acid no spot corresponding to alanine was observed on the chromatogram. The present examinations, in agreement with those of MEINHARDT and SIMMONDS [13, 14] and BOYD *et al.* [3], showed that *E. coli* decomposed serine by deamination into pyruvic acid and ammonia.

E. coli strains were examined as to the connection between their behaviour to serine and their other properties. The action of the strains on carbohydrates, their sensitivity to penicillin, streptomycin and chloramphenicol, their haemolytic and haemagglutinating power and their O antigenic structure have been examined partly by the authors, partly by others. In comparing these properties some association could be demonstrated between the reaction of strains to serine and the fermentation of sucrose. *E. coli* strains decomposing serine generally did not split sucrose ; on the contrary, sucrose was fermented by strains not acting on serine.

The such association between the decomposition of serine and fermentation of sucrose occurred in 80 per cent of the strains. Neither serine nor sucrose was split by 20 per cent of the strains. These observations indicate that the presence of a deaminase system for serine excludes the simultaneous occurrence of an enzyme system active on sucrose.

The action on serine of various *E. coli* strains isolated from the faeces and organs of healthy animals and from animals suffering from enteritis and septicaemia can be summarized as follows.

Some of the *E. coli* strains isolated from the faeces of normal animals and of animals with enteritis decomposed serine while some did not. In septicaemia, from the organs (blood, spleen, liver, kidney) *E. coli* strains not acting on serine were isolated in most cases.

Discussion

From the result of the present experiments it may be concluded that serine as a metabolite has a type determining role in the metabolism of *E. coli*. As regards their behaviour to serine, some of the strains grew in a synthetic medium containing serine, others failed to do so. In the utilization of serine no transient types were found. Thus, according to the presence or absence of an enzyme system for the decomposition of serine, *E. coli* strains may be divided into two definite metabolic types. In the synthetic medium containing serine bacteria can multiplicate only if both the amino acid as a nitrogen donator, and a carbon chain necessary in the synthesis of other amino acids are present. Strains growing in serine medium can produce this lacking carbon chain by deaminating serine to yield pyruvic acid. From pyruvic acid the organisms can synthetize amino acids and nucleic acids. Deamination of serine by bacteria growing in serine-medium was observed in fermentation tubes. On the other hand, organisms not growing in serine-medium did not deaminate the amino acid. The important role of pyruvic acid in the metabolism of *E. coli* is confirmed by our observation that the addition of pyruvic acid supported the growth of strains not multiplicating in serine medium.

The growth of *E. coli* strains in synthetic medium containing serine depends on the presence of a serine deaminase system. This property of the strains has been found very stable.

E. coli strains deaminating serine did not decompose threonine under the circumstances described. This finding does not confirm the statement of WOOD and GUNSALUS that serine and threonine are decomposed by the same enzyme [24]. In accordance with DAWES [5], and BOYD and LICHSTEIN [3], no such enzyme activity could be demonstrated by us.

In *E. coli* strains a regular association was found between the decomposition of serine and the fermentation of sucrose. Strains possessing a deaminase system for serine were unable to ferment sucrose.

In the intestinal tract of healthy animals and animals with enteritis strains not acting on serine, as well as strains decomposing this amino acid were found. However, among strains of non-faecal origin the proportion of those not decomposing serine was much higher than expected from their occur-

rence in the intestinal tract. This high frequency of strains not acting on serine seems to indicate that these bacteria can more easily invade the blood stream, or multiplicate in it.

The result of the present investigation of 220 *E. coli* cultures does not yet allow to conclude that a clear association exists between the decomposition of serine and the pathogenicity of strains. Investigations involving a larger number of strains must be made before the aetiological role of these *E. coli* strains can be determined.

Summary

The utilization of amino acids by 220 *E. coli* strains of various sources has been examined. *E. coli* strains uniformly utilize of glutamic acid, aspartic acid and alpha-alanine. In a synthetic medium containing serine only some of the strains multiplicated. The latter strains, unlike others, deaminated serine in fermentation tubes. In comparing the biochemical properties of *E. coli* strains, it has been found that the action on serine excludes the fermentation of sucrose.

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CORRELATIONS BETWEEN THE ANTIGENIC STRUCTURE AND PHAGE SENSITIVITY OF TYPES OF GROUP SHIGELLA FLEXNERI

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The sensitivity to bacteriophages of the various groups of Shigellae has not been extensively studied, except the group *Sh. sonnei* (now confirmed to be a separate group), which HAMMARSTRÖM [1] has divided into 68 types on grounds of investigations involving 11 phages. *Sh. shigae*, which tends to occur less and less frequently, has not been phage-typed in such a way.

Similarly scarce is the evidence concerning the sensitivity of the Flexner group to bacteriophages. In their investigations on the types V, W, Z, X and Y, BURNET and MCKIE [2] have detected a close relationship between the antigenic type and phage sensitivity of *Sh. flexneri* strains. SARTORIUS [3] subdivided the Flexner group on grounds of phage sensitivity into two groups. The strains H, X, F and D, are sensitive to the phage used by him, and the strains A, B, C, G, Y_1 and Y_2, are resistant to it.

RADOJČIC [4] grouped 11 types of *Sh. flexneri* according to their sensitivity to phage D of SARTORIUS and WINKLER—HELMRICH [5] studied the sensitivity of all Shigella groups to 4 phages and found the Flexner strains to be sensitive to them. Like SARTORIUS, she found individual variations in the grade of sensitivity of Flexner strains, but thought these differences to be unsuitable for classification.

Problems of identification, which have not been solved in the case of the *Sh. flexneri* group even at the Rio de Janeiro conference, are apparently responsible for the incompleteness of pertaining data. It may, therefore, arise whether or not it is reasonable to study in detail the phage sensitivity of that group of microbes, before the complete elucidation of its serological and biochemical relations. We have started research in this direction for two reasons. (i) In recent years most outbreaks of dysentery in Hungary were due to *Sh. flexneri* strains and it was hoped that such investigations would help to solve epidemiological problems. (ii) The Flexner group, being serologically heterogenous, appeared suitable for comparative phage sensitivity and antigenic structure studies, and to yield interesting theoretical and practical data.

Materials and methods

1. *Isolation and choice of phage.* Non-adapted phages, found to be suitable for such work on the basis of their valence even without adaptation, were used.

The phages were isolated from hen's faeces in the following way. A nut-sized piece of recently collected faeces was thoroughly mixed with 10 ml broth. After 4 hours of incubation at 37° C and treatment for 30 minutes in a water bath of 58° C to kill bacteria, the suspension was centrifuged. The supernatant was tested by the usual methods against Flexner strains of different serological types for phage content. 105 of the 150 isolation experiments yielded positive results. The phages capable of lysing all the strains regardless of the serological type and those generally non-lysing were excluded from further study. Fifty phages proved to act differently on the different serotypes. This group was further selected in the first test, because numerous cultures yielded phages of identical valence. Phages lysing all serotypes except types 6 and 4a spec were isolated most frequently. Phages active on types 2a, 2b, 3, 4b, var. X and var. Y were also common. The phages lysing serotype 6 never lysed *Sh. flexneri* strains of the other serotypes.

Subsequently, the phages of different valence were propagated on agar cultures of susceptible strains using one plaque for each transfer until the plaque size had become uniform. The phage thus prepared was then propagated in broth cultures until titres of 10^{-4} or 10^{-5} had been attained. This required usually 5 to 6 propagations. 2.5 ml of the 4-hour broth culture of the susceptible strain and 1 ml of the phage were added to 50 ml of broth. After 18 hours of incubation at 37° C and 30 minutes of inactivation at 58° C the mixture was titrated. If the titre was suitable, the residual suspension was Seitz-filtered, instead of being inactivated. Phage titration was carried out on the agar plate using 10-fold dilution series of phage. The highest dilution causing complete lysis of the sensitive strain was accepted as the phage titre. This dilution was used in subsequent studies. In the course of further propagation some more phages were discarded, because with the rise of titre their valence had widened. Finally, 13 phages proved to be suitable for differentiation of strains. The designations of these phages were, 2, 5, 77, 7, G, 60, 6a, o, p, t, z, g, 1 hb.

2. *Selection of indicator strains.* In the early phase of the investigations, standard type series of *Sh. flexneri* strains were used as the indicator of phage activity. The strains had been obtained from Prof. K. RAUSS. Director, *Institute of Microbiology, Medical University of Pécs*, to whom we express our thanks. Later, however, we used recently isolated strains, especially wishing to study the practical aspects of the problem. These strains, collected from routine isolations made at the *Department of Bacteriology* of this Institute, were antigen-typed in the fresh state by the use of factor sera, prepared as described by RAUSS [6]. As type 5 did not occur in the large material studied, it had to be omitted in phage-typing. The only type 5 standard strain we possess is sensitive to phages 2, 77 and G.

3. *Determination of the sensitivity of strains.* The strains were tested for phage sensitivity by the agar plate method of CRAIGIE and YEN. The following grades of lysis were distinguished. 1. Complete lysis ; 2. complete lysis, with a confluent, homogeneous, transparent secondary culture at the base ; 3. semi-confluent lysis ; 4. isolated plaques of lysis ; 5. no lysis, or the number of plaques was lower than 10. (We found namely that, if less than 10, the number of plaques is not constant and thus cannot be evaluated.) Later, for the identification of the main phage types we have chosen such dilutions of the phage which gave rise either to complete lysis or no lysis at all. Within the main phage types, however, the size of plaques had sometimes also be taken into consideration, if that proved to be persistent and conspicuously different from the usual. Likewise, we designated separately those cases which exhibited the presence of a confluent, homogeneous, transparent secondary culture in the area of complete lysis. When the results of phage sensitivity tests and serological studies had made it necessary, the usual biochemical tests were also carried out.

Results and discussion

1. *Correlations and differences between antigenic type and phage sensitivity.*

By the methods mentioned, 820 strains of *Sh. flexneri* have been tested for phage sensitivity and of these the antigenic type of 734 was determined. The following correlations were found between the phage sensitivity and antigenic type of the strains.

On the basis of their sensitivity to our 13 phages, the *Sh. flexneri* strains were classified into 10 phage types, designated with the letters "a" to "k" (Table I). It is pointed out again that no type 5 strain was tested, because such a strain had not been isolated from the samples. Table I reveals that

Table I

Principal phage types of the strains of the Sh. flexneri group

Serological type	Sensitivity to the phages isolated by us													Phage type
	2	5	77	7	G	60	6a	o	p	t	z	g	1 hb	
Sh. flexneri														
1 a	+	—	—	+	+	—	—	—	—	—	+	—	—	a
1 b	—	—	—	+	+	—	—	+	—	—	+	—	—	b
2 a	+	+	+	—	—	—	—	+	+	+	+	+	+	c
3	+	+	—	—	—	—	—	+	—	—	+	—	—	d
4 a spec.	—	—	—	—	—	—	—	—	—	—	—	—	—	e resistant
4 b	+	+	—	+	+	—	—	+	—	—	+	—	—	f
6	—	—	—	—	—	+	+	—	—	—	—	—	—	g
6	—	—	—	—	—	—	+	—	—	—	—	—	—	h
2 b and var. X.	+	+	+	—	—	—	—	+	+	—	+	+	—	i
group 4 a and var. Y	+	+	+	+	+	—	—	+	+	+	+	+	+	k

Explanation of signs: + = complete lysis
— = no lysis

type "a" strains were sensitive to phages 2, 7, G. z; "b" type ones to 7, G, o, z; "c" type strains to phages 2, 5, 77, o, p, t, z, g, 1 hb. In general, the single phage types represented strains of the same antigenic type. For example, the serotype of "a" type strains was 1a, that of "b" type ones 1b, and so forth. Furthermore, phage type "i" connected serologically the variants 2b and X, and the phage type "k" the group phase 4a and variant Y. RAUSS, deviating from other terminologies, classified variants 2b and X in the same group, on the basis of their common group-antigen [6]. The identical sensitivity to phages also shows these two types to belong to the same group.

The only strain of 4a group phase isolated from routine material showed the same sensitivity as variant Y. The phase variations of type 4a strains have been extensively discussed in the literature [7, 8, 9]. Although on the basis of serological data there is some difference of opinion as to the relationship of 4a group phase strains to variant Y, there is full agreement in that the two serological types stand very close to each other. This is supported by the identity in phage sensitivity.

The remarkable phage resistance of the strains in the specific phase 4a is another interesting phenomenon as regards the relation of serological behaviour and phage sensitivity. In connection with this let it be mentioned that, according to RAUSS [6], in the specific phase the strains of type 4a have a so-called K antigen, and the developing of 4a group phase is a result of minus variation represented by the loss of this surface antigen. It may, accordingly, be assumed that our phages combined with the group antigen of Shigellae. This will make evident why the strains possessing antigen believed to be surface-bound (in the given case K antigen), were resistant to phage. However, we hope to find further phages capable of lysing also the strains 4a spec, it having often occurred that strains, which had been believed to be phage resistant, were lysed when exposed to the action of the adequate phage. For example, type 6 of *Sh. flexneri* was lysed by two phages isolated by us, although SARTORIUS and WINKLER had found the same type to be phage resistant.

Note phage types "g" and "h", dividing serotype 6 into two separate phage types. On the basis of their close serological relationship, BOYD listed the mannite-negative, gas-forming Newcastle type, the Manchester type producing gas from dextrose, mannite and dulcite, as well as the BOYD type 88 not producing gas, into the same group, *Sh. flexneri* 6. Here we refer again to the fact that none of the phages lysing type 6 isolated by us lysed any other type of *Sh. flexneri*, and it were invariably and exclusively the types 6 and 4a spec that were resistant to phages lysing every Flexner type. Further investigations will have to elucidate the property of which this peculiar sensitivity to phage is due.

Eleven of the 15 strains of serotype 6 were classified as phage types "g" and 4 as "h". As it will be seen below, other strains of uniform antigenic structure also showed differences in sensitivity, which were accepted as "variations" and not separate types. As to the justification of accepting the existence of two distinct phage types in the case of serotype 6, the following explanation is put forward. With the variants the differences in sensitivity occurred invariably against the (originally 12) phages used as standards. With serotype 6, however, the difference between types "g" and "h" was manifest against a phage lysing exclusively strains of serotype 6. It became necessary to isolate phage 6a just because strains of serotype 6 resistant also to the original phage "60" had been encountered. The strains of phage types "g" and "h" could not be considered biochemical variants, because their biochemical activity was the same. The role of thermolabile antigens of the strains of serotype 6 in the differences in sensitivity to phage requires further investigation.

In view of the comparatively rare occurrence of type 6, the number of strains of phage type "g" and "h" does not permit conclusions as to the epidemiological significance of the two phage types. The strains had been isolated from patients showing no epidemiological correlations whatsoever.

2. *Variants.* Within the described 10 types of sensitivity to phage we found strains showing some differences in sensitivity, which, for reasons outlined above, have been classified as variants of the type in question. For example, 4 types of variation occurred within type "c", 1 in type "d" and 2 in type "k" (Table II). It is emphasized again that when listing variants,

Table II

Phage type variations

Sensitivity to the phages isolated by us													Phage type variant	Serotype of *Sh. flexneri*
2	5	77	7	G	60	6a	o	p	t	z	g	1 hb		
(+)	(+)	(+)	—	—	—	—	(+)	(+)	(+)	(+)	(+)	(+)	C_1	*Sh. flexneri* 2a
+	+	+	—	—	—	—	+	+	—	+	+	+	C_2	
+	+	a	—	—	—	—	a	a	a	a	—	a	C_3	
+	+	+	+	—	—	—	+	+	+	+	+	+	C_4 (c→k)	
+	+	+	—	+	—	—	+	+	+	+	+	+	k_1 (k→c)	*Sh. flexneri*
+	+	+	+	+	—	—	+	+	+	+	+	—	k_2	var. Y.
+	+	—	—	—	—	—	—	—	—	+	—	—	d_1	*Sh. flexneri* 3

Signs: + = complete lysis
(+) = complete lysis, with homogeneous secondary culture at base
a = pin point sized isolated plaques
— = no lysis.

persistent and constant quantitative differences in lysis and the characteristic size of the plaques were also taken into consideration. The relationship between phage types "c" and "k", and especially that between "c_4" and "k_1" in particular, merit attention. The differences between the two variants are ascribable to their different sensitivities to the phages 7 and G and without serological examination both could be termed either "c" or "k" phage type. The agglutination tests with factor sera showed, however, that the antigenic type of "c_4" was 2a and that of "k_1" the variant Y. This observation is in full agreement with the serological evidence that type 2a and variant Y are closely related serologically, and have many grades of evolution between them. According to RAUSS [6], variant Y may develop from type 2a in 5 steps of phase variations. The discussed variations in sensitivity to phage also confirm this view. It should be mentioned in this connection that we found a Flexner carrier who on the first 4 occasions excreted type "k_1" and later type "k".

3. *Sensitivity of other groups of Shigellae and of other intestinal pathogenic bacteria to the Flexner phages isolated by us.* The sensitivity to our phages of 20 strains of *Sh. sonnei* and 5 of *Sh. schmitzi* was negligible (Table III and IV). The more marked sensitivity shown by some *Sh. sonnei* strains differed in the pattern of lysis from all strains of *Sh. flexneri.*

Table III

Sensitivity of Sh. sonnei strains to the phages isolated by us

Each of the strains shown was resistant to phages 5, 7, G, 6a, o, p, t, z, g and 1 hb

Designation of strain	Sensitivity to the phages isolated by us		
	2	77	60
Sh. sonnei 1	a	—	—
,, ,, 2	a	—	(+)
,, ,, 3	a	+	(+)
,, ,, 4	a	—	—
,, ,, 5	—	+	a
,, ,, 6	a	—	—
,, ,, 7	a	—	—
,, ,, 8	—	—	—
,, ,, 9	a	—	(+)
,, ,, 10	a	a	a
,, ,, 11	—	—	—
,, ,, 12	—	—	—
,, ,, 13	—	—	—
,, ,, 14	—	—	—
,, ,, 15	a	+	(+)
,, ,, 16	—	+	(+)
,, ,, 17	a	+	(+)
,, ,, 18	—	—	—
,, ,, 19	—	—	—
,, ,, 20	a	—	—

Signs as in Table II.

Table IV

Sensitivity of Sh. schmitzi strains to the phages isolated by us

Each of the strains shown was resistant to phages 2, 5, 77, 7, G, 60, 6a, p, g and 1 hb.

Designation of strain	Sensitivity to the phages isolated		
	o	t	z
Sh. schmitzi 1	—	—	—
2	a	a	+
3	+	a	a
4	a	+	a
5	a	+	a

Signs as in Table II.

During the experimental period no *Sh. shigae* strains were encountered in the routine material. The 2 laboratory strains were resistant to our phages.

The 13 strains of *E. coli* dyspepsiae of different serological and phage type were resistant to our phages*, and only a few of the standard *S. typhi* strains of different Vi phage type were slightly sensitive to them (Table V). The strains of Vi phage types B_3, D_1, D_4, D_6, E_2 etc. not included in Table V were resistant to our phages.

Table V

Sensitivity of some standard S. typhi Vi strains of different phage type to the Flexner phages isolated by us

Each of the strains shown was resistant to phages 5, 7, G, 60, 6a, o, t and 1 hb.

Strain (phage type)	Sensitivity to the phages isolated by us				
	2	77	p	z	g
A	K	K	K	—	K
B_1	K	—	—	—	—
B_2	K	K	—	—	—
C_1	a	—	—	—	+
D_2	K	—	—	+	—
D_5	—	—	—	+	—
E_1	+	—	K	—	K
H	—	—	—	+	—
J	K	—	—	—	—
K	K	—	—	—	—
L_1	K	—	—	+	—
L_2	K	K	—	—	—
N	K	—	—	—	—
O	K	—	—	—	—

Signs: K = isolated plaques of lysis, 1 to 1.5 mm in diameter
a = isolated plaques of pin point size
+ = complete lysis
— = no lysis

In Table VI are presented the Salmonella strains showing some degree of sensitivity to our phages. It must be emphasized that the sensitivity of none of the Salmonella strains was identical with that of any of the *Sh. flexneri* strains.

It can be concluded that the phages used for typing the group *Sh. flexneri* proved to be rather specific.

* Phage typing of these strains was done at the *Phage Laboratory of this Institute.*

Table VI

Sensitivity of some Salmonella strains belonging to different serological groups to the Flexner phages isolated by us

Each of the strains shown was resistant to phages 5, 7, G, 6a, o, t and 1 hb.

Strain (serological group)	Sensitivity to the phages we have isolated					
	2	77	60	p	z	g
S. paratyphi B 1. f. (B)	K	—	—	—	—	—
S. essen (B)	a	—	—	—	—	—
S. cholerae suis var. R. 1. f. (C)	K	+	—	+	K	+
S. typhi 0.901 (D)	+	K	—	K	—	+
S. typhi H (D)	K	a	—	—	—	a
S. enteritidis var. Danys (D)	K	—	—	—	—	—
S. dublin (D)	K	—	—	—	—	—
S. panama 1. f. (D)	K	+	K	+	K	+

Signs as in Table V.

4. *In vivo stability of phage types.* The changes in the sensitivity of *Sh. flexneri* strains excreted at different times by the same individual or by patients living in the same community were also investigated. No change in sensitivity occurred, except in one case, in which the *Sh. flexneri* strain excreted 14 times by a patient with chronic dysentery was type "k_1" in the first 4 tests and type "k" in subsequent ones. As to the other cases, strains unchanged in phage sensitivity were recovered from the faeces of the same person twice in 27 cases, thrice in 11, 4 times in 9, 5 times in 3, and 6 times in 1 case. As expected, the strains excreted by individuals living in the same community showed the same sensitivity, as determined by 2 tests in 12 cases, 3 in 5, 4 in 1 and 5 in 1 case.

5. *Type distribution.* Table VII contains the type distribution data for the *Sh. flexneri* strains isolated at the *Department of Bacteriology* of this Institute, in the period from October, 1953, to March, 1955, with special reference to phage and antigenic type. Table VII has the error, common to nearly every similar grouping, that a chronic carrier might have been responsible for the prevalence of some, otherwise less common type. For example, 14 of the variant Y strains tested were isolated from the same individual. Nevertheless, we presented all the Y strains recovered, because a similar disproportion might have occurred with any of the types.

It is clear from Table VII that the dominant serological types of the group *Sh. flexneri* may be typed, as we have described it on grounds of the phage sensitivity. In the case of less common types, such as groups X and 4a, as well as the two phage types of serotype 6, epidemiological studies will

Table VII

Sh. flexneri phage types in the material of the State Institute of Hygiene in the period October, 1953—March, 1955

Phage type (serotype)	Incidence	
	absolute	percentage
a (1a)	1	0.1
b (1b)	16	2.2
c (2a)	596	81.2
d (3)	44	6.0
e (4a spec.)	14	1.9
f (4b)	3	0.4
g (6)	11	1.5
h (6)	4	0.5
i (2b and var. X)	5	0.7
k (group 4a and var. Y)	40	5.5
Total	734	100.0

have to decide the practical value of the method. Furthermore, the significance of the differences in phage sensitivity pointed out as type variations will also have to be determined by further investigation.

Summary

1. As determined with 13 non-adapted phages grown from hens' faeces, 820 strains of *Sh. flexneri* isolated from routine material fell into 10 phage types.

2. Each of the phage types from "a" to "f" corresponded to serotypes *Sh. flexneri* 1a, 1b, 2a, 3, 4a spec. and 4b, respectively. The identical sensitivities (phage types "i" and "k") of variants 2b and X, as well as those of group phase 4a and variant Y have been explained by the close antigenic relationship between them. Serotype 6 could be divided into two phage types ("g" and "h"). The phage resistance of the strains of specific phase 4a, was ascribed to the special antigenic structure of these strains.

3. Other Shigella groups and other intestinal pathogenic bacteria were generally only slightly sensitive to the phages tested.

4. The phage sensitivity of strains excreted by the same individual or by patients living in the same community proved that *in vivo* the phage types were stable.

5. Determination of the phage sensitivity of the *Sh. flexneri* group is suitable for obtaining data on type distribution in the case of dominant types.

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THE INFLUENCE OF IRON UPON OXYTETRACYCLINE PRODUCTION BY STREPTOMYCES RIMOSUS

By

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The examination of the sensitivity of antibiotic production to iron is of a great economic importance. The iron sensitivity of fermentations is systematically investigated in this laboratory. In this connection some interesting observations have been made in the course of oxytetracycline production by *Streptomyces rimosus* [1, 2]. A preliminary report has been published elsewhere [5].

Materials and methods

Strains and preservation of cultures. In the experiments *Streptomyces rimosus* strains B-24 and BS-21 were used. In medium B, strains B-24 and BS-21 produced 900 μg/ml and 1800 μg/ml of oxytetracycline, respectively. The solid medium used for the preservation of cultures was prepared as follows : corn steep liquor (50% total solids) 2.5%, yeast extract 5%, glycerol 5%, agar 2%. pH prior to sterilization, 7.0. This medium was prepared on the basis that sporulation of *Streptomyces rimosus* was enhanced by polyalcohols such as glycerol, sorbitol and mannitol.

In the experiments, however, inoculations were performed with a spore suspension cultivated in shaker flasks, using a rotating shaking machine, and not with spores harvested from the above solid medium. Cultivating the inoculum of *Streptomyces rimosus* in a relatively poor medium in shaker flasks, most of the developing mycelium formed spores and, beside some autolysed and disintegrated mycelium, the culture contained 10^8 to 10^{10} spores per ml. This culture was suitable for the inoculation of fermentations. Storing at — 10° C kept it so for one year. Preparation of this "shake-spore suspension" was performed in the following medium : soybean meal (defatted) 1%, yeast extract 10%, glucose 1%, $CaCO_3$ 0.1%, NaCl 0.3%, pH prior to sterilization, 7.0.

Preparation of yeast extract : 1 kg yeast is liquefied by ethyl acetate ; mixed with 1 liter of water, autoclaved at 121° C for 20 min ; and left to stand for a few hours. Then the somewhat turbid upper part of the solution is used.

Fermentation. Fermentation was carried out at 28° C in 500 ml Erlenmeyer flasks containing 100 ml of medium. The flasks were shaken on a rotating shaking machine. Every point of the shaking table made a circle 2 cm in diameter at a rate of 300 r. p. m. The media were similar to those published in the U. S. Patent [2] :

Medium A : Soybean meal (defatted) 2%, potato starch 2.5% corn steep liquor (50% total solids) 0.4%, NaCl 0.3%, $CaCO_3$ 0.5%, pH prior to sterilization, 7.0.

Medium B : Soybean meal (defatted) 3%, potato starch 0.5%, corn steep liquor (50% total solids) 0.1%, NaCl 0.3%, $CaCO_3$ 0.5%, fat or oil 0.4%. pH prior to sterilization, 7.0. In the 48th, 72nd and 96th hour of the fermentation 0.3 per cent of fat or oil was added, under sterile conditions.

Medium C. The same as medium B, but containing 1 per cent of fat or oil. In this case no oil was added during fermentation.

Sterilization of media was performed at 121° C for 20 min. All three media contained 12 to 16 μg/ml iron, the original quantity contained by the ingredients. Further quantities

of iron were added to the media as an aqueous solution of ferrous sulfate, in some cases before sterilization, in others after sterilization. The Tables and Figures show always the added quantity of iron. The experiments proved that the inhibiting action of iron on oxytetracycline production was the same when the iron had been added before or after sterilization, provided that in the latter case addition was made at the time of inoculation.

Inoculations were made with 1 ml of "shakespore-suspension". The antibiotic content of the cultures was assayed from the 3rd day onwards daily for 5 days. All experiments were made in duplicate and each of them was repeated three times. The Tables present the average antibiotic production of the parallel cultures.

Methods of assay. The assay of oxytetracycline was performed by the method elaborated for chlortetracycline, using *Bacillus subtilis* strain ATCC-6633 [3]. For the assay the culture samples were mixed with an equal part of a 2 per cent oxalic acid solution, then left to stand at room temperature for 2 hours and centrifuged. Dilutions were carried out by an M/15 phosphate buffer of pH 6.2.

For the *determination of oil peroxide* content of the cultures, SIGGIA's method [4] was applied, adapted to the conditions prevailing in this work. The fatty material was extracted from a 100 ml sample with 2 × 20 and 1 × 10 ml of benzene. Extraction of the fermentation fluid by this procedure yielded an unseparable milky emulsion. To the benzene phase, or in the latter case to the emulsion, 50 ml of alcohol was added. The alcohol caused a precipitation in the emulsion, which was separated by filtration. To the filtrate 5 ml of 0.1 N sodium arsenite solution containing 25 g/l sodium hydrocarbonate was added. In case of formation of two phases the elimination of phase difference was performed by adding more alcohol. The solution was so evaporated that finally 30 to 40 ml of aqueous solution free from organic solvents remained. If necessary, water was added during evaporation. The arsenous acid content of the sample was determined by iodometric titration, in the presence of sodium hydrocarbonate. The oil peroxide content of the samples will be given in active oxygen values.

Results

In the first stage of the present experiments it was observed that, using fat-free medium A, 150 to 200 μg/ml iron did not inhibit the production of oxytetracycline. This concentration of iron is much higher than that occurring in fermentors made of iron (20 to 40 μg/ml). In medium B, however, if sunflower oil was added, the production somewhat decreased at iron concentrations usually occurring in iron fermentors, and practically ceased at higher iron concentrations. This observation showed that the inhibitory effect of iron is closely connected with the presence of oil. The experiments carried out with strain B-24 are summarized in Table I.

From Table I it is clear that the inhibiting action of iron depends on the quantity of unsaturated linkages present in the various fatty materials. With sunflower, soybean and other oils containing many unsaturated linkages, iron is highly toxic. On the other hand, iron practically does not decrease the production of oxytetracycline in the presence of substances with a low iodine value, such as palm oil or animal fat. When using other oils and fats having a low iodine value antibiotic production was low. The high softening point of these substances caused a difficulty in their utilization as a source of energy.

During fermentation the amount of mycelium produced, its colour and its morphology were systematically controlled. The amount of mycelium produced did not differ from that produced without the addition of iron, with

Table I

Toxicity of iron

Medium	Fatty material	Iodine number	μg/ml Fe										
			0	20	40	60	80	100	120	140	160	180	200
			μg/ml oxytetracycline										
A	—	—	830	890	910	800	840	800	—	820	—	—	820
B	Sunflower oil	130	870	860	390	250	110	80	—	50	—	30	10
B	Soybean oil	127	760	640	160	70	50	30	—	10	—	10	—
B	Linseed oil	174	910	440	200	40	0	—	—	—	—	—	—
B	Palm oil	52	840	710	760	860	700	760	—	600	—	650	—
B	Animal fat	50	780	870	870	—	920	—	—	750	—	750	—
B	Fish oil	132	800	720	510	—	300	—	—	50	—	0	—
B	Arachis oil	96	780	780	560	160	210	100	—	—	—	—	—
B	Hydrogenated sunflower oil	87	430	430	430	—	460	—	350	—	—	360	320
B	Hydrogenated sunflower oil	5	450	—	570	—	390	—	350	—	—	350	350
B	Palm kernel oil	15	540	600	660	—	770	—	—	540	—	—	580

Strain used : B-24. $FeSO_4$ added before sterilization

the exception of fermentations containing more than 100 μg/ml iron, and those containing oils with an iodine number higher than 100. In these latter cases multiplication was strongly inhibited. A more exact estimation of the degree of inhibition was not possible because of the composition of the medium. The colour of the fermentations changed parallel with the antibiotic production. At the end of fermentation in the presence of iron the usual green colour changed into a greyish-green. With lower yield of oxytetracycline less pigment was produced. Below 200 μg/ml oxytetracycline no pigment production occurred. *Streptomyces rimosus* in the media used at first grew in the typical form of filamentous mycelium, breaking up gradually in the 30th to 40th hour to rod forms, producing gradually spores and disintegrating. Meanwhile, there was continuous new growth. The quantity of mycelium produced reached its maximum only in the 70th to 80th hour. Microscopically, all the morphological stages of growth were simultaneously present. Deviation from this morphology occurred only if growth was so much inhibited that less mycelium was produced than normal. In this case the hyphae were shorter than normally breaking up to bacillary forms, and there was hardly any sporulation.

Using strain BS-21, which yielded more antibiotic than strain B-24, the above results were somewhat altered, as shown by Figure 1.

As it can be seen, the yield by strain BS-21 in medium A was not influenced by the increasing amount of iron, and at the same time it was lower

than in medium B. If using medium B with sunflower oil, the depressing effect of iron was the same as with strains B-24, but this effect occurred at a higher level of oxytetracycline production. On medium B with palm oil, and strain BS-21, on adding more than 50 μg/ml iron, a slight depressing effect was observed, but even with 200 μg/ml iron the yield of oxytetracycline reached 1200 μg/ml. As seen in Table I, with strain B-24 on medium B with palm oil no depressing effect occurred.

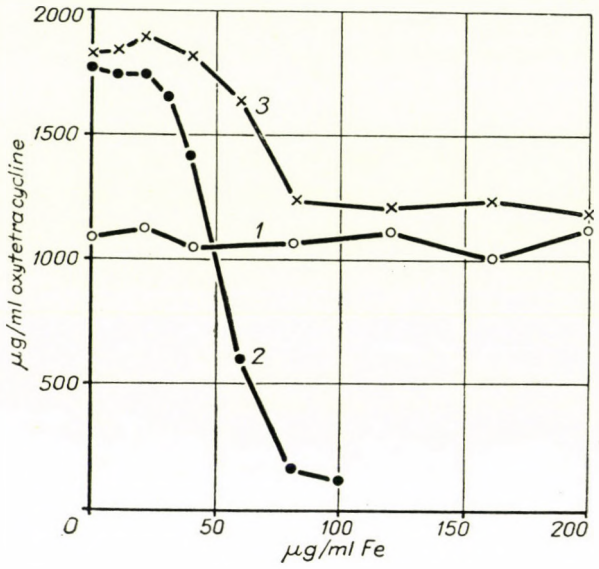

Fig. 1. Toxicity of iron

Explanations :
Strain used : BS-21. $FeSO_4$ added before sterilization.
1 = medium A, 2 = medium B containing sunflower oil, 3 = medium B containing palm oil

The effect of addition of iron in the various stages of fermentation was investigated by using medium C. Medium C containing sunflower oil was used in the oil peroxide determinations. The results are shown in Fig. 2/a. It is clear that, because of the higher oil content at start, iron was more toxic in medium C than in medium B. The later was $FeSO_4$ added to the culture, the less toxic it was. If iron was added in the 72nd hour, its inhibitory action was the same as together with palm oil. (See Fig. 1.)

Fig. 2/b shows the result yielded by the iron-free control flasks. By comparing Fig. 2/a with Fig. 2/b, it will be seen that strong inhibition occurred only in the early stage of fermentation, when production had not yet begun, when the culture was gradually multiplicating and most of the oil added was unchanged and still in the medium.

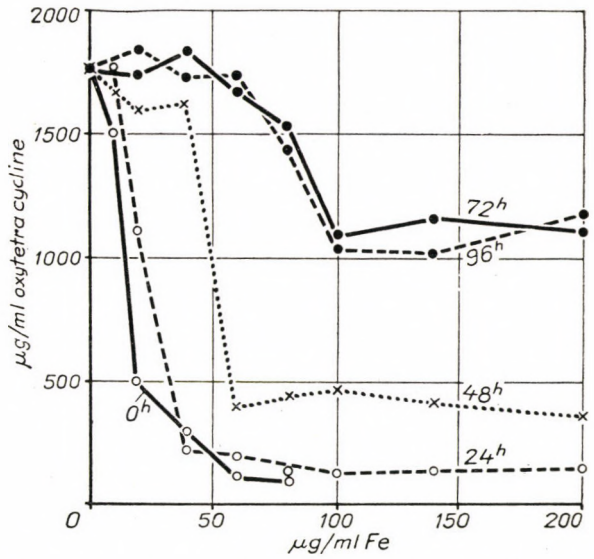

Fig. 2/a Toxicity of iron added at various periods of fermentation
Strain used : BS-21. Medium used : C. $FeSO_2$ added after sterilization

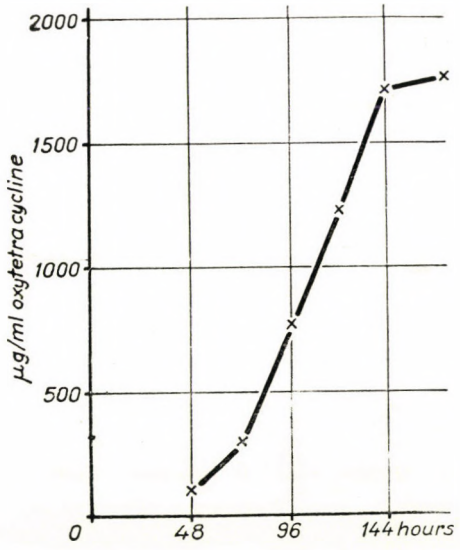

Fig. 2/b. Production of oxytetracycline
Strain used : BS-21. Medium used : C

It is known that the action of heavy metal catalysts on unsaturated oils induces production of peroxide compounds. As shown in Table II, peroxides may be produced by shaking oils with an aqueous solution of iron. Peroxide production in sterile and inoculated medium with sunflower oil was determined during incubation both in the absence and in the presence of 80 µg/ml of iron.

Table II

Formation of oil peroxide by the action of aqueous $FeSO_4$

Time in hours	Active oxygen mg/100 ml
0	0
4	2.5
18	3.8
24	1.7
72	1.0

0.05 g $FeSO_4 \cdot 7\,H_2O$ dissolved in 100 ml distilled water in a 500 ml Erlenmeyer flask with 1 ml sunflower oil added. Flask shaken at 28° C on a rotating shaking machine.

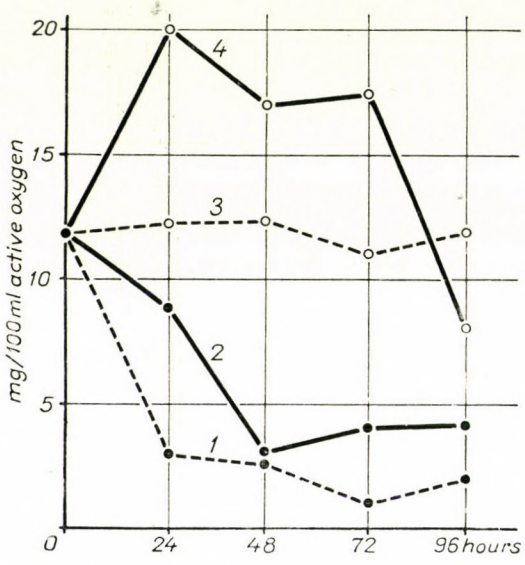

Fig. 3. Formation of oil peroxide in medium C

Explanations :

Strain used : BS-21. Medium used : C, containing sunflower oil. $FeSO_4$ added before sterilization.

1 = uninoculated medium
2 = inoculated medium
3 = uninoculated medium in the presence of 80 μg/ml Fe
4 = inoculated medium in the presence of 80 μg/ml Fe

The results of the determinations plotted aginst time are shown in Fig. 3. In medium C on sterilization 1.0 to 1.2 mg/100 ml of active oxygen was formed regardless whether the medium had been free of iron or had contained 80 μg/ml of it. In sterile medium this value decreased within 24 hours to 20 per cent of the value found at the beginning of incubation. This decrease

although at a lower rate, was taking place also in inoculated medium. In the presence of 80 μg/ml iron, however, the amount of active oxygen that had formed at sterilization remained the same for 5 days in the sterile medium. In the inoculated medium, in the presence of the same concentration of iron, the active oxygen value was doubled within 24 hours. This higher degree of active peroxide production was probably due to a more extensive catalytic surface caused by the growing microorganism.

Discussion

The present examinations revealed that oxytetracycline production by *Streptomyces rimosus* under the circumstances described is very sensitive to iron, provided that the used media contain unsaturated oils as a source of energy. The use of oils with a low iodine value may render iron fermentors suitable for antibiotic production. The inhibitory action of unsaturated oils in the presence of iron is due to the formation of peroxide compounds. It is not yet known whether peroxide compounds act directly or indirectly, through formation of hydrogen peroxide.

This interesting phenomenon was easy to observe, as the medium used contained fatty substances as a main source of energy. Preliminary experiments showed that under proper circumstances the above effect manifests itself also in other fermentations. Heavy metals other than cobalt might also induce a similar phenomenon.

These results are in conformity with observations made in industrial iron fermentors. In the course of sterilization from the wall of an iron fermentor generally 20 to 40 μg/ml iron is released into the medium, provided that the fermentor is used continuously without operating trouble and contamination. During these fermentations, the pH of the medium being nearly neutral, generally no further corrosion of iron occurs. In this case in a fermentation medium identical with medium B with unsaturated oils the depressing effect is slight, but statistically demonstrable. In the absence of a "protective layer" formed on the inside of the fermentors after several fermentations the iron content of the medium so increases that production greatly suffers if unsaturated oil are used. The "protective layer" is missing in newly started fermentors and is removed by brushing.

The results of the present experiments also show that on strains producing more antibiotic iron exerts a direct inhibitory action. This appears at iron concentrations more than 50 to 60 μg/ml, and is not due to peroxide formation. This direct influence of iron can be observed in medium B containing palm oil, and is proved by the fact that iron has the same effect both in the 72nd and 96th hour, and the result correspond to that of the previous experiment.

Summary

1. The influence of iron upon the antibiotic production by *Streptomyces rimosus* has been investigated. Production is strongly inhibited by the simultaneous presence of iron and unsaturated oils. This inhibition can be eliminated by using saturated oils and fats.

2. The inhibitory action of iron and unsaturated oils is due to the formation of oil peroxides.

3. At high fermentation values iron itself produces a direct but moderate inhibitory effect.

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FUNGISTATIC ACTIVITY OF THE SPECIES SPHAEROPSIDALES AND MELANCONIALES

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In the course of research concerned with fungi producing antibiotics, the antagonistic action of many species has already been investigated. A survey of the known microscopic fungi studied in this respect shows that almost each of them belongs to the order Hyphomycetes. Of the species in that order those were studied in the first place which can be isolated from soil or appear in artificial culture media as a result of air-borne infection. Thus, ample evidence is available as to the antagonistic activity of the species Apergillus and Penicillium, as well as of certain other moulds that can be grown readily, such as : Alternaria, Fusarium, Trichoderma, Trichothecium, *etc* . . . However, only a few reports deal with fungi belonging to other taxonomic groups and living under different conditions or being more difficult to cultivate artificially [1, 3, 6, 7].

The antagonistic activity of the species Sphaeropsidales and Melanconiales is less known. For this reason, we have investigated the fungistatic effect of some fungi belonging to this group.

The antagonistic activity of the species of the order Melanconiales has not yet been investigated [1]. Thus far, 7 species of 6 genera of the order Sphaeropsidales have been studied in this respect [1, 7]. CERCOS [3] showed that *Septoria nodorum* had a bacteriostatic activity, KENT [6] reported that *Diplodia zeae* and RIBALDI [7] that a *Camarosporium sp.* had a fungistatic effect.

Experimental

Cultivation of the species tested. Of the herbarium specimens of 48 species of Sphaeropsidales and 10 of Melanconiales ones were selected under a preparative microscope and placed into a wet chamber. The material was incubated for 20 hours at 26° C in order to cause a swelling of the acervuli and pycnidia or to induce the eruption of a conidial mass from the pycnidia. After swelling, from the erupted conidia and — in certain cases —, from the whole acervulus and pycnidium a suspension was made in sterile water. The suspen-

sions were streaked twice in Czapek-Dox and malt agar. In this way pure cultures were obtained from 2 species of Melanconiales and 24 species of Sphaeropsidales. The failures in cultivation were due to non-germinating conidia and to an eventual requirement of some special nutrient substances in some cases, but in many a case the failure was obviously the result of the appearance of extremely large numbers of bacteria, originating from the plant parts. In order to eliminate the contamination, repeated streakings were made in selective media of the following composition,

1. 10 per cent malt agar + 0.4 per cent sodium propionate [5, 8]
2. ,, ,, ,, ,, + 100 μg/ml streptomycin [4]
3. ,, ,, ,, ,, + 100 μg/ml penicillin G
4. ,, ,, ,, ,, + 100 μg/ml chloromycetin [4]

The above media were of different selectivity, depending on the bacterial flora of the host plant. In some cases the presence of 0.4 per cent sodium propionate inhibited also the growth of the fungus to be isolated. In the case of *Phoma herbarum* WEST, for example, streptomycin proved to be the most suitable agent, followed by penicillin, then by the medium containing chloromycetin, whereas sodium propionate inhibited even the germination of the conidium *(Fig. 1)*. By the above method pure cultures were obtained from 39 species.

In vitro studies of the fungistatic activity. To obtain preliminary information, the antagonistic effect of the isolate was tested against 6 test fungi, in two replications in Czapek-Dox and malt agar. The six test fungi were *Colletotrichum atramentarium*, *Colletotrichum lini*, *Rhizoctonia solani*, *Sclerotinia sclerotiorum*, *Aspergillus niger*, *Botrytis allii*. The procedure was as follows. According to the rate of growth, the isolates were divided into groups of four. In Petri dishes, about 10 cm in diameter, the test organism was inoculated in the centre and the four fungi to be tested at equal distances from it. The time at which the test fungus inoculated also depended on the rate of growth. In *Fig. 2* the growth of *Sclerotinia sclerotiorum* is inhibited only by *Phomopsis cordifolia (Brun.) Died.* In *Fig. 3.* the growth of the test fungus *Botrytis allii* is inhibited by both *Phomopsis cordifolia (Brun.) Died.* and *Phoma longissimum West.* Of the 39 species tested, these two exerted a marked inhibitory effect, whereas only the antagonism against certain test fungi was lessened with *Coniothyrium fuckelii Sacc.*, *Microdiplodia henningsii Star.* and *Monochaetia viticola Cavara.* The other species were ineffective against the 6 test fungi involved in this study.

Study of the production of the fungistatic substance. The production of the fungistatic agent was investigated in surface cultures on liquid media of the 5 active species. Into 1000 ml Erlenmeyer flasks each 200 ml of medium

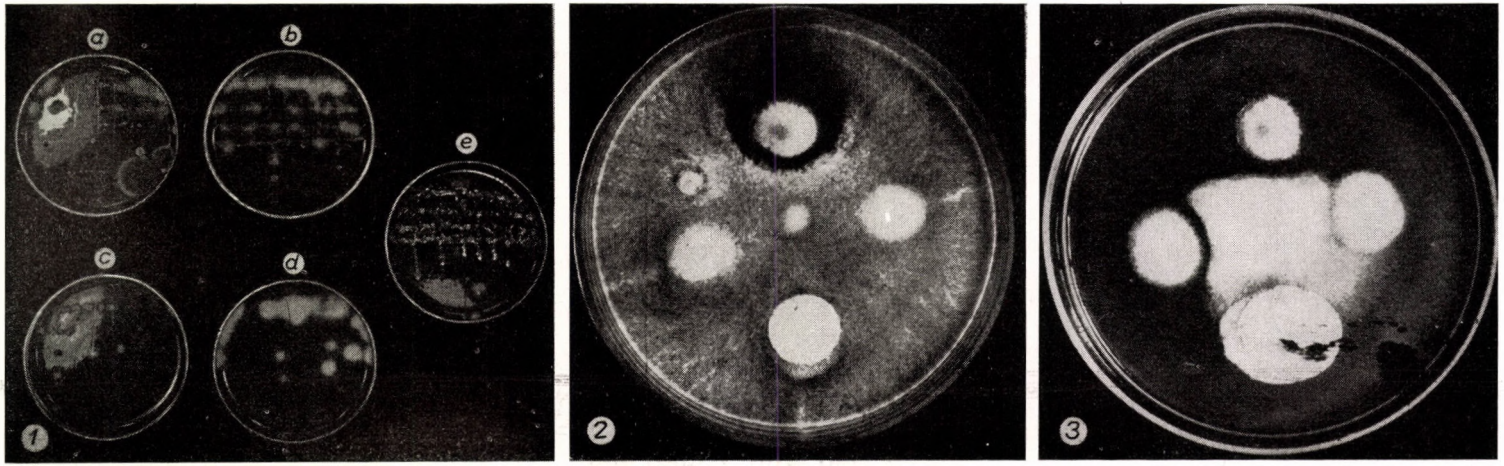

Fig. 1. Streaking of *Phoma herbarum West.* in selective media — *Fig. 2.* In vitro inhibitory effect of *Phomopsis cordifolia (Brun.) Died.* against *Sclerotinia sclerotiorum* — *Fig. 3.* In vitro inhibitory effect of *Phomopsis cordifolia (Brun.) Died.* and *Phoma longissima West.* against *Botrytis allii*

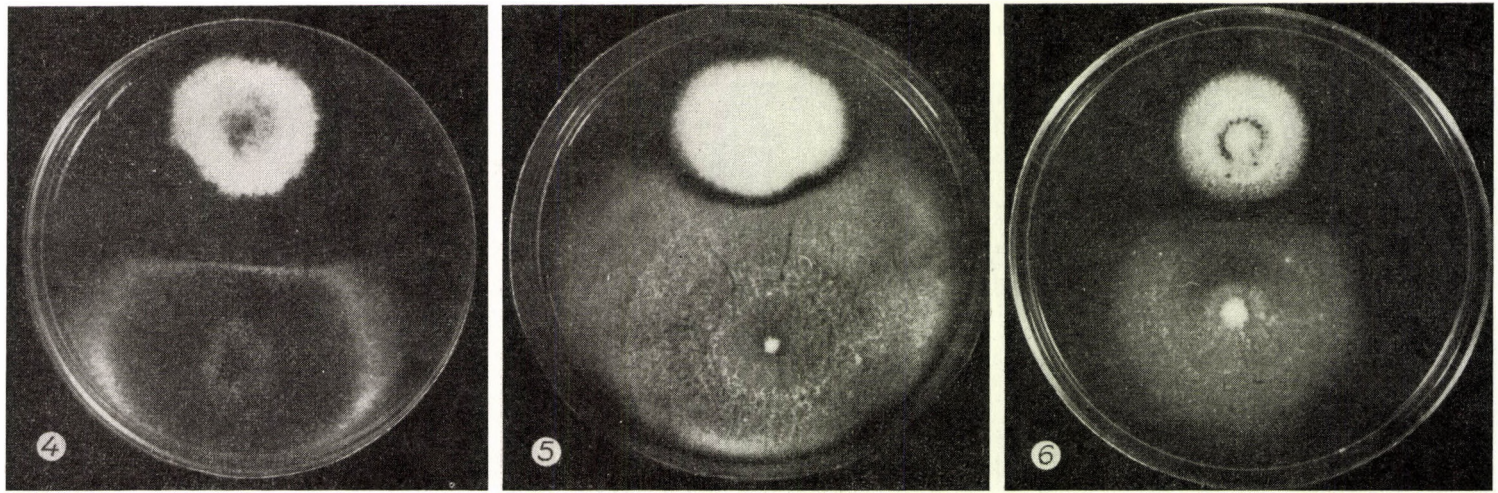

Fig. 4. In vitro inhibition of *Aspergillus niger* by *Phomopsis cordifolia (Brun.) Died.* — *Fig. 5.* In vitro inhibition of *Colletotrichum atramentarium* by *Phoma longissima West.* — *Fig. 6.* In vitro inhibition of *Colletotrichum atramentarium* by *Monochaetia viticola Cavara.*

was poured and the washing of single 14-day slant cultures was inoculated. Cultivation was carried out at 26° C. The culture media used were as follows :

(a) Czapek-Dox medium, pH 5.8, (b) 5 per cent malt extract solution, pH 6.6, (c) g/l ammonium tartarate 2 ; $MgSO_4 \cdot 7\ H_2O$ 0.5 ; K_2HPO_4 1.0 ; KCl 0.5 ; $FeSO_4 \cdot 7\ H_2O$ 0.01 ; sucrose 50.0 ; corn steep liquor 10.0 ; pH 5.3.

Each experiment was repeated 3 times.

Each of the 5 fungi grew best in medium (c) somewhat less in malt and worst in the Czapek-Dox medium. *Coniothyrium fuckelii Sacc.* showed no substantial differences in growth.

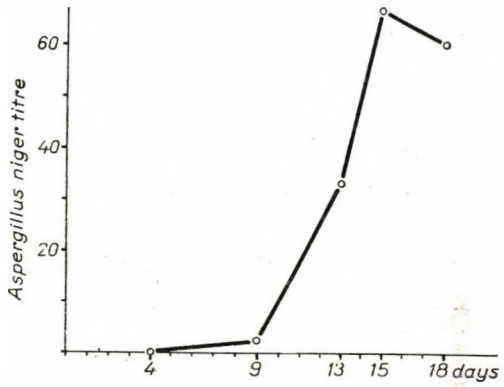

Fig. 7. Active material content of surface cultures of *Phoma longissima West*, at 4 to 18 days of cultivation, expressed in terms of *Aspergillus niger* titre

The cultures were sampled at intervals, usually every fourth day, and the samples were tested for activity in 1 : 2 serial dilutions of the Czapek-Dox medium inoculated with suspensions of *Aspergillus niger* containing 7.5 to 10×10^6 conidia in 0.05 ml, which was the volume of a single inoculum. In the cultures of *Phomopsis cordifolia (Brun.) Died.*, *Coniothyrium fuckelii Sacc.*, *Microdiplodia henningsii Star.* and *Monochaetia viticola Cavara* the *Aspergillus niger* titre did not exceed 8 during the 18 days of culturing. *Fig. 7* shows the active substance content of the cultures of *Phoma longissima West.* in semisynthetic liquid medium between the 4th and 18th days of growth, as determined from samples taken at 4 days intervals and represented in *Aspergillus niger* titre values. The values are the means of 3 repeated tests, the difference between which did not exceed one step of dilution. It is clear that the active agent content reached the maximum on the 15th day of cultivation to decline slowly afterwards.

When passed at about 2-week intervals, *Phoma longissima West.* almost completely lost its fungistatic activity in 6 months in malt agar. The first loss of activity was noticeable in the fourth month, after which the loss progressed rapidly.

Summary

Using selective culture media containing penicillin, streptomycin, chloromycetin and sodium propionate, pure cultures have been made of 39 species out of 48 herbarium specimens of the order Sphaeropsidales and 10 of the order Melanconiales. The fungistatic activity of the isolates was investigated *in vitro*, against 6 test fungi. The fungistatic activity was found to be significant with *Phomopsis cordifolia (Brun.) Died.* and *Phoma longissima West.*, and weak in the case of *Coniothyrium fuckelii Sacc.*, *Microdiplodia henningsii Star.* and *Monochaetia viticola Cavara.* The surface cultures of the five active species were tested for the production of the active agent in 3 different liquid media. The highest *Aspergillus niger* titres were observed with cultures of *Phoma longissima West.*, at 15 days of growth in a semi-synthetic liquid medium. *Phoma longissima West.* lost its fungistatic activity in about 6 months in artificial medium.

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ADENOVIRUSES ISOLATED FROM EXCISED TONSILS

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The latent presence of the so-called adenoviruses in tonsil and adenoid tissues has been known since the first publications of ROWE *et al.* [9, 10, 11]. Certain tissue cultures prepared from tonsillectomy and adenoid biopsy samples were found to exhibit degenerative cytopathic changes after appropriate incubation. The fluid medium of these degenerated tissue cultures, when transferred to normal sensitive cultures, were found to contain a cytopathogenic agent.

Since the first recognition of the Adenoviruses (ARD, RI, APC viruses), 23 groups have been differentiated. Most of these are pathogenic, inducing acute diseases of the upper respiratory tract [2, 3, 11], conjunctivitis [5, 8] or primary atypical pneumonia [4].

In the following we shall report on the isolation of adenovirus from removed tonsils.

Materials and methods

Tissue cultures. 1. Tonsil and adenoid tissue. Tonsils and adenoid tissues obtained by tonsillectomy from children were washed 3 to 4 times with saline containing penicillin and streptomycin. The washed tissues were minced and washed again, and explanted onto a plasma surface in test tubes. The fluid phase of the medium consisted of 58 per cent Hanks' solution, 40 per cent inactivated horse serum and 2 per cent chicken embryonic extract. In certain cases a medium containing 90 per cent bovine amniotic fluid, 5 per cent inactivated horse serum, and 5 per cent chicken embryonic extract was used. To every medium 50 units of penicillin and 100 μg of streptomycin were added per ml. The 1 ml fluid phase in the tubes was changed twice a week. Microscopic control of the cultures was carried out every second day. Degenerated cultures, together with the fluid phase were stored at —70° C until further use.

HeLa : HeLa cultures [12] were maintained in 500 ml Jena glass flasks, with a medium consisting of 40 per cent human serum and 60 per cent Hanks' solution. Tube cultures for inoculation experiments were prepared from the flasks by trypsinization, so as to provide 80 to 100 thousand cells in each tube. Inoculations were carried out after washing the cultures 3 to 5 times with Hanks' solution. The infective material was carried on to the cultures in 0.1 ml volume and after 30 to 40 minutes of preliminary incubation the fluid phase was filled up to 1 ml by maintenance solution. The best results were obtained by a maintenance solution of the following composition : 90 per cent Hanks' solution, 5 per cent rabbit serum, and 5 per cent of a 5 per cent solution of lactalbumin hydrolysate.

