# ACTA MICROBIOLOGICA
## ACADEMIAE SCIENTIARUM HUNGARICAE

ADIUVANTIBUS

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

REDIGIT

G. IVÁNOVICS

TOMUS VI FASCICULUS 1

1959

ACTA MICROBIOL. HUNG.

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## A MAGYAR TUDOMÁNYOS AKADÉMIA MICROBIOLÓGIA KÖZLEMÉNYEI

KIADÓHIVATAL: BUDAPEST V., ALKOTMÁNY UTCA 21.

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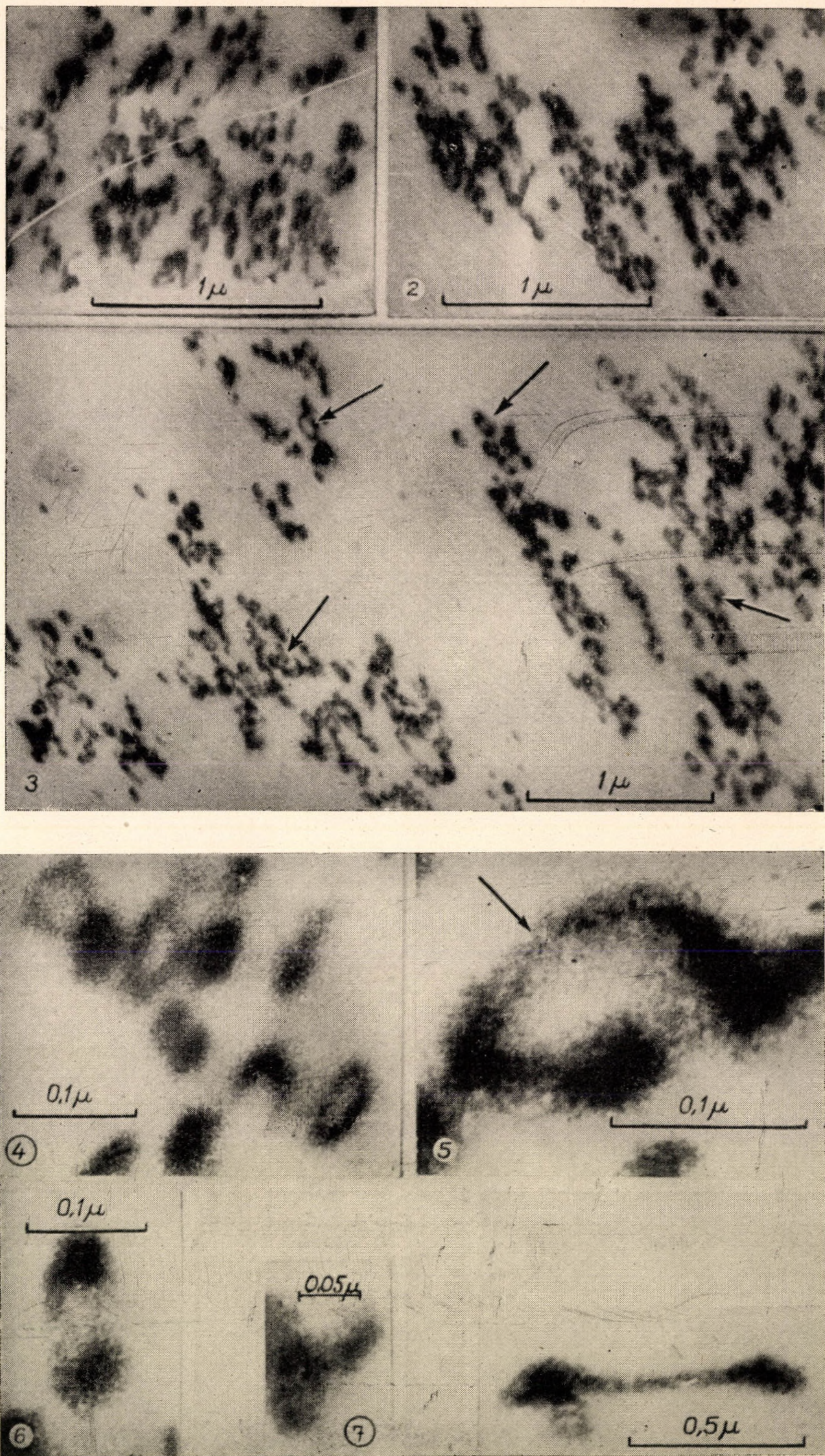


These plates are better copies of the photographs published in Acta Microbiologica Hungarica Vol. 5, pp. 391, 393. Subscribers are requested to insert them in the paper of HOLLÓS, I. and BARNA, A.: Electronmicroscopic examination of complete and incomplete influenza viruses II. Studies of the fine structure of complete influenza virus

# MALONIC ACID AS THE SINGLE CARBON AND ENERGY SOURCE OF PATHOGENIC FUNGI*

By

E. K. NOVÁK

*State Institute of Hygiene, Budapest*

(Received June 2, 1958)

In a previous communication (1955) it has been reported that certain fungi were found able to use malonic acid as the single carbon and energy source.** Similar experiments carried out on *Botryotinia fuckeliana,* together with a description of some initial steps of malonic acid metabolism, have been published last year [1, 2].