Rabbit kidney : Rabbit kidney cultures were made using animals 3 to 4 weeks of age, according to the method of BALDUCCI *et al.* [1]. The medium used consisted of 70 per cent Hanks' solution, 20 per cent rabbit serum, and 10 per cent of 5 per cent lactalbumin hydrolysate solution.

Amnion cells : Human amniotic cell cultures were prepared from fresh aseptically handled placentas, according to the method of LAHELLE [6, 7], with the only difference that we used an 0.25 per cent trypsin solution.

Complement fixation reaction was performed as described by TAKÁTSY [13]. As an antigen the fluid phase of infected HeLa cell cultures was used after the full development of the cytopathogenic changes.

Neutralization tests. Typing of the strains isolated by neutralization tests was performed on HeLa cell cultures. The amount of virus used was so chosen that 0.1 ml of its appropriate dilution cause complete degeneration of the cultures within 3 to 5 days. 0.3 ml of this virus dilutions was mixed with an equal amount of immune serum diluted 1 to 4 and after one half hour of incubation the mixture was distributed into 3 HeLa culture tubes, 0.2 ml each. To the cultures thus incubated 0.8 ml of maintenance solution were given, and incubation was performed at 37° C. The cultures were daily examined under the microscope. The final result of the virus neutralization test was read 24 hours after the complete degeneration of the control cultures inoculated solely with virus. All the dilutions were made with maintenance solution.

The rabbit type specific sera used both for complement fixation and neutralization tests were kindly supplied by DR. R. J. HUEBNER and by DR. U. KRECH, to whom the authors are greatly indebted.

Results

From the 12 tonsillar and adenoid tissue primary cultures, 3 agents were isolated on further passages in HeLa cell cultures (samples 4, 5, 6). In the secondary HeLa cell cultures typical cytopathogenic changes [10] were observed manifesting themselves with rounding and granulation of the cells, disappearance of the confluent cell layers, and appearance of degenerated areas. Most of the rounded cells usually detached from the glass wall. We could maintain all the three cytopathogenic agents by serial passages in HeLa cell cultures. The incubation period of the cytopathogenic effect became shorter on serial passaging. First, degeneration began on the 5th—6th day of the incubation ; later, after 24 to 48 hours.

The three strains were found to cause pathologic changes in rabbit kidney and human amnion cell cultures, essentially similar to those caused by the type strains. Cytopathogenic changes in rabbit kidney resembled those in HeLa cells. Roundening of the cells took place within 2 to 4 days and the appearance of aggregates was followed by their detachment from the glass wall.

In amniotic cell cultures the cytopathogenic effect took place regularly later, *i. e.* in 5 to 8 days, while at that time, though the individual changes closely resembled those described above, no detachment of the cell aggregates was observed within the 8 to 10 days following total degeneration. This observation corresponds to the data in literature [14].

As the complement-fixing antigen is common to the group of adenoviruses, the positive complement-fixation reaction given by our strains was accepted as proof of our supposition that the latter were adenoviruses.

Typing of the strains was performed by neutralization tests. As our strains were isolated from tonsils, type 1, 2, 5 and 6 immune sera were used. The results are presented in *Table I.*

Table I

Neutralization tests

Type specific immune sera (dilution 1 : 4)	Cytopathogenic effect						
	Isolated strains			Type strains			
	tons. 4	tons. 5	tons. 6	1	2	5	6
1	++++	++++	++++	—	++++	++++	++++
2	—	++++	++++	++++	—	++++	++++
5	+++ +	—	—	++++	++++	—	++++
6	++++	+++	+++	++++	++++	++++	—
Virus control	++++	++++	++++	++++	++++	++++	++++

— no cytopathogenic effect
\+ each cross (+) represents degeneration of 25 per cent of the cells in a culture.

As Table I reveals, strain 4 was neutralized by type 2 immune serum ; strains 5 and 6, by type 5.

The positive neutralization test with type 5 strain was supported not only by the lack of a cytopathogenic effect but also by failing in demonstrating the presence of complement fixing antigen in the fluid phase.

Summary

Twelve samples of adenoid tissue from tonsillectomy were cultivated *in vitro* to isolate adenoviruses. 3 strains were obtained, which multiplied readily in HeLa, rabbit kidney and human amniotic cell tissue cultures, causing typical cytopathogenic changes. As revealed by complement fixation and neutralization tests, one of the strains belonged to type 2, 2 of them to type 5 of adenoviruses.

Acknowledgement : We are indebted to Mr. I. Balázs and Mrs. J. Kiszel for the excellent technical assistance.

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INTRACUTANEOUS ADMINISTRATION OF DYSENTERY VACCINE

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The two main problems of endotoxic vaccines, to attain a satisfactory immunogenic effect and to reduce toxicity, seem to be achievable in two ways. One is to bind the antigen to substances which are slowly absorbed, the other is to choose the proper site of administration.

On the basis of the data in the literature, the intracutaneous route of administration appears to offer the most advantages among the latter methods. The intracutaneously administered vaccine produces a slighter inoculation reaction and a better immune response than the vaccines injected subcutaneously or intramuscularly.

The first investigations along these lines in the case of enteral vaccines have been carried out by TUFT [1]. In his comparative studies with subcutaneously and intracutaneously administered typhoid vaccine, a small dose given intracutaneously produced a milder reaction than the subcutaneously injected vaccine, whereas the "H" and "O" agglutinins attained the same level. Tuft therefore recommended intradermal instead of subcutaneous administration.

Like TUFT, VALENTINE *et al.* [2], PERRY [3], as well as WYANDT *et al.* [4], have judged the efficacy of the method on grounds of agglutinin formation. VALENTINE *et al.*, as well as PERRY, found intracutaneous vaccination equipotent with the subcutaneous one carried out with 20 times more material. WYANDT *et al.* also claimed to have obtained better results with subcutaneous vaccination.

In 1940, TUFT [5] reproduced his earlier investigations and found that, also on the basis of the passive mouse protection test, intracutaneous vaccination was superior to the subcutaneous one.

In 1941 VAN GELDER and FISHER [6], then in 1943 NAMMER and NERB [7], immunized large groups of children with typhoid vaccine, both subcutaneously and intradermally. They found the intradermal technique to be only slightly more effective.

In 1943 LEIBOVITZ [8] reported that 1/10 of the subcutaneous dose, as administered intradermally, produced better results and the "O" titres persisted for a longer time.

Mass intradermal inoculations with TAB vaccine were performed by FENNEL [9] in the Hawaii Islands, by TILDEN and ARNOLD [10], and finally by ARGUDIN [11] in Cuba. The reliability of their evidence is lessened by the fact that the comparison was made not on grounds of the protective antibodies, but on the basis of the agglutinin titres.

It has also been suggested that intradermal vaccination would be suitable exclusively for revaccination, but not for initial immunization. In 1939 SILER and DUNHAM [12], and in 1940 LONGFELLOW and LUIPPOLD [13], investigated this problem. According to the latter authors, the intradermal vaccine, which contained 1/10 of the antigen of the subcutaneously administered one, produced on revaccination an equal rise in the protective titre. On this basis they recommended the intradermal route of administration for revaccination.

The problem of the reactions following inoculation was already studied by TUFT, who found that the systemic reaction was less marked after intradermal vaccination.

KAMP [14] immunized nearly 1000 children intradermally. He emphasized that the reactions were negligible and definitely advocated the use of the method.

In another report, LUIPPOLD [15] found the reaction to the intradermally administered vaccine mild and although he recommended the subcutaneous route for the initial immunization, in certain cases he gave preference to intradermal inoculation : reducing the dose may help to avoid serious complications when immunizing older or allergic individuals.

Concerning the intradermal administration of dysentery vaccine, COOPER, KELLER and TEPPER [16] have published data. They gave intradermally a total of 6000 million germs of a monovalent Sh. flexneri 3 vaccine. The local reaction was characterized by erythema of considerable extent, infiltration and, often, by axillary tenderness. Except for the fever, the systemic response was more severe than after subcutaneous vaccination. As far as the immunogenic effect was concerned, passive mouse protection experiments showed the intradermal technique to be effective.

In the light of the conflicting, but to some extent encouraging data in the literature, it seemed interesting to investigate the possibility of administering dysentery vaccine intradermally. The experience obtained is summarized below.

Materials and methods

Antigens. Suspensions of *Sh. flexneri* and *Sh. sonnei*, respectively, dehydrated with acetone, dried and standardized (for bacterial count) by nephelometry, were used as the bacterial antigen. The extract antigens were prepared by BOIVIN's method, were dialysed and Seitzfiltered.

Titration of antigens. The antigens were informatively assayed by the mouse toxicity test and the relative potencies of the antigens and vaccines used were expressed in mouse LD_{50} titres.

Adsorbed antigens. The adsorbent was an $Al(OH)_3$ gel, prepared from 2.5 per cent alum *in statu nascendi*, in every case. The adsorbate was used after washing.

Active mouse protection experiment. Mice were immunized with the minimum immunizing dose, 100 million microbes or 0.003 ml extract, about equivalent with the former. The adsorbed vaccine was injected 3 weeks before infection.

The bacterial vaccine was injected one week apart, the first dose was given 3 weeks before infection. In case of one shot immunization the injection was given two weeks before the challenge.

For infection, we used a suspension in 5 per cent mucin of 6-hour dilutions of a peptone-water culture.

Passive mouse protection experiment. This was made by COOPER's technique [17]. The collected sera, pooled in equal volumes, were suitably diluted and injected 1/2 hour before infection by the intraperitoneal route. Infection was carried out by the technique of the active mouse protection method, with only a single infecting dose. In every case the result was compared with the pre-inoculation protective titre of the same individual.

Complement fixation. Graduated dilutions of inactivated rabbit sera were tested in the usual way, in the presence of a standard antigen containing about 100 million germs/ml, + 100 per cent of complement, by means of the haemolytic system employed in the Bordet-Wassermann test.

Statistical analysis. The results, computed by the method of KÄRBER [18], are given in terms of LD and ED_{50}, respectively.

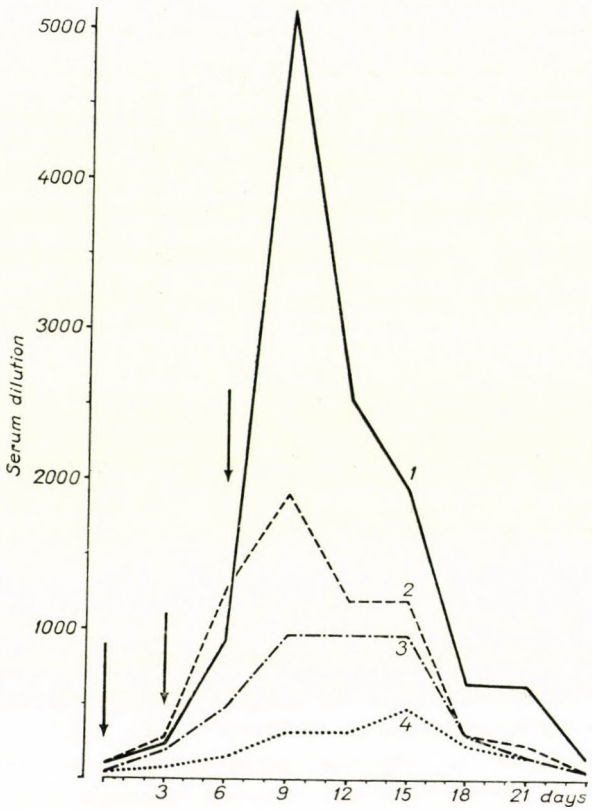
Results

In the first part of the investigations we wished to obtain information as to the agglutinin titres produced by the intradermally administered vaccine, comparing it to different methods of immunization. Following the immunization of rabbits, the agglutination curve of the intradermally inoculated animals ran near to the curve of intravenously immunized animals and significantly surpassed the titre appearing on subcutaneous inoculation. As determined by the complement fixation and the passive mouse protection tests, the sera taken at the time when the peak titres were reached confirmed the results of the agglutination tests. Such an example, selected at random, is presented in *Graph 1.* In this case immunization was carried out subcutaneously and intracutaneously, in three equal doses, one week apart. The total dose was 500 million Sh. flexneri type 1b and an equivalent dose of Boivin extract, respectively.

As far as the bacterial antigen is concerned, the graph shows a remarkable difference in favour of the intradermal technique. This difference is not so marked in the case of the extract antigen.

Since the rabbit experiments yielded such encouraging results, we assayed the intradermal administration of antigen by means of the active mouse protection test.

Thus, we compared the intradermal technique with the subcutaneous one, and the immunizing power of the antigen adsorbed to alum precipitate with unadsorbed antigen. We investigated the relation of the extract to the whole bacterium and the effect of the intradermally inoculated, reduced dose.



Graph 1. Changes in the agglutinin titre of rabbits in response to intradermal and subcutaneous immunization

1 : Bacterial antigen, intradermally
2 : Extract antigen, intradermally
3 : Bacterial antigen, subcutaneously
4 : Extract antigen, subcutaneously.
↓ : Time of immunization.

Finally, the response to a single dose, as compared to that to two inoculations, was also analysed.

The resulting data have been comprised in *Table I.*

It is clear from *Table I* that the highest immunizing titres were induced by the intradermal administration of the full dose of the bacterial vaccine and with the adsorbed Boivin extract. Subcutaneously injected bacterium yielded a much lower titre and this same titre was obtained after the intradermal or subcutaneous inoculation of the extract. Intradermally injected bacterial vaccine containing 1/10 of the immunizing dose had a less marked effect, but this nevertheless attained the immunogenity of the full dose of bacterial vaccine administered subcutaneously on two occasions.

Table I

Immunity of intradermally and subcutaneously immunized mice

Infecting doses dilution ml	Antigen, dose, route and method of immunization							
	Bact. i. d. 1 inoc. 60 µg	1/10 dos. i. d. bact. 1 inoc. 6 µg	Extract i. d. 1 inoc. 0.003 ml	1/10 dos. extract i. d. 1. inoc. 0.0003 ml	Bact. s. c. 2 inoc. 60 µg	Extract s. c. 2 inoc. 0.003 ml	Adsorbed vaccine s. c. 1 inoc. 0.003 ml ant.	Control
10^{-1}	4/5*	1/5	1/4	1/5	1/4	0/4	4/4	.
10^{-2}	4/4	3/5	2/4	2/5	2/4	3/4	2/3	.
10^{-3}	4/4	4/4	4/4	4/4	4/4	4/4	4/4	0/4
10^{-4}	4/4	4/4	4/4	4/4	4/4	4/4	4/4	0/4
10^{-5}								2/4
10^{-6}								4/4
LD_{50}/ml	$>10^{-1.0}$	$10^{-1.7}$	$10^{-1,75}$	$10^{-2.0}$	$10^{-1.75}$	$10^{-1.75}$	$>10^{1.0}$	$10^{-5.0}$
Immunity expressed in LD_{50}	>10 000	3000	2500	1000	2500	2500	>10 000	

* Survivor/infected

The results of the animal experiments, as well as the evidence in the literature made it necessary to extend the investigations to man.

In the informative experiments volunteers, first in groups of 1 or 2 each, then in groups of 10 to 12 each, were immunized intradermally with a polyvalent vaccine. The vaccine contained about 1200 million microbes, representing 0.5 mouse LD_{50}. The control group composed of a similar number of subjects was immunized with adsorbed vaccine at an interval of 1 week. The adsorbed vaccine contained about 5 times as much of antigen as the intradermal vaccine. Before inoculation and 2 weeks after it blood was taken and the pooled sera from both groups were subjected to the passive mouse protection test.

The results are presented in *Table II*.

The data in *Table II* make it clear that the intradermal vaccine representing 1/5 of the antigen of the adsorbed vaccine afforded better (or at least equal) protection than the latter.

The inoculation reactions following vaccination are shown in *Table III*.

The single terms characterizing the grade of reaction, as shown in *Table III*, should be interpreted as follows. Systemic reaction, *mild:* slight rise of temperature, impaired sense of well-being ; *moderate :* fever (not higher than 38° C) for 24 hours, weakness ; *strong :* a temperature above 38° C, vomitus, weakness. Local reaction, *mild :* tenderness, erythema ; *moderate :* pain (not constant), infiltration ; *strong :* constant pain, marked oedematous infiltration, eventually haemorrhage or necrosis.

Table II

Protective value of sera from subjects immunized intradermally and subcutaneously with adsorbed vaccine

Immunizing doses of serum ml	Adsorbed vaccine of 2.5 LD_{50} value 0.5—0.5 ml (1 week)			Intradermal vaccine of 0.5 LD_{50} value 0.05—0.1 ml (1 week)		
	Immunity against...					
	Sh. flexneri		*Sh. sonnei*	*Sh. flexneri*		*Sh. sonnei*
	1b	2a		1b	2a	
0.05	2/4*	2/4	3/4	2/4	3/4	2/4
0.01	1/4	1/4	1/4	2/4	1/4	2/4
0.001	0/4	0/4	1/4	0/4	0/4	0/4
ED_{50}	0.04	0.04	0.018	0.025	0.025	0.025
Rise of protective value in index number	>2.5x	~2.5x	5x	>4x	~4x	3.5x
Challenge doses LD_{50}	250	500	750	250	500	750

* Survivor/infected

Table III

Reactions following inoculation. Comparison of intradermal and adsorbed vaccines

Vaccine	Systemic reaction			Local reaction			Number of test subjects
	mild	moderate	strong	mild	moderate	strong	
Adsorbed, 0.5 ml	4	5	2	5	4	2	11
Intradermal, 0.05ml	6	6	—	—	—	12	12

As to the inoculation reactions, even the small number of cases studied permits one to draw the conclusion that the systemic reaction to the intradermal vaccine is somewhat milder than that to the adsorbed vaccine. On the other hand, the local reactions manifested themselves with considerable infiltration oedema and a tenderness of the axillary lymph nodes. In several cases haemorrhage and necrosis also occurred.

Discussion

The experimental evidence obtained shows that with Shigella vaccines the intradermal route of immunization proved to be superior to the subcutaneous one. As far as the immunogenic effect is concerned, the intradermal technique was found equivalent with the apparently most effective subcutaneous injection of $Al(OH)_3$ adsorbate, both in animal experiments and in man.

Intradermally administered extract did not show the high immunogenity of the whole bacterium, although the results were not inferior to those obtained by immunizing twice subcutaneously with vaccine or extract (see *Table I).* At the same time, it must not be left out of consideration that the subcutaneously administered antigen was 10 times the quantity of that injected intradermally.

Only hypothetical explanations may be put forward as to the lower immunogenic value of the extract. The difference may be ascribed to the protracted resorption of the corpuscular antigen, representing a kind of depot effect, with all its favourable consequences. The adjuvant effect of the inflammatory tissue reaction, which develops on the same basis, may also be taken into account.

The animal and human experiments showed that, as regards immunogenity, intradermal administration was at least equivalent with the adsorbed vaccine. Although these experiments were favourable from the point of view of extending the investigations, the severity of inoculation reactions (necrosis), at least with the present form of the vaccine, contraindicate the use of the method instead of the adsorbed vaccine.

Summary

(i) In rabbits, the agglutinin titres (as confirmed by passive mouse protection tests) produced by the intradermally administered vaccine greatly surpassed those attained by the subcutaneous technique.

(ii) The effectiveness of active immunization of mice with corpuscular vaccine by the intradermal route was equivalent with the adsorbed Boivin antigen, but led to higher immunity than immunization with the same antigens by the subcutaneous route.

(iii) As determined in a small number of volunteers, the blood serum of the subjects inoculated intradermally possessed at least the same mouse protecting titre as that of subjects immunized subcutaneously with five times more adsorbed antigen. The intradermal vaccine may, however, cause necrosis and for this reason the introduction of the method is contraindicated.

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ELECTRONMICROSCOPIC STUDIES OF THE FINE STRUCTURE OF ESCHERICHIA COLI

By

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The fine structure of bacteria, the apparatus that enables the microorganism to meet all its complicated biological functions is revealed only by phase-contrast microscopy or electronmicroscopy. MUDD [1] suggested the bacterial cytoplasm to be a micellar protein (4.5 to 10 Å) lattice with an interstitium of an aqueous protein solution. At the same time he emphasized the importance of the presence of lipid and lipoprotein lamellae.

As to the nucleoid — the nucleus-like substance of bacteria, — electronmicroscopic studies are fairly scarce. In his review, KNAYSI [2] describes the electronmicroscopic appearance of the nucleoid as a vacuole with a special internal structure of poorly defined nature and function. The loose, vacuole-like or more compact appearance of the structure seems to be intimately connected with the actual state of development, growth and multiplication of the microorganism. In aged cultures the role of the nucleoid is so subordinate that BRADFIELD [3] wrote about "nucleoid atrophy". The studies of BIRCH—ANDERSON [4], though very impressive, are the most difficult to reproduce technically. He prepared a stereo-picture of the nucleoid by combining the photographs of a series of sections from a single bacterium.

The thickness of the cell wall was found to be about 100 to 200 Å. The problem, whether or not a cytoplasm membrane exists, has not yet been settled [5].

Both electronmicroscopic and desintegration studies support the view that no organella with definite membranes exist inside the bacteria [6].

Our present studies, made in connection with those known from the literature [7, 8, 9], were concerned with the examination of the fine structure of *E. coli* in its lag and logarithmic phases.

From a methodical point of view we examined (a) the applicability of formalin and OsO_4 fixation [10] to preparates to be embedded in methacrylate, and (b) the effects of polymerization temperature on the formation of artifacts [11].

Materials and methods

The strain of *E. coli* used in the experiments was maintained by serial passages every two or three weeks on broth agar slant medium. (For identifying and supplying the strain, we are indebted to DR. J. SZITA, *State Institute of Hygiene, Budapest.)* The bacterial growth was cautiously scraped off from two 24 hour agar slant cultures, and inoculated into 750 ml broth each. Care was taken to avoid the transfer of broken-off agar debris. Broth cultures were incubated at 37° C for 5 and 24 hours, respectively. At the end of the incubation, samples of about 500 ml were drawn off from each culture, while care was taken not to stir up the sediment. The samples were centrifuged separately at 3000 r. p. m. for 30 minutes. The sediment was washed twice with chilled saline. The final sediment was resuspended in a phosphate buffer of pH 7.2 containing 1 per cent OsO_4. The suspension was then fixed at 4° C for 2 hours. For formaldehyde fixation we added to the bouillon culture a mixture of formalin and phosphate buffer, 1 : 1, to ensure a 1 to 200 formalin concentration in the final mixture. The formalized material was further incubated at 4° C for 24 hours.

When combined fixation was performed, the formalin treatment just described was followed by a single washing of the suspension, and treatment with OsO_4, as described above.

Cells fixed by one of the above methods were sedimented by slow centrifugation. The usual further washings were omitted, to avoid damage to the cells. The homogeneous cell sediment was treated as a homogeneous tissue sample, and not resuspended. Dehydration was peformed in two days, in the usual increasing alcohol series. The night after the first day of dehydration, the material was kept either in 96 per cent or absolute alcohol. On the second day of dehydration the material was embedded by the following method. The material was soaked in an 8 to 2 mixture of methyl methacrylate and butyl methacrylate : placed into methacrylate prepolymerized at 60° C for 4 to 6 hours containing 1 per cent benzoil peroxide. Final polymerization was carried out in glass tubes at 70° C. For experimental purposes certain preparates were polymerized at 45° C, and prepolymerization was omitted.

Polymerization was allowed to run for 24 (in certain cases for 48) hours. This was followed by keeping the embedded material in an exsiccator connected with a forevacuum pump. Next day it was subjected to vacuum of 10^{-4} for some hours, to evaporate traces of alcohol and monomer. Sections were made by an ultramicrotome of our own construction. Of the various ultramicrotome construction described [13 to 24], those adopted to a turn [22, 23, 24] appeared the most readily applicable to our facilities.

The ultra-microtome constructed by us was adopted to a turn. A chromium-nickel-steel bar of 500 mm length and 10 mm in diameter was fixed into the head of the turn. The other end of the bar was made to move in an eccentric bearing on an eccentric disk driven by an electric motor *(Fig. 1)*. The embedded material was fastened on a drill head, mounted onto the eccentrically moving end of the bar. Thus, in our microtome it is the preparate and not the knife that moves.

The 0.1 HP Siemens electric motor revolving at 1400/min. was mounted on a support independent of the turn. The elastic rubber transmission was interchangeable and mostly adjusted to turn the eccentric at 180 r. p. m.

The copper block used for propelling the knife by heat expansion was mounted on the support of the turn. The active length of the block was 120 mm. Heat expansion was achieved by 14 W delivered by a resistance heater of 30 V. The heating was adjusted to ensure 25 000 to 35 000 Å expansion per minute. At such a feed 180 cuts are made in a minute and theoretically the average thickness of the cuts is 140 to 200 Å. In practice the thickness of sections exceeded in certain cases the calculated values.

The mobile knife-holder was fastened to the free end of the copper block. As a knife, a split shop-window glass fragment was used, as suggested by LATTA and HARTMANN [25]. The sections were made to float on distilled water or a mixture of 20 per cent acetone in distilled water. The process of cutting was continuously controlled under a low power stereomicroscope, and the cuts exhibiting Newton phenomenon (gold or silver coloured) were transferred by means of a tungsten loop (wire diameter, 0.1 mm) into another dish containing distilled water or a mixture of 20 per cent acetone in distilled water. On the surface of the fluid the cuts were allowed to stretch for some hours. Apparently appropriate cuts were made to adhere to a formvar membrane mounted onto a microscreen. This procedure was also carried out under the control of a stereomicroscope. The section was then dried by air and examined under the light-microscope. Only appropriate sections were selected for electronmicroscopic study.

The electronmicroscope used was type EM 3 1951 USSR, reconstructed by HOLLÓS and BARNA. Photographs were taken on Agfa Document Film. Primary magnification was × 12 000.

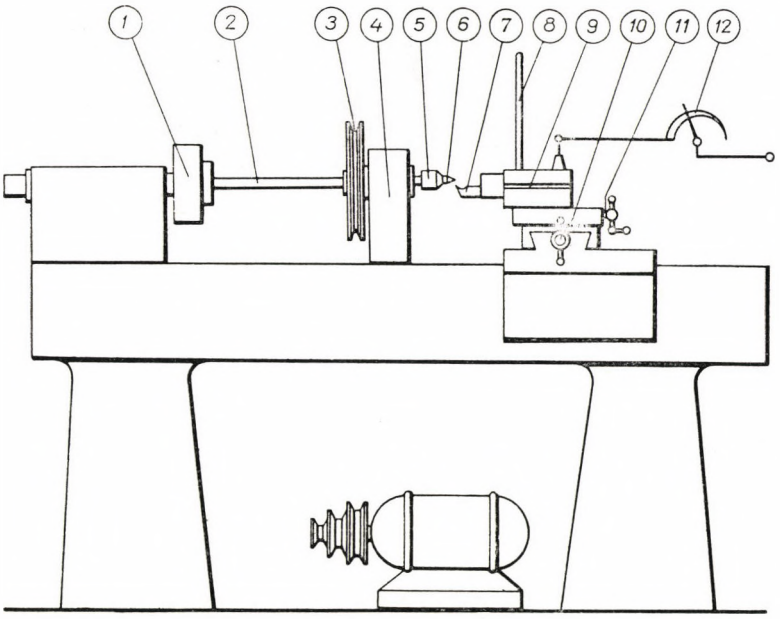


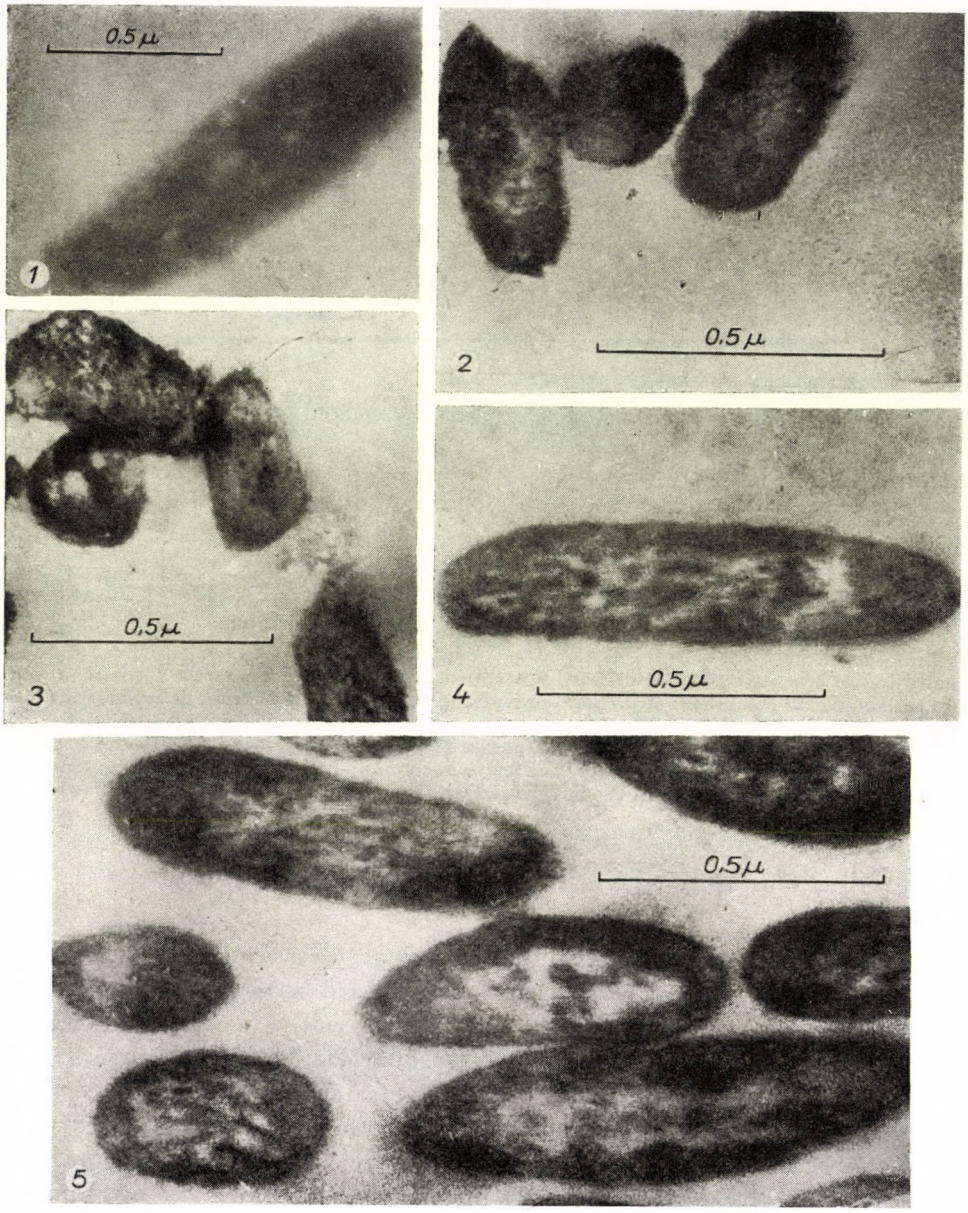
Fig. 1. Scheme of the turn-adopted ultra-microtome

A chromium-nickel steel bar (2) is fastened to the head of a turn (1) and made to move eccentrically by an eccentric disc (4) driven by a transmission (3). The preparate (6) is mounted into the small drill head (5). The preparate revolving at an even rate touches at each revolution the glass knife (7). The heat expansion of the copper block is controlled by a thermometer (8) of 0.1° C sensitivity. The heating current passes through a resistance (12) into the heating wire of the copper block. Before starting the heating, the support is adjusted to the appropriate site by two screws (10, 11). In the foreground, the independent electric motor is shown

Results

At the beginning of our study, in a single case we examined cells of E. coli cultivated in slant agar. Cells thus obtained were found more fragile and more inclined to exhibit artifacts than those cultivated in broth. Similar observations were made earlier by TOMLIN and MAY [9]. For this reason we used in our study only cells of E. coli cultivated in broth.

When examining electronmicroscopically the formaldehyde fixed preparates of E. coli, we found that the cells very rapidly lost their definite fine structure under the electronic beam, a phenomenon described also by MORGAN *et al.* [10]. Nevertheless, fixation during pretreatment had the advantage that the cells were made non-infective and more resistant to damage by centrifugation and other manipulations. In this respect, formaldehyde was superior to OsO_4. As revealed by the electronmicroscopic studies, there was no difference between cells fixed by formalin and OsO_4, and those fixed with OsO_4 alone.



Picture 1. (Photo No 3754). Section from a 24 hour E. coli cell fixed with OsO_4, embedded at 45° C. Both the cell wall and the internal structure are contrastless and damaged. Magnification, about 45 000.

Picture 2. (Photo No 7528). Section of 24 hour E. coli cells fixed with OsO_4, embedded at 70° C : A fine cytoplasmic structure and the coherent cell wall are visible. In the three cells, in spite of their electron-dense structure, no vacuoles are present. Magnification, about 68 000.

Picture 3. (Photo 7526). Section similar to Picture 2 (Photo No 7528). Top, left : cell wall distorted towards the neighbouring cell. The cell beneath has been cut horizontally. It contains

Picture 1 presents a preparate of E. coli fixed with OsO_4 alone and embedded at 45° C. The electronmicrographs exhibited cell with characteristically broken borders and destroyed internal structure, while the whole picture was contrastless and plain. No such cells were ever found in specimens prepolymerized for 6 hours and polymerized at 70° C. Similar observations were made by BORYSKO [11].

We could demonstrate all the characteristical structural elements of E. coli in sections obtained from 24 hour old cultures (Pictures 2 and 3), so the dense cell wall and a light halo inside it ; the micellar structure of the cytoplasm was also observed. A more thorough examination revealed a substance of more marked electronic density inside the spongeous protoplasm. This electron-dense substance was found to be surrounded by a very thin vacuole [7]. The substance of dense structure observable in certain resting cells appeared to be identical with the nucleoid described by BIRCH—ANDERSON. There was a striking morphological similarity between these and certain elements observable in the vacuola of E. coli in the logarithmic phase ; though the former seemed to be more electron-dense than the latter.

Electronmicrographs of young E. coli cells in the logarithmic phase revealed the presence of one or more well-defined vacuoles (Pictures 4 and 5). The density of the substance inside the vacuole corresponded practically to that of the protoplasm, though some comparatively more electron-dense elements were also observed in certain cases. The nucleoid elements in these sections exhibited a complicated three dimensional structure composed of coherent multiple members.

Discussion

It seems important that formaldehyde fixation was not favourable for specimens that were to be embedded in methacrylate before cutting. The intensive electron bombardment during examination resulted in a volatilization of the methacrylate, causing either an internal rearrangement or partial volatilization of the formaldehyde fixed material. No similar phenomenon was observed when fixation had been carried out with OsO_4 alone, or by the method of preliminary formalin treatment followed by OsO_4 fixation.

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a small vacuole with structures, electron-dense material. In the other three cells no vacuole is seen, while each of them contain electron-dense material composed of small granula. Magnification, about 68 000.

Picture 4. (Photo No 7898). Five hours old cells of E. coli fixed with formalin and OsO_4, embedded at 70° C. Inside the cell wall and the cytoplasm a vacuole is clearly observable. It contains electron-dense substance of globular and filamental structure. The electron-density of part of the nucleoid corresponds to that of the plasma, while that of the other part is more dense, similarly to certain aged cells. Magnification, about 80 000.

Picture 5. (Photo No 7888). This picture was taken under essentially similar conditions as Picture 4 (Photo 7898), only it contains somewhat more cells. Magnification, about 80 000.

A proper adjustment of the polymerization temperature was essential in order to avoid formation of artifacts, an observation not sufficiently emphasized in the literature.

In contrast to the observations of TOMLIN and MAY [9], no vacuoles were found in aged E. coli cultures. Our findings appear to confirm those of BIRCH—ANDERSON *et al.* [7].

In 24 hours cultures a great number of cells was still in the phase of division. This finding might satisfactorily explain the fact that the possibly chromatine-like, markedly electron-dense material was not always equally dispersed in the cytoplasm, but in certain cells it has assumed a well-defined structural element. On the other hand, the fairly frequent occurrence of organized nucleoid permits the supposition that it is a constant structural element, whose demonstration depends on the actual site of cutting. As a result of dehydration by alcohol, the bacterial cell loses a great part of its original components. Thus one might suppose that the possible presence of a greater amount of alcohol soluble substances in the cell of young cultures was more apt to lead to the formation of vacuoles than our sections made from aged cells. The minor differences between the dehydration procedure of TOMLIN and MAY [9] and our method might have been the cause why the authors in question found vacuoles in the aged cells. Further studies are required to settle the problem whether or not the vacuoles might be regarded as artifacts.

Summary

(i) The electronmicroscopical structure of resting and of logarithmically multiplying E. coli has been studied by the ultra-thin sectioning technique.

(ii) Difficulties of the electronmicroscopical evaluation of formalin-fixed specimens and the importance of appropriate adjustment of the polymerization temperature during methacrylate embedding has been emphasized.

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THE ANTIGENIC STRUCTURE OF THE INFLUENZA VIRUS STRAINS ISOLATED IN HUNGARY IN 1957

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In Hungary two epidemic waves of influenza were observed in 1957. The smaller wave in February and March involved almost exclusively children under 4 years of age. The antigenic structure of the causative virus was closely related to those influenza A (A-prime) strains which had prevailed in Hungary since 1949. The second wave during the autumn was caused by the Asian virus, as it has been described in a preliminary report [1]. An unusually high morbidity rate and relatively mild clinical pictures were the main characteristics of this epidemic. The antigenic analysis of the virus strains recovered from the two epidemic waves is the object of this paper.

Materials and methods

Viruses. Influenza A, PR8, Paris P. L. 1/49, Budapest 4/49, Budapest 10/51, Budapest 2/52, Budapest 17/53, Budapest 6/54, Singapore 1/57 and the Russian strains of Asian virus, Shvets and Kolet. *Influenza B*, Lee. *Influenza C* 1233. *Influenza D*, Sendai. The Hungarian strains were isolated in this laboratory, the two Russian strains were obtained by the courtesy of the *Virus Institute of the Academy of Sciences USSR (Moscow)*, the remainder kindly supplied by the *World Influenza Centre, London.*

Purified concentrated virus preparations were prepared as described earlier [2].

Hyperimmune sera. Chickens and hamsters were inoculated several times each, with concentrated purified virus, as published previously [3]. To prepare hyperimmune sera, the animals were killed by bleeding 14 days after the last injection.

Haemagglutinin (HA) titrations and *Haemagglutination inhibition (HI) tests* were performed in plexiglass plates. The spiral loop technique was used [4].

Antibody absorption tests. The technique itself has been published in earlier reports from this laboratory [5, 3]. The "optimum" concentration of the purified virus was determined in a preliminary test. "Optimum" was called the lowest concentration of a virus sufficient to absorb all the anti-haemagglutinins active against the same virus strain. On the other hand, in a serum which had been absorbed with the "optimum" concentration of virus no residual HA activity was detectable.

Virus-absorbed sera. Hyperimmune sera (see above) were successively absorbed with the "optimum" concentrations of two or three different strains of virus each [6]. Sera prepared in this manner proved highly specific, having usually no detectable anti-haemagglutinins to any of the virus strains mentioned above, except to the homologous strain. The sera were stored in lyophilized state at $+4^{\circ}$ C until used.

Neutralization tests were performed in minced chorio-allantoic membrane cultures by the semi-micro roller tube method of HORVÁTH [7].

Virus isolation experiments. Throat washings or broth diluted swabs were treated with antibiotics and inoculated into 8 to 9 day-old chick embryos by the amniotic route.

Experimental

Strains isolated from the late-winter epidemic. Twentytwo strains were isolated during the period between February 8, and March 25, 1957. The antigenic analysis was carried out in three steps.

First passage amniotic fluids were used in the first step. Four parallel rows of twofold dilution series of virus were prepared by the spiral loop technique. One drop of a 1 to 50 diluted hyperimmune serum to influenza A (A-prime), B and C, respectively, was added to each of the virus dilutions in rows 1 to 3. In row 4, serum was replaced by physiological saline. All the serum-virus mixtures were tested for HA, using guinea-pig erythrocytes. Table I shows the results of an experiment of this type. As shown in Table I, in the presence of the anti-A (A-prime) serum a 16fold drop in the HA titre of the virus was observed. Anti-B and anti-C sera, on the other hand, caused very slight, if any, inhibition. Essentially the same results were obtained from the tests carried out with any of the 22 strains recovered.

Table I

HA titration with strain Budapest 1/57 in presence of different hyperimmune sera produced in chickens

Sera	Virus dilution					
	1 : 2	1 : 4	1 : 8	1 : 16	1 : 32	1 : 64
Infl. A'	+	—	—	—	—	—
Infl. B	+	+	+	+	—	—
Infl. C	+	+	+	+	+	—
Saline	+	+	+	+	+	—

As the second step, a more exact typing was carried out with amniotic and allantoic fluids of the 2nd passage when sufficient volumes of virus material were available. The usual HI tests were done on the new strains using several standard influenza A hyperimmune sera produced in chickens. The results of tests of this nature are illustrated in Table II. It can be seen that serum prepared against Budapest 6/54 was the only one with titres to the new strains nearly as high as the homologous titre.

In the third step, four of our virus-absorbed sera were tested for HI activity against the new strains. Each of the sera included in this experiment represents the anti-haemagglutinins produced against the dominant antigen of the influenza A virus strains recovered in Hungary from one of the four outbreaks during the period from 1949 to 1956. The results of the HI tests are summarized in Table III. Table III shows that none of the sera except that prepared against Budapest 6/54 was active against the new strains. On the

Table II

HI tests with standard hyperimmune sera

Sera	Homologous titre	Viruses Budapest				
		1/57	2/57	3/57	5/57	7/57
Infl. A PR8	1200*	<12	<12	<12	<12	<12
Inf. A' Paris P. L. 1/49	6400	400	800	800	800	800
Inf. A' Budapest 4/49	6400	800	1200	1200	1200	1200
Infl. A' Budapest 6/54	3200	2400	2400	3200	2400	2400

* Reciprocals of the titres

Table III

HI tests with virus-absorbed sera

Viruses	Virus-absorbed sera			
	4/49 ch^+	10/51 ch^+	2/52 h^+	6/54 ch^+
Budapest 4/49	300*	—	—	—
Budapest 10/51	—⊗	400	—	—
Budapest 2/52	—	—	400	—
Budapest 6/54	—	—	—	200
Budapest 1—22/57 average titres	—	—	—	50
Budapest 1—22/57 extreme values	—	—	—	25 100

* Reciprocals of the titres
⊗ <6

ch^+ = chicken
h^+ = hamster

other hand, the well-defined difference between these heterologous and the homologous titres of the latter serum was sufficient to exclude the identity of the new strains with Budapest 6/54. It can be concluded, therefore, that the strains recovered during the late-winter outbreak were closely related to but not identical with the representative virus of the 1953—1954 epidemic.

In a previous report [6] we demonstrated an antigenic difference between the strains Budapest 6/54 and Budapest 17/53, the latter having been isolated from the same outbreak about a month earlier than the former.

Moreover, Budapest 17/53 showed more similarity to the representative strain of the 1952 epidemic (Budapest 2/52) than Budapest 6/54. According to the supposition that the variation of the influenza virus is always progressive, Budapest 6/54 was expected to be closer related to the 1957 A-prime strains than Budapest 17/53. This question was to be answered by the tests demonstrated in Table IV. As shown in Table IV, the antigenic change was progressive

Table IV

HI tests with virus-absorbed sera

Viruses	Virus-absorbed sera		
	2/52	17/53	6/54
Budapest 2/52	400*	12	—
Budapest 17/53	12	200	25
Budapest 6/54	—⊗	25	200
Budapest 1—22/57 average titres	—	12	50

* Reciprocals of the titres
⊗ <6

also in this instance. It can be seen, however, that our virus-absorbed sera are not strain-specific if very closely related strains are investigated.

Strains isolated from the autumn outbreak. The second epidemic wave reached Hungary late in August. The first victims were young men and women returning from the World Youth Meeting held in Moscow. The first strain was isolated from one of those who had fallen ill on the train. A few days later we observed an epidemic with a morbidity rate of about 35 per cent in an Army unit. From ten throat washings inoculated into 8 to 9 day-old chick embryos five virus strains were isolated.

During the first fortnight of September we examined 8 throat washings obtained from another small outbreak within the Army. No virus was isolated. During the same period one strain was recovered out of 4 sporadic cases.

After the middle of September, more sporadic cases and small outbreaks in the civil population were observed. In a college in West Hungary more than 70 per cent of the students were affected. Surprisingly, no virus could be isolated from 19 garglings taken during the acute phase of the illness. Even serial blind passages were unsuccessful. Nevertheless, in paired sera of the same patients a well-defined anti-haemagglutinin response to the Asian virus was demonstrated. At the end of September and in October the epidemic spread over the country. It came to an end late in November.

Fifteen strains have been isolated altogether, one of them from a *post mortem* specimen.

In order to determine the type of the strains, CF tests were done using infected allantoic fluids as antigens and hyperimmune sera prepared in hamsters (Table V). Only anti-A sera reacted with the new strains which were undistinguishable from the Singapore strain by this test.

Table V

Complement fixation tests with hyperimmune hamster sera produced against laboratory strains

Sera	Laboratory strains							New strains			
	PR8	Paris	Bpest 1/57	Sing	Influenza B	Influenza C	Influenza D	23/57	26/57	29/57	31/57
A PR8	256*	12	12	6	—+	—	—	6	6	6	6
A Paris P. L. 1/49	24	256	64	24				24	24	24	24
A Budapest 1/57	12	64	128	24	—	—	—	24	24	24	24
A Singapore 1/57	6	12	12	64	—	—	—	64	64	64	64
B Lee	—	—	—	—	128	—	—	—	—	—	—
C 1233	—	—	—	—	—	128	—	—	—	—	—
D Sendai	—	—	—	—	—	—	64	—	—	—	—

* Reciprocals of the CF titres
\+ <6

More definite distinction was expected from the HI test. A number of hyperimmune sera produced in chickens were tested against the Singapore strain, the Russian strains Shvets and Kolet, and the Hungarian strains recovered from the epidemic under discussion. Some typical data are shown in the upper right rectangle of Table VI. Among the sera tested, serum to the Singapore strain had the highest titre against our new strains, approximately as high as the homologous titre. The Singapore strain and the Russian strain Shvets (in the following, Singapore-like strains) were, however, not inhibited by the A-prime sera, although these were definitely inhibitory against the new Hungarian strains and the Russian strain Kolet (in the following, Hungarian-like strains).

HI tests with hyperimmune hamster sera also revealed a remarkable difference between Singapore-like and Hungarian-like strains.

This different reactivity might be explained either by an antigenic difference or by supposing that the Hungarian-like strains were more sensitive to the non-specific inhibitors present in some of our hyperimmune sera. To elucidate this question from other sides, cross HI tests, neutralization tests, and finally cross antibody absorption tests were carried out.

A well-defined difference by the cross-HI test is almost generally accepted to be caused by an antigenic difference. An experiment of this type carried

Table VI

Cross haemagglutination inhibition tests

Chicken sera	Virus strains										
	Lee	Infl. C	PR8	Paris	Bp. 6/57	Sing.	Shvets	Kolet	Bp. 23/57	Bp. 26/57	Bp. 33/57
Lee	**1200***	—⊗	—	—	—	—	—	—	—	—	—
Infl. C 1233	—	**400**	—	—	—	—	—	—	—	—	—
PR8	—	—	**3200**	50	—	—	—	—	—	—	—
Paris P. L. 1/49	—	—	25	**1600**	800	—	—	16	64	32	24
Budapest 6/57	—	—	25	800	**1200**	—	—	24	64	128	32
Sing. 1/57 N° 198	—	—	—	—	—	**2000**	1200	1800	1200	2000	2000
Sing. 1/57 N° 204	—	n. t.+	n. t.	12	64	**1200**	600	1000	800	1000	800
Shvets	—	n. t.	n. t.	32	128	800	**800**	1200	1200	1200	1200
Kolet	—	n. t.	n. t.	8	32	300	600	**2000**	2000	2000	2000
Budapest 23/57	—	n. t.	n. t.	12	48	50	100	600	**600**	400	300
Budapest 26/57	—	n. t.	n. t.	6	64	300	600	2000	2000	**2000**	2000
Budapest 33/57	—	n. t.	n. t.	8	24	100	150	800	800	600	**600**

* Reciprocals of the HI titres, ⊗ < 6, + not tested

out with some of the Asian strains is demonstrated in the lower right rectangle of Table VI.

Table VI shows 3 to 12 fold differences between the homologous and heterologous titres of each of the "Hungarian-like" sera, while the differences in relation of the "Singapore-like" sera were not significant.

Chicken sera produced against the Hungarian-like strains (lower left rectangle) showed considerably higher HI titres to the most recently isolated strain Budapest 6/57 than to the Paris strain which had been isolated 8 years earlier.

Neutralization tests revealed a similar difference between Hungarian-like and Singapore-like strains. However, the titres of A-prime sera to the Hungarian-like strains never exceeded 1 : 32 in the presence of 300 to 500 ID_{50} of virus. The same sera showed no inhibitory activity against the Singapore-like strains.

We compared two Hungarian strains with the Singapore strain in cross antibody absorption tests. The results are illustrated in Fig. 1. Fig. 1 shows that after complete absorption of the antibodies capable of inhibiting the HA by the heterologous strain, both Hungarian and Singapore sera had a considerable residue of the homologous titre (1 : 150 and 1 : 38, respectively). Much lower but demonstrable residual titres to the homologous strains were found

in cross absorption tests when our first Asian strain (Budapest 23/57) was tested against one of the later strains (Budapest 26/57).

Serological survey. In July, nearly a thousand human pre-epidemic sera, mostly from blood donors, were tested for anti-haemagglutinin activity to the Singapore strain. No positive samples were found. On the other hand, many of the same sera showed high HI titres (up to 1 : 512) when tested against the Hungarian strains.

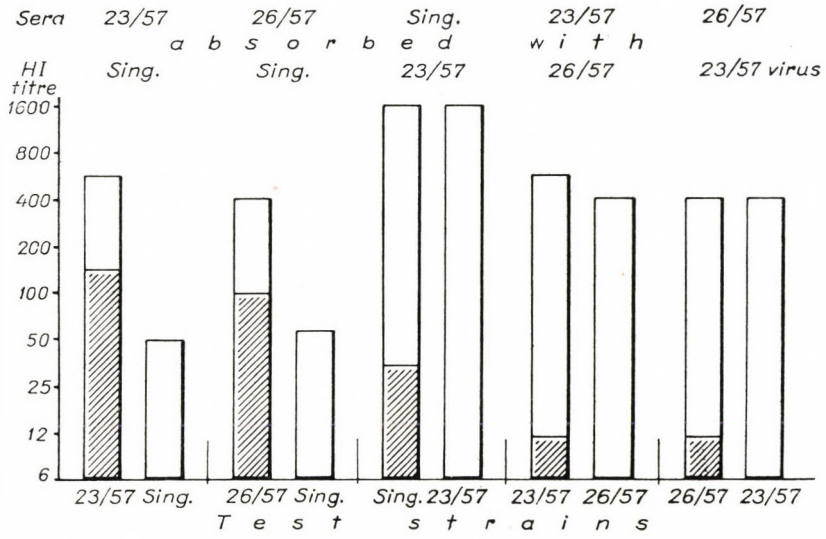

Fig. 1. Cross antibody absorption tests with Singapore and Hungarian strains of Asian virus

Abbreviations:

Sing = Singapore 1/57
23/57 = Budapest 23/57
26/57 = Budapest 26/57

Comparative neutralization tests to several Asian strains were performed with 5 out of the pre-epidemic sera showing high HI titres against the Hungarian strains. Some typical data are shown in Table VII. It can be seen that Singapore-like and Hungarian-like strains behaved differently in the neutralization test, too. The human sera were significantly more active against the Hungarian-like strains.

In November, when the epidemic was coming to an end, serum samples from 9298 recruits were tested for HI activity to the Singapore strain, using the spiral loop dilution technique. The recruits had been called up from different areas of the country. Blood was taken on the second or third day of their joining the Army. The results are summarized in Table VIII.

Table VII

Haemagglutination inhibition and neutralization tests with human pre-epidemic sera

Sera	Virus strains											
	Sing 1/57		Shvets		Kolet		26/57		33/57		37/57	
	HI	N* 50 ID_{50}	HI	N 5000 ID_{50}	HI	N 300 ID_{50}	HI	N 300 ID_{50}	HI	N 300 ID_{50}	HI	N 300 ID_{50}
556	—$^{+}$	4⊗	—	2	256	24	128	48	256	12	512	64
557	—	—	—	—	256	12	128	48	256	6	256	16
640	—	—	—	—	128	6	128	6	256	—	512	8
757	—	—	—	—	256	12	256	24	512	8	512	32
762	—	—	—	—	256	6	128	24	256	12	512	32

* Neutralization titre against 50 ID_{50}, $^{+} < 2$, ⊗ Reciprocals of the titres.

Table VIII

Haemagglutination inhibition tests to the Singapore strain with 9298 serum samples taken from recruits in November, 1957

	Distribution of the HI titres					Positive	Negative
	1 : 2	1 : 4	1 : 8	1 : 16	1 : 16<		
Numerical data	145	766	1572	1276	1126	4878	4420
Per cent	1.5	8.2	16.9	13.8	12.1	52.5	47.5

The data indicate that in Hungary at least 50 per cent of the 21 year-old men had been affected by the epidemic. The real percentage may have been higher, as the immune responses by the convalescents were generally poor. Using the same technique of HI test, 22 of 83 convalescent sera taken during the third week of Asian influenza appeared to be negative.

The rate of sero-positive recruits varied according to the area they had come from. The highest rates were, 122/190 (64.2 per cent) and 708/1129 (62.7 per cent) ; the lowest, 62/194 (31.9 per cent) and 429/1015 (42.2 per cent).

Discussion

The antigenic analysis of the A-prime strains recovered in Hungary in 1957 support the view about the progressive character of the antigenic variation of influenza virus. Owing to the highly sensitive test of cross antibody absorption, it is reasonable to suggest that the winter outbreak was caused by a virus which had originated from the pathogenic agent prevalent at the end of the last wide-spread epidemic in Hungary.

During the interepidemic period, however, probably due to serial passages in partially immune humans, a minor change in the antigenic composition of the virus had taken place. An antigenic variation is thought to be necessary for spreading of a new epidemic within a short period of time. In the present case, the low morbidity rate in the age groups over 4 years might be explained by the fact that the antigenic variation that had taken place during the 3 years of interepidemic period was very slight.

The advantages of our rapid method of antigenic analysis have been discussed earlier [3, 8]. We repeatedly emphasize that using virus absorbed sera, an antigenic analysis, more sensitive than the usual cross HI test, can be performed without the delay caused by the procedure of producing hyperimmune sera against the new strains.

A difference between Singapore-like and Hungarian-like strains of the Asian virus was demonstrated in HI tests performed in hyperimmune sera prepared against Hungarian strains of the virus. A similar difference could not be established using Singapore sera in the same test. Owing to the variable sensitivity of the Asian strains to non-specific inhibitors present in human serum [12], such a one-sided difference in the cross HI test alone does not necessarily denote an antigenic difference between the strains in question. In the antibody absorption test, however, the difference was both-sided, detecting an antigenic component present in the Hungarian strains and lacking in the Singapore strain and *vice versa.*

We are not quite convinced that the antigenic component differentiating the Hungarian strains from the Singapore strain is identical with that rendering the formers to be inhibited and neutralized by A-prime hyperimmune sera and human pre-epidemic sera.

Nevertheless, a relationship of Hungarian-like strains to A-prime strains might be suggested also on the basis of the well-defined neutralizing and HI activity of the "Hungarian-like" sera to the A-prime strains. Since these sera have had higher titres to the earlier strain Paris P. L. 1/49 than to those isolated more recently, the latter seem to be closer related to the Hungarian-like sera than the former. It must, however, be mentioned that some of the "Singapore-like" sera, too, showed a certain activity against the A-prime strains.

On the basis of experimental data by ARCHETTI and HORSFALL [11] and other workers, many authors suppose that the antibodies present in the sera of the affected population may be at least one of the factors responsible for the natural modification of the influenza virus. If so, the modified strain may be expected to be poorer in the antigenic components corresponding to the antibodies present in the population. However,

(i) the Asian virus did not meet related antibodies if not those produced to itself during the course of the infection ;

(ii) the modified strain seem to be related to the old antibodies, while the original strain not.

Thus, the existence of some different modifying factor or factors must be supposed. One of the explanations might be a recombination.

Although the antigenic difference between Singapore-like and Hungarian-like strains seems to be proved, we must not disregard the literary data [12] suggesting the increased sensitivity to non-specific inhibitors of some modified Asian virus strains. Our current experiments also support the important role of non-specific inhibitors in the strong HI activity to the Hungarian-like strains of human pre-epidemic sera and some animal sera.

It has to be decided whether the inhibiting substance effective to the Hungarian-like strains is one of those inhibitors which act also against the earlier strains of influenza virus, or whether it is a fairly distinct substance specific to the Asian virus.

Summary

In Hungary, two influenza epidemic waves were observed in 1957. The winter epidemic involving mostly children under 4 years of age was caused by an A-prime strain. On the basis of the close antigenic relationship the causative agent is supposed to have originated from those strains which had been prevalent at the end-period of the last wide-spread epidemic in Hungary. The autumn outbreak with a high morbidity rate was caused by the Asian virus. Nevertheless, the strains recovered could be distinguished from the prototype strains by cross HI or cross antibody absorption tests. Hyperimmune sera produced in chickens or in hamsters against A-prime strains, as well as human pre-epidemic sera, contain HA inhibiting and neutralizing factors effective against the Hungarian strains and completely inactive on the Singapore strain. One of the two Russian strains at our disposal was rather closely related to the prototype strains, while the other proved to be identical with the Hungarian strains. Beside accepting the role of non-specific inhibitors it is suggested that the Hungarian strains of Asian virus share a minor antigenic component with A-prime strains. This might be explained by assuming a recombination between Singapore-like and A-prime strains.

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THE RESULTS OF INTRACUTANEOUS POLIOMYELITIS VACCINATION IN HUNGARY, 1957

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A wide-spread outbreak of poliomyelitis occurred in Hungary in 1957. Previously, the usual annual incidence varied between 200 and 300 in non-epidemic years and never exceeded 1200 cases even in the epidemic years (Fig. 1). In contrast to this, in 1957, 2334 (23.8 per 100 000) cases of paralytic

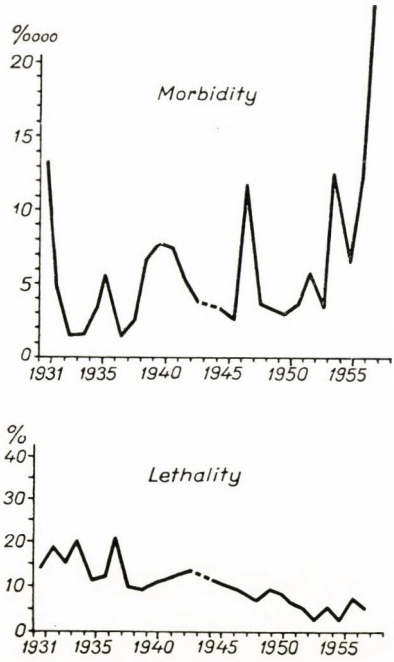

Fig. 1. Poliomyelitis in Hungary

poliomyelitis were reported with a death rate of 6.1 per cent. The outbreak involved the north-eastern part of the country and, to some extent also Budapest. In the other districts the morbidity did not exceed that for the preceding non-epidemic years.

A detailed description of the outbreak will be published in another paper from this Institute [1]. It must be noted, however, that the previous outbreaks had started in June or July, with their peaks in August or September, whereas the curve of the 1957 epidemic began to rise steadily as early as May and, after a peak in July, fell off suddenly. The incidence in the last three months of the year was lower than that during the same season of any of the previous epidemic years. Fiftythree strains of poliomyelitis virus including 46 type 1 strains were isolated at the Virus Department of this Institute during the epidemic season [2].

The vaccination programme

During the 1957 outbreak the usual control measures were completed by introducing active immunization with the Salk vaccine. Because of difficulties in procuring the vaccine, vaccinations were not commenced until the epidemic had reached its peak in July. At that time vaccine was available only for two age groups. A few weeks later, however, further amounts of vaccine were imported. Age groups being in greatest risk were elected for vaccination. According to the official plan, children born in 1955 and 1956 were eligible for the first dose of vaccine in July, while the vaccination of those born in the period 1951—1954 and of infants born in January and February, 1957, was commenced in August. Thus, altogether, children born between January 1, 1951, and February 28, 1957, *i. e.* children aged 0 to 6 years except those under 6 months, were eligible. (Young infants have been barely affected by poliomyelitis in Hungary.) The vaccination campaign was carried out between July 18, and September 25. With the remaining vaccine children over 6 years were immunized. Vaccination of the latter and of those who had escaped vaccination during the campaign went on until the end of the year.

The bulk of the vaccine used in this campaign was prepared by three laboratories, Connaught ; Eli Lilly and Co.; Park, Davies and Co. Smaller quantities made by other laboratories were also used. The vaccination was free of charge and voluntary. Two inoculations were given four weeks apart. Both inoculations consisted of two intracutaneous injections, 0.1 ml each.

The central organization of the vaccination, the import and distribution of the vaccine, and some other administrative measures were done by the Ministry of Health. The local organization in each county and town was provided by the local Public Health Station. The vaccination itself was performed at most places by teams consisting of physicians and technicians expert in intracutaneous administration of vaccine ; in some districts by the district doctors.

Table I illustrates the number of persons eligible for accepting vaccine. The data have been supplied by the Ministry of Health. These show that,

according to the data by the Central Office of Statistics, 1 153 945 children belonged to the age groups which had been elected for vaccination. Of these, 75.7 per cent received two doses until the end of September. The acceptance ratio rose to 80.4 during the last three months of the year. In the Capital, Budapest, the acceptance ratios were much lower, while those for the country districts higher. Of the age group over 6 years, 271 675 children have been vaccinated. Thus a total of 1.2 million children was given two doses of vaccine until the end of the year.

Table I

*Persons vaccinated during the poliomyelitis vaccination campaign, 1957**

		Country	Budapest	Hungary
Persons eligible for vaccination born between 1st January 1951 and 28th February 1957		983 012	170 933	1 153 945
A) Vaccinations in the period July—September 1957				
Of those eligible vaccinated with	single doses	89 144	23 816	112 960
	two doses	792 357	81 554	873 911
Of those eligible unvaccinated		101 511	65 563	167 074
Per cent of children vaccinated with two doses		80.6	47.7	75.7
Persons over 6 years vaccinated with two doses		126 608	10 427	137 035
B) Vaccinations in the period October—December 1957				
Children vaccinated with two doses among those eligible		40 752	12 889	53 641
Persons over 6, with two doses vaccinated		109 326	25 314	134 640
C) Total vaccinations carried out in 1957				
Children receiving vaccine among those eligible for vaccination		833 109	94 443	927 552
Children with two doses, per cent of those eligible		84.8	55.3	80.4
Persons over 6 with two doses		235 934	35 741	271 675
Persons with two doses altogether		1 069 043	130 184	1 199 227

* Figures supplied by the Ministry of Health.

Table I does not contain the data for children vaccinated by private practitioners. Calculated from the quantity of vaccine imported during the year, the number of such children is estimated at least to 80 000, most of them living in the Capital.

Results

The evaluation of the results has been hindered by some circumstances. (a) The campaign was commenced at the peak of the epidemic and was carried out while the attack rate was declining. The comparison on the basis of age incidence has been disturbed (b) by the considerable number of children over 6 having received vaccine ; and (c) the relatively high rate of unvaccinated

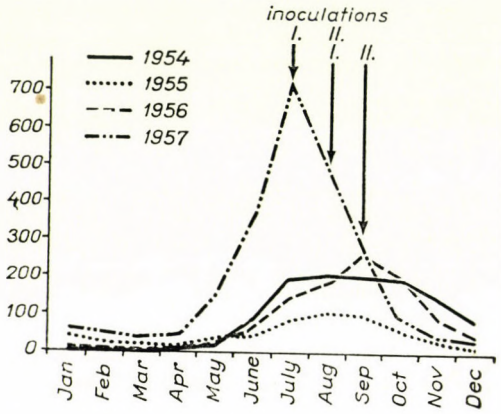

Fig. 2. Poliomyelitis cases in Hungary

children among those eligible for vaccination. The latter, having been spontaneously absent, cannot be taken as an appropriate control group.

Owing to these inevitable sources of error, the accuracy of the evaluation is not quite satisfactory. Since the object of the programme was to overcome an epidemic, the rules of an experimental trial were not followed. Despite the inadequacies of the data, an effort was made to evaluate the results. The evaluation was based both upon the infectious disease reporting cards and data collected by using special forms.

The following facts suggest that the effect of the vaccinations was satisfactory.

(1) The previous outbreaks continued during the autumn months (Fig. 2). In 1957, although the epidemic had significantly exceeded all the epidemics previously observed in Hungary, the attack rate for the last three months of the year was lower than that for the same periods of the preceding two epidemic years.

(2) Fig. 3 presents the comparison of the Hungarian epidemic curve with those for two neighbouring countries where, according to our knowledge, no large-scale vaccination was performed during the epidemic season. All the three curves in the Table being calculated from preliminary data, the Hungarian curve does not conform to that shown in Fig. 2.

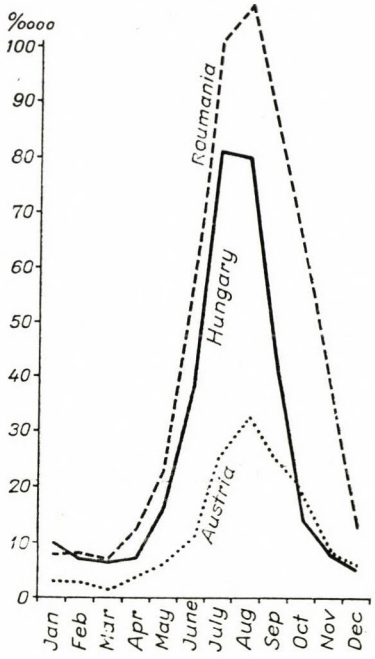

Fig. 3. Poliomyelitis cases in Hungary, Austria and Roumania in 1957 (Preliminary data)

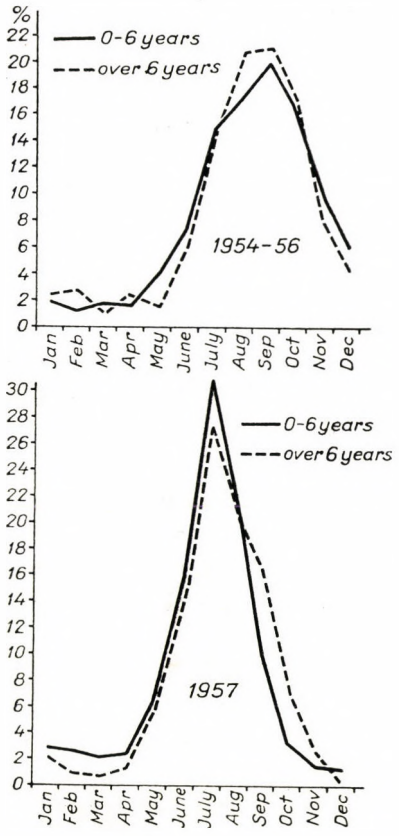

Fig. 4. Poliomyelitis cases in Hungary, by months

As seen in Fig. 3, in Roumania the epidemic, like the earlier outbreaks in Hungary, was protracted for the autumn months. For Austria the seasonal attack rates were considerably lower than for Hungary, except in the last three months when the Hungarian curve fell below the Austrian.

(3) The seasonal attack rate of paralytic poliomyelitis for the age group 0 to 6 years of age was compared with that for the age group over 6 years. The curves in Fig. 4 illustrate the percentual distribution of cases in the two age groups by months. The upper graph illustrates the mean values

calculated from the data of the epidemic years 1954, 1955, and 1956. The lower graph presents similar data calculated from the 1957 epidemic. The curves of the younger and older age groups were nearly parallel in the period 1954—56, while in 1957 the curve of the "younger" group was shifted towards the earlier months *i. e.* the relative attack rate for the younger age group during the autumn months was significantly lower than for the group over 6.

(4) According to the vaccination programme referred to, and disregarding the inadequacies mentioned above, in the last three months the 0 to 6 years age group could be considered as a "vaccinated" group, while the children over 6 years as unvaccinated "controls". As to the attack rates, the two groups are not equivalent because usually about 90 per cent of the diseased occurred in the former group (in the period 1954—1956, 88.1 per cent). Table II presents the monthly distribution for the two age groups of the poliomyelitis cases in Hungary. (To avoid distorsion by small numbers the first four, as well as the last three months have been drawn together.) The same data are illustrated in Fig. 5. In the years 1954 to 1956 during the early and late months the incidence for the group 0 to 6 years was 7 to 8 times that for the group over 6 years of age. This ratio was the lowest for August, about 6 : 1. During the first seven months of 1957, the same ratio varied between 6 and 8. From September onwards, however, there were only about three times more cases in the younger than in the older age group. Assuming that the decrease of this age ratio was due to the vaccination, the month August can be considered as a transitory period when the vaccination might be expected to have exerted some effect. Fig. 5 clearly shows the ratios.

Table II

Distribution of poliomyelitis cases by age groups and months

Month	Number of cases in				Case incidence for children of 0—6 years if that for those over 6 is 100	
	1954—1956		1957			
	0—6 years	over 6 years	0—6 years	over 6 years	1954—56	1957
I—V.	252	32	307	38	787	808
VI.	176	21	310	51	838	608
VII.	373	53	604	101	704	598
VIII.	426	75	410	77	568	532
IX.	501	74	203	62	677	327
X—XII.	802	108	129	42	742	307
Total	2530	363	1963	371	697	

The data in Table II permit the evaluation of the decrease in incidence for the "vaccinated" age group. On the basis of the ratios 6 : 1 or 8 : 1, the

expected number of cases for the "vaccinated" age group can be calculated. During the last four months, 104 cases occurred in the group over 6 years of age. Calculated with the 6 : 1 ratio, 624 cases would have been expected for the "vaccinated" age group during the same period. On the basis of a 8 : 1 ratio, 832 cases would have been expected, in contrast to the 332 cases which had occurred. The last figure makes 53.2 and 40.0 per cent, respectively, of those calculated. Thus, among the persons eligible, during the last three months of 1957, the incidence of paralytic poliomyelitis was reduced by 47 to 60 per cent, when compared with the expected values calculated on the basis of the appropriate data of the preceding three years. If so, the reduction in absolute numbers was 300 to 500.

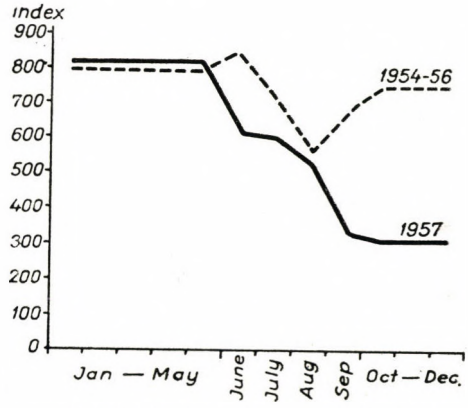

Fig. 5. Case incidence for children 0—6 years, if that for those over 6 is 100

A small number of persons over 6 had also received vaccine, while in the "vaccinated" group infants under 9 months of age and a relatively high proportion of the children eligible for vaccination had not. Thus the calculated values of reduction are to be considered as minima.

(5) The vaccination campaign was commenced in July and the data on the vaccination status of diseased subjects in the "vaccinated" group were collected by individual questionnaires since August. The incidences for the country districts among the persons eligible for vaccination (children born between January 1, 1951, and February 28, 1957) are shown in Table III, those for the Capital are summarized in Table IV. These Tables present the figures for unvaccinated children belonging to the "vaccinated" group, as well as the incidence among those who had received a single dose or two doses of vaccine, and show also the interval between vaccination and onset of the illness (1st fortnight — 2nd fortnight — thereafter). In 6 per cent of the reported cases the data of vaccination were unavailable.

The vast majority of the vaccinations having been completed as late as by the end of September, the full effect of the campaign could not be expected to manifest itself before the last three months of the year. The evaluation of the effect has been hindered by the relatively low incidence during this period. When calculating the attack rates for the different age groups, the number of vaccinations performed until the end of September was used (see Table I).

Table III

Poliomyelitis cases in the country among children eligible for vaccination

Month	Unvaccinated cases	Case incidence among children vaccinated						Total cases
		with single doses			with two doses			
		0—14	15—29	30 and over	0—14	15—29	30 and over	
		days following vaccination						
August	197	60	47	8	29	—	—	341
September	65	17	20	13	26	15	11	167
October	16	1	1	9	4	8	18	57
November	6	1	1	3	—	—	14	25
December	5	—	—	—	—	—	8	13
Total Oct.—Dec.	27	2	2	12	4	8	40	95
Attack rates per 100 000								
	26.6			13.4			5.0	

In the country districts during the last three months of 1957, 27 cases (26.6 per 100 000) of paralytic poliomyelitis occurred among the 101 511 children who had not accepted vaccine. In the same period the incidence beyond 30 days from vaccination among the 88 144 children who had received a single dose was 12 (13.4 per 100 000). Among children who had been given two doses of vaccine (792 357), 40 cases occurred (5.0 per 100 000) from the 30th day onward.

If 14 days are supposed to be sufficient for the evaluation of the full protective effect, the attack rate for these vaccinated with two doses was 6.1 per 100 000 (48 cases). On the basis of the attack rate for the unvaccinated group, in the last three months 230 cases would have been expected to occur in the single- and two-dose-groups altogether. Adding to this the number of expected cases in the Capital yields a sum not significantly different from the result shown under (4). Both calculations show that the attack rate among the children eligible for vaccination who had not accepted vaccine exceeded about five times the rate among children with two doses of vaccine.

Table IV

Poliomyelitis cases among children eligible for vaccination, in Budapest

Month	Unvaccinated cases	Case incidence among children vaccinated						Total cases
		with single doses			with two doses			
		0—14	15—29	30 and over	0—14	15—29	30 and over	
		days following vaccination						
August	25	5	6	—	2	—	—	38
September	4	4	4	3	—	1	2	18
October	2	—	—	1	—	1	1	5
November	—	—	—	1	—	—	2	3
December	—	—	—	—	—	—	1	1

The incidence in the Capital was too low to permit evaluation. This was hindered, in addition, by the undetermined number of non-official vaccinations.

(6) As mentioned above, children born in 1955 and 1956 were vaccinated one month earlier than those born in the period 1951—1954. *Fig. 6* shows the percentual seasonal distribution of paralytic poliomyelitis for one year

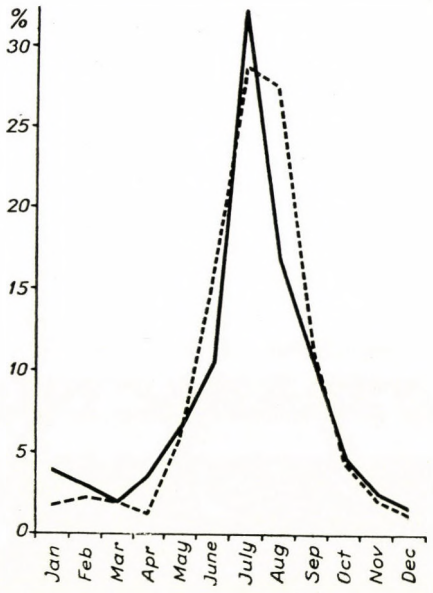

Fig. 6. Poliomyelitis cases in Hungary, by age groups

Explanations:
———— One year old children, vaccinated in July and August
— — — — Three to 6 years old children, vaccinated in August and September

old children belonging to the former group compared with that for the 3—6 year old children. The data for the children 2 years of age could not be utilised since the reporting cards did not show the year of the birth. Thus, children 2 years of age were born either in 1954 or 1955. *Fig. 6* shows that the one month difference in the vaccination was followed by a one month shift in the decrease of the attack rate. The decline setting in in both curves as soon as a month following the first inoculation suggests the possible existence of some protection afforded by a single dose of vaccine.

Table V

Poliomyelitis cases following official vaccinations

Interval between vaccination and onset in day	Number of cases in					
	the country		Budapest		Hungary	
	following the					
	1st	2nd	1st	2nd	1st	2nd
	inoculation					
0— 4	21	22	4	1	25	23
5— 9	27	23	2	1	29	24
10—14	31	14	3	—	34	14
15—19	22	14	3	2	25	16
20—24	28	4	3	—	31	4
25—29	19	5	4	—	23	5
30—39	17	14	—	2	17	16
40—49	9	15	2	2	11	17
50—59	2	7	1	1	3	8
60 and over	5	15	2	1	7	16
Total	181	133	24	10	205	143

(7) The lack of a significant rise of the attack rate following the first inoculation and the consistent decline of the curves after the second dose (Table V) indicate that no provocative effect can be attributed to the vaccination.

Table VI presents the incidence of paralytic poliomyelitis in the period from August through December among the children who had been vaccinated by private practitioners. Private inoculations were mostly administered intramuscularly or subcutaneously. Because of the small number of cases these results cannot be compared with those obtained with the intracutaneous method. It can be concluded, however, that no provocative effect was observed in this group of 80 000 children either.

Table VI

Poliomyelitis cases following vaccinations by private practitioners

(Number of the cases in the period August—December)

Interval between vaccination and onset in day	Number of cases in					
	the country		Budapest		Hungary	
	following the					
	1st	2nd	1st	2nd	1st	2nd
	inoculation					
0— 4	1	—	—	—	1	—
5— 9	—	2	1	1	1	3
10—14	1	—	—	—	1	—
15—19	1	—	—	—	1	—
27	—	—	1	—	1	—
35	—	—	—	1	—	1
115	—	1	—	—	—	1
Total	3	3	2	2	5	5

Discussion

The poliomyelitis epidemic that had started in Hungary in May, 1957, exhibited in June and July attack rates never previously observed. Beside the usual control measures an immunization campaign using Salk vaccine was commenced at the peak of the epidemic. The vaccine was given intracutaneously, a method chosen because of the limited amount of vaccine available. Furthermore, it was hoped that the possible provocative effect of the vaccine would be reduced by using this technique. Choosing the intracutaneous method was consented by the leading experts of poliomyelitis vaccination [3].

The 60 to 90 per cent reduction in the poliomyelitis attack rates due to intramuscular or subcutaneous applications of the Salk vaccine are well known [4, 5, 6, 7].

The Salk vaccine has been administered intracutaneously in some other countries too (Denmark, 1955 ; Czechoslovakia, 1957). In Denmark, the 7-to-12-year age group had been vaccinated by the intracutaneous route [10] but the results could not be evaluated since as few as 7 cases occurred among the vaccinated and control persons altogether during the summer following the vaccination campaign. The results of the intracutaneous vaccinations performed in Czechoslovakia have not been so far available.

The efficacy of our vaccination campaign could not be determined accurately because of certain circumstances. The simultaneous decline of the epi-

demic curve is not a satisfactory indicator of the effect, neither are the facts that the attack rate during the last three months of the year 1957 was lower than those in the neighbouring countries in the same period or in Hungary during the last trimesters of the 3 preceding epidemic years. However, the difference in the attack rates for the vaccinated and unvaccinated children, as well as the fact that in the last months of the year the vast majority of the children who belonged to the age group showing usually the highest morbidity could be considered as protected against poliomyelitis, suggest that the unusually early decline of the epidemic curve was due to the effect of the vaccinations.

The attack rate for the age group of eligible children was compared with that for the group over 6 years of age. On this basis the efficacy of the vaccination has been calculated to 50—60 per cent. The true rate of protection should have been higher because 15—20 per cent of the children eligible for vaccination had not accepted vaccine and a great part of the children belonging to the "unvaccinated" age group had received vaccine by private practitioners.

Slightly more accurate results were expected of the detailed examination of the cases of disease among children eligible for vaccination, since a considerable proportion of the children belonging to that group had not accepted vaccine. Thus, they could be considered as unvaccinated controls. In the last three months of the year the attack rate for the latter was five times more than for the vaccinated children. Evaluation was hindered by the low incidence of paralytic poliomyelitis both in the vaccinated and control groups.

For the children who had received vaccination one month earlier than the rest, the decline of the morbidity curve occurred one month earlier. Thus, even the first dose of vaccine appeared to have a protective effect.

The efficacy of the vaccination could not be determined exactly. It seemed, however, to agree with the protective values obtained in other countries by means of intramuscular or subcutaneous vaccination.

The Salk vaccine was administered intramuscularly or subcutaneously in a number of countries during epidemics [6, 8, 9] without any provocative effect. During our campaign with the intracutaneous technique, neither the first nor the second inoculation was followed by any significant rise in the attack rate. Thus, no untoward effect can be attributed to the intracutaneous technique.

Summary

In Hungary, the Salk vaccine was applied for the first time during the wide-spread epidemic in 1957. The intracutaneous technique was used. The vaccination consisted of two inoculations of 0.2 ml (= two simultaneous injections of 0.1 ml) each at an interval of four weeks. Although the accurate evaluation of the campaign was hindered by a number of inadequate circumstances, several data suggest that it has been effective. The epidemic declined

sooner than in the preceding epidemic years, or in the neighbouring countries in 1957. The incidence for the group included in the vaccination programme was compared with that for the unvaccinated age group. Fifty to 60 per cent reduction in the incidence of paralytic poliomyelitis was observed in the former group. The data concerning the cases of disease occurring among the children eligible, together with the vaccination status of the patients, were collected on special questionnaries. The attack rate for the unvaccinated children eligible for vaccination was five times that for the vaccinated children. The number of cases under observation was too small to permit accurate evaluation of the efficacy of the vaccination. Some decline in the attack rate was detectable following a single inoculation with the vaccine. No provocative effect was observed, although the vaccinations were carried out during epidemic.

Acknowledgements: I wish to thank the Ministry of Health for providing the figures on the vaccinated persons, and Drs. G. BARSY and E. FARKAS for criticism of the manuscript.

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EFFECTS OF FORMALDEHYDE ON INFLUENZA VIRUS II. EFFECTS ON THE INFECTIVITY OF THE VIRUS

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As described in a previous report [1], the inactivation of the haemagglutinating activity of influenza virus by formaldehyde was found to be influenced mainly by three factors, *viz.* the temperature, the pH and the relative concentration of formaldehyde. Results obtained by studying the influence of the same factors on the inactivation of influenza virus infectivity are presented below.

Materials and methods

Virus. The *Paris P. L. 1/49* strain of influenza A-prime virus was used. The strain was maintained by serial passages in 10-day-old embryonated eggs.

Preparaton of purified and concentrated virus suspensions. The RBC adsorption and elution method was used, as described previously [1].

Formaldehyde. A substance meeting the same requirements as described earlier was used [1].

Haemagglutination test. This test was performed according to the method of TAKÁTSY [2], using a 1 per cent chicken red blood cell (RBC) suspension.

Infectivity titrations. Infectivity was titrated by the micro-roller-tube method of HORVÁTH [3]. Minced allantoic membranes of 12-day-old chicken embryos were used as tissue component, and "glucosol" buffered salt solution as the fluid phase. Titrations were carried out in semi-logarithmic dilution series. After 72 hours of incubation at 37° C, virus multiplication was demonstrated by titrating the haemagglutinating activity of the culture fluids.

Evaluation of infectivity titrations. The virus preparations used in inactivation experiments usually exhibited an infective titre of about 10^{-5} to 10^{-6}, and a haemagglutinating titre of about 1 : 250. The formaldehyde concentrations which were found to be sufficient for the inactivation of the infectivity did not alter the haemagglutinating activity even after 72 hours' incubation at 37° C. To avoid errors in evaluating infectivity titrations, each roller-tube-culture was tested for haemagglutination, both at the beginning and the end of incubation. Only an increase in haemagglutinating activity was considered as a sign of virus proliferation. The persistent, unaltered haemagglutination titres never exceeded 1 : 3 to 1 : 9 and appeared only in 10^{-1} to $10^{-1,5}$ dilutions of the infectivity titration series.

In certain cases, especially at acid pH, at low temperatures, we often failed to demonstrate the presence of live virus in lower dilutions ($10^{-1,5}$ to $10^{-2,5}$) of the infectivity titration series, while in the higher dilutions of the same sample the presence of live virus was readily demonstrated. In these cases, we always took the highest dilution of formaldehyde-treated virus initiating the appearance of observable haemagglutination in the culture fluid as the end point of the infectivity titration. As to possible causes of this phenomenon, we refer to the further passages of the present paper.

Construction of the graphs. When presenting graphically the experimental results obtained, the following method was used. Depending on the deviations found, 4 to 6 experiments were performed. Independently of the actual titres found (limits within 1 exponent), the

initial virus concentration was always taken as unit. Decreases in the infectivity on formaldehyde treatment were calculated as the log difference between the starting titre and the actual titres found at different points of time $\left(\log \frac{I_t}{I_0}\right)$. In Fig. 2 the maximum deviations from the mean decrease of the titre are also included. Since grade and character of the deviations were essentially similar in every type of experiments, no extreme values were plotted on the other curves.

General method of inactivation. Stock virus concentrates were diluted by appropriately buffered saline, so as to contain about 10^5 to 10^6 tissue culture infective doses. The diluted virus suspension was divided into two parts, one serving as unformolized control and titrated at the beginning and at the end of the experiment, while the other part underwent formaldehyde inactivation. Except formaldehyde treatment, both control and experimental samples were handled in the same way.

Experimental

Effect of temperature. The effect of temperature on the inactivation of influenza infectivity by formaldehyde was studied at 0.01 per cent concentration of the inactivating agent in saline buffered to pH 6, 7, and 8, respectively. The experimental and control samples were incubated in a water bath of the temperature required. Samples for titration of the formolized virus suspensions were taken every 30 minutes in the 37° C series, and every hour in the 4° C series.

It was found that at 37° C inactivation took place within 30 minutes at pH 8 and pH 7, or within 90 minutes at pH 6. On the contrary, at 4° C the same amount of formaldehyde inactivated the same amount of virus at pH 8 within 12 hours or at pH 7 within 23 hours. Under the same conditions, at pH 6 only a titre decrease not exceeding 2 exponents was realized within 26 hours. Thus the speed of inactivation at given formaldehyde and virus concentrations seems to be remarkably influenced by the temperature. No detectable changes occurred during the experiment in the infectivity titres of the controls at any pH and temperature tested.

Effect of the formaldehyde concentration. The influence of formaldehyde concentration on the speed of inactivation was examined at 37° C at pH 6, 7, and 8, respectively. Samples for titration were taken every ten minutes. Mean values obtained by at least triplicate experiments are presented in Figs. 1/A, 1/B and 1/C.

Regardless of the differences in the speed of inactivation caused by the different pH-s, one can see that the higher the formaldehyde concentration, the more the curve resembles that of a monomolecular reaction. On the other hand, the lowering of formaldehyde concentration was found to cause irregularities in the shape of the curves.

No changes in the infectivity titres of the control groups took place during the experiment.

Effect of the pH. Studies on the effect of the pH on the shape of the inactivation curve were performed at 37° C, using 0.01 per cent formaldehyde.

Samples were taken every ten minutes. The results are presented in Figs. 2/A 2/B and 2/C.

As Figs 2/A, 2/B and 2/C reveal, a shift of the pH towards the acid side causes changes in the inactivation curve that remind very much of those

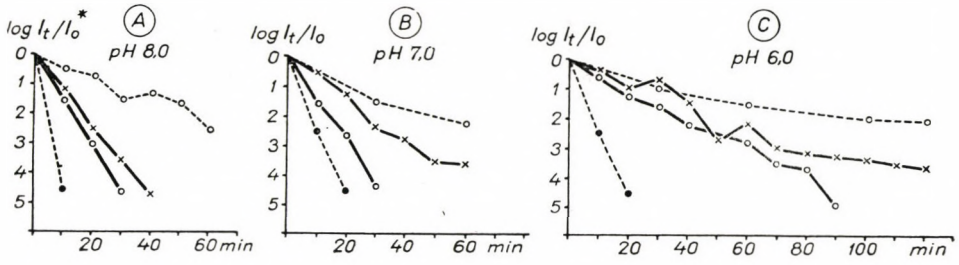

Fig. 1. Effect of the formaldehyde concentration on virus inactivation (Temperature, 37°)

Explanations:

• — — — • 0,030 per cent formaldehyde
○———○ 0,010 ,, ,, ,,
×———× 0,006 ,, ,, ,,
○ - - - - ○ 0,003 ,, ,, ,,

* I_0 = initial infectivity
I_t = infectivity after t minutes of formaldehyde treatment

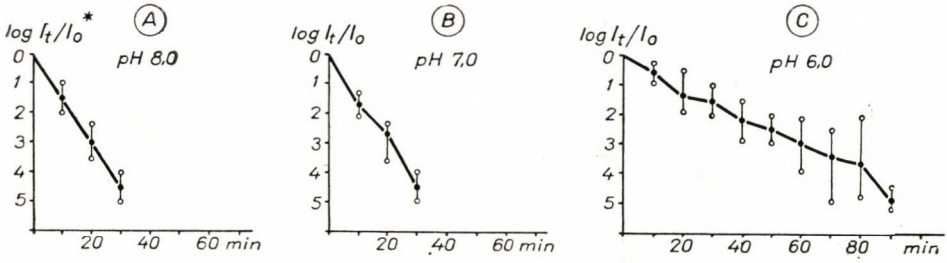

Fig. 2. Effect of pH on the inactivation of influenza virus by formaldehyde (Temperature, 37° C)

Explanations:

•———• mean
○ extreme values

*I_0 = initial infectivity
I_t = infectivity after t minutes of formaldehyde treatment

caused by lowering the formaldehyde concentration at a given pH and temperature.

When examining the shape of the different curves representing mean values and the scattering of values obtained by the actual titrations, one can see that shifting of the pH towards the acid side causes not only a decrease in the reaction velocity, but at the same time an increased deviation of the values also occurs.

Effect of the presence of an amino acid mixture. To study the effect of an amino acid mixture on the inactivating effect of formaldehyde on influenza virus infectivity, we used the Parker 199 tissue-culture medium [4]. The experiment was performed as follows. The pH of the amino-acid mixture

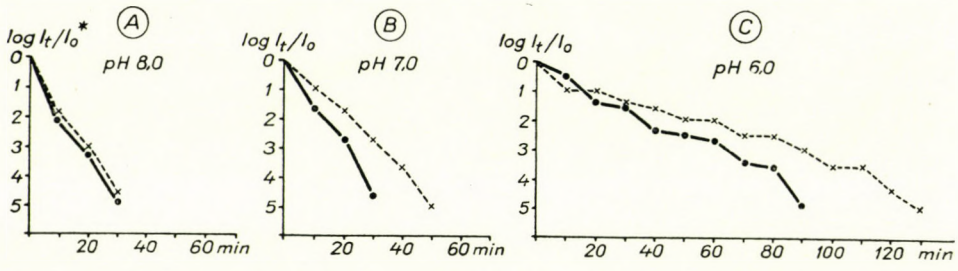

Fig. 3. Effect of an amino acid mixture on the inactivation of virus by formaldehyde. (Temperature, 37°C)

Explanations:

•———• control
× — — — — amino-acid mixture
*I_0 = initial infectivity
I_t = infectivity after t minutes of formaldehyde treatment

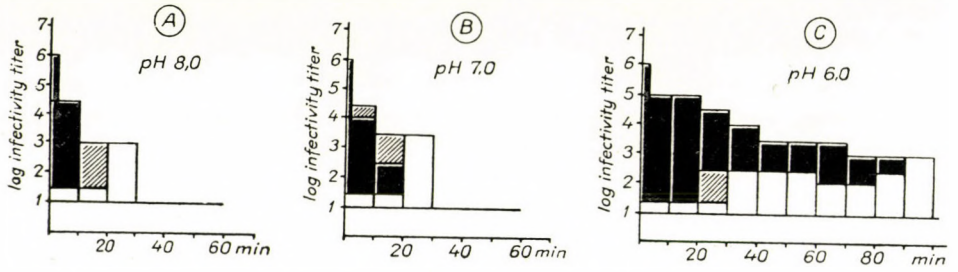

Fig. 4. Apparent "reactivation" of formaldehyde inactivated infectivity (Temperature, 37°C)

Explanations:

■ + haemagglutination
▨ ± haemagglutination
□ ∅ haemagglutination
} in culture fluids after 72 hours of incubation

was appropriately adjusted by adding sodium hydrocarbonate under the control of an electric pH-meter. The stock virus suspension was appropriately diluted by the solutions of different pH. Formaldehyde was added in a final concentration of 0.01 per cent. Inactivation was carried out at 37° C. Controls treated in a similar manner except the addition of formaldehyde were included in the experiment. Results of the inactivation experiment at different pH-s, as compared to those not containig amino acid, are presented in Figs. 3/A, 3/B and 3/C.

As Figs. 3/A, 3/B and 3/C reveal, the presence of a certain amount of amino acids did not influence the course of inactivation at pH 8, while at neutral and acid pH inhibition of the inactivation was observed.

Observations concerning the apparent "reactivation" of formaldehyde-treated virus by dilution. As mentioned above, in special cases an apparent "reactivation" of formaldehyde-inactivated influenza virus has been observed. As an example, we present three typical experiments at different pH-s and at 37° C in Figs. 4/A, 4/B and 4/C.

The phenomenon revealed by Figs. 4/A, 4/B and 4/C consists of certain irregularities observed in the titration of infectivity endpoints following formaldehyde treatment at pH 6.

Discussion

The influenza virus particle is known to bear materials of protein character on its surface, thus its inactivation by formaldehyde may be regarded as a reaction between formaldehyde and protein. The main type of reaction occurring when protein binds formaldehyde is that of an aldehyde amino-group reaction. Were the reaction of an ionic character, it ought to be enhanced by acidification of the environment. On the contrary, it has been found that the reaction prefers an alkaline environment. The reaction that occurs between amino groups and formaldehyde in an alkaline environment is usually an addition that within a certain period of time becomes an irreversible firm bond [5].

The addition character of the reaction seems to be supported by the experimental data presented below. Remarkable differences were caused in the speed of inactivation by relatively moderate changes in the concentration of formaldehyde, especially at neutral and acid pH. The titration results presented graphically in Fig. 4 clearly demonstrate the possibility of virus reactivation under special circumstances. At conditions optimal, or nearly optimal for the addition, the loss of infectivity follows approximately a first order reaction rate (Figs. 1 and 2) and an irreversible inactivation takes place fairly soon. Thus no "reactivation" can be demonstrated on dilution. When conditions are unfavourable for addition, the reaction velocity decreases remarkably, and a period of loose binding appears, as indicated by the "reactivation" phenomenon observable for a fairly long time. Thus the inactivation curves, as presented in the different Figs., represent always a sum of the effects of many different factors. The simultaneous presence of particles of different phases of formaldehyde binding in a mixture, of conditions unfavourable for the virus-formaldehyde reaction, together with the "reactivation" phenomenon appear sufficient to explain the irregularities observed in the corresponding

inactivation curves. Therefore, the rate of influenza virus inactivation by formaldehyde can be followed exactly only when conditions optimal for the reaction are provided for.

Finally, the observations concerning the influence of an amino acid mixture on the speed of inactivation by formaldehyde also support the view that formaldehyde reacts in a different way with influenza virus and amino acids. Acidification seems namely to enhance the inhibiting action of the amino acid mixture, while no such effect can be observed in an alkaline environment.

Although the pH, the formaldehyde concentration, and the temperature appear to be the most important factors influencing the speed of inactivation of influenza virus, the possible role of certain individual differences in the sensitivity of single particles in a normal influenza virus population cannot definitely be excluded.

Further studies on the interactions between formaldehyde and influenza virus are in progress.

Summary

The reaction of influenza virus and formaldehyde appears to be of addition character. The three main factors influencing the speed of inactivation were found to be the pH, the concentration of formaldehyde, and the temperature. Under conditions favourable for addition (alkaline pH, relatively high formaldehyde concentration) the reaction curve resembles very much of that of monomolecular reactions. The less the formaldehyde present and the more acid the pH, the more irregular the curve.

The irregularities observed in the infectivity titrations of formaldehyde-treated virus can be explained partly by "reactivation" of infectivity on dilution.

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PRODUCTION OF OXYTETRACYCLINE IN SYNTHETIC MEDIUM

By

I. HORVÁTH, I. GADÓ and A. SZENTIRMAI

Research Institute of the Pharmaceutical Industry, Budapest

(Received January 23, 1958)

The maximum yield of oxytetracycline is obtained by Streptomyces rimosus when grown in a medium containing soybean meal, starch, and oil [2, 3]. These constituents are insoluble in water and the arising mycelium cannot be separated from them, wherefore attempts to follow up the fermentation process by means of metabolic studies have been of no avail. For this reason, and with a view to studying the metabolic properties of the strain employed, a simple synthetic nutrient medium has been elaborated for the production of oxytetracycline. The present paper describes the experiment undertaken in this direction.

Materials and methods

Strain used. A *Streptomyces rimosus* strain marked BS—21 was used throughout the experiments ; the manner in which it was maintained has been described elsewhere [3]. The flasks were inoculated with 1 ml of a spore suspension produced in shaken culture.

Preparation of inoculum. The inoculum was grown for 96 hours, with shaking, in a medium containing the following constituents. Soluble starch 1.5 g, glycine 0.5 g, NaCl 0.3 g, KH_2PO_4 0.0125 g, palm oil 0.4 ml, tap water to 100 ml. Adjusted to pH 7 before sterilization.

From a four-day vegetative inoculum, the cultures grown in the shaken flask were inoculated with 1 volume per cent, and the submerged fermentations with 10 volume per cent, of the medium.

Conditions of fermentation. In the shaken flask experiments cultures were grown in 500-ml Erlenmeyer flasks containing 100 ml of medium, at 28° C, on a rotary type shaker working at 300 2-cm cycles per minute. The media described in the experimental part of this paper were prepared with tap water. The pH was 7 before sterilization.

Submerged culture experiments were carried out at 28° C in a 25-l spherical glass fermentor of 12 l working volume. For mixing a propeller stirrer with 350 r. p. m. was used. Fermentations were aerated at a rate of 0.5 liter of air per 1 l of medium. Dissolved in tap water, the constituents of the medium used for submerged fermentations were separately sterilized and, prior to inoculation, poured under aseptic conditions into the fermentor to yield a medium of pH 7.

Analytical procedures. The bioassay of oxytetracycline production was carried out by the method of HORVÁTH and WIX [4], using *Bacillus subtilis* strain ATCC 6633 as test organism. The chemical assay was performed according to MIZSEI and SZABÓ [5].

Vitamin B_{12} was determined by the method of HARRISON, LEES and WOOD [6], using the *E. coli* mutant of DAVIS and MINGIOLI [7]. Since the oxytetracycline present in the solution renders this determination impossible, the antibiotic was removed in the following manner. To 5 ml of fermentation liquor 0.5 ml of 1 per cent potassium cyanide were added ; the mixture was autoclaved for 20 minutes at 0.5 atm. pressure ; the mycelium was removed by centrifugation ; to 3 ml of the pure solution 0.3 ml of an alkaline cetylpyridine bromide (Sterogenol) solution were added ; the precipitate arising after mixing was centrifuged ;

the pure solution, after adjusting it with *n* HCl to pH 7, was again autoclaved for 20 minutes at 0.5 atm. pressure. The alkaline Sterogenol solution was prepared as follows. To 1 ml of 10 per cent Sterogenol dissolved in 50 per cent alcohol, 6 ml of 0.5 per cent sodium hydroxide solution were added. The solution was freshly prepared before each determination.

Alpha amino acid was determined with ninhydrin by the colorimetric method of BOISSONAS [8]. The results were obtained in nitrogen from a calibration curve plotted with glycine. Determination of ammonia was carried out by the method of VAN SLYKE and CULLEN [9], and that of total nitrogen by KJELDAHL's method in the WAGNER—PARNASS apparatus.

For determining glucose and starch, the MOLISCH reaction modified for quantitative use by SZÁRA [10] was employed. In a glass-stoppered test tube, to 1 ml of the test solution which may contain 20 to 200 μg of carbohydrate per ml, an equal amount of 2 per cent solution of α-napthol-β-sulphonic acid was added as a reagent ; the test tube was then placed into an ice bath where 8 ml of 87 per cent sulphuric acid (500 ml of concentrated sulphuric acid and 93 ml of distilled water) were added, as another reagent, and shaken together. The cooled samples were placed into a hot bath for 30 minutes, and thereafter cooled to room temperature in another water bath. The colour obtained was measured photometrically and the results were calculated by means of the simultaneously constructed calibration curve. The colour stayed the same for 10 hours.

Examination of the Seitz-filtered culture fluid was carried out by ascending chromatography on *Schleicher—Schüll* paper No. 2045/a at 25° C. One-dimensional chromatogramms were run from 0.1 ml of every sample, using ethanol : water (3 : 1) as a solvent. For two-dimensional paper chromatography the samples were desalted by binding the amino acids to sulphonic acid type resin and eluating them with ammonia ; the solvents adopted were ethanol : water (3 : 1) and *n*-butanol : acetic acid : water (4 : 1 : 5) systems. The dried strips were developed with a 0.2 per cent alcoholic solution of ninhydrin ; this was followed by drying at 60° C for 30 minutes.

Experimental results

Shaken flask experiments. Explorative experiments were first carried out with a view to selecting the appropriate constituents for the synthetic medium. In addition to the nitrogen and energy sources, the nutrient media used in these preliminary studies invariably contained 0.3 per cent NaCl and 0.0125 per cent KH_2PO_4. On the evidence of these experiments, there was no satisfactory growth of *Streptomyces rimosus* strain BS—21 on inorganic nitrogen sources, *i. e.* on nitrate or ammonium salt, or carbamide, but satisfactory growth was observed on numerous natural amino acids and on every combination of these acids ; as a nitrogen source, amino acid combinations were, generally, not found to be superior to individual amino acids in the production of oxytetracycline. It was not possible to increase production either with vitamins or with purine or pyrimidine bases. Where amino acids, individually or in combinations, were used as a nitrogen source, there glucose and starch were equally adequate sources of energy, though subsequent detailed studies revealed that starch was slightly superior in shaken flask experiments, and glucose in fermentation cultures.

Experiments undertaken to establish whether it was possible to influence growth and oxytetracycline production by means of trace elements showed that those present in tap water and the inoculum were sufficient to ensure continued maximum growth and production.

Throughout the preliminary experiments it was observed that the PO_4 content of the medium exerted a marked effect on mycelial growth and oxytetracycline production.

The difficulty that presented itself through the mycelium's growing in coarse granules, was overcome by the addition of Tween 80, of which even as much as 0.2 per cent did not depress growth or diminish oxytetracycline production.

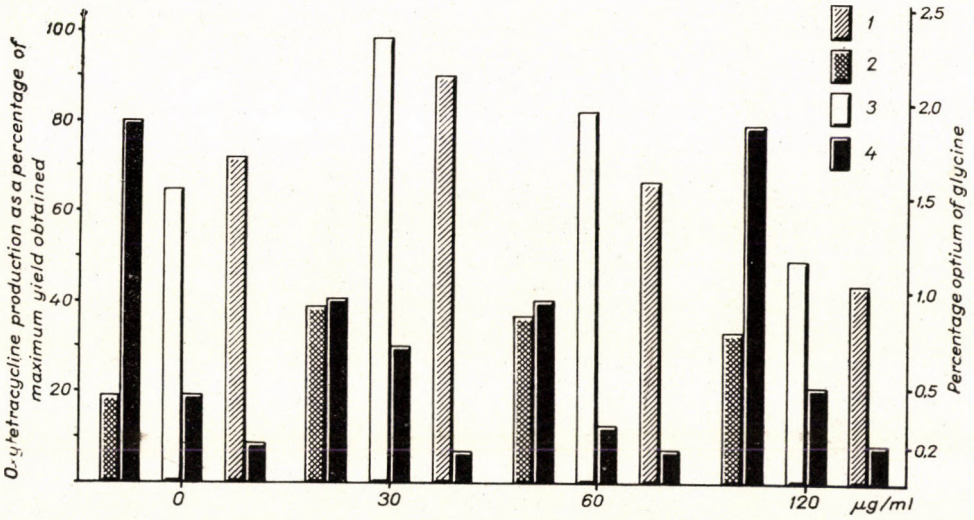

Fig. 1. Synthesis of oxytetracycline in media containing 0,5 (2), 1.5 (3), or 2,5 (1) per cent starch, the optimum amount of glycine (4), and phosphate in one of four different concentrations (abscissa)

The results of these preliminary studies made us use glycine and starch in the media for the detailed experiments, which in addition invariably contained phosphate, 0.3 per cent NaCl, 0.0005 per cent crystalline $Co(NO_3)_2$, and 0.002 per cent Tween 80.

Fig. 1 illustrates the changes in oyxtetracycline production on varying the concentration of starch and phosphate at the optimum glycine concentration. Production levels are expressed in percentages of the maximum production obtained and taken to be 100. Actually, this maximum varied between 450 and 750 µg/ml, and was yielded in the presence of 30 µg P/ml in media containing 0.5 per cent glycine and 1.5 per cent starch.

This graphic presentation shows that production was at its peak in media containing 30 µg P/ml, irrespective of what the starch and glycine concentrations were. It also shows that starch did not favour production in a low, 0.5 per cent concentration, and that while there was no substantial difference in their effect on production between the 1.5 and the 2.5 per cent concentration, the former was usually the more favourable one. The graphs

permit no general conclusion concerning the effect of the glycine concentration. It seems doubtless, however, that the unfavourable effect of a low starch concentration can to some extent be counter-balanced by means of glycine; to achieve this, more glycine is required in a medium to which no phosphate has been added or in which there are 120 μg P/ml present, than in one containing 30 or 60 μg P/ml. It is equally obvious from *Fig. 1* that an increase in the concentration of starch goes hand in hand with a decrease in the optimal glycine concentration.

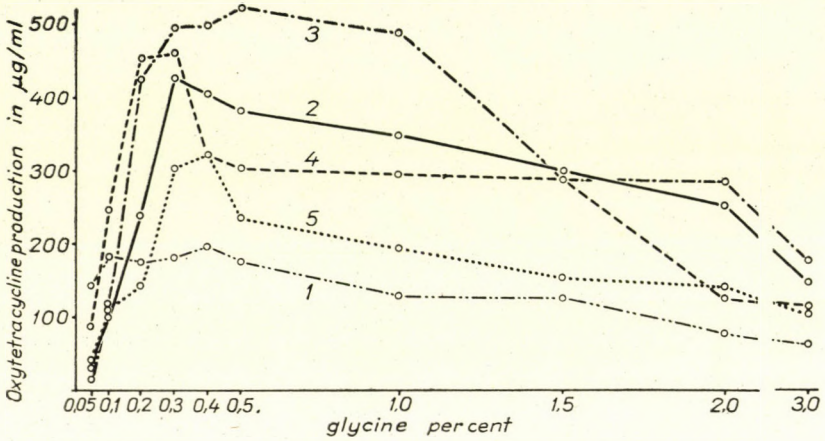

Fig. 2. Effect of glycine in the medium on oxytetracycline production at different phosphate concentrations. 1. Phosphate-free; 2. 10 μg P/ml; 3. 30 μg P/ml; 4. 60 μg P/ml; 5. 120 μg P/ml

The effect which changes in the glycine content were found to exert on the oxytetracycline production are diagrammatically represented in *Fig. 2* for five different phosphate concentrations in media containing 1.5 per cent starch. As seen in this diagram, production was the highest when the glycine content varied between 0.3 and 0.5 per cent, irrespective of the amount of phosphate in the medium. In media containing phosphate in low concentrations, oxytetracycline production showed less sensitivity to changes in the glycine content.

Fermentation experiments. Based on the results obtained in the shaken flask experiments, fermentation experiments were carried out; because of the greater volumes involved, they permitted detailed analyses. The following nutrient medium was found to be the best for use in the submerged experiments. Glucose 1 per cent, glycine 0.5 per cent, NaCl 0.3 per cent, palm oil 0.4 per cent, $Co(NO_3)_2$, crystalline 0.0005 per cent. The amount of phosphate optimal for production was 60 μg P/ml, and was added in the form of potassium dihydrophosphate. The presence of palm oil in this medium meant a deviation

from the one used in the shaken flask experiments. An additional 1 or 2 per cent palm oil were added to act as an antifoam agent, since, unlike in the shaken flask experiments, here Tween 80 proved to be unable to inhibit foaming.

In the first 16 hours of the fermentation process there was pure mycelial growth. About 20 per cent of the ultimate total amount of mycelium, less of course the quantity introduced on inoculation, appeared as a fine filamentous texture, during that phase. Fragmentation started immediately after this short fermentation period : mycelial parts reminiscent of bacilli kept detaching themselves and forming spore chains, thereafter to disintegrate. During this fragmentation process the mycelium naturally continued to increase in amount. This increase lasted for another 40 hours, as shown by the decrease in the total nitrogen of the culture fluid (*Fig. 3c*). Attempts to distinguish after the onset of fragmentation individual fermentation phases underlain by characteristic changes in the morphological picture of the fungus, remained unsuccessful. Oxytetracycline production began simultaneously with the fragmentation process and proceeded almost linearly until the 80th hour, whereafter it slowed down. It should be noted in this connection that on stopping aeration the oxytetracycline production decreased, and within a few hours the bacillary forms disappeared. Most of the sugar and the glycine, these principal energy sources of the fermentation, was consumed between the 16th and the 40th hour, provided the medium contained 60 μg P/ml. The pH governed by the equilibrium of these two constituents of the medium showed a sudden drop immediately before the fragmentation process began, but rose to 7 on the actual onset of that process. This readjustment of the pH was accompanied by intensive liberation of ammonia, which reached its peak in the 60th hour when 40 per cent of the nitrogen introduced was present in the form of ammonia. The peak was followed by a gradual decrease, assumedly because the fat which had been added to act as an antifoam agent, became oxidized. The amount of ammonia liberated was found to depend on the glycine content of the medium and, most remarkably, to be largely proportional to it throughout the fermentation process. Thus, the maximum ammonia production attained in or about the 50th hour was 150 μg N/ml in a medium containing 0.2 per cent, and 1000 μg N/ml in one containing 1 per cent, glycine.

As regards the uptake of phosphate by the mycelium, this was of a very high rate in the first ten minutes after inoculation. Its amount depended on the amount of inoculum used ; where this was 10 per cent, the phosphate uptake was 15 μg P/ml. Subsequently, a second and slower process began, that started growing in intensity at the same rate as the metabolic processes of the fungus kept intensifying ; in 24 hours the medium was deplete of phosphate.