Literary data referred to earlier [2] and some more recent publications [3—9] have dealt with the metabolism enhancing effect of malonic acid in bacteria, plant and animal tissue slices, while concerning fungi no similar studies have been reported.

When performing experiments with malonic acid as the substrate for pathogenic fungi, a double goal has been aimed at : (i) the study of the biochemistry of malonic acid metabolism ; (ii) possible taxonomic characterization of certain fungi according to their ability to use malonic acid as the single carbon source.

## Materials and methods

*Strains* maintained on Sabouraud glucose agar medium in the Type Collection of the Mycological Laboratory of this Institute were used throughout.

*Medium.* The basal medium was composed of 0.5 per cent malonic acid ; 0.4 per cent (or 0.2 per cent) sodium hydroxide ; 0.5 per cent $(NH_4)_2SO_4$ ; 0.1 per cent potassium dihydrophosphate ; 0.05 per cent $MgSO_4 \cdot 7H_2O$. The pH of the medium was 6 or 5, depending on the amount (0.4 or 0.2 per cent, respectively) of the sodium hydroxide added. The medium was used in fluid form or it was solidified by the addition of 3.5 per cent agar-agar. Silica gel plates soaked with the above malonic acid medium were used in some cases. Sterilization was performed in vapour stream at 100° C for 30 minutes. This treatment, even when applied repeatedly, did not damage malonic acid. The control media were of similar composition except for the addition of malonic acid and sodium hydroxide.

The liquid medium was dispensed in test tubes 5 ml each, and before inoculation two drops of yeast extract were added. Agar and silica gel plates were inoculated in appropriate sectors.

* Part of this work was presented at the 1957 *Congress of the Hungarian Microbiological Association.*

** Presented before the Section for General Biology of the *Hungarian Association of Biologists*, May 17, 1955.

## Experimental

The malonic acid media were observed for two weeks after inoculation. Outgrowth of the single species had to be verified by reisolation on Sabouraud agar and identification [10, 11, 12], as only very few of the organisms tested had fructified on the simple malonic acid medium. The results are presented in *Table I.*

**Table I**

*Growth on malonic acid medium*

| Species | Malonic acid silica gel medium | | Fluid malonic acid medium pH 6 | Remarks |
|---|---|---|---|---|
| | pH 6 | pH 5 | | |
| *Monosporium apiospermum (Allescheria boydii)* | + | + | + | fructified on silica gel medium |
| *Hormodendrum compactum* | + | + | + | |
| *Phialophora verrucosa* | + | + | + | |
| *Scopulariopsis sp.* | + | + | + | |
| *Trichophyton gypseum* | — | — | + | |
| *Blastomyces brasiliensis* | — | — | + | |
| *Cephalosporium sp.* | + | + | + | |
| *Aspergillus niger* | — | + | + | |
| *Aspergillus fumigatus* | + | + | + | fructified on silica gel medium at pH 5 |
| *Nocardia asteroides* | + | — | + | |
| *Geotrichum candidum* | + | + | + | |
| *Candida tropicalis* | + | + | + | |
| *Candida albicans* | + | + | + | |
| *Cryptococcus neoformans* | + | + | + | |
| *Candida guilliermondii* | + | + | + | |
| *Candida parapsilosis* | + | + | + | |
| *Candida rugosa* | + | + | + | |
| *Candida stellatoidea* | + | + | + | |
| *Candida krusei* | + | + | + | |
| *Rhodotorula mucilaginosa* | + | + | + | |
| *Helmintosporium sp.* | — | — | — | |
| *Histoplasma capsulatum* | — | — | — | |
| *Microsporum gypseum* | — | — | — | |
| *Cryptococcus luteolus* | + | / | / | Preliminary data from moderate number of experiments |

When inoculated on malonic acid silica gel medium, *Phialophora verrucosa* and *Aspergillus fumigatus* fructified and developed colonies macroscopically identical with those observed on Sabouraud glucose medium.

Of the yeasts examined, *Candida albicans* and *Candida krusei* exhibited the most vigorous growth, while other species of fungi grew only slowly as compared to the yeasts. It is worth mentioning that in two instances remarkable differences were detected in the growth of related species. Thus, *Aspergillus niger* exhibited only moderate growth on malonic acid medium as compared