Each of the *Figs. 3/a* to *3/g* shows the metabolic changes that had taken place at, above, and below the optimum phosphate level in the media. These diagrams allow the following inferences. (i) Phosphate on a level above

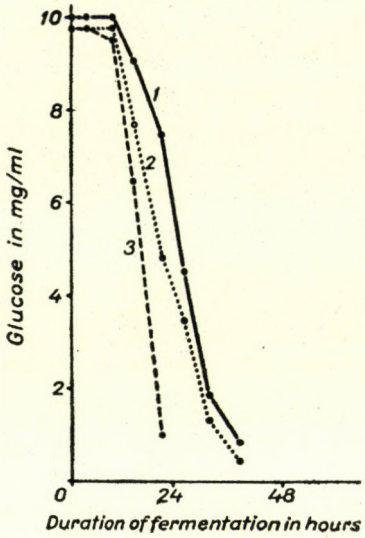

Fig. 3/a

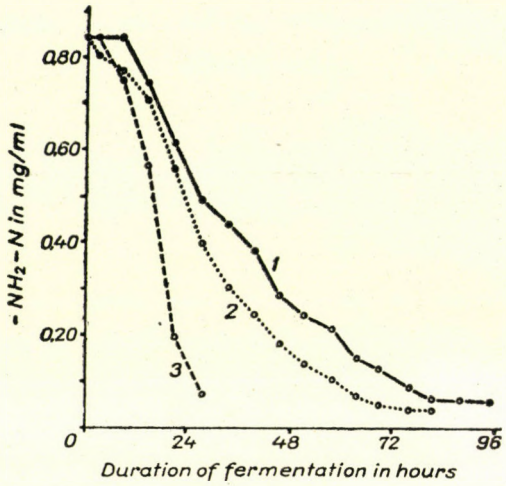

Fig. 3/b

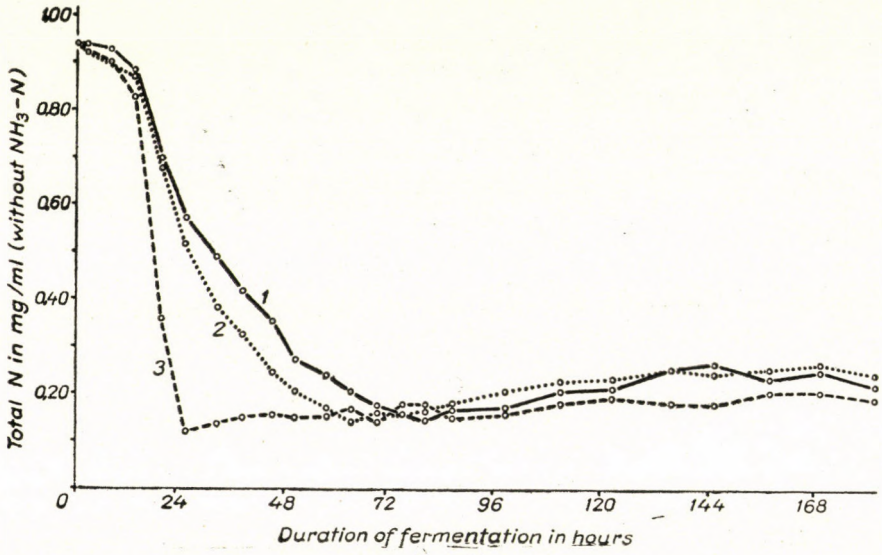

Fig. 3/c

the optimum accelerates, while one below it slows down the consumption of glucose and glycine ; (ii) the decrease of pH taking place immediately before the fragmentation process begins, is independent of the phosphate level ;

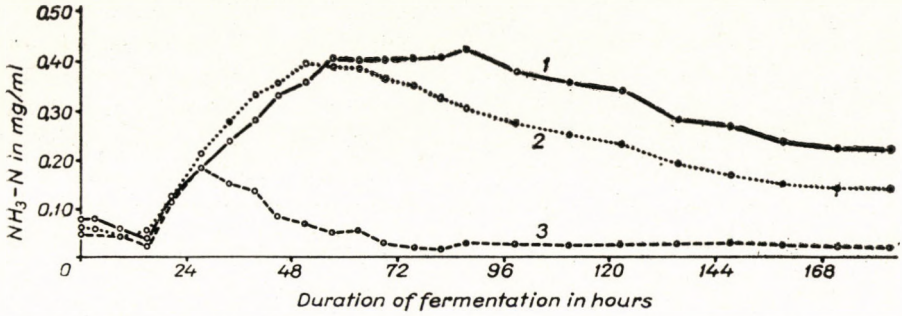

Fig. 3/d

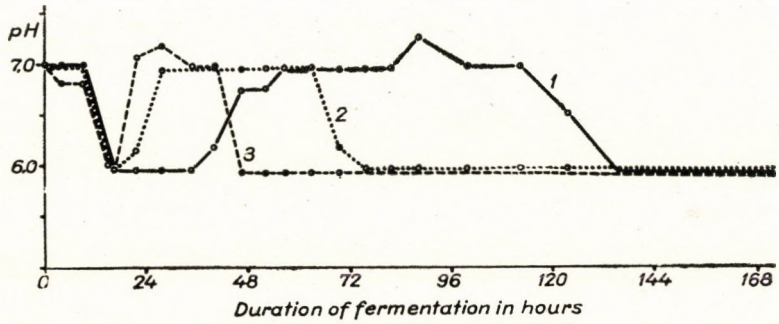

Fig. 3/e

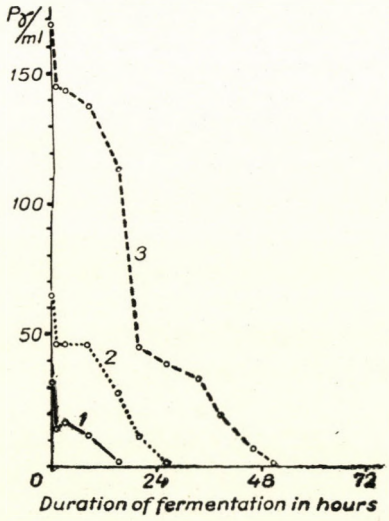

Fig. 3/f

(iii) its rise back to 7 coincides with the beginning of the fragmentation process if the phosphate concentration is above, but occurs much later if it is below the optimum; (iv) a second drop in the pH is the earliest to occur in the presence of 120 μg P/ml, then at the optimum concentration; and lastly at 30 μg P/ml; (v) ammonia starts being liberated at the same time independently of the phosphate level, but the higher rate of liberation seen in the first 24 hours at 120 μg P/ml gives way to a gradual yet rather rapid decrease in the ammonia content; at 30 μg P/ml, ammonia liberation and

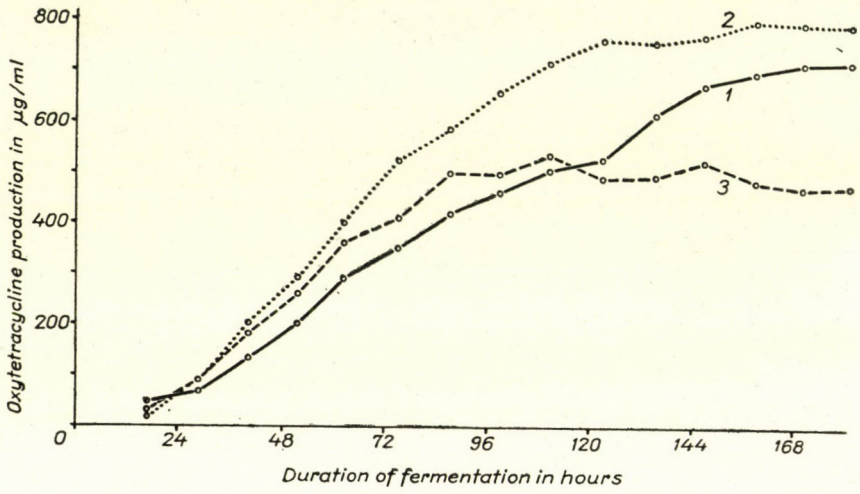

Fig. 3/g

Fig. 3 a — g. Metabolic investigations in fermentation experiments
Explanations: 1. 30 μg P/ml; 2. 60 μg P/ml; 3. 120 μg P/ml

its gradual decrease setting in after 24 hours are appreciably slower; (vi) the three curves for the production of oxytetracycline run essentially parallel, though the one for the optimum comes to lie above the other two; (vii) it is an intriguing phenomenon that the rapid uptake by the mycelium of 15 μg P/ml seen to take place in the first 10 minutes after inoculation, equally occurs at above, and below the optimum phosphate level; afterwards the phosphate disappears from the medium in 50 hours in the case of 120 μg P/ml, and in 15 hours in the case of 30 μg P/ml.

Of the many other experiments carried out to establish what the optimum conditions were, we only wish to mention the one showing that while in the shaken flask experiments the phosphate present in tap water sufficed to ensure a certain rate of growth and oxytetracycline production, it failed to yield either the one or the other in the submerged cultures.

Paper chromatography was used to demonstrate the presence of ninhydrin-positive substances. The numerical data for the results were obtained

by comparing the colour intensity of the spots developed. An amount of 0.1 ml of the filtered solution of each sample was chromatographed in an ethanol: water system. Two-dimensional chromatograms were only run for the purpose of identifying the substances in the concentrated solutions. In the course of these examinations three "marked" spots were observed. On running a 0.1 ml portion of the sample, these corresponded to 5 μg of amino nitrogen. Less than 1 μg of amino nitrogen were contained by the other spots. Of the three marked spots, the first one in the order of migration was found to be hardly running, to be derived from substances of polypeptide or protein nature, and to remain of roughly the same colour intensity throughout the fermentation process. The second spot proved to be due to one of the constituents of the medium, namely glycine ; this disappeared in 80 hours when the phosphate content was 30 μg, in 60 hours when it was 60 μg, and in 45 hours when it was 120 μg/ml. The third marked spot was caused by alanine, an amino acid formed in the course of the fermentation process and present, at the utmost, in an amount corresponding to about 10 per cent of the nitrogen introduced. It appeared, independently of the amount of phosphate, in 16 to 20 hours, *i. e.* after fragmentation and ammonia production had begun, and disappeared, in definite dependence on the increasing phosphate concentrations, in 80, 50, and 24 hours, respectively.

Vitamin B_{12} production was found never to exceed 0.05 μg/ml, *i. e.* between 5 and 10 per cent of the yield obtained in a medium containing soybean meal, starch and oil.

Discussion

A simple synthetic culture medium has been elaborated in which our *Streptomyces rimosus* strain marked BS—21 is capable of producing oxytetracycline at a rate of 500 to 700 μg/ml in shaken flask experiments, and 700 to 800 μg/ml in fermentation cultures. In a medium containing soybean meal, starch and oil, this production amounts to between 2000 and 2200 μg/ml, or roughly to two and a half times the yield obtained in a synthetic medium. However, this cannot be achieved unless the medium is of an equally higher concentration ; that is, of a concentration impossible in a synthetic medium, where the higher glycine concentration is toxic to the fermentation, probably for reasons pertaining to osmotic pressure.

The optimum ratio of nitrogen to energy source has been observed to differ in the shaken flask and the fermentation experiments. The phosphate ions were found to exert a major effect on yield. This has been all the more embarrassing as on the medium containing soybean meal, starch, and oil, and naturally also inorganic and organic phosphate sufficient for the fermentation process, it has proved impossible to enhance production by means of additional, even extremely high, amounts of phosphate. A plausible

explanation of the effect the phosphate ions exert might be that they intensify the metabolic process in *Streptomyces rimosus;* this would be a phenomenon common with microorganisms. Naturally, with the mould's metabolism thus increased, the way in which the medium is used up, might likewise change. This has been pointed out by BORETTI *et al.* [11], and by SHEN [12], who independently of each other, found that while the oxidation of the carbohydrates favourable to production proceeds *via* the hexose monophosphate shunt at a low level of phosphate, it follows the Embden-Mayerhof scheme at higher phosphate levels.

It is difficult to establish the existence of any close correlation between data resulting from minuter metabolic investigations and oxytetracycline synthesis. It is a fact that initial oxytetracycline production coincides with initial fragmentation and the beginning of an increase in the ammonia level and the pH. When this early fragmentation is over, the different morphological forms occur simultaneously and it is not improbable that in some way this phenomenon is after all correlated with production, for in fermentations run at 38° C rapid vegetative growth is followed by a likewise rapid and complete, disintegration, and there is no oxytetracycline production. In our experiments oxytetracycline production was the highest when the phosphate concentration had reached a level where there was substantial liberation of ammonia and synthesis of alanine. When phosphate concentration was increased, these phenomena gradually disappeared and production decreased. Were we to interpret the "balance" between the metabolisms of ammonia and alanine by the extent to which the former is liberated from glycine, or to which a new amino acid is formed, obviously in excess of requirements then this balance would have to be characterized by the fermentation proceeding in the presence of 120 μg P/ml, *i. e.* at a phosphate level which diminishes production. The large quantity of alanine synthesizing at the optimum phosphate concentration might be taken to indicate the possibility of a substantial quantity of active acetate forms, through which the oxytetracycline molecule probably comes into existence.

Summary

In shaken-flask and fermentation experiments, a simple synthetic culture medium has been elaborated for the production of oxytetracycline.

At an unchanging ratio of glycine to carbohydrate, the maximum production depends on the phosphate concentration, which accelerates the depletion of the constituents in the medium.

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THE SPECIFICITY OF THE VACCINIA HAEMAGGLUTINATION-INHIBITION BY THE CEREBROSPINAL FLUID

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Under normal conditions, no antibodies are encountered in the cerebrospinal fluid (CSF). In pathological cases they make their appearance irrespective of whether or not the meninges or the central nervous system are involved in the disease. Their appearance may be due to either of three causes, viz. : — (i) an abnormally high titre of antibodies in the blood, with the blood-cerebrospinal fluid barrier intact ; (ii) disorders in the permeability of the barrier, without an unduly high titre of antibodies in the blood ; (iii) intramural autochthonous formation of antibodies [1]. Numerous data, sometimes contradictory, have been published relative to the antibody content of the CSF, particularly on interferences with permeability in meningoencephalomyelitides (for a detailed review, cf. KAFKA [2]). COLLIER and KRAMER [3] found antihaemagglutinins, in low titres, in the CSF of only one out of their ten patients suffering from acute smallpox or convalescing from the disease, who also displayed neurological and psychiatric symptoms, and whose blood contained antihaemagglutinins. Nor could the authors demonstrate the presence of antibodies in the CSF of revaccinated persons. It has been concluded that neither smallpox nor revaccination have any effect on the permeability of the blood-cerebrospinal fluid barrier. HERRLICH, MAYR, and MUNZ [4] encountered antihaemagglutinins — haemagglutination-inhibiting (HAI) antibodies — in the CSF of some children who had developed encephalitis following vaccination, or were suspect of it.

Our material in which the CSF was examined for HAI antibodies comprised children and adults suffering from various internal or neurological diseases contracted more or less soon after vaccination, and still unvaccinated children of supposedly vaccinated mothers.

Materials and methods

Most of the CSF and blood samples, the latter drawn from the cubital vein, both received in this laboratory within 24 hours, were obtained from the children's department, the rest from the neurological department of the Municipal Hospital of Balassagyarmat. The sera and the centrifuged CSF were inactivated at 56° C for 30 minutes, and kept in the frozen state.

The HAI tests were all performed following TAKÁTSY's [5] micromethod, using the 0.025 ml spiral loop and instillator ; in this laboratory satisfactory results have been achieved with this method for a long time.

The haemagglutinins were obtained from the chorioallantoic membranes of chick embryos infected with vaccinia virus. The chorioallantoic membranes of 10 to 12 day old embryos dropped with the method of BURNET [6] were inoculated with calf lymph vaccinia virus and the infected membranes were harvested 48 to 72 hours later. After 5 to 8 passages, the membranes showing confluent lesions were frozen for storage. When using them, a mixture of several membranes was ground with powdered glass and in 1 ml of phosphate-buffered saline per membrane ; the tissue suspension was then centrifuged for 20 minutes at 1500 r. p. m. The supernatant served for both haemagglutination and haemagglutination-inhibition tests.

Dilutions were made in phosphate-buffered saline.

The vaccinia haemagglutinin-susceptible fowl red blood cells were washed three times with saline and used in 1 per cent suspension.

Haemagglutinin titrations were performed in serial twofold dilutions in three parallels, in a total volume of three drops. Readings were taken after one half hour at room temperature. The highest dilution giving complete haemagglutination was taken as the end point, and regarded as one haemagglutinating unit.

The HAI tests were made with appropriately diluted antigen containing 2 haemagglutinating units. To avoid the non-specific lipid agglutination described by STONE [7], 5 per cent normal rabbit serum, in later experiments a similar amount of horse serum, was added to the virus dilution. The sera and CSF were diluted in twofold series. After addition of the antigen, the slide was covered with a glass plate, and kept at 37° C for an hour. Following the addition of the red blood cell suspension and one half hour standing at room temperature, the reactions were read. The HAI titre was taken as the highest dilution which gave complete inhibition.

Erythrocyte controls, positive and negative serum controls, were arranged for at each titration, as well as virus dilution controls. A standard lyophilized rabbit immune serum served as positive serum control ; its titre was determined for each test separately. Normal rabbit serum was used for negative serum control.

The cell count and protein content of the CSF were determined in the laboratory of the *Balassagyarmat* hospital, the latter with BERGER's [8] sulphosalicylic acid method.

The fractionation of the CSF proteins was carried out as follows. To the CSF an equal amount of concentrated ammonium sulphate solution was added to attain 50 per cent saturation. After 15 minutes, the precipitating globulin fraction was sedimented by centrifugation, and sufficient crystalline ammonium sulphate was added to the supernatant to make saturation complete. After another 15 minutes, centrifugation yielded the albumin fraction. Both protein fractions and the supernatant obtained after complete saturation were then dialysed against distilled water for 24 hours in cellophane sacs.

Results

Part of the CSF was normal, while the rest revealed a variety of pathological changes. The results of HAI tests have been grouped in *Table I* according to whether the CSF had originated from subjects vaccinated less or more than eight weeks before the tests were made, or were still unvaccinated. The total protein values are also incorporated in *Table I.*

We also tested the CSF of ten infants in the 12 to 24 months age group shortly after they had been vaccinated ; the CSF of each of four children was examined on two different occasions. Apart from one low titre (1 : 16), the children's sera were found to contain HAI antibodies in titres a varying between 1 : 64 and 1 : 1024. On the evidence of our earlier findings [9], which agree with the data in the literature, these antibodies in the serum attain their peak concentrations between the end of the second and that of the

Table I

Comparison of vaccinia HAI activity of cerebrospinal fluids of different protein content

Number of CSF tested	Total protein in CSF mg per 100 ml	HAI titre		Vaccination preceded lumbar puncture by
		in CSF 1 :	in serum 1 :	
10	20— 48	∅	16—1024	2 to 8 weeks
2	160— 200	16	128— 256	
42	6— 100	∅	∅— 64	
6	80— 200	4— 8	16	about 6 months
9	160— 720	16— 32	4—128	to 70 years
3	404—1200	64—128	8— 16	
16	16— 64	∅	∅—128	
5	68— 120	4— 8	8— 32	unvaccinated
4	140— 900	16—32	16— 64	
1	580	64	32	

fourth week following the first vaccination ; the titres vary from patient to patient, running up to a maximum of 1 : 1024. Only two of the children's CSF with a high protein content gave a positive inhibition reaction. Both came from infants with meningitis. A few days later the protein content in the CSF of the two patients was found to have become normal ; HA-inhibition was no longer demonstrable in either of them.

The age of the patients vaccinated more then eight weeks before the tests were made, ranged from 2 years to 79 years. With the exception of that of a subject 79 years old, all the sera were found to contain HAI antibodies. No HA inhibition was observed in the CSF containing from 6 to 100 mg protein per 100 ml. To be exact, in one case at a protein level of 80 mg per 100 ml, and in another at 100 mg per 100 ml, inhibition did manifest itself, but only at the lowest titre of 1 : 4. In CSF containing more than 100 mg protein per 100 ml, the inhibition reaction was invariably positive. No relationship could be established to exist between the HAI antibody level of the sera, and the inhibitory titre of the CSF.

The third group in *Table I* presents the test results of 20 allegedly unvaccinated children two of whom were past 5 years of age. (The age limit for the first obligatory vaccination is at present 18 months in Hungary.) The other children were from 1 to 30 months of age. The typical vaccination scars could be detected in none.

The sera of 6 children manifested no inhibition ; the rest had titres between 1 : 4 and 1 : 128. These antibodies were of maternal origin ; they reached the children's organisms in intrauterine life. They remain demonstrable for a long time in the child's blood [10].

As to the CSF, 16 out of 26 showed no inhibition ; the protein levels of these varied from 16 to 64 mg per 100 ml. The others inhibited haemagglutination in titres between 1 : 4 and 1 : 64. These contained protein at levels of 68 to 900 mg per 100 ml. At less than 100 mg levels, inhibition was observed in three cases : in two at 68, and in one at 82 mg protein per 100 ml, in titres from 1 : 4 to 1 : 8. The protein content of the other positive CSF was found

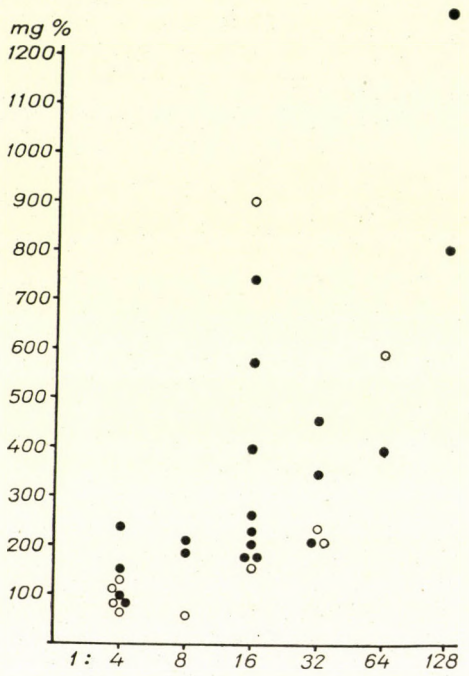

Fig. 1. HAI titres of cerebrospinal fluids with increased protein content (abscissa), and the corresponding values for total protein (ordinate); ● vaccinated, ○ unvaccinated

to have considerably increased. The CSF of a 5½-year old child (the one designated as A. M. in Table II) was tested on 5 different occasions, and gave a positive reaction on each one. No relationship could be detected to exist between the HAI level of the sera and the inhibiting titre of the CSF in this group, either.

On the evidence of the data presented in *Table I* it seems safe to claim that only cerebrospinal fluids exhibit HA inhibition in which an increase in the protein content has taken place. *Fig. 1* shows the values for the total protein in the positive CSF and the corresponding inhibitory titres. It can be seen that widely differing values for protein belonged to the same titre, yet that as a rule higher titres were associated with CSF of higher protein content.

Table II

Changes in the HA-inhibiting capacity and other values of the cerebrospinal fluid of the same patient at different dates during the disease

Age of patient, and diagnosis	Vaccination preceded test by	Date of lumbar puncture	HAI titre in		Total CSF protein mg per 100 ml	Cell count
			serum 1:	CSF 1:		
H. M. 15 months		21 days	256	16	200	480/3
Meningitis sero-purul.	21 days	27 days	1024	0	36	18/3
		46 days*	512	0	28	4/3
P. G. 12 years	about 6	1 day	8	128	1200	2336/3
Meningitis sero-purul.	and 10 years, resp.	10 days**	16	0	100	96/3
M. K. 5 years	about	1 day	16	8	200	
Meningitis sero-purul.	3 years	3 days		8	160	216/3
		5 days		4	140	69/3
		7 days**		4	100	190/3
A. M. 5 1/2 years	unvacci-	1 day	32	64	580	25/3
Meningitis tuberc. recid.	nated	19 days	32	8	84	55/3
		28 days	16	4	112	24/3
		35 days	16	4	120	8/3
		78 days**	8	4	82	2/3

* after vaccination

** after admission to the hospital

To determine which fraction of the CSF proteins possessed HA-inhibiting properties, they were fractionated with ammonium sulphate into two fractions : globulins and albumin.

The results of these investigations revealed that for their capacity to inhibit haemagglutination, the CSF depended on the globulin fraction, since neither in the albumin fraction, nor in the final supernatant was any inhibition demonstrable ; quite like in vaccinia rabbit immune sera, in which, too, HAI antibodies are present in the globulin fractions only [11].

An increase in the protein content of the cerebrospinal fluid is usually associated with one in the cell count, but there is no relationship between cell count and inhibition. In most of the cases manifesting inhibition both the cell count and the protein content were found to be high ; but at low cell counts, too, there was positive inhibition whenever there was at the same time an increase in the protein content. Centrifugation failed to reduce the titre of CSF of a high cell count and positive inhibition.

The HA-inhibiting CSF had originated from patients suffering from various forms of meningitis, poliomyelitis, polyradiculitis, tumour of the

cord, or an eclamptic state. Some patients were examined on several occasions ; the results of such serial tests are illustrated in *Table II.*

Discussion

Our findings tend to show that CSF containing proteins in normal amounts fail to inhibit vaccinia haemagglutination. Of such inhibition, only CSF are capable in which a pathological increase in protein has taken place. Inhibition will set in independently of whether such CSF originates from a patient who has been vaccinated a few weeks or many years, even as many as 70 years, before the test, or from an unvaccinated child between 3 months and 5½ years of age, whose blood can only contain passive HAI antibodies of maternal origin. We were unable to find any closer parallelism between the grade of inhibition and the total protein content of the CSF ; among others, probably for the reason that the sulphosalicylic acid method is not sufficiently sensitive for the reliable quantitative determination of the CSF proteins [12].

Our knowledge of the origin of the proteins in the CSF is incomplete. In diseases involving increased permeability of the blood-cerebrospinal barrier, the serum proteins are supposed to find their way into the CSF ; this means that inasmuch as they are present in the blood, the HA-inhibiting substances likewise have a route by which to enter the cerebrospinal space. Since CSF widely differing in total protein content yield the same inhibitory titre, it is justifiable to assume that the globulin fraction responsible for inhibition can permeate the blood-cerebrospinal barrier not only in a measure corresponding to, but also in one below or above, the proportion in which it is represented in blood protein spectra. Permeability of the demaged blood-cerebrospinal barrier is presently known to change in degrees different for different substances.

We have to question the specificity of the inhibition encountered in the serum and the cerebrospinal fluid of two of the unvaccinated children. The serum of a 2-year-old child was tested on one occasion when it yielded a titre of 1 : 64 ; its CSF gave a 1 : 32 titre. The serum and the CSF of a child 5½ years of age were examined five times, within 78 days and found to be positive each time (patient A. M. in Table II). It is doubtful, indeed, whether these passive antibodies of maternal origin can actually be present in the serum in 1 : 32 to 1 : 64 titres after 2 years and 5½ years, respectively.

Normal serum is also capable of exerting an aspecific effect on the vaccinia virus. Unheated guinea pig serum is known to raise the neutralizing titre of inactivated rabbit immune serum [13]. Normal rabbit serum, too, lowers the infectivity of the vaccinia virus for the rabbit [14]. The serum component exerting the effect is thermolabile, and is destroyed by exposure

to 56° C for 30 minutes ; the HA-inhibiting substance found by us in human serum and CSF is, on the other hand, heat-stable.

Investigations are in progress in this laboratory to obtain a deeper knowledge of the properties inherent in the postulated aspecific inhibition.

Summary

Normal cerebrospinal fluid is unable to exert an effect upon vaccinia haemagglutination. Cerebrospinal fluids containing a pathologically high amount of protein exhibit haemagglutination inhibition, independently of whether they originate from patients who have been vaccinated a few months or many years earlier, or from unvaccinated children in the 3-month to 5 1/2-year age group. No relationship exists between the inhibition titre of the serum and the inhibition observed in the cerebrospinal fluid. It is the globulin fraction that possesses the property of inhibiting haemagglutination. It is assumed that the haemagglutination inhibition observed in some cases was wholly or partly due to the action of non-specific substances.

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CONTRIBUTIONS TO THE PROBLEM OF ANTIBIOTIC-ANTAGONISM

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The increasing number of antibiotics and of microbes resistant to them has focussed attention on the problem of the combined use of antibiotics. Theoretically, the use of combinations is justified

(i) in mixed infections, to widen the range of the antibiotic effect ;
(ii) to prevent the development of resistance ;
(iii) to prevent an eventual change of infection ;
(iv) to ensure a therapeutic effect, which cannot be achieved even by toxic doses of a single antibiotic. (For instance, penicillin + streptomycin = Enterococcus ; streptomycin + aureomycin or chloramphenicol or terramycin = Brucella infections ; etc.)

It should be borne in mind, however, that two or more antibiotics sometimes not only enhance, but also antagonize one another's effect.

JAWETZ [2, 3] grouped the antibiotics as follows. Group I. penicillin, streptomycin, bacitracin, neomycin and polymyxin. Group II. tetracycline group, chloramphenicol, erythromycin, carbomycin, sulfonamides. The members of Group I. are bactericidal, those of Group II. bacteriostatic agents. If an antibiotic is combined with another of the same group, indifferent, additive or synergistic effects may occur, but no antagonism results. When, however, it is combined with a member of the other group, antagonism may be observable in some cases. JAWETZ set up these groups on grounds of experiments *in vitro*, but in some cases he confirmed his results also *in vivo*, in mice.

According to KLEIN *et al.* [4], the effect of an antibiotic combination may vary, depending on the conditions of the experiment and on the strain of bacterium involved : antagonism may result in any combination, beside the synergistic, additive effects.

Somewhat in contrast with JAWETZ, MANTEN [5] claims that the combinations of streptomycin with penicillin, chloramphenicol, the tetracyclines or erythromycin, as well as the combinations of penicillin with chloramphenicol, the tetracyclines or erythromycin have usually antagonistic effects. On the other hand, according to ELLIOT *et al.* [6], antagonism is a rare occurrence.

Similar is the view of MOSONYI [7] and IVÁNOVICS [8], *i.e.* that the problem of combined antibiotic therapy is not yet settled, there being many contradictory data.

The monograph by WALTER and HEILMAYER [9] contains many important statements, of which I should like to point out the following.

Bactericidal synergism is desirable for the widening of the range of effect and for a faster killing of bacteria in severe infections with less sensitive microorganisms. The sensitivity of the pathogenic microorganisms should be within the range of effectiveness of the antibiotics used. Their use should be indicated on the basis of previous laboratory studies. It is naturally unreasonable to prescribe a combination of ineffective preparations if the infection is due to completely resistant microorganisms.

In clinical medicine the occurrence of antagonism is very rare. Penicillin has been reported to having brought no relief in 21 per cent of the pneumococcus meningitis cases tested and penicillin + aureomycin therapy (not intralumbar!) to have failed in 79 per cent of the cases [10]. A combination of aureomycin + streptomycin + sulfonamide is less effective in meningitis due to *Haemophilus influenzae* than the same dose of aureomycin used alone [11]. Apart from these instances, there is neither experimental proof nor clinical observation to confirm the existence of antagonism between antibiotics.

In my own investigations I have been concerned with the problem of antibiotic combinations, with special reference to eventual antagonism or synergism.

Materials and methods

The method employed was based on the one described by PILKINGTON *et al.* [12]. The agar culture medium in a Petri dish was evenly infected with a 1 : 100 dilution of an 18-hour bacterial culture. Subsequently, strips of filter paper impregnated with antibiotic were placed on the media. Two strips soaked in different antibiotic solutions were placed in each Petri dish, crosswise. The results were read after 24 hours of incubation at 37° C.

The antibiotic diffundating from the strip inhibits the growth of bacteria in areas depending in size on the sensitivity of the microorganism. Where the two antibiotics meet, different patterns arise according to the quality of action (neutral, synergistic or antagonistic) they exert on one another. When the effect is neutral, the angle of the growth zone is about 90° (Fig. 1). With a synergistic action, the angle becomes rounded (Fig. 2). When the two antibiotics antagonize each other, the bacteria grow in a sharp angle toward the area in which the two antibiotics meet (Fig. 3). The method is eminently suitable for the qualitative demonstration of antagonism or synergism in the area of bacteriostatic effect.

Results

I have investigated by the above method all the combinations of 6 antibiotics using 30 laboratory bacterium strains. The antibiotics involved were penicillin, streptomycin, chloramphenicol, aureomycin, terramycin and sulfonamide (Superseptyl). The bacteria were ; *Staphylococcus aureus*, 14

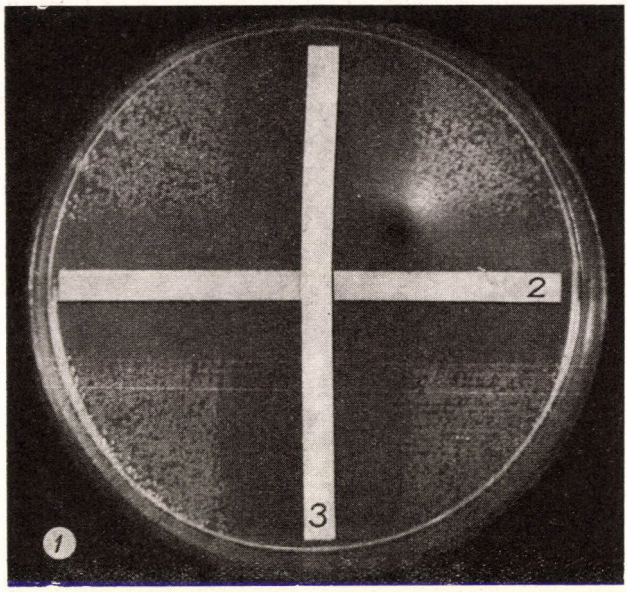

Fig. 1. Neutral antibiotic effect
Bacterium : E. coli (4831)
Antibiotics : 2 = streptomycin, 3 = chloramphenicol

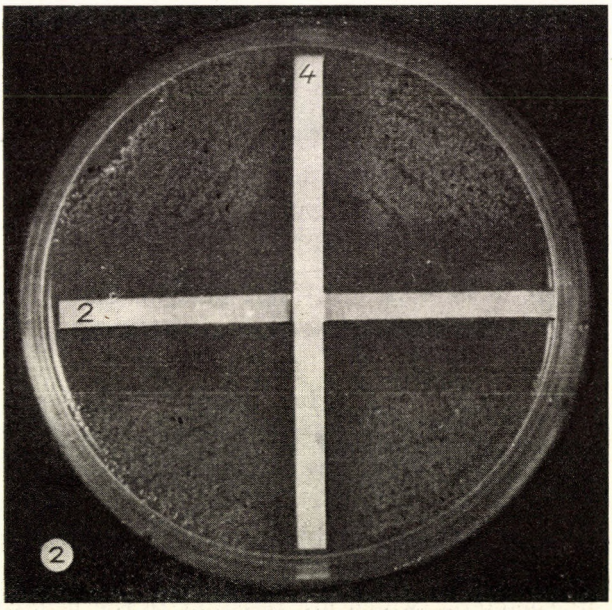

Fig. 2. Synergistic antibiotic effect
Bacterium : E. coli (4831)
Antibiotics : 2 = streptomycin, 4 = aureomycin

strains ; other cocci, 6 ; *E. coli*, 7 ; other bacilli, 3 *(Kl. pneumoniae Friedländer, Ps. pyocyanea, B. subtilis).*

The results are shown in *Fig. 4.*

As to the Staphylococcus strains, the following were observed.

1. Penicillin, combined with the other antibiotics, was neutral or synergistic, and only infrequently antagonistic, notably with the members of the tetracycline group and with chloramphenicol.

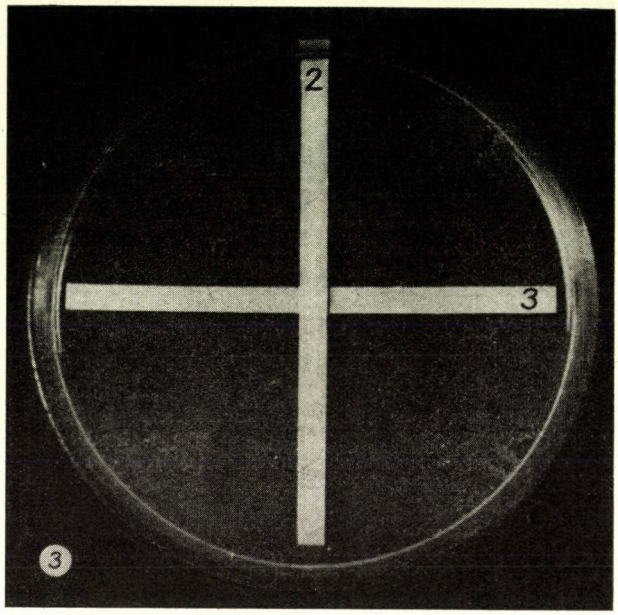

Fig. 3. Antagonistic antibiotic effect
Bacterium : Staphylococcus aureus (127)
Antibiotics : 2 = streptomycin, 3 = chloramphenicol

2. Streptomycin was almost invariably antagonistic to chloramphenicol, very frequently also to the tetracyclines, but never to the sulfonamides, with which it was often synergistic.

3. Chloramphenicol + terramycin + aureomycin + sulfonamide were sometimes neutral, sometimes synergists, but never antagonists.

The results for the other cocci were similar to those obtained for Staphylococcus.

Testing the antibiotic effect with *E. coli*, the following results were obtained.

1. With these bacteria, which are resistant to the usual doses of penicillin, neither synergism nor antagonism was observed in the penicillin combinations.

2. Only with a single strain of E. coli was there noted an antagonism between streptomycin + chloramphenicol. Streptomycin + aureomycin, streptomycin + sulfonamide, chloramphenicol + aureomycin, chloramphenicol + terramycin, aureomycin + terramycin were often synergists.

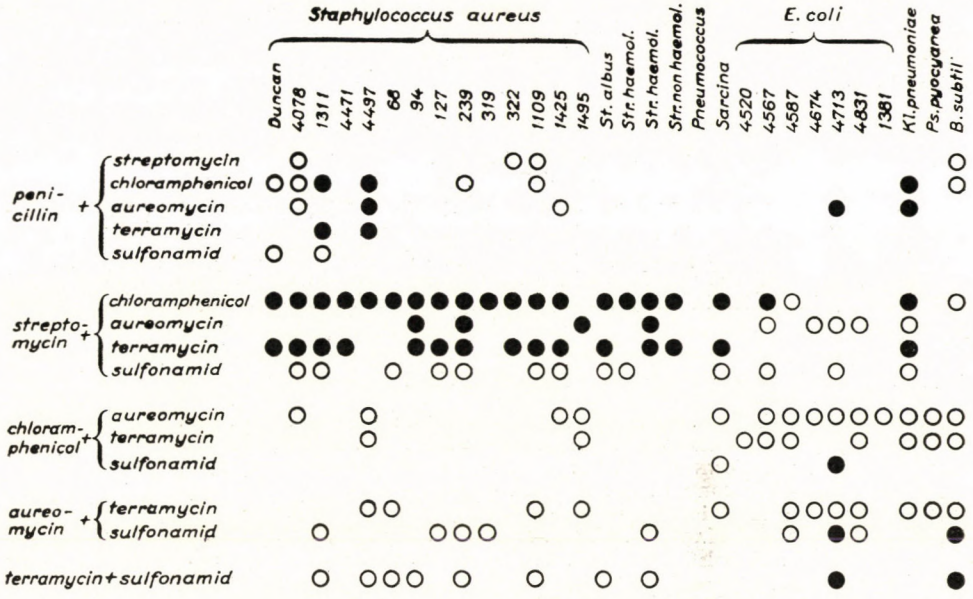

Fig. 4. The results of the experiments
Signs: solid circles = antagonism, empty circles = synergism
The antibiotic pairs not designated by these signs were found to be neutral

Discussion

Our results essentially agree with those by JAWETZ, except for the fact that an antagonism between penicillin and the antibiotics of the second group was seldom noted. The combination of streptomycin particularly with chloramphenicol, often showed antagonism, especially when cocci, in the first place Staphylococci, were the test organism. Different results might be obtained for other species of bacteria, as indicated by the different behaviour of certain *E. coli* strains. It is clear from *Table I* that individual variations may occur even within the same group of bacteria.

It is difficult to draw general conclusions and the best combinations will have to be determined on the basis of careful laboratory studies. It should, however, be always remembered that results *in vitro* cannot be fully applied to the living organism. Extensive investigations are still needed fully to elucidate the problem of antibiotic antagonism and synergism.

In addition, two further points must be emphasized.

1. An antagonism between antibiotics does exist. It is detectable by a suitable technique *in vitro* and it may occur also *in vivo*, under certain conditions. As far as the action on Staphylococcus aureus is concerned, streptomycin antagonizes chloramphenicol and the members of the tetracycline group.

2. A synergism, too, does exist, but it is essential that at least one of the synergistic antibiotics should be effective by itself. No synergism may be expected when none of the antibiotics used is effective on the test microorganism.

Summary

The effect of the combinations of 6 antibiotics (penicillin, streptomycin, chloramphenicol, aureomycin, terramycin and sulfonamide) on 30 different strains of bacteria has been investigated by the agar plate diffusion technique. Streptomycin was found to antagonize chloramphenicol and the members of the tetracycline group in the action on Staphylococci and other cocci. Penicillin seldom antagonized these antibiotics. The E. coli strains varied in behaviour.

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ACQUIRED NATURAL IMMUNITY FOLLOWING RECOVERY FROM KERATOCONJUNCTIVITIS SHIGELLOSA

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Previous studies in this laboratory have indicated [15, 16, 17] that in the sera of experimental animals recovered from keratoconjunctivitis shigellosa there is an increase in the amount of antibodies (agglutinins, mouse protective antibodies), accompanied by a certain degree of clinical immunity.

A number of authors now report that in their hands our procedure yielded results different from ours. The differences can be summarized as follows.

(i) Recovery from the first attack of keratoconjunctivitis shigellosa does not afford immunity; only several (3, 4) subsequent infections are capable of imparting resistance [8, 9].

(ii) Immunity acquired in one eye does not extend to the other [4, 5, 8, 9].

(iii) The eyes of experimental animals recovered from the disease are less susceptible to infection not only with homologous but also with heterologous Shigella cultures; in other words, immunity is non-specific [4, 5, 8, 9].

On these grounds the authors in question deny the suitability of keratoconjunctivitis shigellosa as a model to be used in studies on immunity problems of dysentery [4, 5].

Having repeatedly emphasized that it was just the group of immunological problems that constituted the most important field to which to apply keratoconjunctivitis shigellosa, the need was felt for further investigations to throw more light upon the points disputed.

Materials and methods

Dried cultures. Dried dysentery bacteria were used in all the mouse experiments and some of the conjunctival infections. One-day-old Roux agar cultures were washed with physiological saline, and to eliminate possible agar fragments, the suspensions were passed through a cotton-wool filter and centrifuged at 2000 r. p. m. for one hour. In Petri dishes, the sediment was dried to constant weight at $+4°$ C over calcium chloride in a vacuum exsiccator. The dry bacterial mass of laminar structure was stored in glass-stoppered bottles at $+4°$ C. In one of our preparations, in the second week of storage 300 million, in the 5th to 11th week 100 million, in the 17th to 19th week 50 million, and in the 21st week 20 million Shigella organismus capable of reproduction were counted per one milligram of substance. A measured quantity of the dry preparation suspended in an adequate volume of peptone water was

used as inoculum ; to this, 5 per cent mucin was admixed at the ratio of 1 : 4, when infecting mice. The advantages of using dried Shigellae are obvious : (i) For inoculating mice, no precultivation is required ; an adequate amount of fresh suspension can always be prepared at short notice. (ii) For several days the number of live germs in the unit quantity of dry bacteria remains essentially the same, wherefore it becomes possible to determine the infective dose from the previous germ count. (iii) The virulence for mice of dried Shigella cultures remaining approximately the same for weeks *(Table I)*, the LD_{50} once established can be used throughout an experimental series (but must be controlled repeatedly on every occasion).

Table I

Percentage death rate of mice infected with various doses of dried S. flexneri

Inoculation : 0.5 ml in 5 per cent mucin, intraabdominally
Observation period : 3 days

Storage time in days	Infective dose in μg		
	0.03	0.003	0.0003
18	80	60	53
33	73	47	40
40	87	73	47
47	73	60	27
104	76	64	52

Conjunctival infection. The technique described in earlier papers [15, 16, 17] was employed. Throughout the mathematically analysed tests resuspensions of dried preparations containing the desired germ count per 0.01 ml were used ; with the aid of a graduated pipette, this quantity was instilled in the eyes of guinea pigs, keeping the eyelids of the animals shut for one minute after instillation. Where an infective dose of a higher germ count was to be administered, there instead of instillation a measured quantity of the dry substance was introduced into the conjunctival sac. Due account must always be taken of the fact that the virulence for guinea pigs of dried Shigella organisms is rapidly decreasing during storage.

Mouse protective antibody tests. For passive immunization, 0.1 ml volumes of a mixture of the serum of 15 to 20 guinea pigs secured before conjunctival infection and after recovery from the disease, were used. One hour after the subcutaneous immunization, albino mice weighing 16 to 18 g of our own breed, were infected intraabdominally with 0.5 ml of the mucin-containing suspension prepared in the manner described above. The deaths occurring within the first three days were taken into consideration.

Establishing clinical immunity. In the tests involving mathematical analyses, the control animals agreeing in weight and colour distribution with the conjunctivally infected guinea pigs, were kept in the animal room of the laboratory under the same feeding and nursing conditions as the experimentals. Following recovery, the day after blood samples had been taken for the determination of the mouse protective antibody level, homogeneous groups of both experimental and control animals were infected with gradually increasing doses. The onset and the course of the new attack were recorded ; all the animals were examined daily, and their ocular discharges were cultured on agar and DC.

Evaluation of test results. Most of our test results were analysed by the probit analysis (cf. Tables III, VI, VII, IX, X, XI, XII and XIII).

Results

Clinical immunity of eye recovered from initial keratoconjunctivitis shigellosa, after reinfection of the animal with homologous Shigella strain. A simple way to demonstrate clinical immunity of the recovered eye is to use for the reinfection of the animal after it has recovered from initial keratoconjunctivitis shigellosa, a number of germs approaching that contained in the minimum

infective dose (*e.g.* 1000 million) (Table II). In this manner it can be shown that the dose which after the first conjunctival infection has given rise to the disease in each and every case, fails to act as the MID after the second.

Table II

Clinical immunity of eye recovered from initial keratoconjunctivitis shigellosa and reinfected with homologous Shigella culture

Infective dose : 10^9 germs

Conjunctival infection	In number of cases after	
	first	second
	conjunctival infection	
was followed by keratoconjunctivitis shigellosa	270	98
was not followed by keratoconjunctivitis shigellosa....	—	22
Total	270	120

However, this simple method to demonstrate clinical immunity cannot be applied using an arbitrary germ count. If what has been chosen to be the infective dose, is a weak one, not even the controls will attract the disease ; if on the other hand it is very massive, it may elicit the disease form every one of the eyes that had gone through an initial attack.

Both eyes of ten guinea pigs were infected with *S. flexneri 3*, and, after recovery (on the 30th day following the conjunctival infection), were re-infected by introducing to behind the eyelids a 4-mm loopful of a centrifuged agar culture of the same strain. Keratoconjunctivitis shigellosa developed in all the eyes without exception.

The apparently best way to demonstrate clinical immunity is to infect homogeneous groups of recovered and control animals with regularly increasing doses. In a group of 60 guinea pigs, one eye of each animal was infected with a 2-mm loopful of a 1-day-old agar culture of *S. flexneri 3*. Another group of 60 animals served as the control. After the experimental animals had recovered (in the 7th week following conjunctival infection), their eyes, both the recovered and the spared one, as well as one eye of the controls, were infected with rising doses of our dried *S. flexneri 3* preparation. The results of this experiment, summarized in Table III, furnish evidence that recovery from the first attack of keratoconjunctivitis shigellosa affords very marked clinical immunity.

All estimates of the degree of resistance must be deficient if they fail to take account of a phenomenon analogous to the post-dysenteric state in man [19], which is this : in addition to an increase in the tissue resistance, the alteration in their readiness to react after recovery from keratoconjunc-

Table III

General and tissue immunity after recovery from keratoconjunctivitis shigellosa

Conjunctival infection with *S. flexneri 3* culture
Reinfection with dried *S. flexneri 3* strain

Infective dose mg	Number of eyes left intact in groups of 20 eyes each		
	untreated	conjunctivally infected in the	
		opposite eye	same eye
0.04	10	—	—
0.08	7	12	—
0.16	1	6	—
0.32	—	5	—
1.28	—	—	10
2.56	—	—	2
5.12	—	—	1

Relative resistance

Group of animals	Relative resistance	Fiducial limits
Untreated	1	—
Conjunctivally infected on the opposite eye	2.68	1.39—6.15
Conjunctivally infected on the same eye	24.4	13.7—38.6
Conjunctivally infected on the opposite eye	1	—
Conjunctivally infected on the same eye	8.63	3.58—14.9

tivitis shigellosa, reveals itself in that the tissues respond with inflammatory processes to stimuli which prior to the conjunctival infection have failed to be of any effect upon the eye. Various *E. coli* cultures and killed-off Shigellae have likewise been observed to elicit mild and early subsiding lesions in the period following recovery from keratoconjunctivitis shigellosa. Thus we should regard the post-infectious state as one of the possible consequences of reinfection. In other words, the process called mild conjunctivitis or abortive keratoconjunctivitis shigellosa may be the effect of either of two causes. In some cases, the organism's increased resistance prevents reinfections from inducing inflammatory reactions of more than moderate extension and intensity; in others, the enhanced readiness to react enables the commonly ineffective stimulus to bring forth mild inflammations. Further investigations are needed to shed light on the differing development of the abortive lesions; bacteriological tests were employed for their practical differentiation. The mild lesions are of non-infectious origin, if from the conjunctival exudate Shigella colonies can be grown in small numbers only and for a short time

(not more than a day or two). They are of infectious origin, on the other hand, if daily bacteriological tests continue to show for a considerable period of time a more or less marked multiplication of the pathogenic agent and allow it to be isolated from the exudate.

Table IV

Immunity of eye recovered from a first attack of keratoconjunctivitis shigellosa
Infective dose : 10^7 to 10^9 germs

Effect of conjunctival infection	After first infection		After second infection	
	number of eyes	percentage	number of eyes	percentage
Eye remained intact	474	50.1	95	32.4
Mild conjunctivitis	—	—	13	4.5
Abortive keratoconj. shig.	—	—	147	50.0
Typical or chronic keratoconj. shig.	472	49.9	28	9.2
Atypical keratoconj. shig.	—	—	10	3.5
Shigella sepsis	—	—	1	0.4
Total	946	100.0	294	100.0

It appeared interesting to study acquired natural immunity from the epidemiological point of view, that is, to find out in what the effects of the first and the subsequent conjunctival infections differ from one another, independently of the size of the infective dose and the type of strain. On the evidence of our results shown in *Table IV*, the mere fact that an eye had gone through the disease justifies no expectation of a lowered morbidity in it : more eyes were found to have remained intact after the first than after the subsequent infections. Consequent upon the resistance imparted by the first attack, after the second infection the typical cases were observed markedly to decrease in number, and the fairly uniform syndrome of the first disease to cede its place to a wide variety of clinical pictures, including many mild abortive forms.

In addition to local signs, clinical immunity also revealed itself in that a number of general symptoms, such as pyrexia, anorexia, drowsiness, loss of weight, were of a much milder character, and later even altogether failed to appear, after reinfections. Other moments evidencing immunity have been pointed out in our earlier communications, *e.g.* the fact that subjected to the action of antibacterial preparations, reinfections abate earlier and with greater certainty than do first attacks [17a].

Clinical immunity of the recovered eye after reinfection with heterologous Shigella strain. Since the eye recovered from keratoconjunctivitis shigellosa and reinfected with a Shigella strain different in type or group from the one

Table V

Homologous and heterologous clinical immunity of the recovered eye
Infective dose : 10^8 germs

Effect of reinfection	Number of eyes in which the pathogenic agents used in the first and second conjunctival infections were of			
	the same strain	the same type	the same group	a different group
Eye remained intact	15	12	10	7
Mild conjunctivitis	5	3	2	3
Abortive keratoconjunct. shigellosa	21	25	20	23
Typical or chronic keratoconjunct. shig.	5	7	13	12
Atypical keratoconjunct. shigellosa	3	3	5	5
Shigella sepsis	1	—	—	—
Total	50	50	50	50

used in the first conjunctival infection can be shown to be clinically resistant, the clinical immunity is obviously neither type- nor species-specific *(Table V)*.

While our observations clearly show a definite, though in intensity moderate, local resistance, they can give no information concerning general immunity. If such information is to be obtained, only one of the animals' eyes should be infected and the opposite eye used as an indicator by which to show whether general immunity has or has not developed after the disease had run its course in the infected eye.

Clinical immunity of the spared eye after reinfection with homologous Shigella strain. In the experimental series described above (cf. *Table III*) it was shown that the eye that had been left intact on the occasion of the first conjunctival infection displayed a degree of immunity, which though

Table VI

Clinical immunity of one eye following reinfection of the animal with homologous Shigella culture after recovery of the opposite eye from keratoconjunctivitis shigellosa
Infective dose : 10^7 to 10^9 germs

Effect of reinfection	Number of cases	Percentage
Eye remained intact	56	35
Mild conjunctivitis	2	1
Abortive keratoconjunct. shigellosa	9	6
Typical or chronic keratoconjunct. shig.	82	52
Atypical keratoconjunct. shig.	10	6
Total	159	100

it was lesser than that in the recovered eye, was quite definite in relation to the resistance in the control animals.

Of immunity, in addition to quantitative proofs, qualitative proof, too, can be provided. It has already be shown that in all susceptible animals keratoconjunctivitis shigellosa assumes the typical or chronic form, yet if after recovery from the infection in one eye the animal is reinfected, the originally spared eye will display the same wide variety of disease forms as were observed in the recovered eye *(Table VI)*.

Clinical immunity of the spared eye after reinfection with heterologous Shigella strain. Effects essentially different from those described in the foregoing paragraph can be observed if after recovery from the infection of one eye we infect the opposite eye with a Shigella strain from a different group. In fact, under such conditions no immunity at all can be observed, all successful infections invariably resulting in typical or chronic keratoconjunctivitis shigellosa (*Table VII*).

Table VII

General heterologous immunity following recovery from keratoconjunctivitis shigellosa

First conjunctival infection with S. flexneri 3 culture
Conjunctival infection in spared eye with S. sonnei strain

a) Number of animals in groups of 14 eyes each that contracted the disease

Animal group	Infective dose in mg		
	0.2	0.4	0.8
Control animals	1	2	7
Animals conjunctivally infected in the opposite eye.....	4	5	8

b) Relative susceptibility

Animal group	Relative susceptibility	Fiducial limits
Control animals	1	—
Animals conjunctivally infected in the opposite eye.....	2.16	0.93—20.9

On the evidence of our results it would appear that the qualitative and quantitative differences between local and general immunity reveal themselves, amongst others, in that local immunity is non-specific and of marked intensity while general immunity is species-specific and less marked.

Agglutinins. In keratoconjunctivitis shigellosa, agglutinins make their appearance in the blood serum of the animal [4, 5, 8, 9, 15, 16, 17]. Wishing to find out to what extent they may be regarded as specific, the agglutinating

Table VIII

Agglutinating power of guinea pig sera following conjunctival infection with S. flexneri 3 type culture

Number of sera tested: 22
Method employed: slide test
Serum dilution: 1 : 10
Date of blood sampling: 14 to 31 days after conjunctival infection

Strain used in the test			Number of agglutinating sera
type	antigenic structure, according to RAUSS		
S. dys. 1	—		—
S. dys. 2	—		—
S. sonnei	—		—
S. flexn. 1. a	I	—	—
S. flexn. 1. b	*I. III*	A	20
S. flexn. 2. a	VIII_2 (VIII_3)	D	—
S. flexn. 2. b	VII	D	—
S. flexn. 3	*III. VII* (*VIII_3*)	H	20
S. flexn. 4. a sp.	IV_1 IV_2 (VIII_3)	K	—
S. flexn. 4. a group	IV_1 IV_2 (VIII_3)	—	—
S. flexn. 4. b	*IV_1 IV_2 III*	—	18
S. flexn. 5	VII	G	—
S. flexn. 6	VI	?	—
S. flexn. X var.	*VII*	—	6
S. flexn. Y var.	*VIII_3*	—	3

Table IX

Protective capacity of guinea pig serum against a homologous strain, after recovery from keratoconjunctivitis shigellosa, I

Conjunctival infection: with S. flexneri 3 culture

Method employed (in mouse-protection test): subcutaneous immunization followed one hour later by intraabdominal infection with uniform doses

Infective dose (in mouse-protection test): 0.08 μg of dried S. flexneri 3 in 0.5 ml volume (in 5% mucin)

Observation period: 3 days

a) Number of survivals from groups of 25 mice each

Serum dose, in ml	Rabbit serum agglutinating *S. flexneri 3*	Guinea pig serum	
		after recovery from keratoconj. shig.	untreated
0.1	—	—	13
0.025	—	17	10
0.00625	24	10	6
0.0015625	19	9	—
0.000390615	12	—	—

b) Percentage protective capacity

Serum	Relative potency	Fiducial limits
Rabbit serum agglutinating *S. flexneri 3*	100	—
Guinea pig sera:		
after recovery from keratoconj. shigellosa	4.2	0.61—12.6
untreated	0.61	0.14—1.50

Table X

Protective capacity of guinea pig serum against a homologous strain, after recovery from keratoconjunctivitis shigellosa, II

Conjunctival infection: with S. flexneri 3 culture

Method employed (in mouse-protection test): subcutaneous immunization followed one hour later by intraabdominal infection with uniform doses

Infective dose (in mouse-protection test): 0.08 μg of dried S. flexneri 3 in 0.5 ml volume (in 5% mucin)

Observation period: 3 days

a) Number of survivals from groups of 25 mice each

Serum dose, in ml	First experiment (January 11)		Second experiment (Febr.1)	
	Rabbit serum agglutinating *S. flexneri*	Guinea pig serum after recovery from keratoc. shig.	Guinea pig serum after recovery from keratoc. shig.	untreated
0.1	—	—	—	12
0.025	—	21	15	11
0.00625	24	20	11	4
0.0015625	23	16	6	—
0.000390625 ...	16	—	—	—

b) Percentage protective capacity

Serum	Relative potency	Fiducial limits
Rabbit serum agglutinating *S. flexneri* 3	100	—
Guinea pig serum after recovery from keratoconj. shig.	10.9	1.3—41.2
Guinea pig sera:		
after recovery from keratoconj. shig.	100	—
untreated	14.5	2.2—62.9

capacity of 22 blood serum samples drawn between the 20th and the 31st day after conjunctival infection with *S. flexneri 3* type strain, was determined, using live type strains, and employing a slide test (*Table VIII*). In a 1 : 10 dilution, most of the sampled sera agglutinated the *S. flexneri* 1b,

Table XI

Protective capacity of guinea pig serum against a homologous strain, after recovery from keratoconjunctivitis shigellosa, III

Conjunctival infection : with *S. sonnei* culture

Method employed (in mouse-protection test) : subcutaneous immunization followed an hour later by intraabdominal infection with the same doses

Infective dose (in mouse-protection test) : 0.008 μg of dried S. sonnei in 0.5 ml volume (in 5% mucin)

Observation period : 3 days

a) Number of survivals from groups of 25 mice each

Serum dose, in ml	Rabbit serum agglutinating *S. sonnei* "S"	Guinea pig serum	
		after recovery from keratoconj. shig.	untreated
0.1	—	—	3
0.025	—	9	2
0.00625	21	5	1
0.0015625	9	1	—
0.000390625	7	—	—

b) Percentage protective capacity

Serum	Relative potency	Fiducial limits
Rabbit serum agglutinating *S. sonnei* "S"	100	—
Guinea pig sera :		
after recovery from keratoconj. shig.	3.8	0.9—9.5
untreated	0.2	0.01—0.8

3, and 4 type strains, and some even the X or Y variants, *i.e.* the strains which, according to RAUSS [14], contain the group antigens III and VII, and sometimes VIII_3. Together these factors are encountered in the *S. flexneri* 3 type strain ; accordingly, after conjunctivally infecting guinea pigs with it, in their sera agglutinin fractions will be demonstrable to which the corresponding antigens are present in the strain inducing the infection ; in other words, the agglutinins are specific.

Specificity can also be illustrated by the absorption technique. One of our *S. flexneri* 3 type guinea pig sera, which in a 1 : 10 dilution agglutinated *S. flexneri* 1b, 3, 4b, X and Y variants, was absorbed with the X variant, using the method recommended by RAUSS [14]. The absorbed serum was found to agglutinate only 1b, 3 and 4 type strains, *i.e.*, those containing the group III antigens. On absorbing our serum in addition to the X variant with

Table XII

Protective capacity of guinea pig serum against heterologous strain, after recovery from keratoconjunctivitis shigellosa, I

Serum : pooled guinea pig serum obtained during convalescence following subsidence of keratoconjunctivitis shigellosa induced by means of infection with *S. flexneri 3*

Control serum: pooled guinea pig serum obtained during convalescence following subsidence of keratoconjunctivitis shigellosa induced by means of infection with *S. sonnei*

Method employed (in mouse-protection test): gradual subcutaneous immunization followed one hour later by intraabdominal infection with uniform doses

Infective dose: 0.0008 μg of dried *S. sonnei* in 0.5 ml volume (in 5% mucin)

Observation period: 3 days

a) Number of survivals from groups of 25 mice each

Serum dose, in ml	Guinea pig serum		
	after recovery from keratoconjunctivitis shigellosa induced by		untreated
	S. sonnei	*S. flexneri 3*	
0.1	—	4	3
0.025	10	2	2
0.00625	6	1	1
0.0015625	4	—	—

b) Percentage protective capacity

Serum	Relative potency	Fiducial limits
Homologous guinea pig serum	100	—
Heterologous guinea pig serum.....	1.7	$8.9 \cdot 10^{-6}$ —11.2
Untreated guinea pig serum	0.95	$3.3 \cdot 10^{-15}$—8.5

S. flexneri 1b type culture, H type serum was obtained, which agglutinated none but the *S. flexneri* 3 type strain.

Slide tests were carried out also after infections with *S. sonnei*. They either failed to demonstrate the presence of agglutinins in guinea pig sera or agglutinated (in a dilution of 1 : 5) only the homologous cultures, even without absorption.

The results of our investigations thus permit the conclusion that agglutinins forming in the sera of guinea pigs during keratoconjunctivitis shigellosa are type-specific.

Mouse-protective antibodies. The passive immunizing capacity of serum has been shown to increase during keratoconjunctivitis shigellosa [15, 17].

Table XIII

Protective capacity of guinea pig serum against heterologous strain, after recovery from keratoconjunctivitis shigellosa, II

Serum: pooled guinea pig serum obtained during convalescence following subsidence of keratoconjunctivitis shigellosa induced by means of infection with *S. sonnei*

Control serum: S. sonnei "S" agglutinating rabbit serum

Method employed (in mouse-protection test): gradual subcutaneous immunization followed an hour later by intraabdominal infection with 0.08 µg of dried *S. flexneri 3* in 0.5 ml volume (in 5% mucin)

Observation period: 3 days

a) Number of survivals from groups of 25 mice each

Serum dose, in ml	Rabbit serum agglutinating *S. sonnei* "S"	Guinea pig serum	
		after recovery from keratoconjunctivitis shig. induced by *S. sonnei*	untreated
0.1	10	10	11
0.025	9	9	9
0.00625	4	7	2

b) Protective capacity

Serum	Relative potency	Fiducial limits
Heterologous agglutinating rabbit serum	1	—
Guinea pig sera :		
after recovery from keratoconj. shig.	1.85	0.17—109.4
untreated	1.12	0.23— 5.98

Two series of experiments were performed to investigate into the specificity of the mouse-protective antibodies.

The protective capacity of pooled sera against a homologous strain was tested after conjunctival infection with *S. flexneri 3* and *S. sonnei* cultures, respectively, and was found to be markedly specific *(Tables IX, X and XI).*

The second experimental series aimed at establishing whether the same pooled sera were capable of affording protection against a heterologous culture as well ; the results were negative, no immunizing capacity could be observed *(Tables XII and XIII).*

The body of evidence makes it thus safe to state that the mouse-protective antibodies found in the serum during keratoconjunctivitis shigellosa are specific, at least for the group.

Discussion

According to our findings, while recovery from keratoconjunctivitis shigellosa does impart increased resistance to both the recovered and the opposite eye, in most cases it is unable altogether to protect against the effects of a subsequent infection, but instead reduces the ensuing process in intensity and duration. The immunity of the recovered eye appears to be non-specific ; that of the opposite eye is of a lesser degree, and follows the law of specificity. The agglutinins and mouse-protecting antibodies present in the serum during keratoconjunctivitis shigellosa are specific. The specific clinical immunity of the eye left intact on the occasion of the first conjunctival infection and the specificity of the humoral immunity justify the assumption that it is chiefly the humoral antibodies that underlie the resistance in the spared eye, while in that of the recovered eye tissue immunity likewise plays a significant part.

Our findings had to be analysed in the first line for an answer to the question to what extent the natural immunities acquired in keratoconjunctivitis shigellosa and human dysentery agree in character. Clinical, epidemiological, bacteriological and immunological tests alike produce evidence that dysentery is followed by immunity, although the latter is of low grade only [6, 11, 14, 18]. WALTNER [19] states that dysentery in its early stage runs the course of a more or less severe general infection, while later it becomes more and more local in character. Although in this connection we cannot speak, in the usual sense, of an immunity acquired during the disease, there is little doubt that the capacity of the organism to react has been favourably altered and this may be regarded as a kind of immunity. However, apart from the advantage attaching to it, the alteration in the reactive capacity can also mean a disadvantage, in view of the so-called post-dysenteric state. This same characterization fully applies to the immunity that follows keratoconjunctivitis shigellosa.

In view of the frequent incidence of agglutinins in the normal subject, it is not easy to form a correct opinion of their significance in dysentery. Certain, however, is that the blood of only a number of the bacteriologically verified patients agglutinates the pathogenic agent, and that the agglutinins are specific [14]. The mouse-protective antibodies, too, have been established to possess specificity [2, 12, 13].

While in the aspects discussed above there is complete analogy between the acquired natural immunity after dysentery and that following keratoconjunctivitis shigellosa, apparent differences prevail in respect of the specificity of the clinical immunity, for clinical immunity after dysentery is as a rule regarded as type-specific. However, the relatively low grade of resistance, the post-dysenteric state, chronic dysentery, and the tendency to recurrence

render it a difficult task, and one which involves many sources of error, to measure clinical resistance. This makes it understandable why resistance and humoral immunity are rather consistently held to be the same thing ; *i.e.*, why the principle of the specificity of immunity is by most authors derived from the specificity of the antigen-antibody reactions. Lately, observations have been accumulating which make one to doubt the absolute validity of this principle. In the Flexner group, among the types combined with the dominant group antigens, cross immunity prevails which approaches type immunity ; with Flexner vaccine containing four types a high degree of resistance to all types in the group can be secured [14]. If during the process of immunizing them, caffeine or bromide is administered to mice, both homologous and heterologous immunity can be observed to develop, though the latter to a lessen degree [7]. Clinical and epidemiological observations show that in man, in a considerable proportion of the cases, infection with a single serological type affords immunity also against heterologous types : after reinfection, the symptoms of the disease either fail to present themselves or else they remain mild [3, 10].

On the basis of our above results we full justified in establishing the existence of an analogy between natural immunity acquired through recovery from dysentery and through recovery from keratoconjunctivitis shigellosa, and thereby to verify the model's suitability for the study of dysenteric immunity ; in addition, it permits an attempt to reconcile the many, in part contradictory, observations and concepts of immunity. On the evidence of our findings, immunity developing after recovery from Shigella infections rests upon a twofold material basis : humoral as well as tissue immunity each possesses a significance of its own. Humoral immunity is general, involving the entire organism ; it is type-specific, and of a lower grade than tissue immunity, which is non-specific and restricted to the site of the inflammatory focus. The divergent opinions seem to arise from neglecting or ignoring one or the other of the components that differ in character and intensity. One reason why type-specific humoral immunity cannot in itself be regarded as an indicator of immunity is that non-specific tissue immunity is of greater intensity. On the other hand, the greater intensity of tissue immunity cannot possibly supply the underlying motive to an opinion which denies the significance of humoral immunity. In our view, the natural immunity acquired by those who have recovered from dysentery, is not interpreted realistically unless both of its components are duly taken into account.

In conclusion, we should like to revert to the points in which the observations, and consequently the inferences, of the authors mentioned in the introduction differ from ours [4, 5, 8, 9]. MANOLOV's [8, 9] statement that recovery from a first attack of keratoconjunctivitis shigellosa fails to give rise to immunity is due, we venture to suggest, to his use of an enormous

infective dose, 10 000 million germs. As has been shown by us, there undoubtedly exists a correlation between the appearance of immunity and the quantity of the challenging dose ; and we readily agree with the said author in that where the infection is very massive, the ensuing immunity will prove to be insufficient. To be able to provide evidence of clinical immunity, smaller and gradually increasing doses require to be applied under appropriate experimental conditions. GEKKER *et al.* [4, 5], too, seem to have paid insufficient attention to this essential point. In performing the conjunctival infection, the Soviet authors, assumedly, used a lesser quantity of germs (a loopful of agar culture) than did the Bulgarian workers — and were therefore able to observe clinical immunity following recovery from the first keratoconjunctivitis shigellosa — but they applied experimental conditions under which it is not possible for the eye spared in the first infection to manifest its immunity.

Summary

1. No sound analysis of the phenomena that accompany the natural immunity acquired after recovery from keratoconjunctivitis shigellosa is possible unless the relative experiments are carried out under the appropriate conditions (homogeneous experimental and control groups, regularly increasing infective doses).

2. Recovery from the first attack of keratoconjunctivitis shigellosa imparts clinical and humoral immunity alike, but this immunity is in most cases of a degree too low to protect from massive infection ; it can only mitigate the symptoms of the disease and shorten their duration.

3. Clinical immunity of a higher grade develops in the recovered than in the spared eye. Immunity in reference to the first is non-specific ; to the second, specific.

4. Immunity rests upon a twofold material basis : humoral and tissue immunity each possess a significance of their own. Humoral immunity is of lesser intensity, is general, and type-specific, while tissue immunity is of a higher grade, local, and non-specific.

5. The natural immunity acquired through recovery from keratoconjunctivitis shigellosa being in its essential features the same as that following recovery from human dysentery, keratoconjunctivitis shigellosa represents a model suitable for the study of the theoretical and practical problems of dysentery.

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THE 1957 POLIOMYELITIS EPIDEMIC IN HUNGARY

I. AN EPIDEMIOLOGICAL STUDY

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(Received May 13, 1958)

The 1957 incidence of poliomyelitis in Hungary greatly exceeded the figures for the previous epidemic years. Prior to World War II the most extensive epidemic with 1138 reported cases (13.1 per 100 000) had occurred in 1931 (Table I), and epidemics yielding attack rates between 5 per 100 000 and 8 per 100 000 were experienced in 1936 as well as in the years from 1939 to 1942. After World War II, 1947 was the first epidemic year with an attack rate nearly attaining that in 1931. A smaller epidemic with a total of 500 notified cases occurred in 1952, and all the four years from 1954 to 1957 can be considered as epidemic years. The 1957 record of 2334 cases (23.8 per 100 000) was about double the highest incidence ever experienced in Hungary. Thus, the 5 to 6 year periodicity more or less characteristic of poliomyelitis in Hungary before 1952, has not been observed in the most recent years.

The geographical distribution of poliomyelitis cases in 1957, like in the earlier epidemic years, was somewhat irregular. As seen in *Fig. 1*, the

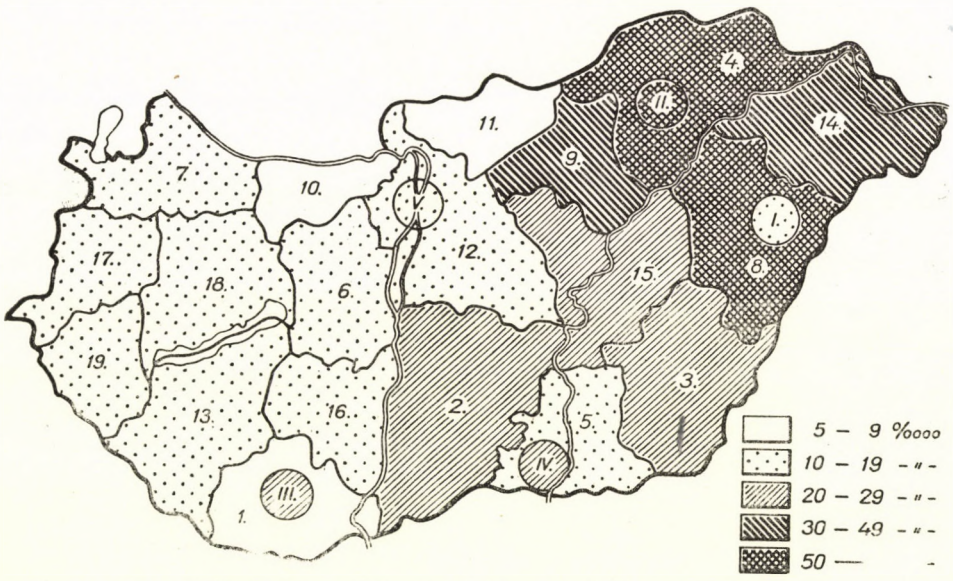

Fig. 1. Notified cases of poliomyelitis per 100 000 population 1957
Note. The names of the counties 1—19 and towns I—V are given in Table I

Table I

Notified cases and deaths from poliomyelitis in Hungary 1931—1957

Year	Cases	Attack rate, per 100 000	Deaths	Death-rate, per 100 000	Case fatality, per cent
1931.....	1138	13.1	156	1.8	13.7
1932.....	392	4.5	71	0.8	18.1
1933.....	112	1.3	17	0.2	15.2
1934.....	125	1.4	24	0.3	19.2
1935.....	267	3.0	29	0.3	10.9
1936.....	470	5.2	57	0.6	12.1
1937.....	127	1.4	26	0.3	20.5
1938.....	302	3.3	27	0.3	8.9
1939.....	572	6.2	48	0.5	8.4
1940.....	706	7.6	74	0.8	10.5
1941.....	682	7.3	76	0.8	11.1
1942.....	485	5.2	64	0.7	13.2
1943.....	328	3.5	45	0.5	13.7
1944.....	.	.	.	.	.
1945.....	280	3.1	32	0.4	11.4
1946.....	227	2.5	22	0.2	9.7
1947.....	1035	11.4	84	0.9	8.1
1948.....	314	3.4	22	0.2	7.0
1949.....	298	3.2	26	0.3	8.7
1950.....	257	2.8	22	0.2	8.6
1951.....	310	3.3	19	0.2	6.1
1952.....	500	5.3	29	0.3	5.8
1953.....	313	3.3	11	0.1	3.5
1954.....	1176	12.1	64	0.7	5.4
1955.....	617	6.3	18	0.2	2.9
1956.....	1100	11.2	77	0.8	7.0
1957.....	2334	23.8	143	1.5	6.1

highest attack rates were recorded in County Hajdu-Bihar (No. 8 in *Fig. 1*), and the counties Borsod-Abaúj-Zemplén (4), Heves (9) and Szabolcs-Szatmár (14), as well as in the town of Miskolc (II). The area east of the river Tisza suffered most and west of the river Danube the incidence was significantly lower than between the two rivers.

Table II shows the geographical distribution of poliomyelitis for the last 5 years.

In 1953, the attack rate for Miskolc (II) rose high above the relatively low rate for the rest of the country. In the epidemic year 1954, the counties Veszprém (18), Győr-Sopron (7) and Szabolcs-Szatmár (14) were most affected. In 1956, Szabolcs-Szatmár displayed a remarkably high attack rate and, to a lesser extent, that for Budapest also exceeded the general rate for that year. In 1957, the disease was most prevalent in the areas where during the

Table II

Notified cases of poliomyelitis per 100 000 population for the counties and towns of Hungary, 1953—1957

County	1953	1954	1955	1956	1957
1. Baranya	0.7	7.5	7.0	3.1	8.7
2. Bács-Kiskun	6.1	7.5	6.8	10.5	23.3
3. Békés	2.8	8.4	2.3	3.6	24.0
4. Borsod-A-Z.	3.5	5.6	7.5	4.5	67.9
5. Csongrád	1.2	5.9	2.8	10.9	13.0
6. Fejér	1.8	16.1	4.9	9.7	13.4
7. Győr-Sopron	0.8	27.8	4.5	6.3	13.6
8. Hajdu-Bihar	1.6	13.8	5.9	3.2	88.2
9. Heves	2.5	10.5	4.3	3.1	40.6
10. Komárom	2.9	16.7	3.6	10.8	7.2
11. Nógrád	0.9	3.8	1.7	6.5	5.7
12. Pest	3.7	12.2	6.2	9.8	17.9
13. Somogy	1.4	8.8	6.1	5.6	11.9
14. Szabolcs-Sz.	2.7	19.5	6.1	47.9	36.3
15. Szolnok	2.9	10.4	7.1	7.3	20.7
16. Tolna	0.4	8.3	2.9	3.3	11.5
17. Vas	1.8	2.8	2.1	8.0	10.0
18. Veszprém	0.3	44.4	7.9	8.4	12.4
19. Zala	0.4	3.3	8.5	2.2	15.6
Counties, total	2.4	12.5	5.4	9.8	25.6
Town					
I. Debrecen	0.8	6.2	6.2	10.8	16.2
II. Miskolc	11.3	5.3	10.0	8.7	69.3
III. Pécs	0.9	7.3	10.0	2.7	20.0
IV. Szeged	—	15.0	5.0	8.0	23.0
Country, total	2.4	12.3	5.6	9.7	26.2
V. Budapest	6.8	11.5	9.2	17.5	13.6
Hungary, total	3.3	12.1	6.3	11.2	23.8

immediately preceding years the incidence had been low. The only exception was the county Szabolcs-Szatmár (14), having been affected also by the 1954 and 1956 epidemics. The frequency by districts in the same county *(Fig. 2)* showed, however, that among the 5 districts yielding attack rates over 30 per 100 000 in 1957, Nyírbátor was the only one which had experienced an epidemic in 1956. In contrast to this, the four county districts having been most affected in 1956, had attack rates under 30 per 100 000 in 1957.

In addition, local outbreaks (over 20 cases) without spreading were observed in a few districts of the less affected counties.

Fig. 3 presents the distribution by month of poliomyelitis in the epidemic years of the period 1945—1957. The relatively high incidence during

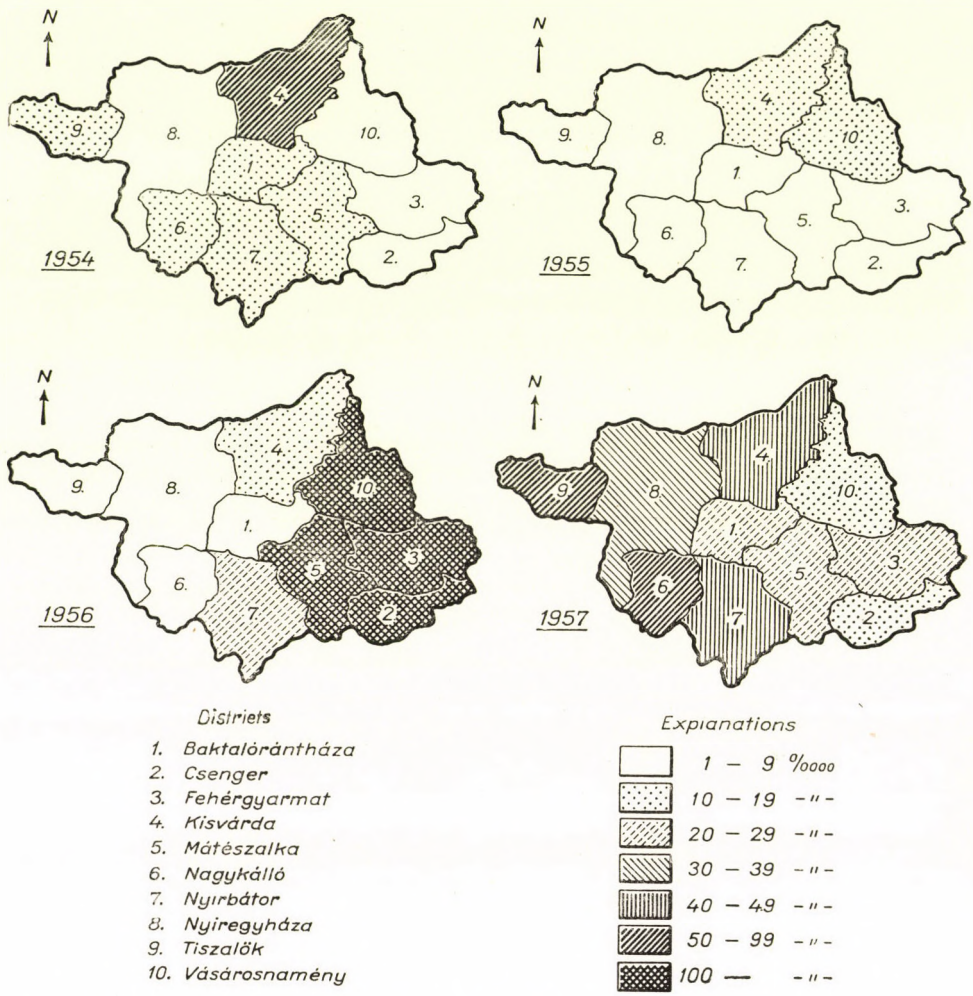

Fig. 2. Notified cases of poliomyelitis per 100 000 population in Szabolcs-Szatmár county 1954—1957

the first months of 1957 had at first been explained as the tail of the 1956 epidemic. The rapid increase during May, however, was a definite sign of the new epidemic. The curve was further rising in June and the highest incidence was recorded in July, then the curve fell suddenly and the case incidence in

September was lower than the corresponding figure for 1956. The morbidity for the last quarter of the year hardly exceeded those for the same season of 1947, 1952 and 1955, and remained under the comparable figures for each of the years 1954 and 1956. Thus, its steeper rise and a sudden fall made the 1957 epidemic curve remarkably different from the curves of the previous epidemics. PETRILLA [1] has demonstrated that the unusual early decline of the epidemic may be attributed, at least partly, to the vaccination cam-

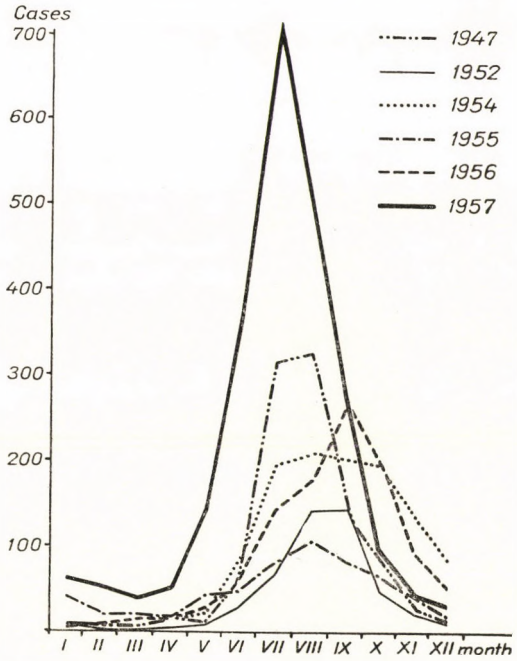

Fig. 3. Notified cases of poliomyelitis by months in the epidemic years of the period 1945—1957

paign organized by the Ministry of Health and performed between the middle of July and the end of September. The age groups 0—6 years were vaccinated two times with Salk type vaccine intradermally.

A comparison of attack rates by month for the epidemic and non-epidemic areas is presented in *Fig. 4.* Prior to the epidemic season already the former yielded higher figures than the latter. The attack rate for the epidemic area began to increase rapidly in May and rose above 100 per 100 000 in July. In that month the attack rate for the county Borsod-Abaúj-Zemplén (4) was nearly 300 per 100 000 and that for Hajdu-Bihar (8) over 250 per 100 000. Incidence in the latter county was still rising in August ; but from that time on it decreased as suddenly as in the other heavily affected counties. Like in the epidemic area, incidence in the non-epidemic area was highest in July, but the attack rate hardly exceeded 50 per 100 000. The decrease was slow

in August, in sharp contrast to the sudden fall in frequency observed in the epidemic areas. During the final quarter of the year, figures for the epidemic and non-epidemic areas were at about the same level.

Fig. 5 presents the distribution by age, of poliomyelitis for 1957. As reported earlier [2, 3], before 1954 the age distribution of poliomyelitis had

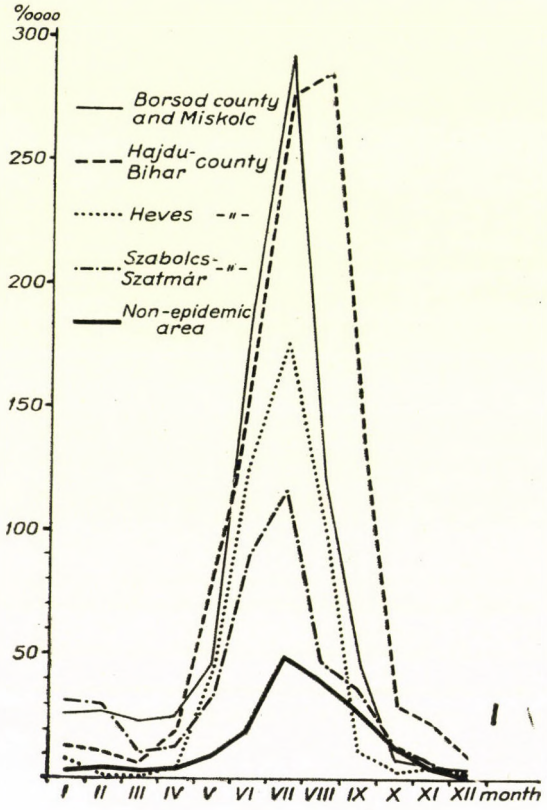

Fig. 4. Notified cases of poliomyelitis per 100 000 population by months for the epidemic and non-epidemic areas 1957

shifted towards the younger groups. This trend was to some extent reversed during the last two years. In 1957, children under one year of age made up only 14.2 per cent of the cases, as compared with the nearly 20 per cent for 1954. Sixtyone per cent of the cases occurred in the age group under two years in 1957, slightly less than in 1954 (67 per cent), or in 1955 (69 per cent). The proportion in the total morbidity of persons over the age of 15 years has not changed since 1954, but that of the children between 3 and 14 years has increased. The shift in age distribution was more definite in Budapest than in the country, as seen in *Figs. 5b* and *c*.

Table III presents the age specific attack rates for the years from 1949 to 1957.

As shown by the Table, through the whole period the 1 and 2 years age group suffered most. In 1957 this age group yielded 284 cases per 100 000 children of the same age. The age specific attack rates for the other age groups

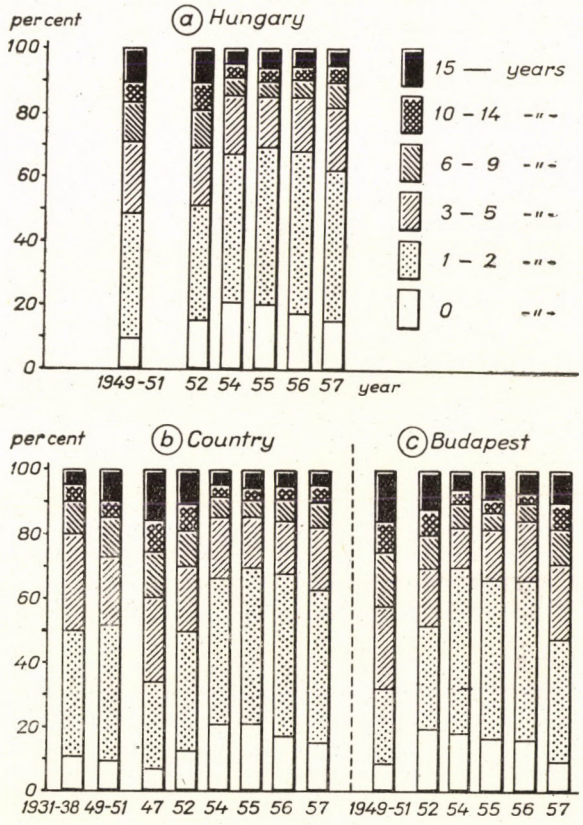

Fig. 5. Percentage distribution by age of notified cases of poliomyelitis *a)* Hungary, *b)* Country, *c)* Budapest

were, 194 for the infants, 73 for children from 3 to 6 years of age, and 19 for those from 7 to 14 years, all per 100 000 children of the same age.

In order to facilitate the survey of the fluctuation in age distribution, index numbers expressed for each age group as the percentage of the age specific attack rate for the 0 year olds have been calculated. The index numbers in the lower part of Table III indicate a gradual shift towards the age groups under 3 years during the period 1951—1955. Since then, however, the index for the age group from 3—6 years has been increasing and since 1956 so has

Table III

Poliomyelitis morbidity by age in Hungary, 1949—1957

a) Age specific attack rates per 100 000

Age group, years	1949	1950	1951	1952	1953	1954	1955	1956	1957
0	10.69	16.14	13.32	39.55	30.71	113.70	57.66	94.52	193.60
1—2	35.66	31.01	33.64	51.86	36.40	156.79	80.04	139.16	283.76
3—6	12.91	11.27	13.99	17.36	9.57	35.15	15.73	32.60	73.41
7—14	3.96	2.52	3.75	6.70	2.82	6.92	3.33	5.47	19.19
15—	0.53	0.33	0.52	0.79	0.43	0.84	0.58	0.86	1.72
Totals ...	3.22	2.75	3.29	5.26	3.27	12.13	6.29	11.19	23.78

b) Age specific index numbers for attack rates, if those for 0 year olds = 100

Age group, years	1949	1950	1951	1952	1953	1954	1955	1956	1957
0	100	100	100	100	100	100	100	100	100
1—2	334	192	253	131	119	138	139	147	147
3—6	121	70	106	44	31	31	27	34	38
7—14	37	16	28	17	9	6	6	6	10
15—	5	2	4	2	1	0,7	1	1	1

the index for children aged 7 to 14 years. The index for the age group over 15 years has not changed since 1953.

Table IV shows the age incidence for the epidemic and non-epidemic areas. It is remarkable that the percentage of patients under 2 years of age was lower while that of patients over 10 years was higher in the non-epidemic areas. This fact might be explained by supposing that the population of the epidemic areas had acquired immunity by natural infection at a younger age.

Table IV

Distribution of the poliomyelitis cases by age in the epidemic and non-epidemic areas of Hungary, 1957
(excluding Budapest)

Age, years	Epidemic area		Non-epidemic area	
	Cases	per cent	Cases	per cent
0	182	15.8	129	13.9
1—2	587	51.0	415	44.7
3—5	217	18.9	192	20.7
6—9	86	7.5	74	8.0
10—	78	6.8	118	12.7
unknown	2		2	
Totals	1152	100.0	930	100.0

Fig. 6 presents the case-fatality of poliomyelitis in the period from 1931 to 1957. Case-fatality had generally decreased since 1945 but some fluctuation was observed.

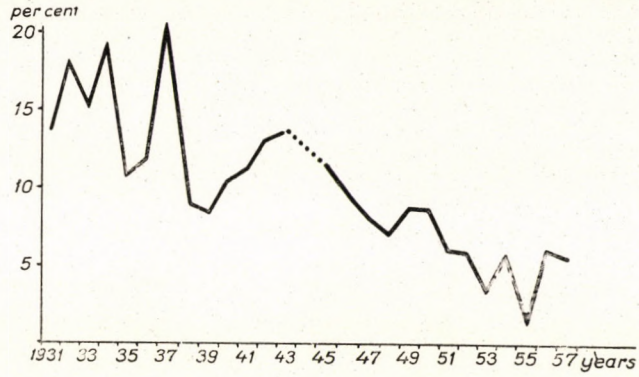

Fig. 6. Case fatality of poliomyelitis in Hungary 1931—1957

The 6.1 per cent case-fatality rate for 1957 corresponded to about the average for the previous years. Counties Borsod-Abaúj-Zemplén (4), Hajdu-Bihar (8) and Heves (9), *i.e.* those which suffered most by the epidemic, had lower case-fatality rates.

Case-fatality for the infants exceeded 9 per cent. It was lower for children, with a minimum in the 10 to 14 years age group. Above 15 years of age the case-fatality rate was significantly higher (10.4 per cent) than in the younger age groups (Table V).

The accompanying report [4] is dealing with the aetiology of the epidemic.

Table V

Poliomyelitis case-fatality by age 1957

Age, years	Cases	Deaths	Case-fatality, per cent
0	333	31	9,3
1	675	44	6,5
2	426	20	4,7
3	243	12	4.9
4	129	8	6.2
5	94	4	4.3
6—9	188	9	4.8
10—14	117	2	1.7
15—	125	13	10.4
unknown	4	—	—
Totals	2334	143	6.1

Summary

A poliomyelitis epidemic involving 2334 cases (23.8 per 100 000) occurred in Hungary in 1957. The epidemic was more extensive in the country (26.2 per 100 000) than in Budapest (13.6 per 100 000). The northeastern part of the country suffered most. County Hajdu-Bihar had the highest attack rate (88.2 per 100 000). The case incidence, which at the beginning of the year had already been relatively high, began rapidly to rise during May and attained its maximum in July. The rapid rise of the curve was followed by a sudden fall. The age specific attack rate was the highest (284 per 100 000) in the group of the one to two years old children. The attack rate was 194 per 100 000 for the infants and 73 per 100 000 for the 3 to 6 year age group. The proportions of the 3 to 6 and 7 to 14 years age groups have increased since 1956. The general case-fatality rate was 6.1 per cent. The age specific case-fatality rate for infants exceeded 9 per cent, and decreased with the increase of age until 14 years of age. The group above 15 years displayed the highest case-fatality rate.

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THE 1957 POLIOMYELITIS EPIDEMIC IN HUNGARY

II. AN AETIOLOGICAL STUDY

By

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(Received May 13, 1958)

In Hungary, the aetiological investigation of poliomyelitis cases by the tissue culture method [1] had started in 1954. Since then, all the three types of poliomyelitis virus, as well as some other types of cytopathogenic enteroviruses, have been recovered. In 1954, type 2 strains were mainly isolated by FÖLDES [2] and PINTÉR *et al.* [3]. In 1955, type 3 seemed to dominate in Budapest, and type 2 in the country [4].

In 1956, owing to circumstances beyond our control, we could not isolate more than 9 cytopathogenic strains [5]. Five of the strains were identified as type 1, one as type 2 and one as type 3 poliomyelitis virus. Two enterovirus strains which could not be neutralized with any of the standard poliomyelitis sera have not been identified properly. The history of the 9 strains is as follows.

Both the type 2 and type 3 strains were isolated from paralytic cases during the first two months of 1956. In April, from a local outbreak yielding 5 paralytic cases at the dermatological department of a children's hospital in Budapest, three type 1 strains were recovered: two from the faeces of the paralytic patients and one from an inpatient who had not developed symptoms of poliomyelitis but one year earlier. A faecal specimen obtained from the same patient one month later yielded an unidentified cytopathogenic strain belonging to none of the three types of poliomyelitis virus. In May, 1956, one type 1 strain was recovered from the faeces of a healthy child in a nursery where a case of poliomyelitis had occurred. The fifth type 1 strain and the second unidentified cytopathogenic strain were isolated from spinal cords obtained in July and August, respectively.

Materials and methods

Specimens. During the twelve months starting in May, 1957, 255 faecal specimens and 4 cerebrospinal fluids were received in order to isolate virus. We usually controlled the clinical diagnosis personally, by revising the case histories. In a few cases, however, diagnose from the reporting cards have been accepted. Sixtyfive of the 255 faeces investigated had originated from patients with paralytic poliomyelitis, 134 from aseptic meningitis clinically undistinguishable from the non-paralytic form of poliomyelitis, and 60 from other diseases. The last cases will not be dealt with in the present report. Most of the samples from paralytic

cases had been sent in by the Central Hospital for Infectious Diseases, Budapest (V),* while the bulk of the specimens from aseptic meningitis cases by the County Hospital, Debrecen (I).*

Preparation of faecal specimens. The procedure described earlier [4] was modified by adding aureomycin to the suspending fluids. In the case of mycotic contamination, the suspending fluid was completed by adding 0.1 mg crystal violet per ml, and further tissue cultures were inoculated. By these procedures both bacterial and mycotic contaminations were eliminated.

Inoculation of tissue cultures. The faecal suspensions were stored at —20° C, thawed rapidly and let to warm up the room temperature, then each suspension was inoculated into both trypsinized monkey kidney cultures and fibroblastic cultures of human embryotic skin-muscle tissue fragments. The tubes were re-incubated for 5 to 7 days, whilst examined daily under the microscope. If cytopathic changes had been observed, the culture fluid was immediately harvested and stored in a deep freezer until subpassaged. Negative culture fluids were subjected to three blind passages.

Neutralization tests. The cytopathogenic strains were identified in neutralization tests. The type 1, 2 and 3 poliomyelitis standard sera had been kindly supplied by Professor H. v. PETTE, *Hamburg.*** The Coxsackie A9, B1—5 and ECHO 9 immune sera had been prepared in mice and in rabbits, respectively, at this Department by Dr. I. DÖMÖK. A 1 : 20 dilution of each serum was mixed with equal volumes of 1 : 50 diluted virus suspensions. After shaking, the serum-virus mixtures were placed at 37° C in an incubator for one hour before inoculation of three tissue cultures with each of the mixtures in the same manner as described above. Results were read on the 3rd, 4th and 7th days following inoculation. If none of the three standard poliomyelitis sera had neutralized the test strain, this was tested against a mixture of the three sera, as well as against the Coxsackie and ECHO sera mentioned above and, finally, if necessary, against a mixture of the latter sera.

Results

We examined 65 cases of poliomyelitis and 134 of aseptic meningitis out of the cases occurred in Hungary in 1957. Sixtyfive cytopathogenic strains were isolated in monkey kidney cultures, while only 29 in human embryonic skin-muscle cultures. Table I gives the results for the paralytic cases. The results for Borsod-Abaúj-Zemplén County (4)* are separately presented in Table I. This county was one of those having been most affected by poliomyelitis in 1957. Table I shows that type 1 poliomyelitis virus was isolated from 60 per cent of the paralytic cases. This percentage was particularly high (87 per cent) for County (4)*. Thirtythree strains were identified as type 1 poliomyelitis virus, while 5 strains belonged to type 2. One specimen contained both type 1 and 2 viruses. In this case the culture fluid could not be neutralized but with a mixture of type 1 and 2 sera.

In contrast to the paralytic cases, only 19 per cent of the aseptic meningitis cases submitted for examination yielded virus and only 54 per cent of the strains (14/26) proved to be type 1 poliomyelitis virus, as seen in Table II. Six strains belonged to the type 9 of ECHO viruses, while 6 could not be identified with any of the sera available at the time of the investigations. (The proper identification of the strains is in progress. Moreover, DÖMÖK

* The meanings of the numerals in brackets see in Hungary's map presented in Fig. 1 of O. RUDNAI's accompanying paper [6]. Each arabic numeral means a county, each Roman numeral a town.

** We are greatly indebted to Prof. H. v. PETTE for supplying the sera.

Table I

Viruses recovered from cases of paralytic poliomyelitis in Hungary, 1957

Faecal sample	Samples received from			
	Borsod county (4)*	Other counties and towns	Hungary No	Hungary per cent
Cytopathogenic	20	19	39	60
Non-cytopathogenic	3	23	26	40
Totals	23	42	65	100
Type of the strain recovered				
Polio type 1	17	16	33	85
Polio type 2	3	2	5	13
Polio type 1 and 2	—	1	1	2
Totals	20	19	39	100

Table II

Viruses recovered from aseptic meningitis cases occurred in Hungary, 1957

Faecal sample	Samples received from			
	Hajdu-Bihar county (8)*	Other counties and towns	Hungary No	Hungary per cent
Cytopathogenic	22	4	26	19
Non-cytopathogenic	90	16	108	81
Totals	114	20	134	100
Type of the strain recovered				
Polio type 1	11	3	14	54
ECHO 9	5	1	6	23
Unidentified	6	—	6	23
Totals	22	4	26	100

is investigating the suckling mouse pathogenicity of the unidentified strains, as well as of the specimens which had failed to cause cytopathic changes in tissue cultures.)

The age specific incidence rate of poliomyelitis in 1957 was the highest for children aged 1—2 years, as demonstrated by RUDNAI [6]. According to PETRILLA's data [7], after introducing the Salk vaccination,

* See footnote p. 370

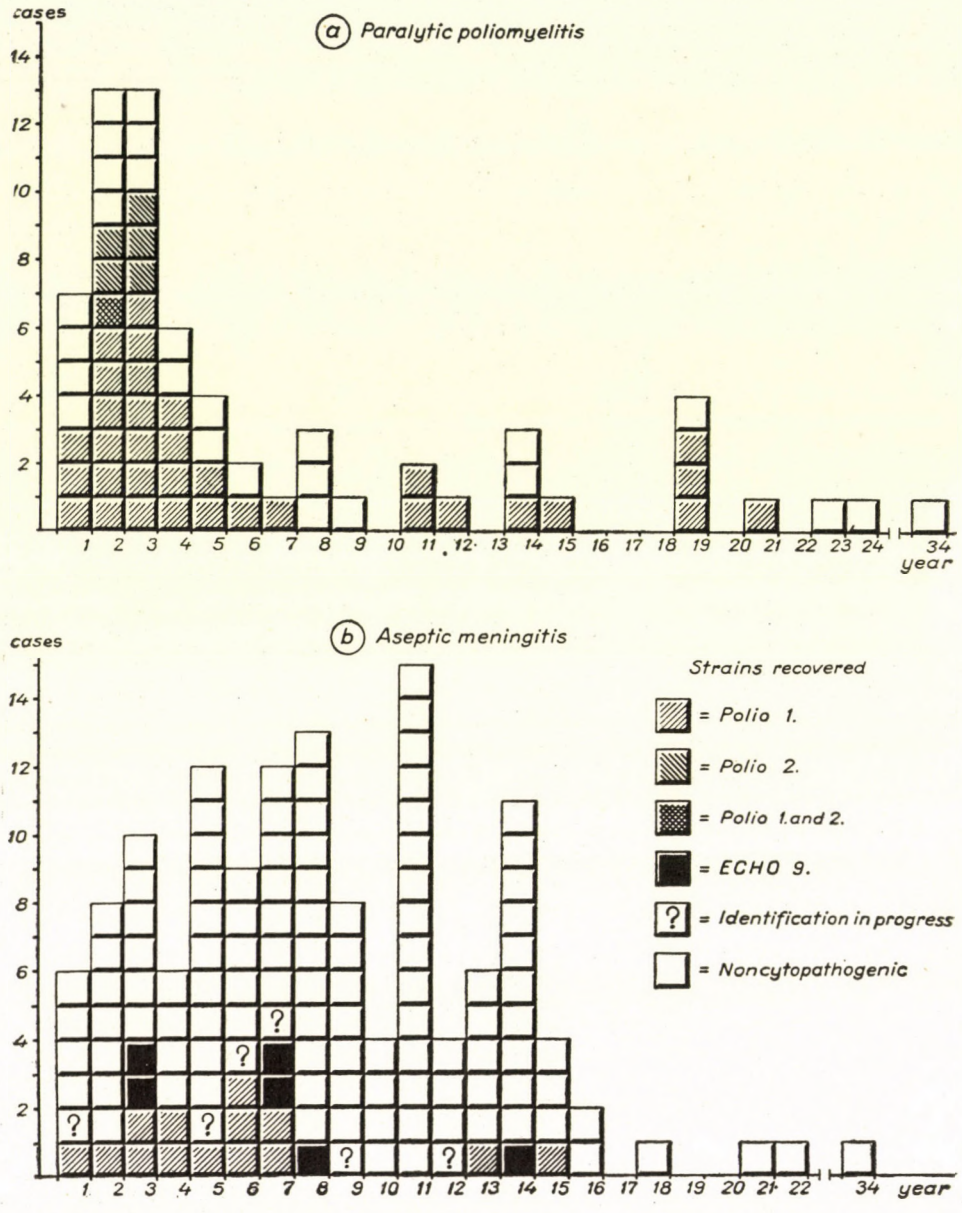

Fig. 1. Distribution of the isolated viruses by age of the patients

at the peak of the epidemic, some change in the age distribution could be demonstrated. It has therefore, to be emphasized, that most of our specimens were obtained in the period preceding the vaccination. *Fig. 1* shows that the age distribution of the paralytic cases submitted for examination was very similar to that of the total cases occurred in Hungary in 1957, and the proportion of the cases yielding virus showed no remarkable fluctuations in the different age groups. All the five type 2 strains had originated from children 1—2 years of age.

As to the geographical distribution, the great majority of the paralytic cases investigated by us belonged to the epidemic areas in Northeast Hungary, and in Budapest and its surroundings. Thus, with respect to geographical as well as age distribution, the strains recovered seem to be a true mirror of the strains that had caused the epidemic.

As mentioned above, the bulk of the faecal specimens of patients with aseptic meningitis were obtained from county Hajdu-Bihar (8).* A few specimens were received from the counties Tolna (16),* Pest (12),* Szabolcs-Szatmár (14),* Borsod-Abaúj-Zemplén (4),* Heves (9)* and Nógrád (11).* In contrast to the paralytic cases, the 134 patients with aseptic meningitis were nearly equally distributed in the age groups 0—4 years, 5—9 years, and 10—14 years. Only six of them were over 15 years of age. Type 1 poliomyelitis virus was isolated more frequently from non-paralytic patients aged 0—6 years (12/63), as compared with those over 7 (2/71). As shown in *Fig. 1*, there was a similar but less significant difference between pre-school and school-children concerning the isolation of ECHO 9 and unidentified viruses (8/63 and 4/71, respectively).

Discussion

As shown by the virus strains isolated during the epidemic the 1957 poliomyelitis epidemic in Hungary was caused by type 1 poliomyelitis virus.

A simultaneous cumulation of mild abacterial meningitis cases was observed in several counties, with a particularly high frequency in county Hajdu-Bihar (8)* that was most affected by the poliomyelitis epidemic.

During poliomyelitis epidemics the meningeal form of the disease generally does not attain more than one third of the cases. If it runs to 50 per cent or more, like in some counties of Hungary in 1957, the partial aetiological role of other pathogenic agents, *e. g.* ECHO viruses, is to be supposed. This suspicion is supported by the following facts.

(i) The age distribution of the aseptic meningitis cases was fairly distinct from that of the paralytic poliomyelitis cases ;

* See footnote p. 370.

(ii) poliomyelitis virus was isolated from 60 per cent of the faeces of paralytic cases, while only from 10 per cent of the patients with meningitis ;

(iii) ECHO 9 and unidentified strains were recovered from 9 per cent of the patients with meningitis and from none of the paralytic cases.

No virus was isolated from 81 per cent of the specimens received from patients with abacterial meningitis in County Hajdu-Bihar (8).* These might have excreted ECHO viruses in a concentration insufficient to infect even the relatively susceptible monkey kidney cultures. On the other hand, Leptospirae might have been involved in some cases of meningitis, as supported by the presence of Leptospirae agglutinins in the convalescent sera of 9 out of 25 patients yielding no virus. (The tests were performed at the *Institute of Microbiology, Medical University, Budapest.)*

The causative role of ECHO viruses in some epidemics of aseptic meningitis has been proved by a number of authors. GODFREDSEN and V. MAGNUS [8] isolated ECHO 9 virus from the cerebrospinal fluid of a patient with aseptic meningitis. In addition, on the grounds of virus isolations and immune responses, the primary aetiological role of the ECHO 9 virus in the wide-spread aseptic meningitis epidemic in 1956 has been proved in many countries, including Hungary [9]. However, HAMMON *et al.* [10] have shown that ECHO viruses may be present as concomitants in poliomyelitis patients. In the experiments of the same authors, ECHO viruses were able to interfere with the growth of poliomyelitis virus in tissue cultures, except when the quantity of the latter exceeded 100 times that of the former. Unfortunately, we had no means to perform serological tests and that is why we could not definitely establish the aetiological role of the ECHO viruses in the meningitis epidemic.

Trypsinized monkey kidney cultures were remarkably more sensitive to enteroviruses than fibroblastic cultures of human embryonic skin-muscle tissues.

Summary

1. Faecal specimens from 65 cases of paralytic poliomyelitis and 134 cases of aseptic meningitis were examined in order to isolate virus.

2. Sixtyfive cytopathogenic strains have been isolated in trypsinized monkey kidney cultures, while only 29 strains were isolated from the same material in fibroblastic cultures of human embryonic skin-muscle cultures.

3. In monkey kidney cultures 60 per cent of the paralytic patients' faeces showed cytopathogenicity. Eightyfive per cent of the strains belonged to type 1, the remainders to type 2 poliomyelitis virus. One specimen yielded both type 1 and type 2 viruses.

4. Only 19 per cent of the faeces of patients with aseptic meningitis proved to be cytopathogenic. One half of the strains was type 1 poliomyelitis virus, a quarter belonged to the type 9 of ECHO viruses, the remainders have not been identified.

5. ECHO 9 virus might have had a causative role in part of the cases of aseptic meningitis.

* See footnote p. 3.

Acknowledgements: The author is indebted to Miss E. CSONKA and Mr. P. RUZICSKA for providing the trypsinized monkey kidney cultures, to Mrs. A. OROSZ and Mr. J. VÁMOS for the human embryonic skin-muscle cultures and to Dr. I. DÖMÖK for preparing and supplying the Coxsackie and ECHO immune sera.

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STUDIES ON THE CYTOPATHIC CHANGES CAUSED BY DIFFERENT TYPES OF THE ADENOVIRUS GROUP IN HUMAN AMNION AND DETROIT-6 CELL CULTURES

By

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Since the introduction of Detroit-6 and human amnion cell cultures into virus research [1—4], investigations have been concerned with the possible existence and characteristic appearance of the cytopathic changes exhibited by some groups of viruses. A number of authors have reported adenoviruses [5, 6] to be cytopathogenic [3, 7, 8] in these cultures. In the present report, data will be presented on the comparative cytopathogenic effects (in the following, CE) of some adenovirus strains belonging to different types of the adenovirus group.

Materials and methods

Virus strains. One type 1, two type 2, three type 3, one type 4, four type 5, one type 6 and two type 7 strains of the adenovirus group have been examined. Four strains were of Hungarian origin. One type 2 and two type 5 strains were isolated from tonsils by us [9] while one of the type 3 strains, recovered by BÉLÁDI and KAHÁN [10], had originated from an epidemic outbreak of pharyngoconjunctival fever in Szeged, 1956.

Stock virus suspension containing 10^6 TCD50/ml had been prepared from HeLa or Detroit-6 cultures. When examining the CE, 0.1 ml of undiluted or diluted stock suspensions were used as inocula.

Tissue cultures. The procedures followed in the preparation of rabbit kidney cultures and trypsinized continuous cultures of HeLa cells have been described in another paper [9].

The continuous cultures of *Detroit-6* cells (a cell line derived from the sternal bone marrow of a patient with lung cancer) [4] were prepared in Roux flasks of 1 or 2 litres, essentially in the same manner as described for the HeLa cells [9]. For virus studies trypsinized cultures in Jena glass tubes were used. The nutrient medium was composed of 20 per cent human serum, 8 per cent of 5 per cent lactalbumin hydrolysate in Hanks' solution and 72 per cent Hanks' balanced salt solution. Eagle's medium with 10 per cent horse serum was used as maintenance solution.

Human amnion cultures were prepared as follows. Amniotic membranes of fresh placentae were separated and two or three times washed with saline ; large blood clots were removed. The amniotic tissue was placed in 0.25 per cent trypsin solution and incubated in a water bath of 37° C for 30 minutes while shaken gently from time to time. The first trypsin solution containing mainly mucus and erythrocytes was discarded. The trypsin solution was changed 3 to 4 times in intervals of 30—60 minutes. The collected trypsin solution of high cell count was centrifuged at 1500 r. p. m., the cells were washed two or three times with Hanks' balanced salt solution and resuspended in the culture medium (see below) to a cell count of 200 000 to 300 000 cells per ml. This suspension was distributed in tubes, 1 ml in each. Stationary cultures prepared in this manner formed a confluent layer of cells after incubation for 4 to 8 days. The growth medium consisted of 20 per cent human serum, 10 per cent of a 5 per cent solution of lactalbumin hydrolysate, and 70 per cent Hanks' balanced salt solution. The composition of the maintenance solution was 5 per cent rabbit serum, 5 per cent of 5 per cent lactalbumin hydrolysate and 90 per cent Hanks' solution.

Type specific immune serum. Rabbits were immunized by 5 injections of 1.5 ml of culture fluid from virus infected rabbit kidney cultures. The rabbits were killed by bleeding one week after the last injection.

Neutralization tests were performed in human amnion and Detroit-6 cultures, as described earlier [9].

Results

When investigating the cythopathogenic spectra of 14 strains (see above) belonging to the first seven types of the adenovirus group, in human amnion cultures two distinct types of tissue degeneration were observed. The CE of the type 3 and type 7 strains (in the following, "type 3 degeneration") was fairly different from that brought about by the type 5 and type 6 strains (in the following, "type 5 degeneration"). Each of the remaining immunological types could be grouped into one of the two degeneration types.

"*Type 3 degeneration*" starts as a change in the refraction of the cells. The cells become more conspicuous, showing more contrast under the light microscope. This is soon followed by rounding and, to some extent, shrinking of the cells at the edge of the cell layer. The cell margins remain sharp, the cells do not tend to separate. Starting at the edge of the culture, the completely coherent culture *(Fig. 1)* becomes converted step by step to a network of rounded cells *(Figs. 2 and 3)*. At a later stage the network transforms into small islands consisting of 3 to 10 rounded cells each. Isolated cells are scarcely seen *(Figs. 4 and 5)*. In the cells of some cultures granulation is observable.

In cultures inoculated with 10^5 TCD50 of virus the complete degeneration developed in 6 to 8 days. The progress was slower and depending on the degree of dilution in cultures which had been inoculated with more dilute virus suspensions. The CE by type 3 strains could be neutralized with type specific immune sera.

The pattern of CE exhibited by each of the two type 7 strains was almost identical with the "type 3 degeneration". That caused by the type 4 strains was similar in appearence but developed definitely slower.