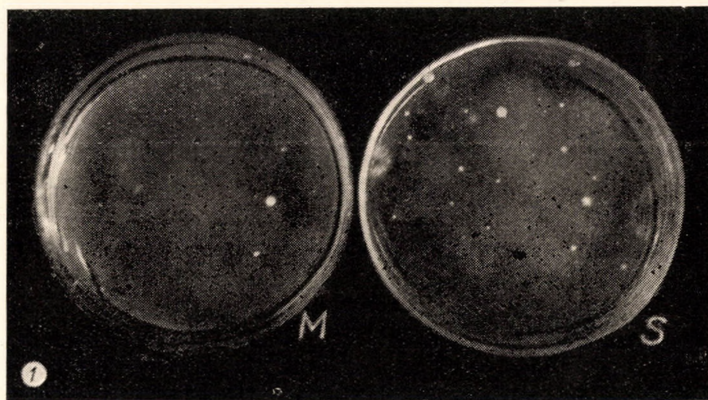


*Fig. 1.* Contamination test on malonate acid agar medium
M = Malonic acid agar medium of pH 6
S = Sabouraud glucose agar medium of pH 5.6

to *Aspergillus fumigatus*, moreover at pH 6 the former fungus did not grow at all. *Aspergillus fumigatus*, on the other hand, grew fairly vigorously under both pH conditions, producing even conidia at pH 5. As to the Cryptococci, *C. luteolus* developed colonies in two or three days, in contrast to *C. neoformans* which exhibited the slowest growth rate in the group of yeasts and developed hardly realisable colonies within the same period. Data concerning the growth of *C. luteolus* were derived of a moderate number of experiments.

As the malonic acid medium was found practicable in cultivating and isolating fungi, its sensitivity to bacterial contamination had to be determined. Four plates each of malonic acid medium of pH 5 and pH 6 were exposed to open air for eight hours. As a control, four plates of Sabouraud glucose medium (pH 5.6) were similarly exposed. Two plates of each group were incubated at 26° C and two at 37° C. The incidence of bacterial contamination was generally 2 to 3 colonies in each plate of malonic acid medium. As the number of the plates used in the experiment did not suffice for evaluating the results statistically, we only present a photograph of the most heavily infected malonic acid medium together with one of the Sabouraud media from

the above experiment. Each plate had been incubated for 3 days at 26° C before the photograph was taken.

On the basis of the above results we are using the malonic acid medium in routine diagnostical work and have obtained satisfactory results.

## Discussion

Successful cultivation of 20 pathogenic fungi on a medium containing malonic acid as the single carbon source has proved these organisms to be able to metabolize this compound as the single carbon containing substrate. Similar results had been obtained earlier with *Botryotinia fuckeliana* as the test organism.

As to the different pathways of malonic acid metabolism, no data have been presented in the present report and we only mention the results of some Warburg studies showing that in few of the 20 organisms mentioned above oxidation seemed to be the initial step of malonic acid degradation, in contrast to some earlier results obtained with *Botryotinia fuckeliana.* Examination of the dehydrogenase activity also suggested differences between *Botryotinia fuckeliana* and the fungi in the present studies. This would mean that in fungi there occur different forms of malonic acid metabolism.

## Summary

Of a total of 24 different fungus species tested, 20 were found able to use malonic acid as the single carbon and energy source.

In related species differences were demonstrated in the metabolization of malonic acid. This may serve for differentiating certain related organisms.

The use of malonic acid medium is recommended for routine diagnosis in medical mycology.

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# A QUANTITATIVE SEMI-MICRO TISSUE CULTURE METHOD AND ITS USE IN MICROBIOLOGY

## II. USE OF THE METHOD IN TITRATING THE VIRUS OF AUJESZKY'S DISEASE

By

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(Received June 11, 1958)

The apparatus used for tissue culturing in our novel quantitative method described in an earlier paper [8] seemed to suggest itself for a trial in the titration of viruses that give rise to degenerative changes in cells. The virus of Aujeszky's disease (Ay virus for short) appeared to be the most suitable for use in these titration experiments. This virus has been subjected to numerous experiments in this Institute [1—7], and CSEREY *et al.* [1] were early in demonstrating that, being capable of causing marked degenerative lesions in chicken-heart fibroblasts, this virus is titratable in tissue culture.

The present report deals with investigations into the applicability of the semi-micro method for titrating and neutralizing the Ay virus.

### Materials and methods

1. *The Ay virus strain.* Our viral strain marked XXV, originally isolated from pig brain, has been maintained in Maitland-type cultures prepared from chick-embryonic tissue [1]. The strain adapted to this tissue was passaged, again in Maitland-type culture, in a nutrient fluid to be described later in this paper. Passages were performed every other day. Tissue extract triturated in the liquid nutrient and cleansed by centrifugation was used as the inoculum in a dilution of 1 to 100. Of this dilution a quantity of 0.1 ml was measured to 5 ml of culture fluid containing tissue fragments from a 12-day-old embryonated egg. Material extracted from tissue, taken up in the supernatant, and freed from the sediment by centrifugation, was invariably used as the viral suspension. The viruses were stored in the frozen or lyophilized state at —20° C.