The first phase of the "*type 5 degeneration*" is also characterized by a change in the refraction of, and the appearence of a dense granulation, in the cytoplasm of most of the cells. At a later stage, the majority of the cells separate, some of them become rounded with blurred cell margins and nuclear outlines. In contrast to the network characteristic of the "*type 3 degeneration*" the whole culture appears to be loose and deranged *(Figs. 6 and 7)*. When the changes progress to complete degeneration, rounding and destruction of the cells is more prominent and, finally, only single and paired cells, often with blurred outlines, are seen *(Fig. 8)*. The degeneration was complete within 6 to 8 days if undiluted inocula had been used. Dilution of the inoculum prolonged the completion of the degeneration but did not influence

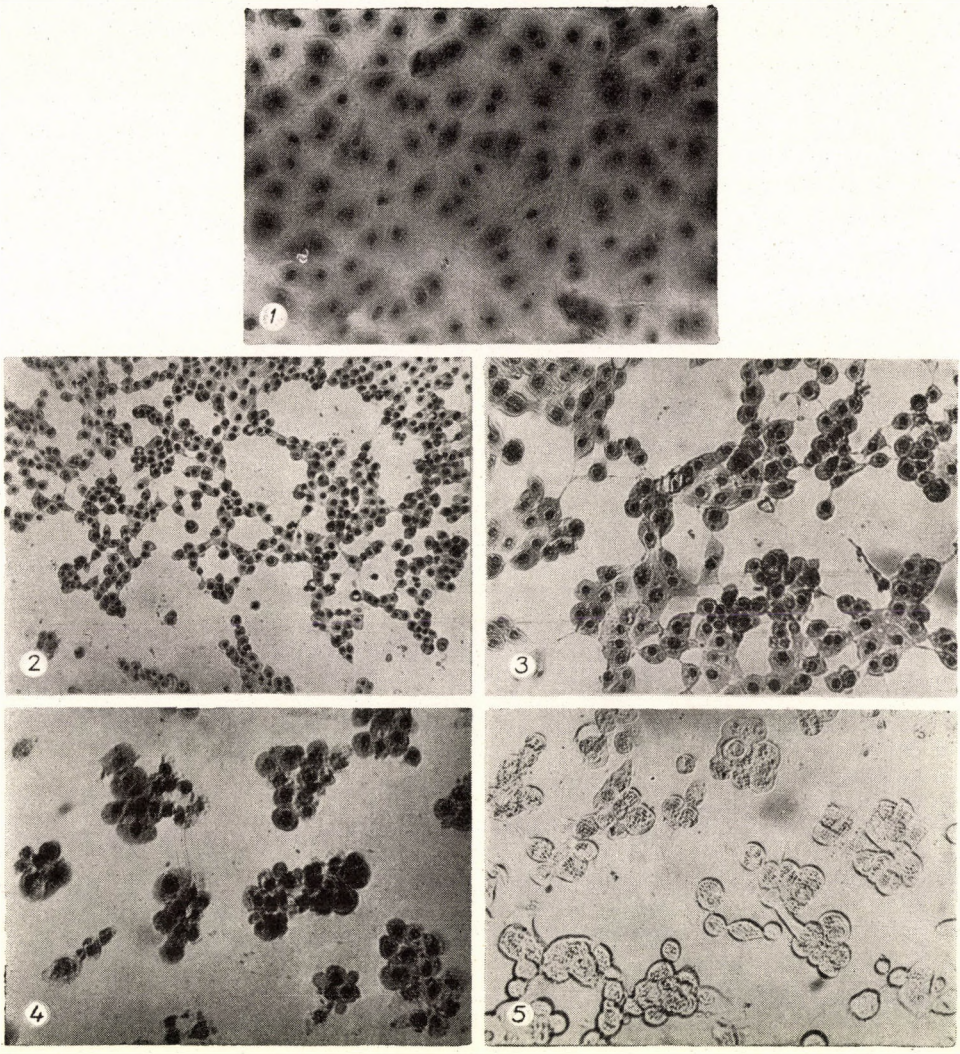


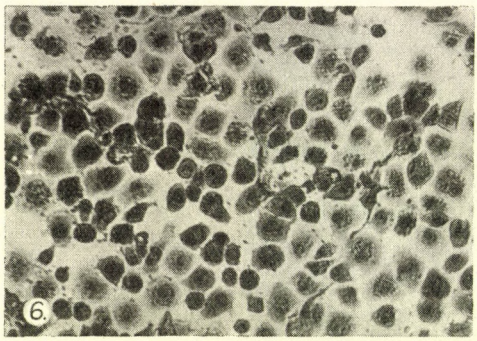
Fig. 1. Normal amnion culture. Giemsa, × 150

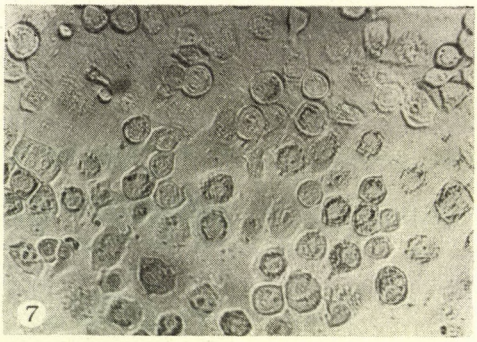
Fig. 2. Human amnion culture 4 days after inoculation with type 3 adenovirus. Giemsa, × 70

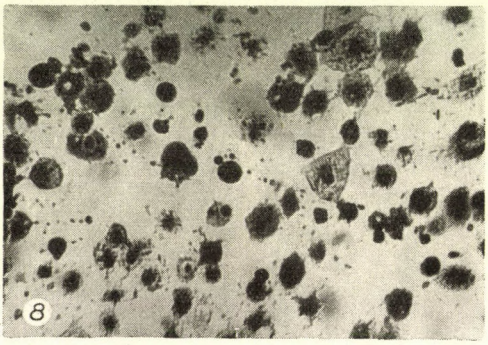
Fig. 3. The same, × 150

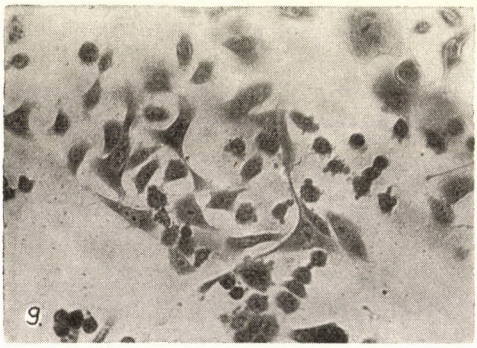
Fig. 4. Human amnion culture 8 days after inoculation with type 3 adenovirus. Giemsa, × 150

Fig. 5. The same. Unstained. × 150









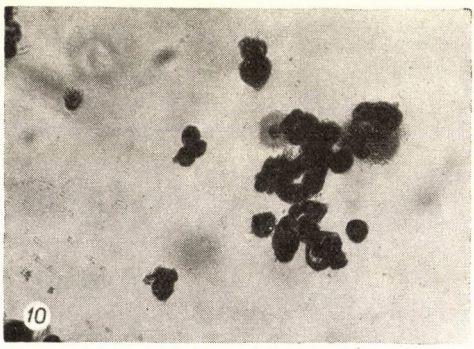


Fig. 6. Human amnion culture 4 days after inoculation with type 5 adenovirus. Giemsa, × 15
Fig. 7. The same. Unstained, × 150
Fig. 8. Human amnion culture 10 days after inoculation with type 5 adenovirus. Giemsa, ×15
Fig. 9. Normal Detroit-6 culture. Giemsa, × 150
Fig. 10. Detroit-6 culture 5 days after inoculation with type 3 adenovirus. Giemsa, × 150

its pattern. The characteristic CE could be neutralized by type specific immune sera.

Regardless of the type of the degeneration, it was characteristic of all the strains examined that the majority of the degenerated cells did not detach from the glass wall even during an observation time of several weeks. Similar observations concerning type 2 and 5 strains have been reported by us [9].

The adenovirus strains mentioned above have been examined for cytopathogenicity in Detroit-6 cultures, too. Cytopathogenic effects in some extent similar to those brought about in the HeLa cells were observed. The cells became rounded, the clumping degenerated cells formed islands which detached from the glass wall at the final stage of destruction. All the strains examined produced the same cytopathic changes.

Discussion

It is remarkable that the immunological types of the adenoviruses eliciting the "type 3 degeneration" are proved pathologic agents in pharyngoconjunctival fever, atypical pneumonia and other acute respiratory diseases [11,12]. In contrast to this, the human pathogenicity of the immunological types producing "type 5 degeneration" is rather dubious; most of the strains belonging to these types have been isolated from spontaneously degenerating tissue cultures of human adenoids and tonsils [13]. Thus, the existence of a closer biological relationship might be supposed between the different immunological types exhibiting similar CE. The pattern of cytopathic changes in human amnion cultures seems to be suitable also for distinguishing types or subgroups within other virus groups, *e. g.* DÖMÖK [14] observed fairly distinct patterns of the cytopathogenic changes brought about by the different types of ECHO viruses.

Summary

The cytopathogenic effect of 14 strains belonging to the types 1 to 7 of adenoviruses have been studied. In human amnion cultures types 3, 4 and 7 have been found producing almost identical cytopathogenic effects, which definitely differed from those brought about by the strains belonging to the types 1, 2, 5, and 6. The two patterns of degeneration have been described in detail. In Detroit-6 cultures undistinguishable changes were produced by all the 7 immunological types.

Acknowledgements: We are indebted to Mr. BALÁZS and Mrs. KISZEL for their excellent technical assistance.

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GENETIC TRANSDUCTION IN THE SALMONELLA GROUP

By

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In 1952, ZINDER and LEDERBERG described a new method of great genetic significance, by which hereditary properties could be transferred from one Salmonella type to the other. This new kind of genetic recombination has been termed transduction. Using the filtrates of Salmonella strains free from viable bacteria, numerous workers have subsequently carried out transduction of nuclear components. Changes concerning the nutrient requirement, drug resistance, biochemical properties and antigenic structure of the recipient strains have thus been demonstrated.

The substance responsible for transduction was at first thought to be desoxyribonucleic acid. Later investigations, however, made it probable that temperate phages of Salmonellae were concerned. The role of phages may be interpreted as that of a vector, as phage particles released from the lysogenic strains or propagated on the donor strain are provided with genetic factors responsible for one or more hereditary properties of these bacteria. The adsorption of these phage particles on the recipient strain will cause the incorporation of the genetic material which is probably a fragment of desoxyribonucleic acid. The cell populations unharmed by the lytic action, however, small their number, will thus offer means for studying the result of transduction on the basis of their new properties.

The great number of serotypes and the frequent occurrence of lysogenic strains make the Salmonella group most suitable for transduction studies. Following the classical work of ZINDER and LEDERBERG [1], a series of reports were published on the transduction performed by phage PLT 22 obtained from LEDERBERG's lysogenic *S. typhi murium* strain LT 22. STOCKER, ZINDER and LEDERBERG [2] and KAUFFMANN [3] carried out successful transduction of flagellar antigens. BAILEY showed that stable O forms could be converted into motile bacteria by phages [4, 5]. Further studies on this problem were made by EDWARDS, DAVIS and CHERRY [6], LEDERBERG, EDWARDS [7], STOCKER [8]. Transduction of the somatic antigen was carried out by Japanese authors [9]. The latter workers, and also BARON *et al.* [10], transduced streptomycin resistance together with the change of the somatic antigen.

In the present investigations a series of experiments were undertaken to find lysogenic strains in Salmonella groups A, B and D for obtaining transducible phages, and to transduce flagellar antigens to strains belonging to the same groups.

Materials and methods

Cultures. The lysogenic strain *S. typhi murium* LT 22 and the indicator strain *S. typhi murium* LT 2 were obtained from Dr. J. LEDERBERG. The following strains were of the stock culture collection of our laboratory.

Monophasic strains : *S. paratyphi A* (4 strains) ; *S. enteritidis* (2 strains) ; *S. essen*, *S. derby* (2 strains) ; *S. budapest*, *S. berta*, *S. dublin*, *S. rostock*, *S. moscow*, *S. montevideo*, *S. typhi* (4 strains).

O strains : Ty 0901 ; *S. paratyphi B; S. paratyphi A.*

Freshly isolated strains : *S. typhi* (30 strains), *S. typhi murium* (10 strains).

Immune sera. O and H sera were prepared from standard strains in this laboratory. Absorbed sera were prepared by KAUFFMANN's method [11].

Culture media. Semisolid medium containing 0.8 per cent agar was distributed in 5 ml amounts into U tubes. Other media were used in plates.

Preparation of phage lysates. The above-listed strains of Salmonella groups A, B and D were tested for the production of transducing phages. The same strains were used in antigenic transfers. The phages used in the work were isolated by growing each Salmonella strain together with the other strains in every possible combination. The broth cultures were then freed of viable bacteria by passing through Seitz filters. The lysates were tested for phage action by the plating method. Of the lysates those only giving plaque counts 10^7 to 10^9 per ml were used. With lysates giving lower plaque counts the sufficiently high titre was reached by propagation upon the corresponding indicator strain. In the refrigerator the titre of the phages remained unaltered for months and in sterility tests they proved to be free from contaminants.

Phage lysates. From more than one hundred symbiotic cultures only some lysates proved suitable for the transduction experiments. They were as follows.

PLT 22 : Symbiotic phage of the internationally used *S. typhi murium* LT 22 strain. Indicator strain : *S. typhi murium* LT 2. Uniform plaque morphology.

PE 269 : Symbiotic phage of *S. enteritidis* 269. Indicator strain : *S. enteritidis* 64. Two different plaque forms.

PES : Symbiotic phage of *S. essen.* Indicator strain : *S. enteritidis* 269. Uniform plaque morphology.

PPA : Phage PLT 22 propagated upon *S. paratyphi A.* Phages PE 269, PES and PPA were prepared in the course of the experiments, by using the various cultures listed above.

Transduction technique. In one arm of an U tube containing 5 ml of semisolid agar, 0.5 to 1.0 ml of phage lysate was pipetted. From a 24 hour agar slant culture a loopful of the recipient strain was inoculated in the same arm. Swarming of the culture was indicated by growth in the other arm of the U tube. By subculturing this growth on agar plates, the cultures were identified by agglutination or other methods. All experiments were controlled by using an adequate number of tubes containing no phage.

Results

The results of successful and reproducible transduction experiments in the A, B and D groups of Salmonellae are given in *Table I.*

On employing lysate PLT 22, from *S. paratyphi B* stable O form swarming cultures could be isolated. The cultures in phase 1 contained either *b* or *i* H antigens. Other cultures possessed phase 2 antigen *1, 2.* The transferred flagellar properties were hereditary and corresponded to the rules of phase variation.

Table I

Results of transduction experiments

Antigenic structure of recipient strain	Phage lysate	Donor strain	Antigenic structure of recombinant strain	Mechanism
1, 4, 5, 12 :- (*S. paratyphi B* "O")	PLT 22	*S. typhi murium*	1, 4, 5, 12 : b	flagellation
			1, 4, 5, 12 :-1, 2	flagellation
			1, 4, 5, 12 : [i]	transduction
	PE 269	*S. enteritidis*	1, 4, 5, 12 : [gm]	transduction
	PES	*S. essen*	1, 4, 5, 12 : [gm]	transduction
9, 12 :- (*S. typhi* 0901)	PLT 22	*S. typhi murium*	9, 12 : d	flagellation
1, 9, 12 : gm (*S. enteritidis*)	PPA	*S. paratyphi A*	1, 9, 12 : [a]	transduction

Explanation :

[ ] means transduction of H antigen of the donor strain

Phage PE 269 isolated from *S. enteritidis* also had a transducing effect on *S. paratyphi B* O form. In this experiment a recombinant was formed containing the somatic antigens of *S. paratyphi B* and the flagellar antigens of *S. enteritidis*, thus corresponding to the antigenic structure of *S. essen*. A similar result was obtained with phage PES propagated upon *S. essen*. The antigenic character of the hybrid produced by this phage was the same as that of *S. essen*.

The hybrids formed by transduction from *S. paratyphi B* O strain were, however, only antigenically affected. Slime wall formation *(Fig. 1)*, as well as biochemical reactions and mouse pathogenicity of the recombinants were the same as those of the parent strain. Both the recipient and the recombinant strains were lysed by phage lysates responsible for the transduction.

Phage PLT 22 caused the flagellation of the stable O form of Ty 0901 in about 40 per cent of the experiments. This phage had no lytic action on other *S. typhi* strains and was indifferent for them.

Phage PLT 22 had no transducing action on other strains. The proportion of successful transductions and flagellations with the characteristic H antigen is presented in *Table II*.

Of the monophasic strains, a *S. enteritidis* strain was transduced by phage PLT 22 propagated on *S. paratyphi A* into a strain containing the somatic antigen of the recipient and the flagellar antigen of the donor strain. Thus, the antigenic structure of the hybrid was identical with that of *S. sendai* in phase 1.

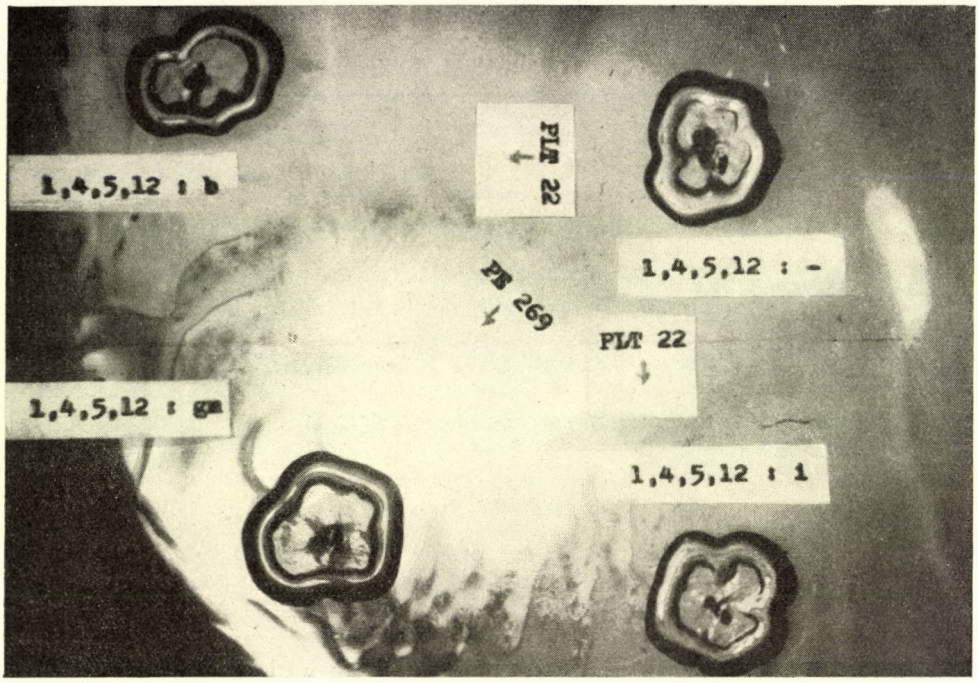

Fig. 1. Slime wall production by the initial and transduced strains of S. paratyphi B on agar plates incubated for 3 days. (Magnification, × 5) Key: PLT 22 and PE 269 = phages, 1, 4, 5, 12 : b, etc. = antigenic structure

Table II

Frequency of transductions in one series of experiments

Recipient strain	Phage lysate	Recombinant strain	No. of tubes	No. of successful transductions	per cent
S. paratyphi B "O"	PLT 22	1, 4, 5, 12 : b	20	6	30
	PLT 22	1, 4, 5, 12 : [i]	20	4	20
	PE 269	1, 4, 5, 12 : [gm]	18	3	16.6
	PES	1, 4, 5, 12 : [gm]	14	2	14.2
	Control*	—	10	0	—
S. typhi 0901	PLT 22	9, 12 : d	16	7	43.7
	Control*	—	10	0	—
30 freshly isolated strains of *S. typhi*	PLT 22	—	10 each	0	—
	PE 269	—	10 each	0	—

Explanations :

* Tubes without phage

[ ] means transduction of H antigen of the donor strain

The above results agree with those in the literature as to the transducing and flagellating capacity of phage PLT 22. New recombinants were, however, produced with lysates prepared from *S. enteritidis*, *S. essen* and *S. paratyphi A*.

Discussion

The great number of serotypes, the complexity of antigenic structure, the phase variation, the phenomenon of induced phases and the easy demonstration of transduction make the Salmonella group most suitable for investigations into bacterial genetics. The experiments have shown that most phages of Salmonellae are able to produce flagellation of non-motile cultures and to transfer genetic material from one strain to the another.

According to BAILEY, the occurrence of phages with transducing capacity among *S. typhi murium* strains was 12 per cent [4]. EDWARDS, DAVIS and CHERRY [6] showed that a variety of freshly isolated Salmonella strains of groups C, D, E and G was capable of producing such phages. The existence of new genotypes produced by transduction may suggest that phages are responsible for the natural production of serotypes. While the transduction experiments made so far have been able to transfer only one genetic property, closely related natural serotypes may markedly differ in their biochemical behaviour. In the present experiments, the transduced *S. paratyphi B* strain possessing *i* antigen of *S. typhi murium* retained its initial fermentation reactions, virulence and slime wall formation (see *Fig. 1*). The same was true for the recombinant of *S. paratyphi B* with the antigenic structure of *S. essen*. Chemical analysis of substances playing a role in transduction may solve this biologically important phenomenon, in which in the absence of close contacts, the transfer of genes is performed by a filterable agent.

The epidemiological significance of transduction is not yet clear. The transduction experiments of FURNESS and ROWLEY [12] on transferring virulence and those of FREEMAN [13] on the transduction of toxin production by *C. diphtheriae*, show the possible role of phages in the determination of pathogenicity. The observation of experimentally transduced drug resistance suggests the role of transduction in acquiring resistance under natural conditions.

The technique of transduction is of practical importance, since by transmitting motility it is possible to determine to which types certain stable O forms belong.*

* Since this report was submitted, the author has been able to examine a freshly isolated non-motile Salmonella strain containing somatic antigens 4, 5. Through the action of phage PLT 22, antigen *i* was transduced in 20 per cent, while strains possessing antigens *b* and 1, 2 were obtained in 60 per cent of the experiments. Using the same O strain and phage PE 269 from *S. enteritidis*, transduction of H antigen *gm* was observed in 50 per cent.

It should be emphasized that in nature transduction through phage action is probably rare and in experiments a highly selective method must be employed for obtaining transduced forms. Thus it is obvious that transduction does not decrease the diagnostic value of the Kauffmann-White schema ; on the contrary, it was that schema that has made possible the discovery and investigation of that phenomenon.

Summary

(1) Phage lysates used in transduction experiments were prepared by Seitz-filtration of symbiotic cultures of Salmonellae. Transfer of antigens was performed in U tubes cultures containing semisolid agar and phage lysate.

(2) With LEDERBERG's phage PLT 22 the motility of stable O forms of *S. typhi* and *S. paratyphi B* was produced. Flagellar antigen *i* was transferred from the donor strain to *S. paratyphi B* O form.

(3) With phage lysates from *S. enteritidis* and *S. essen*, flagellar antigen *gm* was transduced to *S. paratyphi B* O form, resulting in an antigenically new recombinant.

(4) To a freshly isolated non-motile untypiable Salmonella strain — containing O antigens 4, 5, — *i* phase in 20 per cent, *b* and *1, 2* phases in 60 per cent were transduced by phage PLT 22. To the same strain phage PE 269 from *S. enteritidis* transduced the *gm* phase.

(5) By phage PLT 22 propagated upon *S. paratyphi A* a hybrid strain corresponding to *S. sendai* phase 1 was produced from *S. enteritidis*.

(6) The recipient strains were lysed by phage PLT 22. The antigenic recombination did not affect the other initial properties of the strains.

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ELECTRONMICROSCOPIC EXAMINATION OF COMPLETE AND INCOMPLETE INFLUENZA VIRUSES

II. STUDIES OF THE FINE STRUCTURE OF COMPLETE INFLUENZA VIRUS

By

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The problem of the fine structure of influenza virus has not been settled. There is no generally accepted hypothesis as to the relation of fine-structural elements to the biological functions of the particle, in spite of the extensive physical, morphological, chemical and biological examinations carried out on complete and incomplete influenza virus particles [1—8]. Earlier studies [9, 10/a—d] by direct visualization without shadowing did not reveal remarkable data on the internal structure of influenza virus. An important progress was the adoption of the methods of chemical and enzymatic pretreatment and ultra-thin sectioning. In particles disintegrated with ethylether, HOYLE *et al.* [11/a, b, c] found globules of different composition and function inside the lipid cover. These intracellular globules develop in a well-defined chronological sequence. VALENTINE and ISAACS [12/a, b] demonstrated in particles belonging to the Myxovirus group by means of combined trypsin and ribonuclease treatment a trypsin-resistant ribonucleoprotein ring in which the RNA filaments were arranged in a spiral form.

The studies carried out on ultra-thin sections of influenza virus were mostly concerned with the role of filamentary forms in virus multiplication. Less attention has been paid to the very structure of the virus [13/a, b, c]. HARFORD *et al.* [14] demonstrated the presence of inclusions, containing elementary bodies lacking any fine structure, in the bronchiolar epithelium of mice. MORGAN *et al.* [15] reported impressive observations on the intracellular development of influenza virus. On the electron micrographs published by these authors a definite structure can be distinguished inside the developing and the fully developed virus particles present on the surface of the host cells. Nevertheless, no evaluation of these structural elements has been given in the text.

The present report deals with examinations of the internal structure of the spherical form of purified complete influenza virus in ultra-thin sections. Some observations on the filamentary forms [10d, 12a, 16] have also been made.

Materials and methods

Virus. The Paris PL 1/49 strain of influenza A virus, kindly supplied by the *World Influenza Centre, London*, was used throughout. The strain was maintained by serial passages in the allantoic sac of 11 days old embryonated hen's eggs. As an inoculum, 0.1 ml of a 10^{-4} virus dilution was used and the infected eggs were incubated for 36 hours. Harvesting was made after appropriate chilling.

Characterization of the virus material. Before preparation for electronmicroscopic examination, the ratio of infectivity and haemagglutinating activity (I/HA) was determined. Infectivity titrations were made by the micro-roller-drum method of HORVÁTH [17]. ID 50 was calculated according to REED and MUENCH [18], haemagglutination titres were determined by the spiral loop method of TAKÁTSY [19]. Was the I/HA ratio characteristic of complete virus ($\geq 10^6$), the suspension was purified and concentrated by the method of TAKÁTSY [20/a, b] and the final product was suspended in saline. The samples thus obtained had usually a haemagglutination titre of about 200 000.

Preparation for electronmicroscopic examination. The purified material was fixed at 4° C by 1 per cent OsO_4 in a phosphate buffer of pH 7.2 according to PALADE [21]. Fixed particles were thrown down by centrifuging for 30 minutes at 3000 rpm. Further treatment was made as described earlier [22]. The dehydrated material was embedded in a mixture of 8 volumes methyl-methacrylate and 2 volumes butyl-methacrylate, and polymerized at 70° C, as suggested by BORYSKO [23]. The ultra-thin sections were made by the ultra-microtome constructed by one of us (A. BARNA), using a glass splinter for cutting. The theoretical thickness of the cuts was about 100 to 170 Ä.

The samples were examined with an EM 3 USSR 1951 type electron microscope readjusted by HOLLÓS and BARNA, with a centerable objective aperture of 40 μ, 60 to 65 KV voltage. Micrographs were taken on AGFA document film, with a primary magnification of 12 000.

Results

Figs. 1 to 3 reveal a great variability of virus particle diameters due to cutting the single particles at different levels. The ellipsoid form of the particles was due to the unilateral distortion caused by the pressure of the knife during cutting.

The border of the particles was blurred, while inside them a more electron-dense lamellary structure was observed.

Some particles revealed an internal structure composed of spots of different electron density, while some appeared as spots of high but homogeneous electron density, and exhibited an internal structure when the exposition time of the ma nification was short. Other particles seemed to contain no electron-dense material. As to the situation of the electron-dense spots within the particles, they were mostly eccentric, while their number and diameter varied. The eccentricity of the electron-dense material was not found to be related to the direction of cutting, as even in a single section there was a great variety of localization.

In the central part of *Fig. 1* there is a single particle that had burst on its right side during the preparation procedures. In contrast to VALENTINE and ISAACS [12/a, b], who often found such particles in their trypsinized preparations, in our material the phenomenon was rare.

Fig. 4 represents part of *Fig. 1* at greater magnification. The internal structure of the elementary bodies is well-visible in this picture. The external

border is indistinct, while inside there is a lamellar internal border separated from the innermost lamella by a less electron-dense layer. The innermost

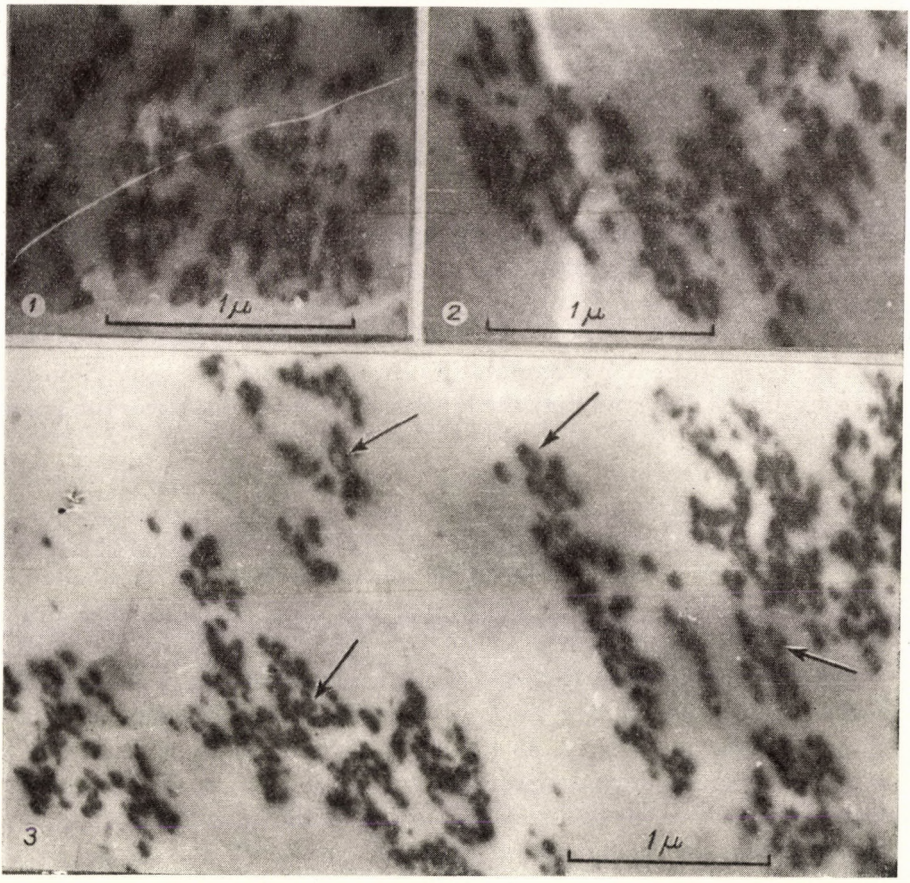

Fig. 1. (Photo 4235.) The white line across the photograph was a defect in the film emulsion. The ellipsoid shape of the particles was due to the pressure caused by the edge of the knife. The remarkable differences in diameter resulted from cutting the single particle at different levels. The elementary bodies are surrounded by an indistinct structureless material. Inside of this, an electron-dense lamella is seen. In certain particles an additional internal lamella, while in some particles near the poles an electron-dense spot was detectable. Magnification, about 33 000

Fig. 2. (Photo 6163.) Same as *Fig. 1.* Magnification, about 36 000

Fig. 3. (Photo 6177.) This picture generally corresponds to *Fig. 1.* The bipolar electron-dense spots in the elementary body shown by the arrow at the bottom on the right side are connected by an undulating fine filament. Magnification, about 26 000

border is best seen in the right upper part of the particle. It appears as of this border consisted of two different lamellae, but the thickness of the supposed separating layer is at the resolution limit of our microscope.

Fig. 5 exhibits the particle shown by the arrow in the left upper part of *Fig. 3*, at greater magnification. The outer loose border is fairly marked and the electron-dense material appears to have concentrated in three different parts of the virus particle. The structure of the outer electron-dense lamella is at the part to which the arrow points. Left downwards, the internal lamella is seen with an apparently double structure. The outer lamella was generally present in each particle, while the internal one could be found only in some parts of a few particles.

Fig. 6 represents a detail of *Fig. 3*, at greater magnification. The particle is shown by the arrow on the upper right side of the picture. On the left side of the electron-dense substance, at the upper pole of the particle, the ending of the internal lamella is visible. The outer lamella runs outside the electron-dense spot.

Fig. 7 presents the particle signed by an arrow on the left side at the bottom of *Fig. 3*, at greater magnification. In this elementary body, 5 electron-dense spots can be observed near the border of the virus. This was the greatest number of electron-dense spots observed in a single particle.

In the particle shown by the arrow on the right side at the bottom in *Fig. 3*, the two electron-dense spots of bipolar localization are connected by a thin line.

As to the order of magnitude of the structural elements described above, some measurements have been performed but their small number did not permit statistical evaluation.

The distance between the inner and the outer lamella was found to be 85 to 100 Å (mean, 95 Å). The thickness of the outer lamella was 60 to 80 Å ; that of the inner, 60 to 85 Å. As to the duplicated nature of the inner and outer lamellae, no reliable proof could be obtained since the resolution of our microscope did not reach the necessary order of magnitude.

The elctron-dense material in complete particles was found to vary in diameter. In shape, it was either circular or ellipsoid. The internal lamella was often found to end in the electron-dense material. This latter seemed to be compact and inside it no structural details were revealed at the resolution of our microscope. Electron-dense material of a loose internal structure was found to occur only in incomplete particles [27].

In the present series of examinations, filamentary particles cut along their longitudinal axis were found only in three instances. One of them is presented in *Fig. 8*. The structure of the right polar electron-dense spot is remarkably looser as compared to the left one or to that of the ordinary complete virus particles. The filamentary particles has only one single border lamella. Nevertheless, the small number of our observations does not permit to draw conclusions as to the structure of the filamentary particle.

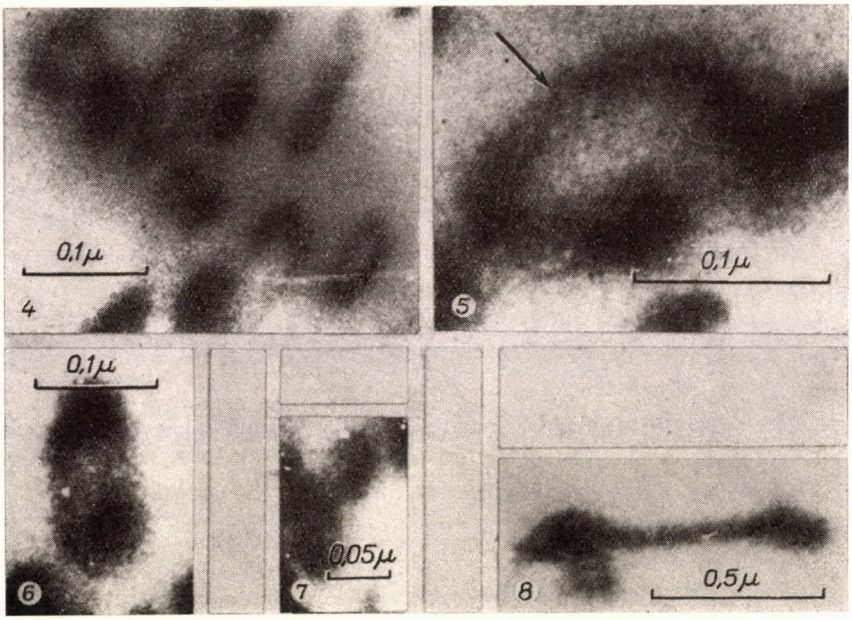

Fig. 4. (Photo 4235.) Detail of *Fig. 1* at greater magnification. In the triangular deformed particle, two lamellae and a less electron-dense separating layer are clearly apparent. Magnification, about 165 000

Fig. 5. (Photo 6177.) The particle shown by the top left arrow in *Fig. 3* is presented at greater magnification. The elementary body is surrounded by a structureless material with indistinct borders. Inside this, a lamellar structure can be observed. At the area to which the arrow points the structure of the outer lamella is clearly seen. The internal lamella is situated to the left, below the other one. The duplicate nature of the internal lamella appears at certain parts of the particle. Electron-dense material occurs in three places in the elementary body. Magnification, about 262 000

Fig. 6. (Photo 6177.) The particle shown by the top right arrow in *Fig. 3* is presented at greater magnification. The elementary body contains bipolarly situated electron-dense material. The upper electron-dense spot is surrounded by the outer lamella, while the inner one appears to end in the electron-dense material itself. Magnification, about 157 000

Fig. 7. (Photo 6177.) The particle shown by bottom left arrow in *Fig. 3* is presented at greater magnification. There are five electron-dense parts near the periphery of the particle. Magnification, about 175 000

Fig. 8. (Photo 39 600.) A filamentary form cut along its longitudinal axis is presented. The filament is surrounded by a single lamella only. There are two bipolarly situated electron-dense parts in the particle. No other structural elements can be seen inside the filament. The electron-dense part left resembles the similar elements of the complete influenza virus particle, while that right is of a remarkably looser structure, than the former. Magnification, about 47 000

Discussion

Except certain phages and the tobacco mosaic virus, the internal microstructure of viruses is scarcely known. Although the electronmicroscopic examination of the larger viruses, *e. g.* pox viruses [24], does not present technical difficulties, no exact data on the fine structure even of these particles

are available. The complicated technique involved in the studying of small viruses, *e. g.* the poliomyelitis virus [25], explains why the ultra-structure of these agents has not been examined.

On the basis of the experimental data available, we venture to present a tentative hypothesis for the fine structure of the influenza virus particles. The results described above are in agreement with the observations of MORGAN *et al.* [15], as to the structureless, poorly defined outermost surrounding area of the elementary bodies. Inside of this cover takes place an external lamella. This external part of the virus particle has been found to be destroyed by ether treatment [11] or to be digested by trypsin after HCl pretreatment [12]. These data suggest this protecting structural element to be of lipoprotein character.

ADAMS and PRINCE [26] described that when Newcastle disease virus had penetrated into Ehrlich ascites tumor cells, not only the cell opened but also the virus lost its protecting lamella.

On the grounds of the pertaining literary data and the electronmicrographs presented in this report, we suggest the following hypothetical structure of the particle. The ribonucleoprotein part of the virus is situated about 84 to 100 Å deeper than the bordering and protecting lipoprotein layer. The ribonucleoprotein component resembles a hollow hemisphere with an undulating circumference. At the edge of the hemisphere there are the ribonucleic acid chains arranged either circularly or in a spiral form. The supposed structure is schematically presented in *Fig. 9* in which the different possibilities of cutting and the schematic presentation of the electronmicroscopic pictures thus obtainable are also shown.

When cutting the hemispherical shell at the level of the ribonucleic acid containing marginal line (a), eccentrically situated electron-dense spots surrounded by an outer lamella will be seen in the electronmicrograph. The more the plane of the cutting coincides with the edge of the hemisphere, the more waves can be demonstrated (b). If the shell is cut above the RNA chain, only the two lamellae are found (c). There is also the possibility to cut the shell diagonally. In this case the electronmicrograph will reveal in the particle a double lamella on one side and an electron-dense spot bordered by a single lamella on the other (a). In certain other cases the shell may be cut away as a whole and only the lipoprotein containing structureless part of the virus will be bordered by the single outer lamella (d). The occurrence of some relatively small particles exhibiting a single central electron-dense core suggests the possible existence of an electron-dense area at the top of the shell (e). This type of cut particles is rarely met with and they always measure less in diameter than those exhibiting double lamellae or bipolarity. We are inclined to suppose that the electron-dense substance present at the top of the hemispherical shell may be the central starting point of the RNA chain.

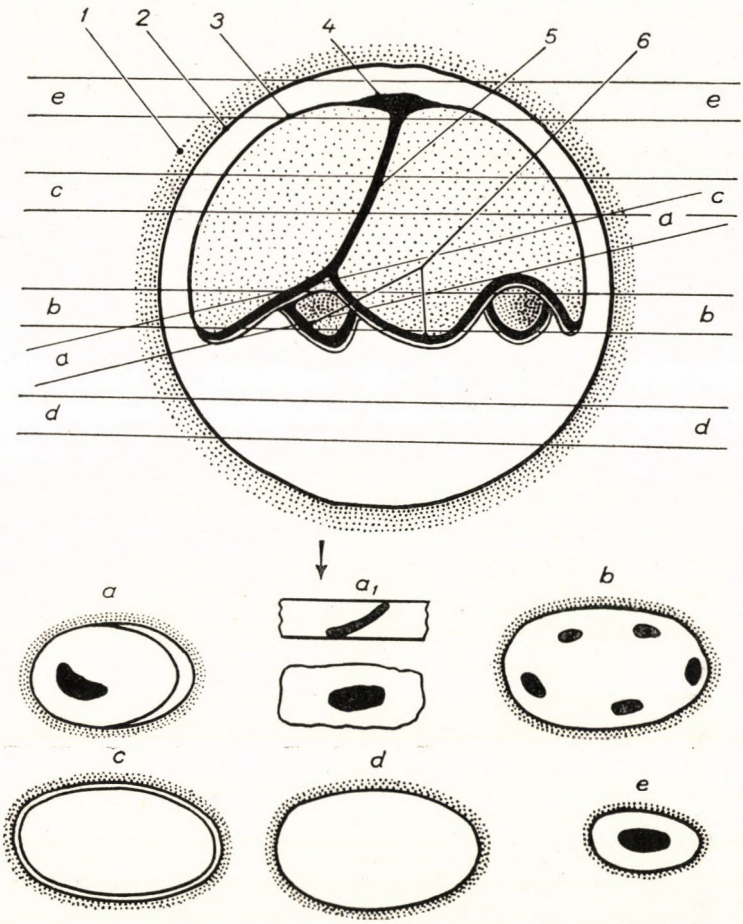

Fig. 9. Schematic presentation of the hypotethic structure of a complete influenza particle, and its possible sections

1. The particle is surrounded by loose material with indistinct borders.
2. The outer bordering lamella, supposedly [11, 12, 26] of lipoprotein character,
3. The ribonucleoprotein hemisphere,
4. The starting point of RNA at the pole of the hemisphere,
5. The RNA filament on the surface of the protein hemisphere,
6. The RNA filament running parallel to the wavy edge of the hemisphere,

a) When cutting the hemispherical shell at the level of the RNA filament, a micrograph exhibiting eccentrically situated electron-dense materia! bordered by the outer lamella is obtained. In certain areas the internal lamella is visible,

a/1. The electron-dense filament is not parallel to the plain of cutting. In the micrograph, the oval shape resulted from overlapping

b) The more parallel to the wavy edge of the hemisphere the cut is made, the greater the number of the wave peaks revealed electron-dense spots. We could demonstrate a maximum of 5 wave peaks

c) When cutting the particle where it contains the ribonucleoprotein hemisphere not containing RNA, the internal structure revealed consists only of the two lamellae,

d) When the elementary body was cut on the part where it contains neither the protein part not the RNA chain of the ribonucleoprotein hemisphere, the electronmicrograph obtained was structureless round or ellipsoid element, bordered only by the outer lamella,

e) We suppose that particles exhibiting a single central electron-dense core represent those cut at the level of the central RNA spot at the pole of the hemisphere. Elementary bodies of this structure measured remarkably less in diameter than those usually met with, and they were very rarely observed on our preparates

By chance we succeeded in cutting the central core and the marginal chain together with the connecting filament in a particle presented in *Fig. 3*, where the two polar spots are connected by an electron-dense filament running across the middle of the particle.

Our hypothesis agrees well with the data both of VALENTINE and ISAACS [12/a, b] and POWELL and POLLARD [28]. The latter authors supposed from the effects of ionizing radiation on the interfering capacity of influenza virus that the latter must be connected either with a spherical shell or a sheet-formed structure.

Our observations of the filamentary are too few to permit discussion. Let it be tentatively noted that the filamentary forms were found to possess only a single bordering lamella. Thus it appears that any part of the filamentary form may be able to adsorb to appropriate cells. The internal structure, *i. e.* the double lamella and an organized electron-dense substance enabling the particle to multiply were observed to occur in certain parts only of the filamentary form. This observation is supported by the data of DONALD and ISAACS [29], who found that ultrasonic irradiation of a virus suspension containing mostly filamentous forms resulted in an increase in the haemagglutinating titre, while the infectivity was unaltered.

Summary

The fine structure of purified complete influenza virus particles has been studied in ultrathin sections.

On the grounds of the pertaining literary data and our electronmicrographs it has been attempted to outline a hypothesis on the inner structure of the complete form of influenza virus.

Acknowledgements. We are indebted to dr. GY. TAKÁTSY *(State Institute of Hygiene, Budapest)* for kindly supplying the purified, concentrated virus material.

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STUDIES ON THE OPTIMUM CONDITIONS OF MEGACIN PRODUCTION IN SYNTHETIC CULTURE MEDIA

By

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Strains producing megacin, the antibacterial principle of *Bacillus megaterium*, commonly occur in soil [1, 2, 3]. The genesis of that antibacterial substance, which belongs to the group of bacteriocins, is in correlation with the lysis of bacterial cells [3, 4]. The massive lysis of the cells of megacinogenic strains and the megacin formation associated with it can be induced by exposing young cultures to ultraviolet light. This phenomenon bears close resemblance to what is often mentioned in the literature as the LWOFF effect and which is due to an induction of the prophage of lysogenic strains.

The conditions of the induction of megacin production in cultures grown in complete media have been elucidated in several details [2, 3, 4]. IVÁNOVICS and ALFÖLDI [4] could not induce megacin formation in a minimum medium, in which the optimum growth of the strain was also unsatisfactory. ALFÖLDI [5, 6] showed that only such synthetic culture media are suitable for the induction of megacin formation which contain sufficient quantities of Mn^{++}.

In order to obtain concentrates suitable for investigating the properties of megacin we made use of cultures grown in synthetic media. For this reason we have studied in detail the optimum conditions of megacin formation in synthetic media.

Materials and methods

Megacinogenic strains. The strains involved in the present study were *Bacillus megaterium* 216 [1, 2] and further 16 megacinogenic strains of *Bacillus megaterium* [3] isolated from soil. The strains were maintained in YDC agar, with passaging at intervals.

Culture media: YDC (yeast extract-casein-digest). For the details, the reader should consult earlier papers from this laboratory [2, 4].

Synthetic A. NH_4Cl, 1.0 g; K_2HPO_4, 0.7 g; KH_2PO_4, 0.3 g; Na_2SO_4 (dry), 0.1 g; glutamic acid, 0.5 g; $MnCl_2 \cdot 4\ H_2O$, M/50 000; $MgSO_4 \cdot 7\ H_2O$, M/5000; $FeSO_4 \cdot 4\ H_2O$, M/100 000: dissolved in 1000 ml of water adjusted to pH 7.2, and autoclaved. Immediately before use, 5 ml of a 20 per cent, autoclaved glucose solution was added to 100 ml of the culture medium [5].

Synthetic M. Citric acid, 2.0 g; $(NH_4)_2HPO_4$, 1.0 g; NH_4Cl, 0.5 g; Na_2SO_4 (dry), 0.4 g; KCl, 0.1 g; $MgSO_4 \cdot 7\ H_2O$, 0.5 g; $MnSO_4$, 0.04 g; $(NH_4)_2MoO_4$, 0.002 g; $ZnSO_4$, 0.02 g; ferriammonium citrate, 0.03 g; $CoNO_3$, 0.004 g; dissolved in 1000 ml of distilled water, adjusted to pH 7.2, and autoclaved. As the source of carbohydrate, 5 ml of a sterile 20 per cent glucose solution was added to 100 ml of the culture medium [10].

Culturing. Twenty ml volumes of nutrient fluid in 100 ml Erlenmeyer flasks were inoculated with 20 hour cultures of the megacinogenic strain grown on YDC agar slope. The inoculum was 0.2 ml of the suspension of washed off bacteria, adjusted to an optical density of 0.400. Aerisation and measuring of the growth of cultures were carried out as described earlier [2, 4]. The temperature was 35° C. The rate of growth is shown in terms of optical density.

Induction. Ten ml volumes of the suitably developed culture were measured into Petri dishes. The about 2 mm thick fluid layer was irradiated with ultraviolet light under constant, gentle shaking, using a low-pressure, 20 W mercury vapour lamp of the "Hanau" type. Irradiation was made from 50 cm distance in the optical axis. As measured on the basis of $T_{2r}+$ phage inactivation by the method of LATARJET [8], the output of the lamp at that distance was 9.1 erg/square mm/sec. of 2537 Å rays.

Titration of megacin. Megacin was titrated by using the phage-resistant B. megaterium (Mutilate-C) strain as an indicator, as already described [4].

Experimental

Experiments with the strain B. megaterium 216

Determination of the optimum dose of irradiation required for induction. Ten ml samples of the cultures of strain *B. megaterium 216* in medium "A" (optical density 0.400; about 3.8 to $4.0 \cdot 10^7$ colony forming units) were irradiated with different doses of ultraviolet light. Subsequently, the samples were carried over one by one into 100 ml Erlenmeyer flasks and were re-incubated at 35° C under gentle shaking. By the end of the experiment (after 8 and $^1/_2$ hours) the cultures were centrifuged and in the supernatant the amount of megacin was estimated. The results are shown in *Fig. 1.*

As seen in *Fig. 1*, the energy delivered in 25 sec, *i. e.* 227.5 erg/sq. mm, proved to be the optimum under the experimental conditions employed, giving rise to the most marked megacin formation and lysis. These experiments showed clearly that the grade of lysis and megacin formation are virtually parallel with each other and induction is achieved with small doses, which cause hardly any primary damage to the viability of cells.

The significate of the age of the bacterium culture with respect to lysis and megacin formation. The age of the irradiated culture is of considerable significance, as far as the optimum megacin yield is concerned. In the earliest log phase the cultures were lysed gradually after the optimum irradiation, but the megacin yield was low. When the cultures were irradiated at the end of the log phase, there was no appreciable lysis and the megacin yield was very low. The optimum conditions occurred during approximately the first third of the log phase, in the range of 0.250 to 0.600 o. d., equivalent to $4.0 \cdot 10^7$ to $1.3 \cdot 10^8$ bacterium cells per ml (*Fig. 2*).

In such young cultures megacin production was significant even when lysis was only partial (*Fig. 3*).

In spite of the high end values for optical density, the changes of cytological nature introducing megacin production or lysis occurred in most cells

[IVÁNOVICS and ALFÖLDI]. The induction of cells was confirmed by the fact that on explanting the cells of such cultures together with the suspension of the indicator bacterium strain into soft agar, exclusively such empty plaques were obtained as are characteristic of induced megacinogenic cells [7].

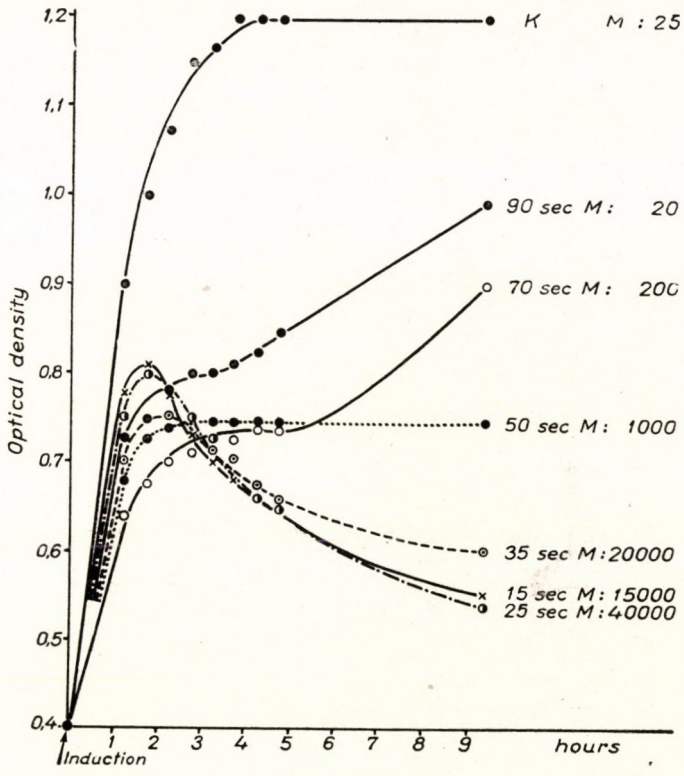

Fig. 1. Megacin production by strain B. megaterium 216, in response to irradiation with different doses of ultraviolet light

Irradiation with UV light marks the beginning of the experiment (induction). The data for the single curves are: the duration of irradiation and the reciprocal value of the titre of megacin formed (K = non-irradiated control culture)

The observations made in culture medium M were in satisfactory agreement with the above results. Medium M differs in composition from medium A. The traces of Co and Zn and the complex-forming citrate had no influence on either lysis or megacin formation. For this reason, both culture media appear to be suitable for the production of megacin in synthetic medium.

Experiments with other megacinogenic strains

Similar experiments were performed with further 16 *B. megaterium* strains, which were all well inducible in complete culture media.

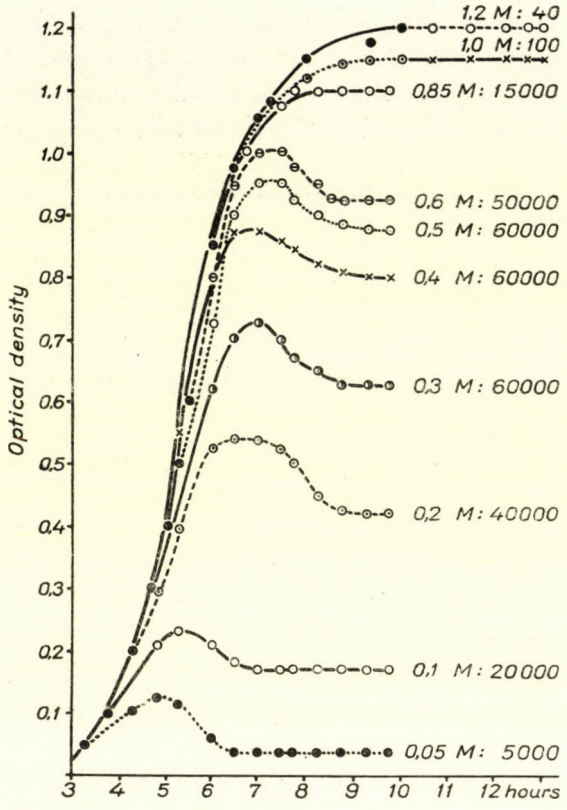

Fig. 2. Megacin production by strain *B. megaterium* 216, following the irradiation of cultures of different ages with the same dose (227.5 erg/sq. mm) of UV rays
The abscissa shows the age (development) of culture at the time of irradiation

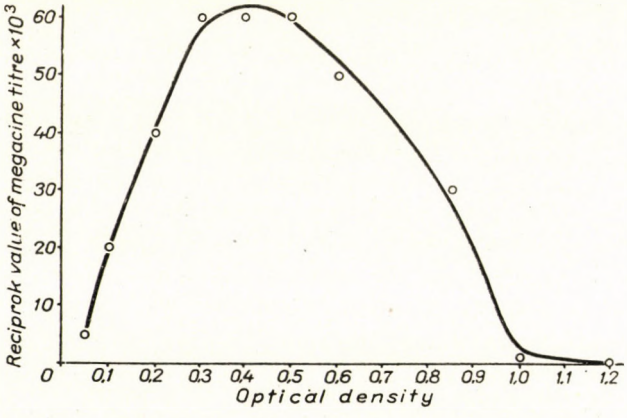

Fig. 3. Lysis and megacin production by strain *B. megaterium* 216. Cultures of different ages irradiated with the same dose (227.5 erg/sq. mm)
The values for the single curves are : development of culture in o. d. at the time of induction and the reciprocal value of the megacin titre

In spite of the identical experimental conditions, the megacin yield was very low in these cultures. The maximum megacin titre was 1 : 1000. Nevertheless, after ultraviolet irradiation there was practically no difference in behaviour between these strains and *B. megaterium 216*. Residual growth was followed by different grades of lysis. Megacin production was identical in both culture media, thus it appears that conditions other than external are responsible for the differences mentioned. Changes in the age of cultures and in the dose of ultraviolet light had practically no influence on the yield of megacin. These observations are in agreement with those made earlier in complete culture media [3]. It appears that strain 216 of *B. megaterium* has a special megacinogenic activity, which surpasses that of all the strains we had selected from 200 strains of *B. megaterium* on the basis of megacinogenic property [3].

Discussion

The successful induction of lysogenic bacterium cultures is known to require a certain optimum amount of energy [9]. Likewise, the induction of megacin production is greatly influenced by the dose of ultraviolet irradiation. In the present experiments, in synthetic media the dose of energy required was found to be 227.5 erg/square mm, about half of the amount required in complete media (unpublished experimental data). It should be borne in mind, however, that complete culture media absorb more of the ultraviolet rays and this was apparently responsible for the above difference. The ultraviolet energy required for the induction of lysogenic strains varies from culture medium to culture medium [9].

Megacin production is influenced by the age of the irradiated culture. A significant yield is expectable only when after irradiation the culture is continuing to grow satisfactorily. This is due to the fact that megacin production is introduced by a suitable rearrangement of the cellular substance, taking place during 2 or 3 periods of division and growth, respectively [IVÁNOVICS and ALFÖLDI].

Strain 216, which has a very high megacin yield, occupies a special position among the 17 strains of *B. megaterium* tested. Although they all show induction and lysis, the maximum antibacterial titre of the cultures of the other strains varies from 1 : 100 to 1 : 1000, as compared with the 1 : 40 000 to 1 : 60 000 titres obtained for strain 216.

Summary

In megacinogenic bacterium cultures, optimum megacin production in synthetic media can be induced by small doses of ultraviolet light. Only sufficiently developed young cultures produce high yields of megacin in response to such irradiation. The megacinogenic strains vary in regard to the amount of megacin produced. The greatest amounts were yielded by strain *B. megaterium* 216.

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AN EFFECTIVE METHOD FOR ISOLATION OF ANTHRAX PHAGES

By

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Information concerning phages active against *B. anthracis* is still scarce. Most of the authors failed to demonstrate the presence of any transferable agent in the transparent plaque-like changes frequently observable in aged cultures of *B. anthracis*. This problem will not be discussed in the present report; for details we refer the reader to Sobernheim's monograph [1].

The isolation of an anthrax phage strain from sewage was reported by Cowles [2] in 1931, but no further characterization of the agent was given. Concerning anthrax phages, the studies of McCloy [3] are most remarkable. She isolated a highly specific anthrax phage from a lysogenic strain of *B. cereus*. This strain designated as *B. cereus W* was found to release several different, but genetically closely related, temperate phage mutants, each of which have been adequately characterized. The anthrax phage isolated by McCloy is so specific that it was successfully used for identifying the bacterium [5]. Using the same phage strain, Iványovics and Földes [6] succeeded in differentiating *B. anthracis* from "pseudo-anthrax" *(B. cereus)* strains.

The investigations made by one of us (Iványovics, to be published) with McCloy's temperate phage necessitated the isolation of some further anthrax phage strains for comparative studies. As *B. cereus* is known to be the most common mesophilic, spore bearing bacillus in the soil, the isolation of lysogenic strains similar to the phage W has been attempted. To obtain the temperate phages directly from the soil the following method was applied.

Samples of cultivated soil suspended in broth were incubated at 37° C for a period of a few hours, presumably sufficient for spore germination and for a moderate number of initial cell divisions resulting in the release of phages from the eventually present lysogenic bacteria. Selective enrichment of the small amount of phage thus obtained can be achieved by suppressing the growth of concomitants by streptomycin and by using streptomycin-resistant host cells to support phage multiplication, this process being undisturbed by the presence of the drug.

This procedure makes a filtration of the primary cultures superfluous. The isolation of phages is further facilitated by seeding the culture onto a

streptomycin containing indicator plate. Pure lines can be obtained by re-isolation from the plaques obtained on the indicator plate.

The method has proved successful for the isolation of anthrax phages. A total of 13 soil samples has been examined ; 10 supplied 1 or more phage strains.

Materials and methods

Bacterium strains. B. anthracis strain DAVIS was used as an indicator organism. (The original strain and its streptomycin-resistant variant were kindly supplied by Dr. ELINOR W. MCCLOY.) The identification of *B. anthracis* and *B. cereus* strains used has been described earlier [6]. The identity of these strains was doubtlessly stated. Strains of *B. megaterium* were isolated in this laboratory [7].

Media. Yeast extract pepton fluid medium (YP) was prepared, as described earlier [8]. When solid medium was required, 1.5 per cent agar-agar was added.

Preparation of indicator plates. To each ml of melted YP agar 25 μg of streptomycin were added and made up to plates in Petri dishes. Suspensions of streptomycin resistant *B. anthracis* DAVIS cells were prepared by an appropriate suspending fluid from 16 hours old agar slant cultures. The optical density of the suspension was adjusted to 0.2 to 0.3 (tube diameter, 16 mm). Following the addition of 50 μg/ml of streptomycin, 1 ml of the suspension was added to an equal amount of melted and cooled (60° C) agar medium and the mixture was spread on the surface of a streptomycin containing agar plate.

Indicator plates containing the different other strains were prepared similarly, except for the addition of streptomycin.

Phage titration. Plaque counting was performed by the usual method in a soft agar layer. Titration of lysates was performed by spreading one drop of a decimal dilution series each onto the surface of separate indicator plates.

Diluent. As a diluent, a mixture of 1 volume YP broth and 4 volumes of saline was used.

Isolation of phage strains. Four to five g of cultivated soil was suspended in 15 to 20 ml of tap water and allowed to stand for 2 days at room temperature. This was followed by vigorous shaking. After allowing the shaken suspension to settle for half an hour, 5 ml of the supernatant was added to 5 ml YP broth and incubated at 37° C for 4 hours under gentle rocking. This was followed by the addition of 50 μg of streptomycin to each ml of the mixture and by the inoculation of 5 ml young *B. anthracis* DAVIS culture containing the same amount of streptomycin per ml. After 6 or more hours of re-incubation, loopful samples of the culture were seeded onto the surface of separate streptomycin containing indicator plates. The plates were incubated at 37° C. After appropriate incubation the appearence of isolated plaques, uniform or different, could be observed. More effective isolation was achieved if different dilutions of the primary cultures were added to the indicator cell suspension and the mixture was made up to a soft agar overlay containing streptomycin. Phage strains obtained from the plaques were transferred to cultures of streptomycin resistant Davis cell cultures for further subculturing. Plaque countings were carried out in the lysates and the selected plaques were re-isolated.

Results

Morphology of plaques isolated. According to plaque morphology, several phages were usually differentiated in the different soil samples tested. Eight strains isolated from 4 soil samples were examined more precisely. Their plaque morphology, when cultivated on *B. anthracis* DAVIS, was as follows,

cs (clear, small) : clear, sharply marginated plaques 1 to 3 mm in diameter (see *Fig. 1*).

cg (clear, great) : clear plaques 3 to 4 mm in diameter. Plaques of that type were observed not only in primary isolations but appeared also as variants on later transfers in Davis host cells (see *Fig. 2*).

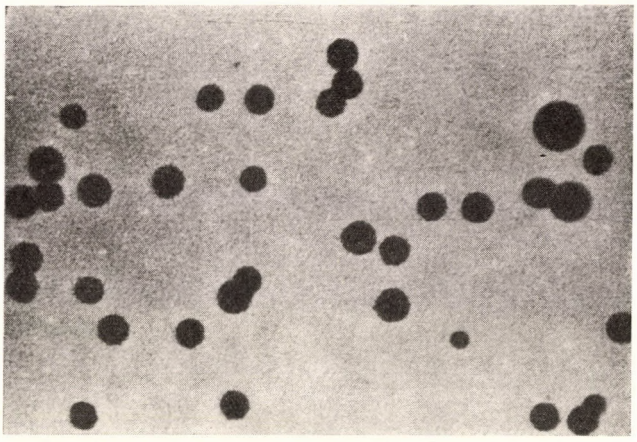

Fig. 1. Clear small plaques (from soil sample 26)

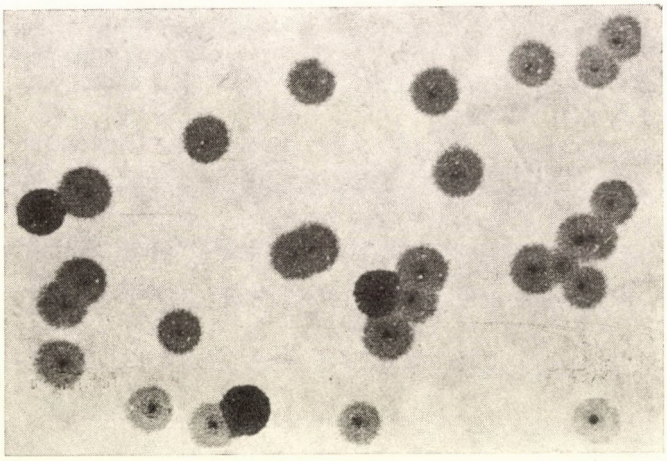

Fig. 2. Target like and clear great plaques (from soil sample 26)

tl (target-like) : plaques 3 to 4 mm in diameter, with a clear central area surrounded by a wide, homogeneously turbid halo. In subcultures of these plaques 1 to 5 per cent *cg* mutants appeared (see *Fig. 2*).

cr (clear center with peripheral ring). Clear plaque 1 mm in diameter, with no cleared up surroundings. A ring-like loosening was observed a few

millimetres from the centre of the plaques (see *Fig. 3*). Plaques of similar appearance occurred in some subcultures of *cg* plaques (see *Fig. 4*). The mutants could have been subcultured in pure lines (see *Fig. 3*).

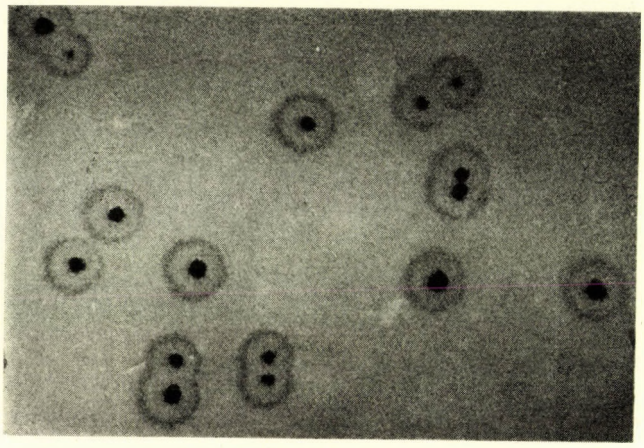

Fig. 3. Clear plaques with peripheral ring (from soil sample 26)

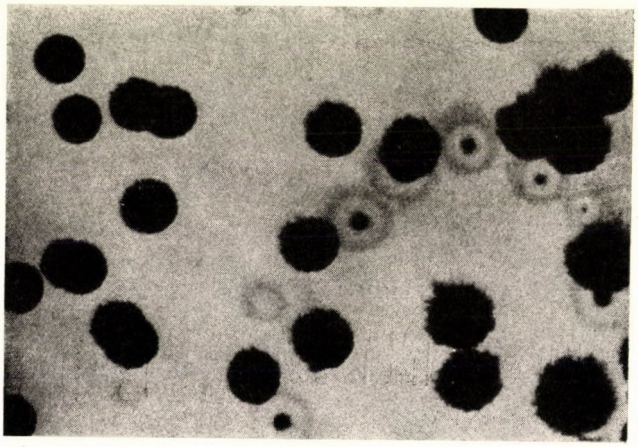

Fig. 4. Mutation of clear plaques with peripheral ring into great clear plaques (from soil sample 29)

ts (turbid, small) : homogeneously turbid plaques 1 to 3 mm in diameter. In subcultures usually, *cs* mutants appeared.

Host range of the phages isolated. Of four different soil samples eight phage materials were prepared on the basis of morphological differences with in the *B. anthracis* DAVIS host cells. Part of the phage materials contained

two kinds of particle of different plaque morphology, as a result of mutation occurring during cultivation. The phage materials contained 10^7 and 10^{10} particles per ml. The results obtained on inoculating indicator plates with 0.02 ml each of a decimal dilution series of the phage materials are presented in Table I.

Table I

Sensitivity of Bacillus anthracis strains to phage-materials

B. anthracis strain	Phage materials and their plaque forms							
	26 *cs*	26 *tl+cg*	26 *cr*	29 *cg+cr*	27 *cr*	27 *ts+cs*	28 *cg*	28 *tl+cg*
No. 68	5	5	4	5	5	5	6	6
No. 69	5	5	4	5	5	4	6	6
No. 71	5	5	4	5	5	6	6	6
No. 77	5	5	4	5	5	6	6	6
No. 89	5	5	4	5	4	5	6	6
No. 100	6	6	4	7	5	4	6	5
XWS	5	5	4	7	1	1	5	5
NPA R	5	7	4	7	4	4	5	5
Vollum R	6	6	4	7	3	4	6	6
Weybridge	4	4	0	5	0	0	3	0

Note: The figures represent the reciprocal values of the logarithm of the dilution (*e.g.* 6 = 10^{-6} titre); 0 indicates that the stock lysate was ineffective

As Table I reveals, the strains *B. anthracis* Weybridge and XWS (from our own collection) did not exhibit the same sensitivity against each of the phage strains, and the XWS strain was generally less sensitive against each of the phage materials.

Table II

Number of Bacillus cereus strains sensitive to different phage materials

Number of strains examined	Number of strains sensitive to different phages							
	26 *cs*	26 *tl+cg*	26 *cr*	29 *cg+cr*	27 *cr*	27 *ts+cs*	28 *cg*	28 *tl+cg*
16	8	9	4	5	1	4	5	2

The same phage materials were found to have a slight or no effect against the 15 *B. cereus* strains tested. As presented in Table II, part of the strains were absolutely resistant to certain phage samples; in these cases lysis did not result even on the addition of undiluted phage material. Strains designated in Table II as sensitive formed plaques only on the addition of a great number

of phage particles : lysis was observed only in areas treated with undiluted material or with a dilution of at most 1 to 100.

The plaque morphology of the different *B. cereus* strains used as host cells will not be dealt with. Suffice it to mention that their plaques were usually less clear than those of the anthrax strains.

The specificity of the phages isolated has been further supported by tests made on some other bacteria. All of the 8 phage materials were ineffective on the following bacteria. (The figures in brackets represent number of strains tested.) *B. subtilis* (8) ; *B. megaterium* (10) ; *Sarcina flava* (1) ; *Diplococcus pneumoniae* (3) ; *Staphylococcus aureus* (4) ; different *Salmonellae* (4) and *Shigellae* (2) ; *Pseudomonas aeruginosa* (5).

Conclusions

The method presented above has proved suitable for isolating anthrax phage strains from different soil samples. All the samples were collected in *Szeged*, but the frequent occurrence of anthrax phages suggests their ubiquitous character. The phages isolated are most probably temperate ones and inclined to develop virulent or semi-temperate mutants. The activity spectrum of our phage strains is very similar to that of the phage isolated by McCLOY [3] from *B. cereus* strain W. Further studies are in progress in our laboratory to demonstrate whether or not the strains isolated by our method were different as to antigenicity and other characteristics.

Summary

Phage strains highly specific to *B. anthracis* were readily isolated from soil samples without any filtration, by using a streptomycin-resistant *B. anthracis* strain as host cell. The method seems to be suitable for isolating other phages.

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THE USE OF CARBON TETRACHLORIDE IN THE PRODUCTION OF COX-TYPE TYPHUS VACCINE

By

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The observations of COX [1] on the growth of typhus Rickettsiae in the yolk-sac of embryonated hen eggs afforded a new way for the large-scale production of typhus vaccine. The original type of this vaccine similarly described by COX [2], was later modified by several authors [3, 4].

The present report deals with the application of CCl_4 to large scale typhus vaccine production. The use of CCl_4 was found to simplify the procedure and to yield a final product of satisfactory purity.

Materials and methods

Rickettsia strain. The Hungarian strain U/43 of *Rickettsia prowazeki* isolated in the guinea pig in 1943 [4] and maintained since that time by serial yolk-sac passages was used.

Preparation of the infected yolk-sac suspension was performed according to the method of FARKAS (cited by DREGUSS and FARKAS [4]), with the only modification that the yolk-sacs were homogenized in a Turmix-type homogenizer, with the addition of an equal volume of saline containing 2.5 per cent phenol.

Carbon tetrachloride (CCl_4). The solvent used was of analytical purity, produced by the *Chinoin Pharmaceutical Works, Budapest.*

Testing of immunogenic effect was made according to the official Hungarian prescription for testing commercial typhus vaccine in guinea pigs. Groups of 5 male animals weighing about 250 g were inoculated 3 times, 1 ml each with 1 to 20, 1 to 40, 1 to 80, 1 to 160, and 1 to 320 dilutions of control or test vaccine, respectively. 3 weeks after the last injection each animal was challenged by 1 ml of a 1 : 200 suspension in saline of Rickettsia infected guinea pig brain. Unvaccinated animals infected simultaneously with the same material served as controls. The rectal temperature of the animals was registered daily for 3 weeks.

Complement fixation test. This was performed according to the micromethod of TAKÁTSY [5], using 1.25 per cent sensitized sheep erythrocyte suspension, 4 units of guinea pig antibody and 2 units of complement.

Nitrogen determination. The PREGL modification of the KJELDAHL method was used.

Determination of CCl_4 was made by the method of FUYIWARA [6].

Experimental

Extraction of antigen from phenolized yolk-sac suspensions in the presence of various amounts of CCl_4. To samples of phenolized suspensions of *R. prowazeki* infected yolk-sac homogenisates, prepared as described above, different proportions by weight of CCl_4 were added. After thorough mixing the suspension was diluted by adding 4 volumes of saline containing 1.0 per cent phenol.

The mixture was homogenized by vigorous shaking. The milky material thus obtained was centrifuged for 20 minutes at 3000 r. p. m. As a result of centrifugation, the suspension separated into 3 layers : a supernatant slightly opalescent aqueous phase containing the antigen ; a creamy layer of debris ; and the intensively yellow coloured CCl_4 at the bottom. The top layer was carefully collected. The two other layers were homogenized again and extraction was repeated, using the same amount of saline with 0.5 per cent phenol. As 72 per cent of the total complement fixing antigen content was yielded by the first, and 22 per cent by the second extraction, no further extractions were usually made. After being tested for antigen content by complement fixation, the two extracts were pooled. The minimum antigen content of the two extracts was required to guarantee a minimum of 1 : 64 complement fixing titre in the final product. As the phenol content of the final product was usually about 0.5 to 0.7 per cent, no further preservatives were added. After being appropriately tested for sterility, safety and immunogenic effect, the vaccine was ready for use.

As a reference standard for the evaluation of immunogenic effect of our preparation, a control vaccine was simultaneously prepared by the method of FARKAS [cf. 4], using the same lot of infected yolk-sac suspensions.

Effects of different amounts of CCl_4 on the complement fixing antigen and the dry substance content as compared to the control vaccine, are presented in *Table I.*

Table I

Comparison of CCl_4 treated and control vaccines

CCl_4 per cent	Cf antigen titre					Dry substance mg/ml	Decrease in dry substance per cent
	4	8	16	32	64		
10.....	4*	4	4	4	3	12.3	24
25.....	4	4	4	4	—	12.0	26
50.....	4	4	4	1	—	10.4	36
Control	4	4	4	4	—	16.2	—

* The figures represent the grade of positive fixation

As *Table I* reveals, the complement fixing antigen content of the material was not remarkably affected by treatment with 10 to 25 volume per cent of CCl_4. Moreover, the slight increase of antigenicity observed in samples extracted in the presence of 10 per cent CCl_4 was regularly present. Concentrations higher than those mentioned above appeared to cause damage to the antigen. The dry substance content was the lower, the higher concentra-

tion of CCl_4 was applied during extraction. Concentrations of CCl_4 less than 10 per cent were found to be less effective in lowering the dry substance content. No remarkable differences were detected in the N content of extracts obtained by using 10 to 50 per cent CCl_4. Thus, vaccines for further studies were always prepared by using 10 per cent CCl_4.

It being known that ethyl-ether treatment causes the release of soluble antigen from the surface of Rickettsiae [7], experiments were carried out to establish whether CCl_4 had any similar effect. Twenty ml samples of the control and the CCl_4-treated vaccine each were centrifuged at 8000 r. p. m. for 1 hour. The complement-fixing antigen content of both the original material and the supernatant was determined without any further treatment. The sediment was made up to the original volume by saline and the complement-fixing antigen content of the suspension was determined.

The soluble antigen content of both the CCl_4-treated and the control vaccine (the latter not involving any treatment with solvent) was equally found to be at least 87 per cent. Thus CCl_4 treatment did not seem to cause remarkably more soluble antigen to be released than treatment with phenol [4]. The soluble antigen content of our preparations is very similar to that of certain ether-treated commercial typhus vaccines prepared abroad [8].

Determination of the immunogenic effect of CCl_4 treated typhus vaccines. This was performed according to the method described above. A typical experiment out of the several determinations performed is presented in *Table II.*

Table II

Immunogenic effect in guinea pigs

Vaccine	Undiluted	Vaccine dilution inoculated				
		1 : 20	1 : 40	1 : 80	1 : 160	1 : 320
10 per cent CCl_4	0/5*	0/5	0/5	0/5	0/5	0/5
Control	0/4	0/5	1/5	0/5	2/5	3/5
Unvaccinated animals		8/8				

* Numerator of the fractions designs number of animals with fever reaction, the denominator of those inoculated

From the data of *Table II* the conclusion can be drawn that the immunogenic activity of the CCl_4-treated vaccine was somewhat superior to that of the control vaccine in common use in Hungary.

Testing the final product for CCl_4 content. The sensitivity of the FUYIWARA test [6] permits the demonstration of 1 mg CCl_4/ml vaccine. None of our preparates tested did ever contain an amount of CCl_4 demonstrable by this method. This is readily explained by the poor solubility in water of the solvent at room

temperature (maximum, 0.8 mg/ml) and by its high affinity to the lipids abundantly present in the yolk-sac suspension.

Testing the final product for possible untoward effect in animals. As our procedure makes use of a solvent which has not been employed for a similar purpose, it seemed necessary to test the product for its possible untoward effects. The tests were performed in guinea pigs and mice, as follows. Two groups of guinea pigs, of five animals each, were used. In the first group each of these animals was given 5 ml of the vaccine subcutaneously, while the animals in the second group received the same dose intraperitoneally. When calculated by kg body weight, this amount represents about 1000 times the human dose. All the animals remained well and normal and gained weight during the period of observation. Two groups of mice, 10 animals each, were given 1 ml vaccine per animal intraperitoneally and subcutaneously, respectively. This 1 ml represents about 4000 times the human dose when calculated per kg body weight. No untoward effects whatever were observed during the 14 days' period of observation.

It could, accordingly, be stated that no untoward effects are to be expected when giving the vaccine to humans. Experimental vaccinations of volunteers did in fact never reveal any undesirable effects.

On the basis of our experiments and of the official trial, the procedure has been accepted and authorized for production of typhus vaccine in Hungary.

Discussion and summary

The use of CCl_4 in the extraction and purification of infected yolk-sac suspensions for the production of typhus vaccine seems to be justified on the following grounds.

The procedure is remarkably simplified.

The vaccine, prepared by extraction with a saline solution containing phenol in the presence of 10 per cent by volume of CCl_4, contains 24 per cent less dry substance than the vaccine previously used in Hungary.

No residual CCl_4 was demonstrated in the final product by FUYIWARA's test (sensitivity, 1 mg CCl_4 in 1 ml vaccine).

The antigenicity of the vaccine prepared by the CCl_4 method was found satisfactory when tested in guinea pigs.

No untoward effects were observed after the vaccination of humans, or on injecting 1000 times and 4000 times the human dose by kg body weight to guinea pigs and mice, respectively.

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A QUANTITATIVE SEMI-MICRO TISSUE CULTURE METHOD AND ITS USE IN MICROBIOLOGY

I. QUANTITATIVE SEMI-MICRO TISSUE CULTURE METHOD

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Different quantitative tissue culture methods have been described, each more or less expressive of the amount of fibroblasts growing out from the explanted fragments of tissue [1—11]. Though based on different principles, each of them has been found to fail in practice, for none ensured quantitatively exact routine work. They have been conspicuous for the following major deficiencies: (i) failure of the curves illustrating cellular multiplication in dependence on time to express the true dynamics of fibroblastic multiplication; (ii) failure to eliminate the differences in growth due to differences in the size of explants; (iii) comparatively few parallel tests have been made by the authors. Although fibroblasts grown out from individual pieces of tissue have sometimes been observed and characterized with great accuracy, what has actually been described was nevertheless not "fibroblastic multiplication" but the multiplication of the fibroblasts of one explant.

The present report will deal with our attempts at the elaboration of a semi-micro quantitative tissue culture method based on the statistical evaluation of nearly 300 000 data obtained from more than 50 000 tissue explants.

Materials and methods

1. *The apparatus.* Rolling drums of the type applied in measuring the infectivity of influenza virus, and described earlier [12], were used in these experiments, each drum holding 100 of 2-ml ampoules. Lately, in tissue culture experiments we have given preference to short-necked 2-ml vials because they are easier to handle and their use reduces to a minimum the risk of aerial contamination *(Fig. 1)*.

Working under glass, each drum was placed vertically on an aluminium drum stand. This was essential for two reasons: first, it almost completely precluded fungal infection; secondly, an adequate amount of culture fluid added to the explants in the tubes in horizontal position prevented the tissues from desiccation during the explantation of tissue fragments.

With the plasma bed prepared, the ampoules were placed on a heat-sterilized slanting aluminium plasma-tube stand whence, one after the other, they always rolled to the same place within easy reach.

A platinoid loop set in a short aluminium haft was used to smear the plasma and the embryo extract (EE).

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2. *Cleaning and sterilizing the glass vessels.* After use, the 2-ml vials were rinsed with tap water and allowed to stand for one day in chromous sulphuric acid. After subsequent rinsing, the vials were left to stand for one day in tap water, then two days in distilled water, dried, placed in drums and, before use, sterilized in dry heat. The other glass vessels were cleaned and sterilized in a similar manner.

3. *Obtaining plasma.* Plasma was obtained from cocks starved for one day, by adding to 10 ml of their blood 0.1 ml of 0.1 per cent heparin dissolved in physiological saline. Following centrifugation with 500 *g* for 10 minutes the plasma was removed by suction, and after the addition of 100 μg/ml of both streptomycin and penicillin it was distributed into ampoules at the rate of 1 or 2 ml and stored in the frozen state at —20° C. Prior to using it, possibly formed insoluble materials were separated by centrifugation and the pure plasma was kept in small-sized agglutination tubes.

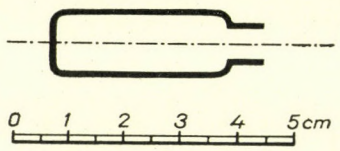

Fig. 1. Cross-section of glass vessels in present use

4. *Preparation of embryonic extract.* EE was prepared from 10-day-old chick embryos. Following decapitation and removal of the abdominal organs, the embryos were washed with culture fluid, weighed, and after the addition of 2 parts of liquid nutrient for 1 part of embryo, homogenized in a Waring blendor. To the extract thus obtained, 100 μg of both streptomycin and penicillin were added, for each ml. The mixture was allowed to stand for one hour at room temperature, and thereafter frozen. Next day, after thawing it, the material was centrifuged with 800 *g* for 10 minutes, distributed into ampoules at the rate of 1 or 2 ml, and then stored. Before use, the possibly precipitated parts were separated by centrifugation, and the pure liquid was poured into small-sized agglutination tubes.

Plasma or EE left over in ampoules unsealed was discarded.

5. *Preparation of solutions.* From the chemicals of analytical purity, concentrated solutions of a composition described below, were prepared in bisdistilled water, distributed into ampoules sterilized at 105° C, and stored at room temperature. Before use, the liquid removed from the ampoule was diluted to the amount required with sterile bisdistilled water. Thereafter 20 μg of each streptomycin and penicillin were added for 1 ml of the culture fluid.

6. *Obtaining pieces of chicken heart.* Chicken hearts were obtained from 12 to 13 day-old embryonated eggs, incubated at 38,5° C ; the large blood vessels were cut off completely : the hearts were placed in a Petri dish containing a little culture fluid and, using sharp scissors, cut into pieces of the desired size : these were then washed two or three times with a buffered salt solution to be described later in this communication.

7. *Cleaning and sterilizing rubber stoppers.* Unused rubber stoppers were boiled first for 30 minutes in *n* HCl, and after washing out the acid, for another 30 minutes in *n* NaOH. After washing out the alkali, the stoppers were kept in ethanol for a week. From time to time, the alcohol was replaced. The rubber stoppers were thereafter carefully washed with distilled water. Before use, to ensure sterility, the stoppers were once more boiled, this time in a buffered salt solution contained in a glass vessel. Even after such scrupulous preliminary treatment the stoppers are of no avail for tubes in which the liquid comes into contact with them : no toxic effect might be observed in the first few days, yet later the cells in culture will nevertheless be found to degenerate.

I. Measuring the dynamics of tissue proliferation

1. *The principle of the method.* If we were to obtain a reliable idea of the dynamics of fibroblastic multiplication, we would have to restrict our observation to the multiplication of a single explanted fibroblast at a time. We would then find that the divided cells grew in a circle around the explanted cell. The variations of the circular area in dependence on time would yield

a multiplication curve which might be regarded as a reliable indication of the dynamics of fibroblastic growth. This was the principle we attempted to apply, and to do so on the strength of the following consideration. When a piece of tissue is placed on a plasma bed, fibroblastic growth begins on the surface of contact between it and the plasma coagulum. The cells along the line of adhesion grow radially to roughly the same length, wherefore the growth ring around the explant is of fairly the same width. Assumedly, for their width the growth rings are not considerably dependent on the size of the tissue ; so if the increments in width are measured from the edge of the explant, *i. e.* if the tissue diameter is not considered, then the average of the measurements may be regarded as a radius with which to compute the area of a circle ; this area, it can be claimed, is then characterizing the dynamics of the multiplication of fibroblasts growing out from the pieces of tissue. In this manner, what we arrive at is not the actual number of fibroblasts grown, or some value proportionate to it, but a value which in some degree is independent of the size of the tissue. Later in this report it will be shown that by this means the differences in growth which are consequent upon differences in tissue size, can to a large extent be reduced ; were we to add up the fibroblasts actually grown around an explant, the values obtained would much more substantially deviate, in dependence on the size of the explant.

Denoting the radius of the explant with r, and that measured after fibroblastic multiplication with R, the formula $(R - r)^2 \pi$ will underlie the computations.

In practice, provided an adequate number of parallels is examined, cellular proliferation is determined as follows. Using an ocular micrometer magnifying from 20 to 30 times, the growth increments are measured from the edge of the explants, and recorded. All the measurements are taken on the same side of the explants, irrespective of whether the other side shows a greater or lesser rate of growth. Taking the arithmetical mean of all recorded values to give the radius of a circle, the area of that circle is computed which, ultimately, expresses fibroblastic multiplication in sq. mm.

2. *Preparing tissue cultures.* In the experiments, HANKS' solution [13] had been used until, with the aid of the method, optimal concentrations were established for each substance.

Tissue was explanted under glass *(Fig. 2)*. The rolling drums were placed on the drum stand ; their lids were removed ; using forceps, three ampoules, one after the other, were taken out. Thereafter, with Pasteur pipettes first plasma, then EE, (about 0.01 ml of each) were placed into the tubes, one next to the other, and were smeared with a sterilized loop over one side of the vials. The tubes were then placed on the plasma-tube stand. Fifteen to 20 plasma tubes were prepared at a time. Since only one side of the ampoules had been coated, there arose a layer about 0.1 mm thick. There was no need

to mark the site of the plasma bed, for if the ampoules were held appropriately the refraction of light was distinctly visible. Using a Pasteur pipette, with its end bent and so constricted as to prevent the tissues from reaching the capillary part, the tissue pieces were put on one by one. In each ampoule 4 pieces of tissue, about 1 mm in diameter, were placed next to one another. The tissues were put on the surface of the plasma bed in a manner which ensured that the third of the ampoules nearest their orifice remained unoc-

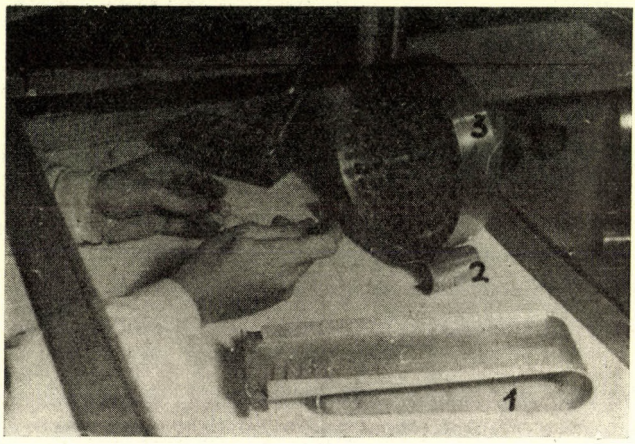

Fig. 2. Explanting pieces of tissue
1. Plasma tube stand. 2. Drum stand. 3. Rolling drum

cupied. Such tissue cultures were put back into the drums, taking care that the pieces of tissue invariably occupy the bottom part. Using a 5-ml pipette, 0.5 ml of culture liquid were measured into the tubes. Then the other tissue cultures were prepared. The tissues were never left without culture fluid for more than 10 or 15 minutes, as drying up had been found greatly to reduce the cells' tendency to multiply. To prepare a drum with 400 explants took about 80 working minutes.

When the tissue cultures in one drum were ready, the drums were mounted on a shaft driven by a gramophone motor to make 5 or 6 revolutions per hour. Each motor rotated three shafts with, in all, 21 drums on them. The same apparatus is used by us for titrating influenza virus [12].

Tissue growth was controlled from time to time with the use of an ocular micrometer. Reading the growth increments of 200 to 300 pieces of tissue required 20 to 30 minutes. Although the individual ampoules were not stoppered, and the drum was open when the tissue cultures were prepared and read, not a single case of bacterial contamination was encountered. Even in the case of repeated readings fungal infections occurred but very rarely and never before the 6th day ; their number was less than 1 per thousand.

3. *Multiplication and disintegration curves.* In the manner described above, the fibroblastic multiplication of pieces of 288 12-day-old chick embryo hearts, cultured in HANKS' solution containing 2 per cent EE, was controlled on 11 occasions within 20 days, during which the culture fluid in the vials had not been changed. Cellular destruction was estimated from the microscopic picture showing cells rounded off, disintegrating, and undergoing granulation. The following is a diagrammatic representation of multiplication and disintegration *(Fig. 3)*.

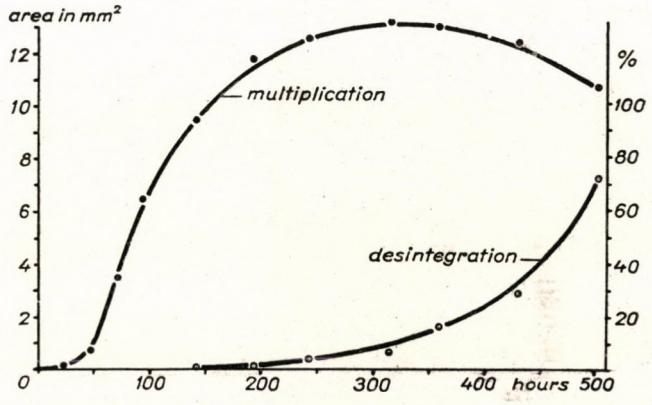

Fig. 3. Curves showing fibroblastic multiplication and disintegration in pieces of 12-day-old chick embryo hearts

The multiplication curve is reminiscent of that for bacterial proliferation. The same stages are encountered, namely, the resting, logarithmic, stationary, and diminishing stages. In the diminishing stage the cells perish, and some of them drop off the walls of the ampoules. The curve shows that logarithmic multiplication lasts for 8 to 10 days without changing the liquid, even if no surplus EE has been put into the culture fluid. As a layer of gossamerlike delicacy, and in a stripe of fairly uniform width, the cells densely circumgrow the explants.

In all experiments efforts should be made to determine the whole curve, wherefore, when chick tissue is used, the observations are to last for at least 9 to 11 days. One of the characteristic points of the curve is the point of maximal growth, which projected to the ordinate is obtainable in sq. mm.

The other curve is the disintegration curve illustrating the percentage destruction of fibroblasts. It shows that disintegration, called also "spontaneous disintegration", begins at a low rate which increases gradually. This type of disintegration curve is entirely different from that produced by Aujeszky's virus [14], which is a steeply ascending sigmoid curve.

Experiments similar to the foregoing were carried out on four occasions, each time involving 288 parallel tissues ; each time they produced the same multiplication and disintegration curves.

In the course of the subsequent experiments only the multiplication curves were determined, up to the point where active cellular division ceased.

4. *Establishing the limits of error.* Whenever multiplication curves were determined from the observation of only a few tissues, they greatly differed

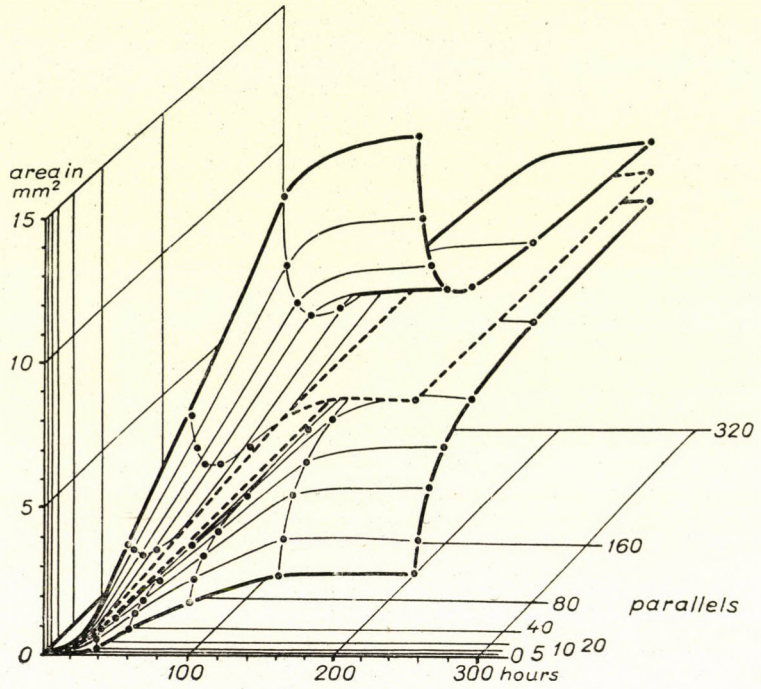

Fig. 4. Double standard deviation of the multiplication curve with varying numbers of parallels

from one another. In order to establish the exactness attainable with varying numbers of parallels, 1080 parallel explants were kept under observation in an EE-free buffered salt solution, the composition of which will be given later in this report. These explants were roughly of the same size, and averaged 1.31 mm in diameter. From the readings, the average result of the experiment was computed, and the multiplication curve, as well as the double standard deviations of the average values yielded by a varying number of parallels, were drawn *(Fig. 4)*.

The sigmoid surface in the middle of *Fig.* 4 illustrates the average results. The asymmetric surfaces below and above it show the double standard deviations with the use of varying numbers of parallels. Practically reliable, only

slightly deviating, values are obtained on working with as few as 80 to 160 parallels. Where it is desired to demonstrate very slight differences there, besides increasing the number of tissue pieces, the diameter of the explants must necessarily be taken into account.

5. *Fibroblastic multiplication with the tissue pieces varying in size.* The purpose of one of our experiments was to establish whether or not the halo

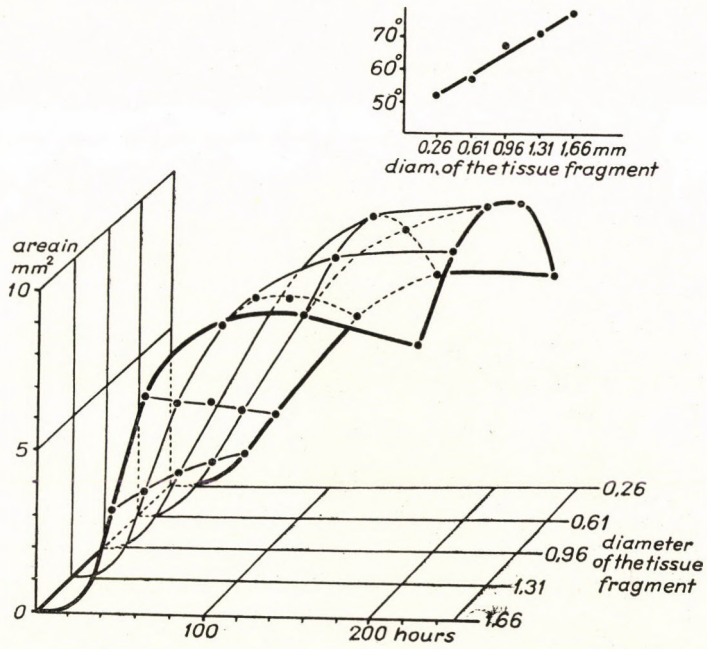

Fig. 5. Fibroblastic multiplication in chicken heart on explanting tissue pieces varying in size. Upper right hand corner indicates the inclination of the various multiplication curves in the logarithmic stage, expressed in angles

of fibroblasts growing around tissue explants differing in size was of the same width. The pieces of chicken heart varied markedly in size, and the tissues were explanted into the individual vials without particular picking. HANKS' solution was used in the experiment. At the end of the experiments the data were grouped according to the diameters of the explants, and thus the values for multiplication at different times were calculated from 50 to 110 data per group. In the individual groups the difference between the calculated and actually found average diameters of tissue explants was less than ± 0.08 mm ; tissue pieces with values beyond this limit were discarded. *Fig. 5* shows the curves obtained for the different tissue sizes.

On the evidence of *Fig. 5* the tissues small in size multiply but slowly, and those of about 1 mm yield maximum growth. Still larger tissues grow even more rapidly, but no longer yield as high multiplication values as those

1 mm in size. Following a period of sudden growth, extremely large-sized tissues die early. The growth rates of the individual groups are clearly indicated by the rises in the logarithmic stage, expressed in angles. This is illustrated in the upper right-hand corner of *Fig. 5*. With tissues varying in size, the steepness of the logarithmic stages depends on the size of the tissue, probably within limits. This experiment shows that tissue size is not without effect on the multiplication curve.

Finding the peaks of the curves shown in *Fig. 5*, we illustrated the maximum multiplication values thus obtained, using our method in computa-

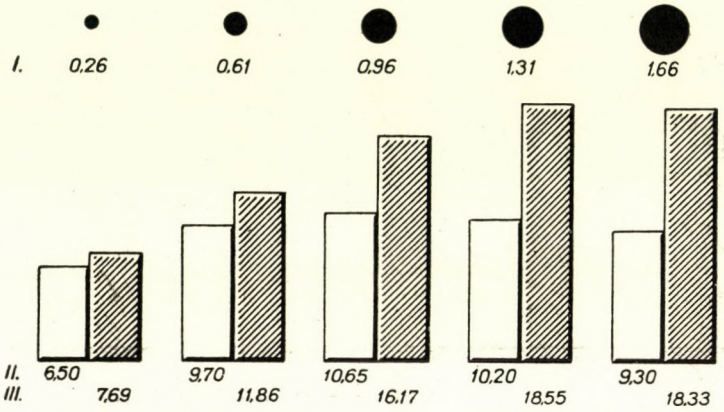

Fig. 6. Maximum multiplication in the experiment as per *Fig. 5*, calculated with two different methods

I. Average diameters of tissue pieces in mm
II. Maximum growth values in sq. mm, calculated with our method
III. Actual growth increment in sq. mm

tion. In addition, we calculated the area overgrown by the fibroblasts ; this was done by deducting the area of the explant from the area of the circle calculated with the diameter of the entire tissue growth, *i. e.* growth was calculated by the formula $(R^2 - r^2)\,\pi$. These values are represented in *Fig. 6* by the shaded rectangles.

It can be seen that by using the latter method of calculation, great differences are obtained in the values for the multiplication of the fibroblasts grown from the various differently sized explants. In using our own method in the calculations, on the other hand, the divergence due to differences in size is very small.

We should like to compare our method with two other methods of calculation, by representing with them the experimental values obtained in the 228th hour as in *Fig. 5*. The data obtained by means of the formula $(R^2 - r^2)\,\pi$ refer to the area actually overgrown by fibroblasts. The data calculated accord-

ing to the formula $\frac{R^2 - r^2}{2r}$ indicate multiplication per cell, since this formula expresses the ratio between the actually overgrown area and the circumference of the explant. The results obtained by the three different methods of calculation are illustrated in *Fig. 7*.

It can be seen that the data obtained with our method fall between those produced by the other two calculatory procedures, and that from one another our data differ but little, or at least not as widely as in the other

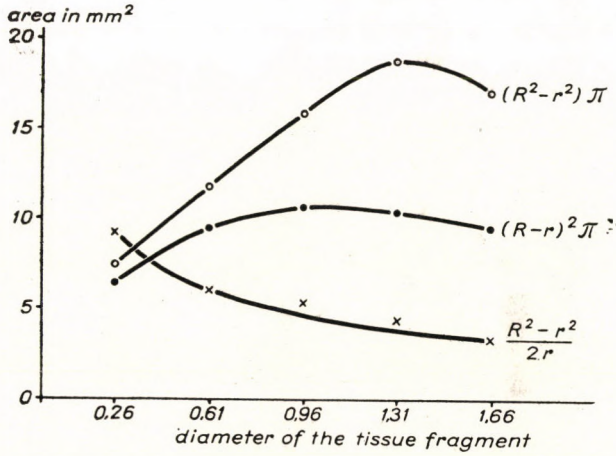

Fig. 7. Values obtained in the 228th hour of the experiment as per *Fig. 5*, calculated with three different methods

two cases. The differences in multiplication obtained with explants varying in size offer parallels to the radially measured width of the growth rings.

We refrained showing the values [6] calculated by the formula $\frac{R^2 - r^2}{r^2}$ indicating the relation between the overgrown areas and the areas of the explants, because they seemed improbable.

If we consider that in the experiments we can explant, without any particular picking, tissue pieces fairly equal in size, then, on the application of our method, the difference due solely to differences in tissue size may be found so very slight, in relation to the other sources of error present, as to be altogether negligible. In a number of experiments, each involving 200 to 500 parallel tissue pieces, the average tissue diameters differed from one another by from ± 0.03 to 0.1 mm only.

II. Optimal conditions of various factors

In this chapter we shall briefly report the experimental results, one after the other, obtained while investigating the optimal conditions of various factors.

1. *Use of plasma and EE.* When used while still fresh, the plasma coagulum was frequently found to dissolve early, a feature that interfered with cell adhesion and multiplication. No dissolution was observed to take place when the plasma was used after freezing for a week or ten days. Experiments carried out with a trypsin inhibitor showed the latter to be dispensable [15, 16].

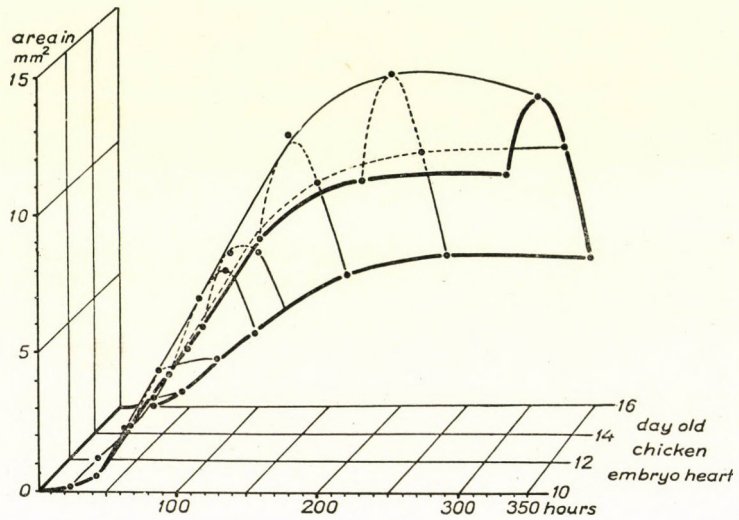

Fig. 8. Density per sq. mm of fibroblasts grown on chicken heart

If stored at −20° C, both plasma and EE remained fit for use for 3 or 4 months, without showing any significant differences in fibroblastic proliferation.

2. *Optimal age of chick embryo hearts.* An experiment was performed to study fibroblastic multiplication in pieces of the hearts of chicken embryos removed from 10, 12, 14, and 16-day-old eggs, respectively *(Fig. 8)*.

Fig. 8 shows that the embryo heart pieces obtained from eggs incubated for 12 days yielded the highest rate of growth.

It is not all the same at what time after their removal the chicken hearts are used for the purposes of the experiment. For 2, 4, 8, and 16 days, washed pieces of heart were kept at +4° C in HANKS' solution, which was applied as a thin layer on the cut-up pieces placed in a Petri dish. *Fig. 9* is a graphic representation of the maximum growth in tissues explanted at different times.

This curve plainly demonstrates that tissue preserved in the manner described cannot be used after the 3rd or 4th day. Remarkably, tissue kept in this manner is viable for 10 to 12 days only, and will not live or multiply beyond this period of time, even if cultured at 37° C.

3. *Effect of rotation on tissue multiplication.* Involving 268 parallel tissues, an experiment was made to establish if, and in what measure, rotation was a factor indispensable, or negligible, in tissue cultivation. For the first few days, fibroblastic multiplication was found to be about the same in tissues in still-standing drums and drums making 5 or 6 revolutions per hour. Later, after the 5th or 6th day, the non-rotated tissues altogether ceased multiplying,

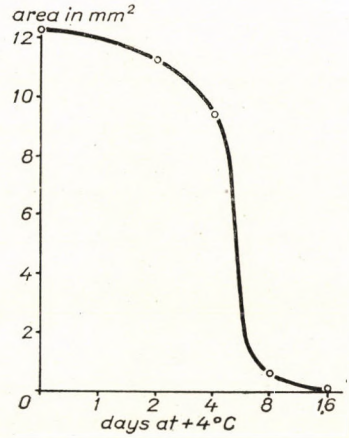

Fig. 9. Fibroblastic growth on cheicken hearts removed from eggs incubated for varying periods of tim

while in the rotated tissue active multiplication was observed for another 2 or 3 days.

4. *Optimal concentrations of the substances used in the buffered salt solution.* The optimal concentrations of the substances dissolved in bisdistilled water were determined separately. Dilutions were prepared from each, and for each dilution an independent experiment was set up involving 80 to 100 parallel tissues. The highest concentration yielding the longest multiplication curve was regarded as the optimum concentration. To the tissues placed on the plasma coagulum, only the buffered solution was added, with no extra serum or other substance in it. The optimum concentrations of the substances used were, NaCl, 0.8% ; KCl, 0.08% ; anhydrous, $CaCl_2$, 0.02% ; $MgSo_4 \cdot 7H_2O$, 0.02% ; $Na_2HPO_4 \cdot 2H_2O$, 0.006% ; KH_2PO_4, 0.006% ; glucose, 0.1%; $NaHCO_3$, 0.035% ; and phenol red, 0.001%.

From among these substances, $NaHCO_3$ and the buffers with phenol red were each ampouled separately in a hundredfold concentration of the

above quantities. The other substances were ampouled together in a twenty-fold concentration. Sterilization in the autoclave at 105° C lasted for 30 minutes. Before use, the ampouled substances were diluted to the required concentration with sterile bisdistilled water ; this brought the pH of the solution to 7.6. Antibiotics were added to the solution in the amounts mentioned earlier in this report. The amount of KCl in the solution was twice the usual concentration. For this very reason, experiments involving 250 parallel tissues were repeatedly carried out, and on each occasion fibroblastic multiplication was found to differ significantly from the control, which contained 0.04% KCl only.

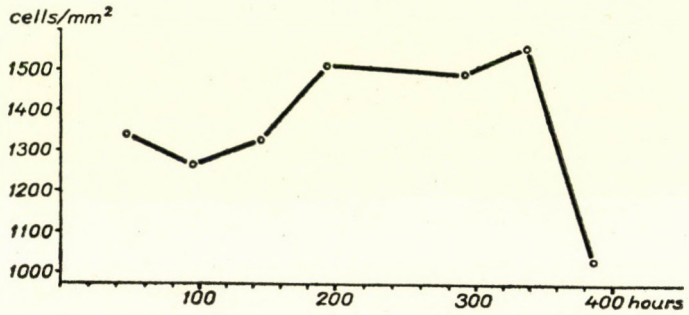

Fig. 10. Maximum growth in pieces of 12-day-old chicken heart, following storage at +4° C for varying periods of time

5. *Comparing the buffered salt solution with other solutions.* Our solution was compared with a number of earlier approved solutions. Titrated on chicken-heart fibroblasts, our solution yielded the highest growth rate, and was followed by HANKS' solution. Much lower growth rates were observed in tissues in GEY's [17], SIMMS' x_7 [18], and Tyrode solution. Small differences were found to occur between the two first-mentioned solutions.

6. *Fibroblastic multiplication in sealed and unsealed tubes.* The individual vials in the rolling drums were left unsealed, and their contents were in contact with the air within the drums. Under such circumstances acidification began on the 6th or 7th day only, and was a very slow process. In sealed tubes the solutions acidified as early as the 2nd day ; even so, fibroblastic growth was satisfactory. No differences, however, were noted between the multiplication curves.

7. *Cellular density.* Since cellular density changes with changes in the cultural conditions, it was deemed essential to throw light upon the role of this factor. For fibroblastic density, we first examined a physiological solution elaborated by ourselves and containing 2% EE. After staining them with Harris' haematoxylin, photographs were taken of the fibroblasts [19]. In evaluating them, always the fibroblasts present in the new-grown strip were counted, computing the result for sq. mm. *(Fig. 10).*

Each of the 7 prints in *Fig. 10* was determined by the average of 4 counts. Their respective positions show that following a slight rise of the number of cells in the first days, cellular distribution remains fairly even, until cells begin to die off and there accordingly is a steep drop in their number.

In another experiment, three different culture fluids were used in order to obtain three different multiplication curves, in which to study changes in cellular density. One contained 2% EE and produced the highest rate of growth, another contained no EE, a third one contained 10% cattle serum and gave the least multiplication *(Table I)*.

Table I

Density per sq. mm of fibroblasts grown in three different solutions, as counted at three different points of time

Buffered salt solution containing	Maximum growth in sq. mm	Cellular count per sq. mm read at hours		
		66th	136th	185th
2% EE	13.1	940	1220	1223
10% cattle serum	6.8	1095	—	1317
0.....	11.4	780	883	998

On this evidence, cellular density was the lowest in tissues cultured in buffered salt solution without any additions, while in those with added EE or cattle serum it was on nearly the same higher level.

8. *Effect of sera and ultrafiltrates on the chicken heart fibroblasts.* Since difficulties often present themselves in the use of animal sera and ultrafiltrates, a number of them were examined in varying concentrations to study their effect on tissue growth. Bovine, equine, and chicken sera were examined after inactivation at 56° C for 30 minutes. Concentrations of 0.5, 1, 2, 4, 8, 16, and 32% were prepared in the buffered salt solution. Experiments with 90 to 100 parallel tissues were made for each dilution.

The bovine sera obtained on different occasions displayed differences in behaviour. In one cattle serum, for example, the fibroblasts showed definitely enhanced multiplication in concentrations up to 8%, while in another serum a concentration as low as 2% failed to stimulate tissue growth.

Ultrafiltrates had a roughly similar or perhaps slightly more unfavourable effect on the tissues than the sera from which they had been prepared. *Fig. 11* graphically presents the action on tissue growth of a cattle serum, and *Fig. 12* that of the ultrafiltrate prepared from it.

It merits mentioning that it might have been difficult to demonstrate the differences displayed in *Figs. 11* and *12*, particularly if the current tissue culture methods had been used with only a few parallels.

In addition to the cattle sera, some horse sera were subjected to examinations. In one of them the growth rate was found to increase at concentra-

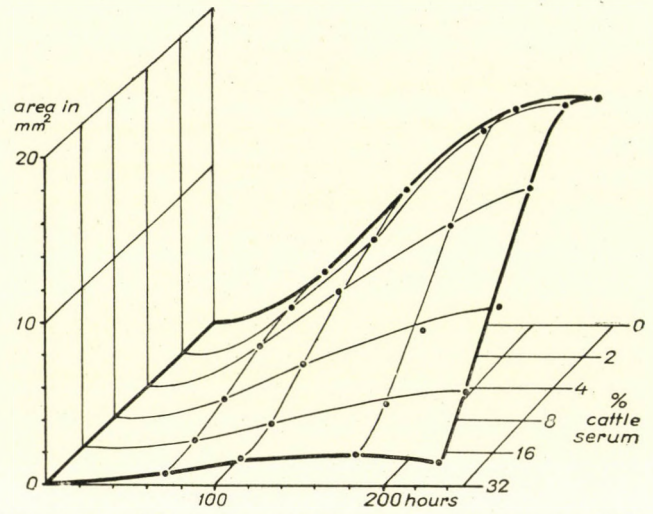

Fig. 11. Fibroblastic growth in pieces of chicken heart cultured in buffered salt solution containing bovine serum

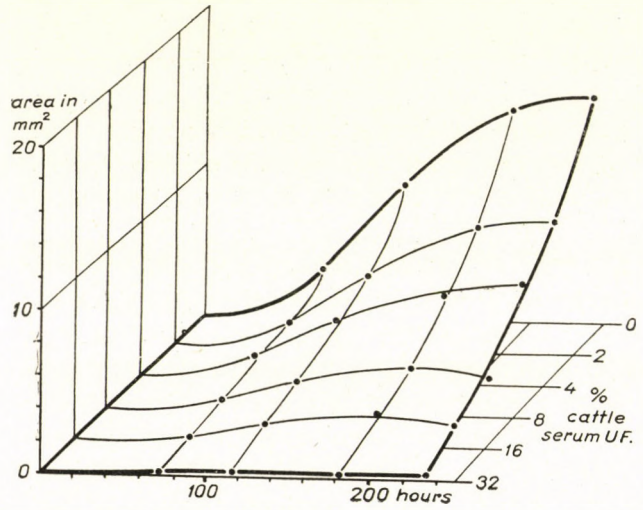

Fig. 12. Fibroblastic growth in pieces of chicken heart culture in buffered salt solution containing the ultrafiltrates of bovine sera at different concentrations

tions below 2%, while in concentrations above this level the fibroblasts grew at a lesser pace. Disintegration during this experiment has been recorded and tabulated as follows.