2. *The immune serum* was obtained from a guinea pig repeatedly inoculated with virus inactivated by means of ultraviolet irradiation and subsequently challenged with infective virus [6]. For the guinea pig immunized in this manner we are indebted to DR. BÉLÁDI. Into this guinea pig 1 ml of viral suspension obtained from tissue cultured for two days was injected subcutaneously on each of two additional occasions. Two weeks after the last injection the serum of the blood drawn from the guinea pig was stored away in 1 ml ampoules, at —20° C. On using them, the immune sera were inactivated at 56° C for 30 minutes.

3. *The tissue culture and its inoculation with virus.* The methods used in preparing and maintaining tissue cultures, the equipment applied, and the procedures followed in cleaning and sterilizing glass vessels, have been described in previous papers [8, 9]. Chicken-heart fragments obtained from 12 to 13-day-old embryonated eggs were used for titrating the virus. In routine titrations three tissue fragments were placed on top of the thin plasma coagulum smeared over the wall of each of a number of 2 ml phials ; thereafter culture fluid was measured into them and they were rotated in drums in an incubator.

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As a culture fluid, our own buffered solution [8] was employed to which 0.5 per cent egg white was added from a stock solution. The stock solution was prepared from fresh eggs, dissolving 20 per cent egg white in the buffered salt solution ; after mixing, this was kept for one day in the refrigerator at +4° C, then centrifuged, distributed into ampoules, and stored in the frozen state at —20° C. The stated quantity of egg white is conducive to the proliferation of fibroblasts, as can be seen in *Fig. 1.* Another reason why we used just that quantity of egg white in the culture fluid was that it had been found to stabilize the Ay virus to a readily demonstrable degree. The method of measuring tissue proliferation has been described and diagrammatically represented in our previous communication [8].

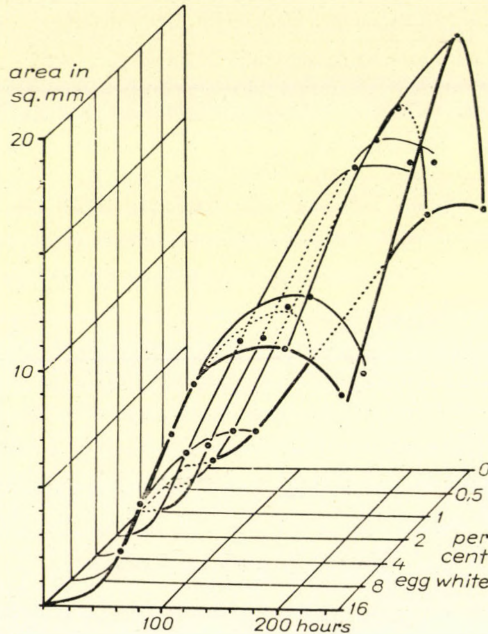


*Fig. 1.* Growth of chicken-heart fibroblasts in buffered salt solution containing egg white in different quantities

Precultivation for two days preceded the inoculation of the tissues with virus. The drums were placed vertically on a drum stand, with the tissue cultures in them well-protected against infective fungal spores from the air. Using TAKÁTSY's spiral loop [10], the virus was diluted in agglutination tubes containing the solution employed in the preparation of semi-micro tissue cultures. Of the viral suspensions 0.1 ml amounts were measured with the aid of 1 ml pipettes into each glass vessel. Infective titres were calculated according to Kärber's method [13].

## Experimental

1. *Selection of the liquid medium for use in preparing Maitland-type cultures.* Three different kinds of culture fluid were tested, *viz.* Hanks' solution [11], Simms' $x_7$ solution [12], and a buffered solution currently used by us in titrating influenza virus [9]. To each of them 0.5 per cent egg white was added. The Maitland-type cultures were inoculated with Ay virus from the 178th passage. The quantities of virus present in the supernatants were checked by titration during cultivation. In Hanks' solution viral pro-

liferation was found to cease rather soon, with an early shift of the pH in the acid direction. In the other two solutions the pH changed slowly. Simms' $x_7$ yielded viral infectivity about one exponent higher than the other solutions. Accordingly, Simms' $x_7$ solution containing 0.5 per cent egg white was made the solution of choice for cultivation.

2. *Inactivation of the Ay virus under various differing conditions.* In testing the virus for inactivation, one portion of the tissue extract containing virus from the 185th passage was sealed in ampoules, the rest was lyophilized in small tubes. Both viral materials were stored at —20° C. Using them at

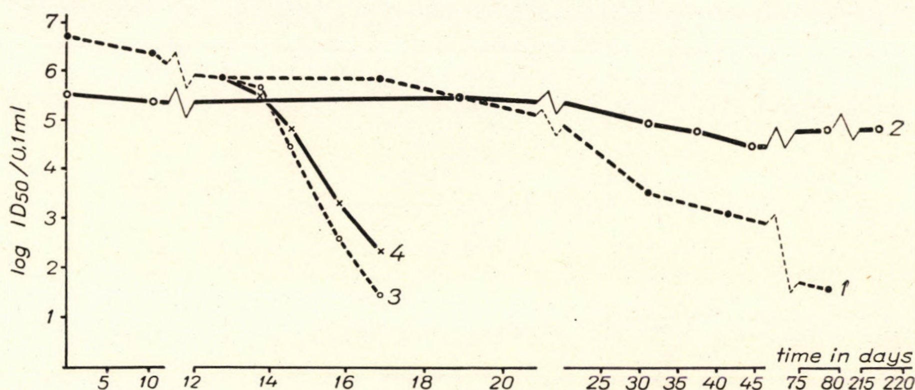


*Fig. 2.* Inactivation of the Ay virus under different conditions

1. Non-lyophilized material stored at —20° C. — 2. Lyophilized material stored at —20°. — 3. Inactivation at +37° in Simms' solution — 4. Inactivation at +37° in Simms' solution containing egg white

different dates, they were titrated in five parallel tubes per dilution. In one experiment inactivation at +37° C too was studied in Simms' solutions containing no egg white and 0.5 per cent egg white, respectively. The results are illustrated in *Fig. 2.*

Even in the frozen state the virus remained viable for several months. In the lyophilized state there was hardly any perceptible reduction in infectivity, not even after 6 to 8 months. For this reason preference was given to lyophilized preparations, the infective titres of which were known with dependable accuracy.

*Fig. 2* shows that inactivation at +37° C required several days to complete. The curves are indicative of definite, though not very considerable, viral stabilization.

3. *The interrelation of infective titre and the number of tissue fragments per tube.* For each dilution 15 tubes were used in parallel, and 1, 2, 4, and 8 tissue fragments, respectively, were explanted per glass vessel in each group. Thereafter, without precultivation, the tubes were inoculated with virus

from the 185th passage. The results were read five days later. They are shown in *Fig. 3*.

On statistically analysing this experiment, the coefficient of regression was found to be quite near the limit of significance (P = 0.05). On the other hand, a significant difference (P = 0.01) was seen to prevail between the two extreme titre values.

It appears safe to state that — probably within certain limits — the infective titres are functions of the number of tissue fragments, respectively the quantity of tissue. In culture fluids with 1 or 2 tissue fragments in them

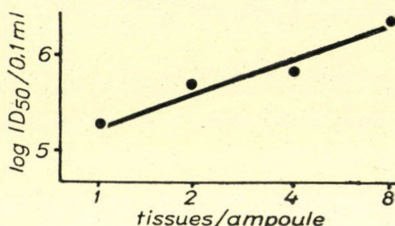


*Fig. 3.* Changes in the infective titre in dependence on the number of tissue fragments

relatively low titres, in those containing more tissue higher titres were obtained. In *Fig. 3* a difference of about one exponent can be seen in dependence on whether 1 or 8 tissue fragments have been explanted per ampoule. This is no negligible difference, particularly not with viruses of otherwise rather low infective titres.

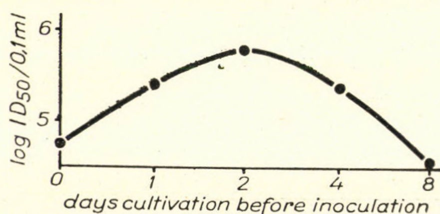


*Fig. 4.* Changes in the infective titre in dependence on the duration of precultivation

4. *The interrelation of infective titre and precultivation.* In one of our experiments the tissue cultures were precultivated for varying periods of time before inoculation with the virus. In one group infection with the virus was preceded by no precultivation, in another by precultivation for 1 day, in a third for 2 days, in a fourth for 4 days, and in a fifth for 8 days. The results of this experiment carried out in 15 parallel tubes per dilution are presented in *Fig. 4*.

It can be seen that different periods of precultivation yielded different infective titres. The highest titre was obtained after precultivation for 2 days

only. With the tissues inoculated on the 8th day the infective titre was found to be lower. The fact that after 8 days' precultivation there still was viral proliferation, is proof of the fibroblasts' viability for at least another 2 or 3 days.

Statistical analysis revealed significant differences in the infective titres of the tissues precultivated for 0 and 2 days ($P = 0.01$), as well as between those precultivated for 2 and 8 days ($P = 0.05$).

Since growth was observed in practically all the tissue cultures, it seemed a justified question why precultivation for some length of time should at all precede infection with the virus ; was it not probable that subsequent absence of growth or minimal growth would be due to the degenerative action exerted by the virus? On the other hand, there is the fact that the infective titre obtained after two days' precultivation was by about one exponent higher than that obtained when the tissue culture was inoculated with the virus without any previous precultivation. This is a difference that cannot possibly be ignored.

In addition, it was of interest to establish how the high infective titre develops at different cell counts. The height of titres is not determined exclusively by the number of cells. Were the titre dependent on the cell count alone, we would not, in our case, have obtained the highest titre from the tissues precultivated for two days, but from those which, for example, had been precultivated for 4 or 8 days. For its proliferation the virus apparently depends more on the momentary viability than upon the number of the cells. The optimal duration of precultivation being two days, the time for inoculation coincides with the beginning of the logarithmic stage of fibroblastic multiplication.