The data in *Table II* supply evidence of the rate of disintegration being in direct ratio to time and serum concentration. *Fig. 13* illustrates the dis-

Table II

Disintegration percentage in buffered salt solution containing horse serum

Reading taken at hours	Serum %							
	0	0,5	1	2	4	8	16	32
40	0	0	0	0	0	0	0	0
89	0	0	0	0	0	2.5	36.8	38.0
136	0	0	5.0	11.8	22.8	31.0	67.0	62.0
188	0	0	2.5	10.3	25.3	36.8	86.0	89.0
232	0	0	5.1	14.1	22.8	38.5	94.0	100.0

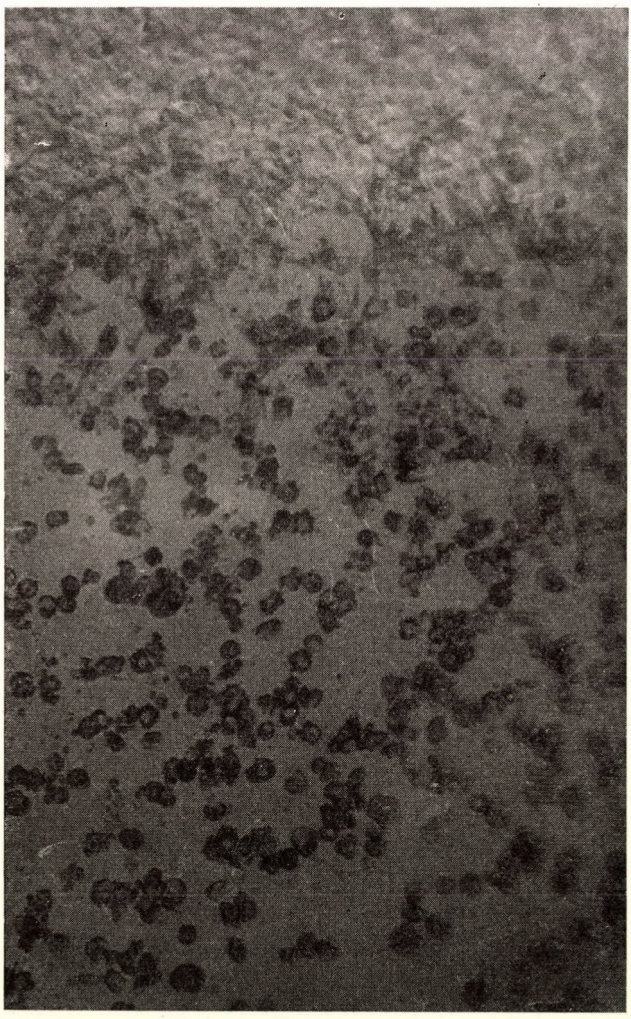

Fig. 13. Disintegration of fibroblasts grown for 9 days in buffered salt solution containing 32% horse serum

integration in tissue cultured for 9 days in a liquid nutrient containing 32% horse serum.

As regards chicken sera, fibroblastic growth was found to decrease in culture fluid of more than 2% serum concentration. In dilutions weaker than this, tissue growth was more intense than in the controls.

Increased attention appears to be needed in obtaining and selecting the appropriate sera, for they may differ greatly even if seemingly obtained in a similar manner. It is obviously expedient to convince ourselves of the usability of the sera before experimenting with them.

Discussion

A method has been elaborated with a view to characterize, within fairly wide limits, by nearly identical values the fibroblastic growth measured simultaneously in a number of explants differing in size.

There are two earlier methods by which multiplication can be measured ; they are underlain by the two formulas already mentioned, namely, $(R^2 - r^2)\,\pi$ and $\frac{R^2 - r^2}{2r}$.

When the actually overgrown area is represented, the area covered with fibroblasts increases, and does so rather intensively, in the same direction as the tissue increases.

In the first days the growth value per cell is largely the same for all the explants, irrespective of their size, but in the subsequent culture stage the growth area is in inverse ratio to the size of the explant. Thus the explant surrounded by the least number of cells yields the highest value, and conversely.

Attempts were made to equalize with our method of calculation the extreme values obtained by means of the two earlier methods mentioned above ; *Fig. 7* is the graphic representation of these attempts. It was thought expedient to characterize by nearly identical values the growth rates measured simultaneously in a number of explants of the same type and cultured under similar conditions, but differing in size. Experimental data show that the radially measured growth rings are the widest in the tissue explants about 1 mm in diameter. Thus with our method of calculation, tissues of this size yield the highest growth values.

In each of our experiments 500 to 800, sometimes even more than 1000, pieces of tissue were used. Thus with the aid of our method of calculation, and on using an adequate number of parallel tissue pieces, it was always possible to work with the desired accuracy.

Fibroblastic wreaths were seen to appear around each and every piece of chicken heart cultured under the conditions described. Only an exceptional

few of the 50 000 tissue explants failed to grow, and there was usually a peculiar explanation for such failure ; for example, the explant came to lie not on the plasma but on the glass, or pieces of the non-growing large arteries were the cause of the absence of cellular multiplication. The fibroblastic wreath circumgrowing the tissue piece was fairly even in width, yet readings were always taken on one side only. Readings on the top and bottom parts of the tissue did not improve the exactness of the experiments. The fibroblasts formed a dense gossamerlike film with no granulation or vacuoles perceptible in it, although they were sometimes cultured as long as 9 or 10 days.

Provided the fibroblasts are cultured and their multiplication is observed for at least 9 or 10 days, it is possible to decide on delicate differences. Readings need not be taken frequently. In routine tests readings taken at 3 days intervals, *i. e.* on the 3rd, 6th, and 9th day, have proved to be sufficient. Naturally, if it is desired to determine the multiplication curve with greater accuracy, the growth may be estimated more frequently.

At first sight it seemed that the use of unsealed small glass vessels involved a substantial risk of airborne bacterial and fungal contamination. As long as horizontally placed drums were employed the percentage incidence of fungal infection was indeed 8. But since the introduction of drum stands which keep the drums in a vertical position, this incidence rate has dropped to below 0.1%. Not a single case of bacterial infection has been encountered.

The method described seems to offer several advantages, *viz.*:

1. It is objective and practically eliminates, within fairly wide limits, differences in growth due to differences in tissue size.

2. It takes little place and small quantities of plasma, EE, and culture fluid to work up a great number of tissue pieces.

3. Since each and every explant is certain to grow, their handling is quick and simple.

4. By using open glass vessels we save the work of sealing them ; besides, we ensure that the pH change slowly during cultivation.

5. The method can be applied in several fields of microbiological work. It permits testing certain biological substances, extracts, vitamins, amino acids, etc. for their inhibitory or stimulating effect on the cells of explanted tissues. In addition, the tissue cultures are suitable for use in measuring the tissue toxicity of certain substances. Provided we use the appropriate tissues, we can easily titrate various viruses.

The results achieved by the method in these fields will be reported in subsequent papers.

Summary

A new quantitative tissue culture method has been elaborated ; its principle is that we regard the average value of all the fibroblastic growths, measured radially from the margin of the explants, as the radius of a circle, and compute the area of that circle to use it for expressing fibroblastic multiplication in sq. mm. This method, within fairly wide limits, practically eliminates differences in growth due to differences in explant size.

Pieces of tissue were placed on thin plasma coagulum in open short-necked 2-ml glass vessels. After the addition of culture fluid, these were placed in an aluminium drum to rotate in a thermostat.

The part played by various factors has been examined with this method. The conditions of fibroblastic multiplication and disintegration have been studied ; the experimental limit of error on the use of a varying number of parallels has been determined ; cellular density and the effect of rotation have been investigated : the optimal concentrations of the substances in the culture fluid have been established, etc.

The method has the following advantages over the current ones, *viz.* :

1. It is objective and practically eliminates, within fairly wide limits, differences in growth due to differences in tissue size.

2. Little place and small quantities of plasma, EE, and culture fluid are needed to work up a great number of tissue pieces.

3. Since each and every explant is certain to grow, their handling is quick and simple.

4. By using open glass vessels we save the work of sealing them : besides, we ensure that the pH change slowly during cultivation.

5. The method may offer advantages in several fields of microbiological work. It permits testing extracts, amino acids, vitamins, etc. for their inhibitory or stimulating effect on the cells of explanted tissues, it can be used in measuring the tissue toxicity of substances and for titrating viruses.

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ACTA MICROBIOLOGICA

ТОМ V.

РЕЗЮМЕ

МЕТОД, ОПРЕДЕЛЯЮЩИЙ ВЫДЕЛЕНИЕ АНТИБИОТИЧЕСКИХ ВЕЩЕСТВ ВАРИАНТАМИ STREPTOMYCES GRISEUS

Г. САБО и Т. ВАЛЬИ-НАДЬ

Авторы выработали количественный метод определения абсолютных величин для изучения одноклеточных колоний Streptomyces.

Метод применяется:

1. Для количественного изучения способности выделения антибиотических веществ Streptomyces griseus (и других микроорганизмов), как для изучения изменчивости, наблюдаемого в мире микроорганизмов;

2. Для исследования взаимоотношений между морфологическими свойствами колоний и способностью выделения антибиотических веществ единичных вариантов.

3. Для выделения вариантов, продуцирующих больше стрептомицина (или других антибиотиков).

БИОЛОГИЧЕСКОЕ ИЗУЧЕНИЕ СИНТЕЗА СТРЕПТОМИЦИНА. I. АКТИВНОСТЬ α-МАННОЗИДАЗЫ ИЗУЧЕНА В КУЛЬТУРАХ STREPTOMYCES GRISEUS

Д. КОЛЛАР

1. Автор экспериментально подтвердил определение Hockenhull и его сотрудников, по которому в культурах S reptomyces griseus расщепление стрептомицин-В и α-фенилманнозида производится α-маннозидазой.

2. Автор изучил влияние разных соединений (углеводов, ингибиторов) на активность энзима и определил оптимум pH и температуры энзима.

3. Автор рассмотрел ряд гипотез в связи с появлением активности α-маннозидазы и результат обзора показал, что появление энзиматической активности не объясняется процессом активации, мутацией или освобождением внутриклеточных форм. Условия появления энзима показывают индуктивный характер.

БИОХИМИЧЕСКОЕ ИЗУЧЕНИЕ СИНТЕЗА СТРЕПТОМИЦИНА. II. ОБРАЗОВАНИЕ α-МАННОЗИДАЗЫ У STREPTOMYCES GRISEUS И ЕЕ РОЛЬ В БИОСИНТЕЗЕ СТРЕПТОМИЦИНА

Д. КОЛЛАР

1. Автор, исследуя возникновение активности α-маннозидазы в культурах Streptomyces griseus нашел, что энзим синтетизируется в мицелиях под влиянием различных α-маннозидов индуктивно.

2. Автор определил оптимум температуры и pH синтеза энзима и исследовал влияние разных соединений (клеточных ядов, амминокислот, углеводов и т. д.) на процесс синтеза.

3. Автор установил, что синтез энзима задерживается внутренним торможением, связанным с эндогенным обменом веществ и объясняет роль синтеза стрептомицина появлением α-маннозидазы.

4. Результат исследований показал, что синтез α-маннозидазы не всегда — по крайней мере не у всех штаммов — индуктивного характера. Энзим в небольшом количестве может появляться в мицелии и конституционально. У α-маннозидазы нельзя провести резкой границы между двумя характерами.

РЕЗУЛЬТАТЫ ВАКЦИНАЦИИ ПРОТИВ КОКЛЮША

А. ПЕТРИЛЛА и Д. БАРШИ

В Венгрии в 1953 году ввели обязательную вакцинацию против коклюша. По существующему методу вакцинации подлежат грудные дети в возрасте от 6 до 11 месяцев вакциной di-per-te (2 прививки на протяжении 4-х недель) и через год прививку надо повторить. Обязательные прививки надо сделать ежегодно весной и осенью. Кроме того, надо привить вакциной di-per-te детей первого класса начальной школы, значит практически детей в возрасте 6 лет.

Эффективность вакцинации против коклюша исследована на основе снижения заболеваемости привитых возрастных групп по сравнению с заболеваемостью непривитых.

Имеющиеся до сих пор результаты прививок показывают, что эффективность вакцинации составляет 20—30% у привитых один раз в возрасте одного и трех лет и 50% у привитых дважды в возрасте двух лет. Вакцинация в возрасте 6 и 7 лет пока совершенно не эффективна. Для того, чтобы заболеваемость коклюшем значительно снизилась, вакцинацию грудных детей надо начать рано, не позже, чем в возрасте 3—4—5 месяцев.

ВЫДЕЛЕНИЕ ФАГОВ, ВЛИЯЮЩИХ НА МИКОБАКТЕРИИ

Э. ВАНДРА и Ф. ГАЛ

Из 28 проб почвы в 5 случаях удалось двумя разными методами выделить фаги, в двух случаях влияющие на Mycobacterium phlei, в остальных случаях влияющие на Mycobacterium minetti, smegmatis и на friburgensis.

РЕАКТИВАЦИЯ ПРИ НИЗКОЙ ТЕМПЕРАТУРЕ STREPTOMYCES GRISEUS ИНАКТИВИРУЕМОГО УЛЬТРАФИОЛЕТОВЫМИ ЛУЧАМИ

Г. САБО

Часть спор Streptomyces griseus, обработанных ультрафиолетовыми лучами, снова приобретают способность к размножению и образуют колонии после сохранения спор при низкой температуре.

Степень реактивации при 0° С больше, чем при 10° С. Реактивация при 17° С не наблюдается.

ЗАРАЖЕННОСТЬ СОБАК ЛЕПТОСПИРАМИ В БУДАПЕШТЕ

М. ФЮЗИ и Й. КИСЕЛ

Для выявления зараженности собак лептоспирами в Будапеште в 1955 г. были сделаны массовые серологические исследования. Проведена реакция агглютинации-лизиса с сыворотками 104 собак. При реакции применялись следующие типы лептоспир:

L. grippotyphosa, L. canicola, L. icterohaemorrhagiae, L. hebdomadis, L. sejrő, L. pomona, L. mitis, L. bataviae, L. australis-A, L. australis-B, L. ballum.

Положительный результат был получен всего в 21 случае (21,1%), в большинстве случаев авторы нашли зараженность L. canicola (16), но получили положительный результат и с L. sejrő (3) и L. pomona (2).

Несмотря на значительную зараженность собак L. canicola, до сих пор в столице у людей canicola — лихорадка не наблюдалась, хотя такие же случаи в провинции нередки. Характерная провинциальная локализация заражения людей L. canicola объясняется в первую очередь тем, что там надо учитывать роль в распространении заражений и других домашних животных (свиней, рогатого скота, лошадей), а также окружающие условия, более благоприятных в распространении заражения.

РОЛЬ ВОЗРАСТА ПРИ НЕКОТОРЫХ ЗАРАЗНЫХ БОЛЕЗНЯХ

Д. БАРШИ и О. РУДНАИ

1. Авторы определили возрастную заболеваемость пяти инфекционных болезней (кори, коклюша, скарлатины, дифтерии и полиомиелита) на протяжении 1931—38 и 1949—54 годов. Они установили, что заболеваемость всеми исследуемыми инфекционными заболеваниями показывает ясный сдвиг в сторону более младших возрастных групп.

2. Авторы определили возраст, при котором заболеваемость упомянутыми пятью заболеваниями достигает максимума. Отклонение максимума заболеваемости к более младшей возрастной группе, наблюдаемое не только в средних величинах обоих исследуемых сроков, но особенно бросается это в глаза в последующие годы после второй мировой войны. Это отклонение наиболее выражено в случаях коклюша и полиомиелита. Абсолютный максимум заболеваемости коклюшем появляется уже на первом году жизни, и поэтому было бы целесообразным вакцинировать против коклюша в более младшем возрасте.

3. Авторы проанализировали связь между возрастом и циклическим движением эпидемических кривых и установили, что в циклах эпидемические кривые единичных возрастных групп приблизительно в равных размерах изменяются у всех пяти заболеваний. Это наблюдение, повидимому, противоречит общепринятой теории циклического движения заболеваний дыхательных путей.

СИСТЕМАТИЧЕСКИЕ МИКОЗЫ В ВЕНГРИИ

А. ЧИЛЛАГ

Автор сообщает случаи систематических микоз, диагносцированных в прошедших двух годах в Государственном Институте Гигиены в Будапеште. Во всех случаях автор отмечает клинический диагноз, вписанный при посылании исследуемого материала, данные исследования мазков, результаты культивирования, морфологические животнопатогенные особенности выделенных грибков, а также — в отдельных случаях — другие обследования, подтверждающие микоз.

ВОПРОС О ФИЛОГЕНЕЗЕ B. ANTHRACIS

Д. ИВАНОВИЧ и Й. ФЕЛДЕШ

Штаммы B. anthracis и B. cereus, а также и их варианты, можно с точностью отдифференцировать друг от друга в тех случаях, когда морфология клеток, образование колоний и патогенность не дают достаточных основ для какой-либо дифференциации. Хорошо развитая система фосфатазы (лецитин и фенофталеин-фосфат, расщепляющий энзим, и гемолитическая активность) совместно с активностью пенициллиназы B. cereus дает дифференциальный диагноз—при благоприятных экспериментальных условиях — довольно достоверно. Дальнейшую возможность для дифференциации можно найти в чувствительности обеих бактерий к фагу. Рассматривая эти особенности, обе бактерии мож-

но принять за разные специесы, и поэтому не обосновано назвать B. anthracis вариантом B. cereus. Наличие одного специфического глюкозамин-галактоз полимера, описанного раньше, как компонента оболочки B. anthracis, можно было найти во всех наших (26) изучаемых штаммах B. anthracis. В этом отношении этот тип кажется гомогенным. Тождественный специфический материал можно было найти у нескольких штаммов B. cereus. На основе этого, один из типов B. cereus имеет такой же полисахаридный компонент, как B. anthracis. Этот тип B. cereus резко отличный от тех штаммов, из которых мы выделили полисахарид с другими химическими и серологическими свойствами. Полисахарид последнего типа состоит из 1 мол. глюкозы и 3 мол. аминосахара. Аминосахар состоит из глюкозамина и, вероятно, из так называемого «не идентифицируемого гексозамина». Молекула дополняется уксусной кислотой (ацетил) и частью пептида. В пептиде удалось определить α, ε — диаминопимелин кислоту, аланин, глютаминовую кислоту и аспарагиновую кислоту.

ИЗУЧЕНИЕ ВЗАИМООТНОШЕНИЙ МЕЖДУ ВИРУСАМИ КОКСАКИ И ПОЛИОМИЕЛИТА. II. ПОЗДНЯЯ НЕВОСПРИИМЧИВОСТЬ К ПОЛИОМИЕЛИТУ МОЛОДЫХ МЫШЕЙ, РАННЕЕ ЗАРАЖЕННЫХ ВИРУСОМ КОКСАКИ B1

И. ДЕМЕК

1. Мыши, зараженные вирусом коксаки B1 в возрасте 9 дней, более невосприимчивые к заражению вирусом Лансинг, по крайней мере, в течение 60 суток, чем раннее незараженные мыши в том же возрасте.

2. Как результат невосприимчивости, количество погибших снижается, и средний инкубационный срок удлиняется.

3. С точки зрения возникновения невосприимчивости не играет роль способ прививки вирусом коксаки B1.

4. Относительная эффективность заражения вирусом Лансинг после 36 или 60 дней заражения вирусом коксаки B1 в среднем 3,6%.

5. Гуморальные антитела не играют роль при поздней невосприимчивости. Обсуждает возможное объяснение на основе интерференции и других возможностей.

ИЗУЧЕНИЕ В ТКАНЕВЫХ КУЛЬТУРАХ ЦИКЛИЧЕСКОГО РАЗМНОЖЕНИЯ ВИРУСОВ ГРИППА И ND

И. ХОРВАТ

Были изучены условия адсорбции и элюции двух штаммов гриппа и одного штамма ND. В модельном опыте автор регистрировал циклическое размножение вирусов в тканевой культуре, без применения гетерологичного вируса, облученного УФ лучами. Размножение в случаях исследуемых вирусов началось через 3—4 часа после лаг. фазы. Логарифмическое размножение в случаях первого цикла продолжалось $1^1/_2$—2 часа, затем после некоторого более короткого спокойного периода начался второй цикл, который был несколько более короче, чем первый.

ИЗОСЕРОЛОГИЧЕСКОЕ ИССЛЕДОВАНИЕ АНТИ-О АГГЛЮТИНИНА, ВЫЗЫВАЮЩЕГО ГЕМОЛИТИЧЕСКОЕ ОСЛОЖНЕНИЕ ПРИ ПЕРЕЛИВАНИИ КРОВИ

Э. ХОРВАТ

Описано серологическое исследование анти-0 антитела, вызывающего гемолитическое осложнение. Изучаемые антитела показали некоторые серологические особенности, существенно отличающиеся от обыкновенных; это в некоторой степени говорит за воз-

можности перехода переходных форм антител в иммунный тип. Последнее кажется подтверждается появлением и повышением титра антител и тесной связью между этим, как предполагаемыми антигенными стимулами, и беременностями, заканчивающимися абортами.

БЫСТРАЯ ОЦЕНКА РЕЗИСТЕНТНОСТИ СОРТОВ РИСА К PIRICULARIA С ТЕПЛИЧНОЙ ПРОБОЙ

Й. ШЮДИ и Й. ПОДХРАДСКИ

Автор сообщает новую, хорошо повторяемую и относительно быструю (приблизительно требующую 15—20 дней) тепличную пробу для определения резистентности сортов риса к Piricularia.

По данным исследования 20 сортов, резко резистентные и резко восприимчивые сорта этой пробой можно легко отличить.

Эта проба не дала возможность достоверно дать оценку таких сортов, где чувствительность имела переходящий характер, все-таки среди и таких сортов можно было поставить хорошо репродуцируемый ступенчатый ряд.

На основе иных экспериментальных и литературных данных более подробно авторы изложили то мнение, что возрастное изменение резистентности риса к Piricularia, с одной стороны, и резистентность сортов, с другой стороны, существенно разные явления.

БЫСТРЫЙ ПРИЕМ ДЛЯ БИОЛОГИЧЕСКОЙ ОЦЕНКИ ОКСИТЕТРАЦИКЛИНА И ХЛОРТЕТРАЦИКЛИНА

Л. НИРИ

Автор сообщает быстрый метод для биологической оценки окситетрациклина и хлортетрациклина.

Применяя очень простую питательную среду, которая содержит ростовой фактор (фолиевая кислота или индолуксусная кислота в количестве 0,0014%), оценку вышеупомянутых двух антибиотиков можно определить на протяжении 3—4 часов со штаммом ФДА 6633 Bacillus subtilis.

Применение ростовых факторов, сокращающих время, необходимое для некоторой биологической оценки, кажется применяемым методом и для других биологических оценок.

ИЗМЕНЕНИЯ ЧУВСТВИТЕЛЬНОСТИ ПАТОГЕННЫХ БАКТЕРИЙ К АНТИБИОТИКАМ В 1953—1956 ГГ.

Л. ВАЦИ, Д. БАРШИ и М. КУБИНИ

Авторы статистически обрабатывали данные исследований бактериологического отделения за 1953—1956 годы. Они оценили результаты чувствительности к антибиотикам 8908 патогенных микробов, выделенных из 6765 исследуемых материалов. На основе статистического материала можно было установить следующее:

1. В упомянутом сроке частота наличия штаммов Klebsiella в разных исследуемых материалах значительно увеличивалась.

2. Можно было определить, что за срок исследования резистентность стафилококков к пенициллину, пневмококков к стрептомицину и резистентность E. coli и Ps. pyocyanea к хлорамфениколу в значительной мере увеличивались.

3. Существует зависимость среди чувствительности к разным антибиотикам отдельных патогенных микробов. Это кажется противоречит тому нашему предыдущему определению, что изменение резистентности мы могли обследовать только по антибиотикам, когда к другим антибиотикам осталась неизмененной. Это явление объясняется только

тем, что образование резистентности имеет не только одну, а разнообразные причины, и часть из них кроется в генеральных и общих особенностях бактерий, другая часть касается чаких свойств бактерий, которые связываются с одним определенным антибиотиком.

ПРИМЕНИМОСТЬ МЕТОДА ГЕМОЛИЗА ПО MIDDLEBROOK-у ДЛЯ ОПРЕДЕЛЕНИЯ ТУБЕРКУЛЕЗА У ЧЕЛОВЕКА И ЖИВОТНЫХ

Н. КЕРТАИ

Автор сообщает об исследовании туберкулезной зараженности населения и крупного рогатого скота одного села в связи с исследованиями взаимоотношений туберкулеза у людей и у животных. Результаты, полученные аллергической пробой, автор сравнивал с результатами серологической пробы, чтобы установить практическую оценку последней. С этой целью он применил помимо внутрикожной туберкулиновой пробы реакцию гемолиза по Middlebrook-у. У 453,4% исследуемого населения реакция Манту была положительная со старым туберкулином в разведении 1 : 10 000, но с реакцией гемолиза серологическая положительность была 27%.

После кожной пробы туберкулин положительными были 26,7% животных, но реакцией гемолиза 47,1%. Только 1/3 часть всех сывороток людей и животных с активным туберкулезом содержала гемолизирующие антитела. 68,5%. Манту-положительных людей и 63,1% туберкулин-положительного крупного рогатого скота были серологически отрицательными, когда 29% Манту-отрицательного населения и 51,2% туберкулин-отрицатльных животных гемолитической пробой оказались положительными. Реакцию, полученную с разведением сыворотки 1 : 8 и выше, считали положительной.

Из результатов опытов следует, что серологический метод по Middlebrook-у в настоящей форме не заменяет аллергическую пробу и не способен для выявления зараженности туберкулезом человека и животных. Что касается специфичности и целенаправленности, отстает от кожной туберкулиновой пробы, не удовлетворяет практическим требованиям, даже ее специфичность сомнительная.

СПОСОБНОСТЬ SARCINA FLAVA В РАЗНЫХ ЦИКЛАХ ЕЕ ЖИЗНИ ФЕРМЕНТИРОВАТЬ САХАР

И. НАС

Автор сообщает процесс регенерации фильтрующихся форм одного штамма Sarcina flava. Автор изучал способность ферметировать сахар в разных циклах процесса и нашел редко наблюдаемое явление, что исследуемый штамм Sarcina, который вначале не ферментировал никакого сахара, в ранних стадиях регенерации фильтрующихся форм показал ферментативную активность со многими углеводородами и только после окончания регенерации потерял постепенно эту способность.

ЭТИОТРОПНОЕ ЛЕЧЕНИЕ ЭКСПЕРИМЕНТАЛЬНОГО ШИГЕЛЛОЗА

Б. ШЕРЕНЬ

Автор сообщает о результатах лечения 300 больных (хлорамфениколом, окситетрациклином, стрептомицином, сульфаметилфтиазолом или сульфагуанидином), проведенного в 500 случаях шигеллозного кератоконъюнктивита. Экспериментальный шигеллоз подобно дизентерии человека очень хорошо реагирует на каузальное лечение, но в достаточно большой части случаев не удается достигнуть лечением желаемого результата. Причинами неудачи терапии могут быть: резистентность возбудителя, концентрация химиотерапевтических средств в очагах недостаточная или мало прочная, антибактериальные защищающие свойства организма, не способные уничтожить возбудителя. На основе своих исследований автор приписывает причинным медикаментам in vivo только бактериостатическое влияние и предполагает, что уничтожение возбудителя лежит на бактерицидных веществах организма. За неудачу современных причинных лечений ответственна слабость защитных свойств организма. Рекомендует применение циклического лечения и иммуно-химиотерапию.

ОЦЕНКА НА ЧЕЛОВЕКЕ ВАКЦИНЫ НОВОГО ТИПА ПРОТИВ СВИНКИ

Д. ФЕЛКАИ

Автор исследовал иммуногенность в человеке одной, к гелью адсорбированной, концентрированной, убитой формалином вакцины свинки.

В группе I. были привиты 91 человек отрицательной кожной пробой два раза с дозами 0,5 мл со сроком 6 недель. В группе II. были вакцинированы 13 людей одной дозой 0,5 мл.

Иммунный ответ был установлен определением содержания антител крови, взятой в начале иммунизации, на 3 и 6 неделях пробами ЗГА, РСК (с обоими антигенами S и V), а также после 3 месяцев, когда кожная проба была повторена.

В обеих группах I и II быстрый и стойкий иммунитет возникает в 3 неделю без заметного понижения титра до конца 3 месяца. После иммунизации наличие положительных кожных проб значительно увеличивается.

ИССЛЕДОВАНИЕ ЛИПОИДОВ КИШЕЧНЫХ БАКТЕРИЙ

Л. ВАЦИ и П. ИНЦЕ

1. Авторы исследовали состав липоидов, экстрагируемых петрол-эфиром, эфиром и хлороформом из штаммов S. paratyphi B и E. coli 0 : 111, чувствительных и резистентных к хлорамфениколу.

2. Резистентные варианты обеих бактерий содержат больше липоидов, чем чувствительные.

3. Состав липоидов резистентных бактерий отличается от такового чувствительных. Липоиды резистентных бактерий содержат значительно больше фосфора и фосфатидов и из последних количество кефалиновидных во много раз увеличивалось.

4. Подобное изменение состава липоидов в пользу фосфатидов, в том числе и кефалина, было определено у штаммов E. coli 0 : 111, резистентных к антибиотикам in vivo.

5. Фосфатиды резистентных бактерий содержат в $1\,^1/_2$ раза больше азота и фосфора, чем фосфатиды чувствительных.

6. Сравнивая с предыдущим, штамм Streptococcus haemolyticus «A» содержал очень мало липоида фосфатидов и кефалина.

7. Можно предполагать, что повышение резистентности к антибиотикам является результатом изменения состава поверхностных липоидов бактерий.

БЫСТРЫЙ МЕТОД ДЛЯ ОПРЕДЕЛЕНИЯ ЧУВСТВИТЕЛЬНОСТИ К АНТИБИОТИКАМ, ПРИМЕНЕНИЕМ БУМАЖНОЙ ПОЛОСКИ, ПРОПИТАННОЙ ТРИФЕНИЛ-ТЕТРАЗОЛИУМ-ХЛОРИДОМ

Э. ЙЕНЗИ и Э. ХОРВАТ

Авторы выработали метод определения чувствительности бактерий, первоначальных патологических материалов к антибиотикам с применением бумажной полоски, пропитанной трифенил-тетразолиум-хлоридом. Положив полоску на зареженную питательную среду-агар, на дисках, пропитанных антибиотиками, красный цвет редуцированного трифенил-тетразолиум-хлорида показывает зону задержки в 5 часов инкубации.

ДАННЫЕ К СИМПТОМАТОЛОГИИ ФЕНОМЕНА ШВАРЦМАНА

Л. КЕСТЮШ, Э. САБО, Д. БОТ и И. ЙОКАИ

Установлено, что при возникновении феномена Шварцмана из неорганических ионов сыворотки, концентрация K и Ca значительно понижается и под влиянием подготовляющего внутрикожного эндотоксина появляется гипогликемия, а после разрешающей дозы содержание сахара крови повышается. Вместе с гипергликемией чрез-

мерно повышается активность фосфогексозизамеразы, причиной этого повышения является поражение мышц. Итак, в расстройстве обмена углеводородов в первую очередь заинтересована мускулатура. Симпатиколитические вещества (особенно эрготамин) укрепляют разрешаемость феномена Шварцмана и это делает невероятным то, что симпатикомиметические эффекты эндотоксинов являются первопричинными факторами.

ИССЛЕДОВАНИЕ ОБМЕНА УГЛЕРОДА У BOTRYOTINIA FUCKELIANA И ЕГО СВЯЗЬ СО СЛАДКИМ ГНИЕНИЕМ. I. ОРГАНИЧЕСКИЕ КИСЛОТЫ, КАК ЕДИНСТВЕННЫЕ ИСТОЧНИКИ УГЛЕРОДА ГРИБКОВ

Э. К. НОВАК и Д. ВЕРЕШ-ФЕЛКАИ

Исследуемый штамм плесневого грибка **Botryotinia fuckeliana** Wetz. из 16 испробованных органических соединений единственным источником углерода и энергии употреблял следующие десять:

молочная кислота, молочнокислый Na, лимонная кислота, винокаменная, янтарная кислота, уксуснокислый Na, фумаровая, малоновая кислота, аскорбиновая кислота и этилалкоголь.

Муравьиная кислота, пропионовая кислота, щавелевая кислота, щавелекислый Na и уксусная кислота дали отрицательные результаты.

Сравнивая результаты с литературными данными, опыты, с одной стороны, подтверждают функцию цикла Кребс, с другой стороны, применение малоновой кислоты противоречит тому. Здесь вероятно дело в том, что из разных возможных путей обмена веществ условия (например, дача малоновой кислоты) определяют функцию одного или другого. Этот вопрос объясняется в следующей статье.

ИССЛЕДОВАНИЕ ОБМЕНА УГЛЕРОДА У BOTRYOTIANIA FUCKELIANA И ЕГО СВЯЗЬ СО СЛАДКИМ ГНИЕНИЕМ. II. УПОТРЕБЛЕНИЕ МАЛОНОВОЙ КИСЛОТЫ И ЕЕ ВЛИЯНИЕ НА ОБМЕН ВЕЩЕСТВ ГРИБКА

Э. К. НОВАК

Исследования употребления малоновой кислоты грибком **Botryotinia fuckeliana** показывают то, что малоновая кислота, декарбоксилированная уксусной кислотой, окисляемая через янтарную кислоту щавелевой кислотой. В освобождении энергии и в процессах биологического синтеза грибка, культивированного на малоновой кислоте, важную роль играет, вероятно, кислая фракция, определяемая, как гидроксамовая кислота. Эту кислотную фракцию можно найти также в грибке, культивированном на сахаре.

Малоновая кислота на сахарной среде в концентрации, 0,005 М и даже ниже оказывает стимулирующее действие на потребление кислорода и рост грибка.

РОЛЬ СЕРИНА В ОБМЕНЕ ВЕЩЕСТВ ШТАММА ESCHERICHIA COLI

Л. ПРОГАСКА и Ф. МУРАНИ

Авторы исследовали в синтетических питательных средах и в ферментативных пробирках потребность аминокислот 220 различными штаммами E. coli. Штаммы ведут себя с одинаковой биохимической активностью из исследуемых аминокислот только к глютаминовой кислоте, аспарагиновой кислоте и альфа-аланину. В питательной среде, содержащей серин, только некоторые штаммы E. coli развивались. Эти штаммы в ферментативных пробирках расщепляли серин с дезаминированием, остальные нет. Сравнивая способность расщепления серина штаммов E. coli с другими биохимическими свойствами нашли то, что расщепление серина штаммами исключает ферментирование сахарозы.

СВЯЗЬ МЕЖДУ АНТИГЕННОЙ СТРУКТУРОЙ И ЧУВСТВИТЕЛЬНОСТЬЮ К ФАГУ ТИПОВ ГРУППЫ SH. FLEXNERI

Д. ГАШПАР

1. На основе исследований штаммов Sh. flexneri, происходящих из 820 обычных материалов, группу Sh. flexneri, с помощью 13 выделенных из куриного помета и не адаптированных фагов, можно разделить на 10 фаготипов.

2. Фаготип от «a» «f» соответствует 1a, 1b, 2a, 3, 4a спец и 4b серотипам Sh. flexneri. Тождественная чувствительность 2b и X вариантов и групповой фазы 4a и варианта У (фаготипы «i» и «k») объясняется тесной связью антигенной структуры. Серотип 6 можно разделить на два самостоятельных фаготипа «h» и «g»). Резистентность к фагам штаммов в фаге 4a спец можно приписать к специальной антигенной структуре штаммов.

3. Чувствительность прочих штаммов Shigella и других патогенных кишечных бактерий, применимых к фагам, в общем была низкая.

4. Чувствительность к фагу выделенных штаммов тождественными лицами или больными, живущими в тождественном коллективе, доказывает in vivo стабильность фаготипов.

5. Определение чувствительности к фагам группы Sh. flexneri, способное регистрировать данные распределения типов с точки зрения доминирующих типов.

ЧУВСТВИТЕЛЬНОСТЬ К ЖЕЛЕЗУ ФЕРМЕНТАЦИЙ ОКСИТЕТРАЦИКЛИНА, СДЕЛАННЫХ С STREPTOMYCES RIMOSUS

И. ХОРВАТ, К. МАДЬЯР, И. ГАДО, Й. САНТО и Т. ВАДКЕРТИ

1. Авторы исследовали влияние железа на ферментацию Streptomyces rimosus. Они установили, что присутствие железа и непредельного масла сильно задерживает производство. Эту задержку можно избежать применением более предельных масел и жиров.

2. Причиной задержки производства в питательной среде, содержащей железо и непредельные масла, является токсическое влияние возникших соединений маслопероксида.

3. При высоких производственных показателях железо имеет также одно «непосредственное» производствозадерживающее влияние.

ИЗУЧЕНИЕ ФУНГИСТАТИЧЕСКОЙ АКТИВНОСТИ ВИДОВ SPHAEROPSIDALES И MELANCONIALES

Й. ВЕРЕШ

Из гербарийных экземпляров 48 видов Sphaeropsidales и 10 видов Melanconiales с применением селективных питательных сред, содержащих пенициллин, стрептомицин, хлоромицетин и натрийпропионат, автор выделила чистые культуры 39 видов. Она исследовала фунгистатическую активность выделенных материалов in vitro к 6 грибкам. Фунгистатическая активность была значительная у видов Phomopsis cordifolia (Brun) Died. и Phoma longissima West. и слабая у Coniothyrium fuckelii Sacc., Microdiplodia henningsii Star. и Monochaetia viticola Cavara. В поверхностной культуре 5 активных видов автор исследовала на 3 различных питательных средах производства действующего вещества. Самые высокие титры Aspegrillus niger она наблюдала при культурах Phoma longissima West. поставленных на 15-дневной полусинтетической питательной среде. Phoma longissima West. культивированный на искусственной питательной среде, через $^1/_2$ г. потеряет фунгистатическую активность.

АДЕНОВИРУСЫ, ВЫДЕЛЕННЫЕ ИЗ ТКАНИ МИНДАЛИН

И. НАС, М. ТОТ и А. ЛЕНДЕЛ

Авторы обработали тканевые культуры 12-и миндалин и аденоидов — полученных при тонзилэктомии — для выделения аденовируса. Удалось выделить три штамма вируса,

которые проявляют характерный цитопатогенный эффект в культурах клеток HeLa, почек кролика и человеческого амниона. Реакцией связывания комплемента и вируснейтрализующей пробой установили, что из трех штаммов один относится ко 2 типу, остальные два — к пятому типу аденовирусов.

ИССЛЕДОВАНИЕ ВНУТРИКОЖНОГО ПРИМЕНЕНИЯ ВАКЦИНЫ ДИЗЕНТЕРИИ

И. КЕТИ

1. В опытах, проведенных на кроликах, титры агглютинина, образующиеся под влиянием вакцины, привитой внутрикожно — подтверждены пассивной защитной пробой на мышах — значительно превышали титры, полученные подкожным методом.

2. Активная иммунизация мышей, сделанная внутрикожно корпускулярной вакциной, равноценная сделанной с Boivin антигеном, связанным с осадком, но подкожная иммунизация с тождественными антигенами дает менее защитный эффект.

3. При иммунизации небольшого количества добровольцев, сыворотки внутрикожноприви тых имели, по крайней мере, тождественную защитную способность, как и сыворотки, иммунизированных в 5 раз большим количеством антигеном, адсорбированным к осадку. Некротизирующее действие внутрикожной вакцины противоречит приме нению этого метода.

ИССЛЕДОВАНИЕ ПОД ЭЛЕКТРОННЫМ МИКРОСКОПОМ ТОНКОЙ СТРУКТУРЫ E. COLI

И. ХОЛЛОШ и А. БАРНА

1. С применением техники ультратонких срезов изучена под электронным микроскопом тонкая структура E. coli в спокойной и логарифмической стадиях размножения.

2. Авторы указывают на трудности электронно-микроскопического изучения ультратонких срезов материалов, фиксированных формалином, и на важность постановления подходящей температуры полимеризации.

АНТИГЕННАЯ СТРУКТУРА ШТАММОВ ВИРУСА ГРИППА, ВЫДЕЛЕННЫХ В ВЕНГРИИ В 1957 Г.

Д. ТАКАЧИ, К. БАРБ и Э. ФАРКАШ

В Венгрии в 1957 г. были две волны эпидемии гриппа. Причиной эпидемии в конце зимы, которая затрагивала детей в возрасте моложе 4 лет, был такой штамм A′, происхождение которого надо искать на основе близкого антигенного родства в штаммах, выделенных в конце эпидемии, бывшей тремя годами раньше. Причиной осенней эпидемии, которую сопровождала высокая заболеваемость, был азиатский вирус, но выделенные штаммы в перекрестной реакции ЗГА и в перекрестной пробе истощения с сыворотками куриц показывали отличие от прототипных штаммов. Иммунные сыворотки, приготовленные в хомяках и курицах против штаммов A′ и много человеческих сывороток, взятых перед эпидемией, содержат ГА ингибиторы и вируснейтрализирующие факторы, которые эффективны с венгерскими штаммами, но совсем не эффективны со штаммом Сингапур. Из 2 исследуемых советских штаммов один стоит ближе к штамму Сингапур, а второй является тождественным с венгерским штаммом. Авторы, помимо того, что признают роль специфических ингибиторов,предполагают, что венгерские штаммы азиатского вируса имеют общие антигенные компоненты со штаммами A′. Это, может быть, объясняется тем, когда бы мы предполагали, что случилась рекомбинация между штаммами Сингапур подобными и A′.

РЕЗУЛЬТАТЫ ВАКЦИНАЦИИ ПРОТИВ ПОЛИОМИЕЛИТА В 1957 Г.

А. ПЕТРИЛЛА

В Венгрии первый раз в связи с эпидемией полиомиелита в 1957 г. была применена вакцина по Салк-у. Прививки дали внутрикожно, по дозам 0,2 мл (на 2 вкольные места 0,1 0,1 мл), которые были повторены через 4 недели. Точную эффективность прививки определить по причине разнообразных затруднений не было возможно, однако, из многих данных можно сделать вывод, что вакцинация была эффективная. Эпидемия раньше снижалась, чем в других эпидемических годах или чем в соседних странах. Сравнивая заболевания, обследуемые в привитой возрастной группе с теми, которые появились в непривитых возрастных группах и рассчитывая на этой основе, в тех месяцах, когда уже можно было ожидать влияние прививок, по крайней мере, на 50—60%меньше заболеваний наблюдал в привитой группе, чем в непривитой. В форме индивидуальных анкет автор собирал данные, связанные с заболеваниями и прививкой, встречающимися в группе, назначенной на вакцинацию. Из этого исследования можно было определить, что в группе, назначенной на вакцинацию, заболеваемость среди непривитых была ровно в 5 раз больше, чем среди привитых. Обследования ограничиваются относительно небольшим количеством случаев, и поэтому расчет эффективности вакцинации можно принимать только приблизительным. Заболеваемость уже после первой прививки показала некоторое снижение. Прививки сделали во время эпидемии, но провоцирующее влияние не наблюдали.

ВЛИЯНИЕ ФОРМАЛЬДЕГИДА НА ВИРУС ГРИППА. II. ВЛИЯНИЕ НА ЗАРАЗИТЕЛЬНОСТЬ

Ш. КОХ и Е. ЧЁНКА

Реакция между формалином и вирусом гриппа кажется имеет аддиционный характер. На реакцию инактивации влияет 3 главных фактора, то-есть pH, относительная концентрация формальдегида и температура. При благоприятных условиях (щелочная среда или относительно высокая концентрация формалина) кривая реакции очень напоминает мономолекулярную. Чем ниже концентрация формалина и чем более кислая среда, тем больше отклоняется кривая от мономолекулярной.

Некоторые нерегулярности, которые были обследованы в связи с титрацией заразительности вируса, обработанного формалином, дают возможным сделать вывод, что при разведении существует некоторая реактивация заразительности.

ПРОДУКЦИЯ ОКСИТЕТРАЦИКЛИНА В СИНТЕТИЧЕСКОЙ ПИТАТЕЛЬНОЙ СРЕДЕ

И. ХОРВАТ, И. ГАДО и А. СЕНТИРМАИ

Авторы выработали просто составленную питательную среду при ферментативных опытах для производства окситетрациклина.

При постоянной пропорции глицина и углевода достигнутая максимальная продукция зависит от концентрации фосфата, который ускоряет использование ингредиентов питательной среды.

СПЕЦИФИЧНОСТЬ ЗАДЕРЖКИ ГЕМАГГЛЮТИНАЦИИ ВАКЦИНИИ СО СПИННОМОЗГОВОЙ ЖИДКОСТЮ

Й. САТМАРИ и Ш. ХОЛИК

Нормальная спинномозговая жидкость не влияет на способность вируса вакцины агглютинировать эритроциты куриц. Патологическое нарастание белка в спинномозговой жидкости задерживает гемагглютинацию независимо от того, что взята ли от больных,

привитых вакциной оспы несколькими неделями или многими годами раньше или даже от непривитых. Между титрами задержки сывороток и задержками, наблюдаемыми в спинномозговых жидкостях, нет соотношения.

Задержка, в общем, тем сильнее, чем больше количества белка в спинномозговой жидкости. Задержка является способностью глобулиновой фракции. Авторы предполагают, что причинами задержки гемагглютинации в сыворотке и наблюдаемой в единичных случаях в спинномозговой жидкости, являются полностью или в частности специфические вещества.

ДАННЫЕ К ВОПРОСУ АНТАГОНИЗМА К АНТИБИОТИКАМ

Й. ЛАЗАР

Автор исследовал действие комбинаций 6 антибиотиков (пенициллин, стрептомицин, хлорамфеникол, авреомицин, террамицин и сульфонамид) на штаммах 30 различных бактерий методом диффузии в пластинчатом агаре. По результатам стрептомицин ведет себя антагонистически к хлорамфениколу и к членам группы тетрациклина в случае стафилококков и других кокков. Пенициллин только редко действует антагонистически к этим медикаментам. Штаммы E. coli ведут себя различно.

ХАРАКТЕР ЕСТЕСТВЕННОГО ИММУНИТЕТА, ПРИОБРЕТЕННОГО ВСЛЕДСТВИИ ПЕРЕЗАБОЛЕВАНИЯ KERATOCONJUNCTIVITIS SHIGELLOSA

Б. ШЕРЕНЬ

1. Соответствующий анализ явлений, связанных с приобретенным естественным иммунитетом, вследствии перезаболеваний Kc. sh., возможен на основе необходимых опытных условий (подопытные и контрольные группы по составам гомогенные, регулярно восходящие заразные дозы).

2. Однократное перезаболевание Kc. sh. с точки зрения клинической защиты и иммунологии равным образом сопровождается иммунитетом, который, однако, слабый, и в большинстве случаев не в состоянии устранить патогенный эффект массивного заражения, только уменьшает явления и сокращает срок заболевания.

3. Клиническая защита выздоровленного глаза важнее, чем глаза незатронутого при первом заражении конъюнктивы. Иммунитет, относящийся к первому, неспецифичный, к последнему — специфичный.

4. Иммунитет имеет двойную материальную основу: гуморальный и тканевой иммунитет. Из них каждый имеет свое значение. Гуморальный иммунитет слабее, общий и типо-специфичный; тканевой иммунитет более сильнее, имеет местное значение и не специфичный.

5. Важнейшие характеристики естественного иммунитета, приобретенного перезаболеванием Kc. sh. может служить моделью для изучения теоретических и практических проблем иммунитета дизентерии.

ЭПИДЕМИЯ ПОЛИОМИЕЛИТА В ВЕНГРИИ В 1957 Г. I. ЭПИДЕМИОЛОГИЧЕСКОЕ ИЗУЧЕНИЕ

О. РУДНАИ

В Венгрии в 1957 г. была эпидемия полиомиелита с заболеванием 2334 (23,8/100 000). Эпидемия была более распространена в провинциях (26,2/100 000), чем в Будапеште (13,6/100 000). Наиболее затронута была северо-восточная часть страны. Заболеваемость в области Хайду-Бихар была наивысшая (88,2/100,000). Количество заболеваний было сравнительно большое уже и в начале года, быстро увеличивалось в мае, самую большую величину оно достигло в июле. За быстрым повышением следовало внезапное падение. Возрастная специфичная заболеваемость была наивысшая (284/100,000) в возрасте 1—2 лет, за ним следовал грудной возраст с заболеваемостью 194/100,000 и затем дети от 3 до 6 лет с 73/100,000. Пропорция участия в заболеваемости групп от 3 до 6 и от 7 до 14 летных с 1956 г. увеличивалась. Летальность в среднем была 6,1%. Возрастная специфич-

ная летальность в грудном возрасте превышала 9%, в детском возрасте с повышением возраста снижалась до 14 лет. Летальность была наивысшая среди заболевших в возрасте старше 15 лет.

ЭПИДЕМИЯ ПОЛИОМИЕЛИТА В ВЕНГРИИ В 1957 Г. II. ЭТИОЛОГИЧЕСКОЕ ИЗУЧЕНИЕ

Э. МОЛНАР

1. Для выделения вируса были исследованы испражнения 65 случаев паралитических полиомиелитов и 134 асептических менингитов.

2. В трипсинизированных культурах почек обезьян выделено 65 цитопатогенных штаммов и из этих же материалов только 29 в кожно-мышечных культурах человеческих эмбрионов.

3. В культурах почек обезьян 60% из испражнений паралитических больных являлись цитопатогенными, 85% штаммов типизированы, как 1. тип, остальные принадлежат к 2. типу вируса полиомиелита. Из одного исследуемого материала были выделены как 1. так 2. типы вируса полиомиелита.

4. Из кала больных асептическим менингитом только 19% являлись цитопатогенными. Половина штаммов была вирусом полиомиелита 1. типа, четвертая часть принадлежала к 9. типу вирусов ECHO, остальные еще не идентифицированы.

5. Патологическую роль вируса ECHO 9 в ряде случаев асептических менингитов можно предполагать.

ИЗУЧЕНИЕ ЦИТОПАТОГЕННОГО ЭФФЕКТА АДЕНОВИРУСОВ РАЗНЫХ ТИПОВ В КУЛЬТУРАХ ТКАНЕЙ ЧЕЛОВЕЧЕСКОГО ЭМБРИОНА И КЛЕТОК ДЕТРОИТ 6.

И. НАС и М. ТОТ

Авторы изучали цитопатогенный эффект 14 штаммов, принадлежащих к первым 7 типам аденовирусов на тканевых культурах человеческого эмбриона и клетках Детроит 6. Они установили, что тканевая дегенерация, вызванная штаммами 3., 4. и 7. типов, по характеру различается и хорошо отделима от тканевой дегенерации, вызванной штаммами 1., 2., 5. и 6. типов. Оба типа дегенерации излагаются подробно. В тканевой культуре Детроит 6 не удалось наблюдать значительного отличия дегенерации клеток, вызванной штаммами разных типов.

ОПЫТЫ ТРАНСДУКЦИИ В ГРУППЕ SALMONELLA

Г. КЕЛЕМЕН

1. Фаговые лизаты, необходимые для трансдукционных опытов Salmonella, автор приготовил Зейц — фильтрованием симбиотических культур штаммов. Техникой трансдукции он применял культивирование в изогнутой «u» образной трубке, наполненной жидким агаром и фаг-лизатом.

2. С фаг-лизатом Lederberg PLT 22 он наблюдал появление жгутиковых у стабильных O-штаммов S. typhi u S. paratyphi B и также жгутикого антигена («i») у донорского штамма.

3. От S. enteritidis и S. essen с фаг-лизатом выделенным нами, удалось передать жгутиковый антиген вышеупомянутому O-штамму S. paratyphi B, приготовляя по антигенной структуре новый рекомбинированный штамм.

4. Среди обычного исследуемого материала автор нашел один не типизированный и не подвижный штамм с соматической антигенной структурой 4,5, у которого с фагом PLT 22 ему удалось в 20% случаев получить трансдукцию «i» фазы, а в 60% появление „b" и „1,2" фазы. В то же самое время с фагом (PE 269) выделенным из S. enteritidis в 50% случаев появилась трансдукция жгутиковой фазы «gm».

5. Автор с фагом (PLT 22) размножившимся на штамме S. paratyphi A получил из штамма S. enteritidis штамм, соответствующий по антигенной структуре S. sendai.

6. Фаг PLT 22 растворял воспринимающие клетки. У рекомбинированных штаммов антигенная структура изменилась, но первоначальные свойства сохранились.

ЭЛЕКТРОННО-МИКРОСКОПИЧЕСКОЕ ИЗУЧЕНИЕ КОМПЛЕТНОГО И НЕКОМПЛЕТНОГО ВИРУСА ГРИППА. II. ТОНКАЯ СТРУКТУРА КОМПЛЕТНОГО ВИРУСА ГРИППА, ИССЛЕДОВАННОГО НА УЛЬТРАТОНКИХ СРЕЗАХ

И. ХОЛЛОШ и А. БАРНА

Авторы изучали тонкую структуру очищенных частиц комплетного вируса гриппа А на ультратонких срезах. На основе литературных данных — которые были в распоряжении авторов — и на основе собственных исследований они пытаются установить гипотезу о внутренней структуре комплетного вируса гриппа.

ИССЛЕДОВАНИЕ ОПТИМАЛЬНЫХ УСЛОВИЙ ПРОДУКЦИИ МЕГАЦИНА В СИН ТЕТИЧЕСКОЙ СРЕДЕ

Э. НАДЬ

В культурах мегациногенных бактерий оптимальную продукцию мегацина в синтетической среде можно индуцировать низкой дозой УФ лучей. Высокая продукция мегацина достижима только в случае облучения удовлетворительно развитых молодых культур. Мегациногенные штаммы в отношении количества продукции мегацина различные. Очень мегацино-продуктивный штамм 216 B. megaterium.

ЭФФЕКТИВНЫЙ МЕТОД ДЛЯ ВЫДЕЛЕНИЯ ФАГОВ СИБИРСКОЙ ЯЗВЫ

Г. ИВАНОВИЧ и Й. ЛАНТОШ

С применением штамма, резистентного к стрептомицину, B. anthracis в присутствии стрептомицина очень просто и без фильтрации очень часто можно выделить из почвы штаммы фагов, сильно специфичные к B. anthracis. Авторы верят в то, что основы метода можно применять и для получения других фагов.

ПРИМЕНЕНИЕ ЧЕТЫРЕХХЛОРИСТОГО УГЛЕРОДА В ПРИГОТОВЛЕНИИ ВАКЦИНЫ СЫПНОГО ТИФА ПО ТИПУ КОКС-А

Ш. КОХ и А. БЕНКЕ

Применение четыреххлористого углерода в массовом производстве вакцины сыпного типа обосновывают следующие результаты:

1. Метод является очень простым.

2. Экстракт желточного мешка в присутствии 10% четыреххлористого углерода в физрастворе с фенолом содержит на 24% меньше сухого вещества, чем раньше применяемая подобная вакцина.

3. В выпускном продукте четыреххлористый углерод не доказывается.

4. В пробах эффективности на морских свинках антигенность вакцины, обработанной четыреххлористым углеродом, является лучшей, чем вакцины, употребляемой до сих пор.

5. 1000 кратная и 4000 кратная доза на один кг веса тела вакцины, приготовленной новым методом, не имеет неприятного влияния в мышах и в морских свинках.

КОЛИЧЕСТВЕННЫЙ ПОЛУ-МИКРОМЕТОД КУЛЬТИВИРОВАНИЯ ТКАНЕЙ И ЕГО ПРИМЕНЕНИЕ В МИКРОБИОЛОГИИ. I. КОЛИЧЕСТВЕННЫЙ ПОЛУ-МИКРОМЕТОД КУЛЬТИВИРОВАНИЯ ТКАНЕЙ

И. ХОРВАТ и В. БАЛАЖ

Авторы выработали новый количественный метод культивирования тканей, по которому средние результаты роста фибробластов, измеряя от края эксплантата в направлении радиуса и считая это радиусом одного круга, вычислением площади этого круга, сравнительным числом можно характеризовать рост фибробластов в мм². Этим методом практически, между довольно широкими пределами, устраняли разницы роста, получившиеся из величины эксплантатов тканей.

Кусочки тканей авторы положили на тонкий коагулат плазмы, находившейся в открытой 2 мл пробирке с суженным горлышком, затем пробирки, содержащие кусочки тканей, положили в барабан из алюминия и после дозировки питательной среды, вращая в инкубаторе, культивировали.

С помощью метода исследовали роль разных факторов. Изучали отношения роста и дезинтеграции фибробластов, определили погрешность опытов в случае применения параллель в разных количествах, исследовали гущу клеток во время культивирования, роль вращения, определили оптимальные концентрации веществ питательной среды, и т. д.

Преимущества метода можно суммировать в следующих пунктах:

1. Метод является объективным и практически устраняет, между довольно широкими пределами, разницы роста, возникающие из величин эксплантатов.

2. На небольшом месте с применением небольшого количества плазмы, эмбрионального экстракта и питательной среды, можно работать с большим количеством тканевых культур.

3. От того, что все эксплантаты растут, работа с ними уверенная, быстрая и простая.

4. Применением открытых пробирок не только выгадывают закрытие пробирок, но тогда же наступает медленное изменение pH.

5. Метод можно еще применить, при опытах упомянутых в статье, также для исследований стимулирующего или задерживающего влияния аминокислот, витаминов и т. п. на рост фибробластов, для распределения токсичности веществ в тканевых культурах и для титраций вирусов.

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