5. *The effect of rotation upon the infective titre.* Using 15 tubes per dilution, one of our experiments was designed to find out if there were changes in the infective titres in dependence on whether or not the tubes were rotated after inoculation. The results are presented in Table 1.

**Table I**

*Changes in the infective titre in dependence on different conditions*

| Precultivated for | After infection | $ID_{50}/0.1$ ml |
|---|---|---|
| 2 days | rotated | $10^{5.81}$ |
| 2 days | not rotated | $10^{5.50}$ |
| 0 day | rotated | $10^{5.18}$ |

Virus from the 196th passage was used in this experiment. *Table I* shows that tissues not rotated after inoculation yielded a sligthly lower titre

than those rotated, and that, as is congruent with what has been said above, a still lower titre was obtained from tissue not precultivated.

Although the statistical evaluation revealed no significant difference in the infective titre between the rotated and non-rotated tubes, in several individual experiments infectivity was nevertheless found to be higher in the viruses titrated in the rotated tubes. The rotated tissue cultures undoubtedly outliving the non-rotated ones by 2 or 3 days, their use in the titration of the virus is obviously advisable.

6. *Determination of the limit of error in titration, and plotting the degeneration curve.* In the experiment designed to determine the infective titre, 30 parallel tubes were used per dilution in a series of viral dilutions prepared at differences of 0.33 log. In addition, a dilution containing a higher infective dose ($10^{-4}$) was included into the experiment, in order to study fibroblastic multiplication under such conditions as well. Virus from the 196th passage was used. With the viral dilutions the tissues were infected after 69 hours of precultivation. Subsequently, along with tissue growth, the number of positive tubes was recorded, *i.e.* of those in which fibroblastic disintegration was complete. The changes in their number are shown in *Table 2.*

**Table 2**

*Changes during cultivation in the disintegration of tissues infected with different dilutions of Ay virus*

| Viral dilution — log | Hour of readings | | | | |
|---|---|---|---|---|---|
| | 96th | 115th | 144th | 185th | 234th |
| 4.00 | 4 | 30 | 30 | 30 | 30 |
| 5.00 | 0 | 17 | 29 | 30 | 30 |
| 5.33 | 1 | 5 | 18 | 22 | 22 |
| 5.66 | 0 | 3 | 12 | 18 | 18 |
| 6.00 | 0 | 2 | 7 | 11 | 11 |
| 6.33 | 1 | 1 | 2 | 3 | 3 |
| 6.66 | 0 | 0 | 2 | 2 | 2 |
| 7.00 | 0 | 0 | 0 | 1 | 1 |

Number of tubes — out of a total of 30 tubes — displaying disintegration due to viral action

One of the curves in *Fig. 5* indicates the percentage of disintegration in viral dilutions up to $10^{-5}$—$10^{-7}$. The number of positive tubes in the 234th hour was taken to be 100 per cent, for it was by then that viral disintegration appeared to have come to its termination.

Our objective in studying the time required for disintegration, was to determine the minimum length of time which, when titrating the Ay virus

under these conditions, must be allowed to elapse before the experimental results can be read. To this end, only the area was included in the evaluation where in one of the dilution series every tube was already positive up to the dilution when every tube became negative, respectively when in the experiment only a single one became positive. This zone may be called the transition zone. In this area positivity will develop at the latest point of time only, because it is here that the quantity of the inoculated virus is the least, and so in

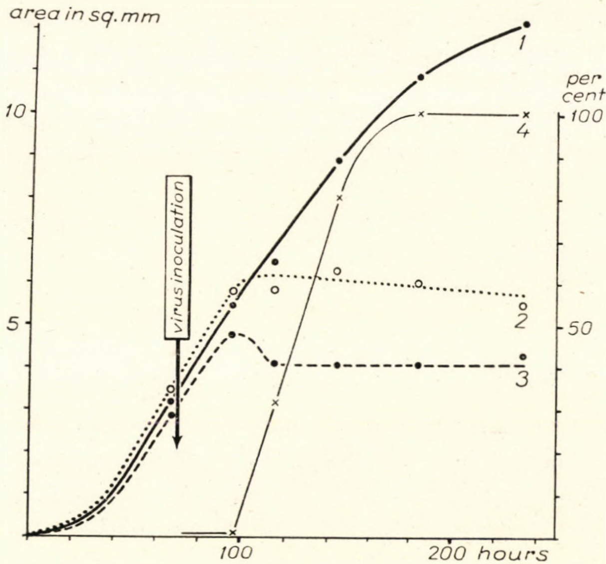


*Fig. 5.* Growth of fibroblasts infected with Ay virus and development of the disintegration curve

1. Fibroblastic growth in all the negative explants within the transition zone.
2. Fibroblastic growth in all the positive explants within the transition zone.
3. Fibroblastic multiplication in explants infected with viral suspension of $10^{-4}$ dilution.
4. Disintegration curve.

calculating the virus titre this area is of the greatest importance. The disintegration curve shows that if it is desired to obtain 100 per cent positivity in the experiment, then the readings are to be taken at least 4, but preferably 5, days after the infection.

Following the principles outlined in our previous communication [8], fibroblastic multiplication was calculated for each and every positive or negative tissue found in the transition zone ; the relative proliferation curves can be seen in *Fig. 5.* The curve for the tissues grown in the negative tubes runs the course usual for non-infected tissues, while the curve for the tissues grown in the positive tubes shows that logarithmic fibroblastic multiplication continues for nearly 30 hours following infection, whereafter it suddenly

breaks off, never to be resumed again. Largely the same course is run by the curve for fibroblasts inoculated with virus suspension of $10^{-4}$ dilution.

Logarithmic growth following viral inoculation does not mean that the cells infected with the virus have grown. In these dilutions only a few $ID_{50}$ have found their way into the tubes ; assumedly not more than a few cells have been infected. However, the few destroyed cells are not noticed, and disintegration is only found to be marked, when all the fibroblats show the usual picture. It follows that only the cells non infected with virus have participated in the active growth observed for 25 to 30 hours. It merits mentioning that although on some occasions a very massive inoculum ($10^{4.39}$ $ID_{50}$/0.1 ml) had been introduced into the tubes, active fibroblastic multiplication was still observed on the first day. It may be assumed that following infection the cells are still capable of growth, or perhaps even to undergo one more additional division.

The $ID_{50}$ calculated from the data of the above-mentioned experiment corresponded to $10^{5.80}$/0.1 ml, while the calculated standard deviation was found to be $\pm 0.056$. On using serial dilutions and parallels different from those applied in the above experiment, a considerably higher dispersion must naturally be taken into account.

Availing ourselves of one of FINNEY's methods [14] and of the kind assistance of L. CSIZMÁS (from the *Human Vaccine Production and Research Institute)*, we calculated from the data obtained in the above experiment the values corresponding to Poisson's expectable distribution (E/r). These are represented in the diagram in *Fig. 6*, together with the values (r) obtained in the experiment.

On the evidence of *Fig. 6* there was hardly any difference between the values calculated and obtained ; this proves that the dose-response curve, *i.e.* the relation of viral concentration to the number of positive tubes, can be closely approximated by the curve for Poisson's distribution.

In our experiments nothing but the number of parallels seems to have influenced the exactness of the titrations. The calculated $\chi^2$ corresponded to 45 per cent, which shows that the values obtained do not differ essentially from those arrived at by means of the Poisson's distribution.

7. *Comparing titration by means of the semi-micro method with an earlier procedure in use in this Institute* [6]. Formerly, the viral dilutions were first precultivated for three days in Huang-type cultures whereafter, following tissue explantation in hanging-drop culture, the inoculated tubes were examined for viral proliferation.

In our experiment, 10 Huang-type chicken-heart tissue cultures were used. Three days after infection with the viral dilutions, 0.1 ml of the fluid in the tubes was added to the precultivated tissues in each of the rotating tubes, in order to find out the liquid of which culture contained the virus.

At the same time, three of the heart fragments in the tubes were made to continue growing in each of a number of hanging-drop cultures. The infective doses found were, $10^{6.00}$ $ID_{50}/0.1$ ml, with titration in rotating drums of precultivated tissues; $10^{5.28}$ $ID_{50}/0.1$ ml in Huang-type tissue cultures; and $10^{5.16}$ $ID_{50}/0.1$ ml, in hanging-drop cultures. This means that following precultivation according to HUANG, the infective titre was by about one exponent

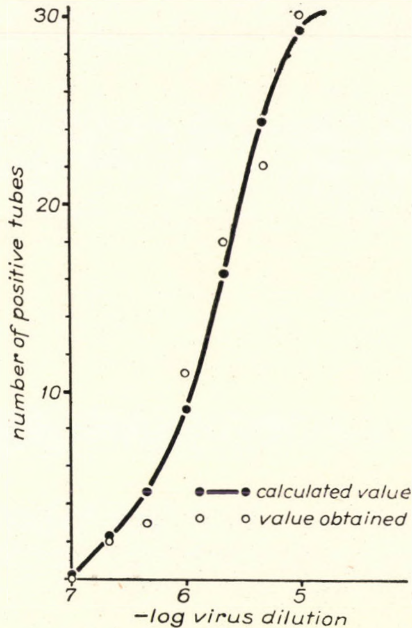


*Fig. 6.* Illustration of the Poisson distribution on the evidence of the experimental data

lower. This agreed with the difference that had been found when titrating tissues infected with the virus without precultivation and after precultivation for two days.

8. *The neutralization test.* Several communications from this Institute have dealt with questions concerning immunization against the Ay virus [2, 4, 7]. In the present paper we wish to discuss only that neutralization test which can be carried out with the semi-micro tissue culture method.

To serial dilutions prepared from inactivated guinea pig immune serum equal amounts of various dilutions of the Ay virus were added. Allowing them to stand for one hour at room temperature, these mixtures of serum and virus were measured into six parallel tubes per dilution containing culture precultivated for two days. From the data obtained five days after immunization, and calculated according to REED and MUENCH [15], the curve shown in *Fig. 7* was plotted.

The extent of neutralization was calculated in accordance with the principles laid down in our earlier work concerning the influenza virus [9]. With the Ay virus, it is no longer expedient to calculate neutralization for 100 $ID_{50}$, as can be done with the influenza virus [9] ; to narrow down the deviation, neutralization is better calculated not for 100 $ID_{50}$ but for 1000 $ID_{50}$. Since in the experiment to which *Fig. 7* refers, the infective titre of the virus was $10^{6.00}$ $ID_{50}$/0.1 ml, the serum dilution neutralizing 1000 $ID_{50}$ corresponded to $10^{-2.4}$.

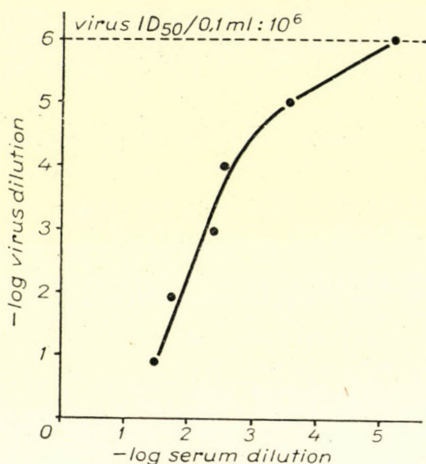


*Fig. 7.* Neutralization of the Ay virus with the immune serum of a guinea pig

In neutralization experiments three data must be determined simultaneously before one can proceed to calculate the neutralization titre. One is the number denoting the infective capacity of the virus used ; the other two derive from neutralization experiments carried out with two different quantities of the virus. The concentration of the suspensions containing the two different virus quantities should be so chosen that the one be lower and the other higher than the quantity — *e.g.* 100 $ID_{50}$ — for which it is desired to calculate the neutralizing serum dilution.

From the three values obtained in the experiment it will then be possible to arrive by means of interpolation at the neutralization titre calculated for the desired $ID_{50}$.

The neutralization values obtained from the data-pairs, of which one will be above and the other below the $ID_{50}$ line, will display a deviation hardly more considerable than that found when infectivity is measured with the method used in titration.

## Discussion

Proving fairly stable, the Ay virus presented no peculiar difficulties in the experiments. Inactivation at +37° C was in some measure reduced by the 0.5 per cent egg white added to the culture fluid. Incidentally, egg white more readily stabilizes the influenza than the Ay virus [9]. Addition of 0.5 per cent egg white failed to bring about a change in the result of titration : on titrating 15 parallels of each of a number of several virus samples, the infectivity of the culture fluid was found to be the same with as without egg white. It is remarkable that even in a culture fluid containing 2 per cent embryo extract — which definitely intensifies fibroblastic multiplication — no increase in infectivity could be demonstrated.

To certain factors particular attention must be paid in determining the conditions optimal for titration : in addition to the rotation of the cultures, major importance attaches to the number of tissue fragments in the culture fluid and to the length of time for precultivation. The maximum change called forth in the infective titre by any one of these factors is expressible by a difference of one over of magnitude. Of course, these changes can only be demonstrated if, instead of the customary 1 or 2 tubes, an adequate number of parallels is involved in the experiment for each dilution.

After the lapse of a certain optimal period of time, either each of the tissue fragments in the individual phials displayed degeneration, or no degenerative lesions at all were encountered in the fibroblasts growing around the tissue explants. This made the negative tubes readily distinguishable from the positive ones, since in the non-infected tissues the degenerative process begins but slowly, and not before the 9th or 10th day [8]. On comparing the curves for degeneration as it develops in tissues infected and not infected with the virus, we find that cellular destruction due to viral action is represented by a suddenly ascending sigmoid curve *(Fig. 5)*, while the other type of degeneration is characterised by a curve ascending slowly from the 9th or 10th day onward [8].

The conclusion is that by using tissue cultures, conditions can be created which permit titrations far more exact than those obtainable in the most carefully conducted animal experiments, as the dose-response curve, *i. e.* the relation of viral concentration to the number of positive tubes, can be closely approximated by the Poisson distribution curve.

## Summary

A semi-micro tissue culture method has been studied for its applicability in titrating the Ay virus and in the neutralization test. It has been found that tissue cultures provide more favourable conditions for titration than do animals. As regards neutralization, it is better to calculate the titre for 1000 $ID_{50}$ instead of 100, for this narrows down deviation.

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# A QUANTITATIVE SEMI-MICRO TISSUE CULTURE METHOD AND ITS USE IN MICROBIOLOGY

## III. USE OF THE METHOD IN STUDYING THE ACTION OF DIVERSE CHEMICAL SUBSTANCES AND GAS GANGRENE BACILLUS TOXINS ON CHICKEN-HEART FIBROBLASTS

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(Received June 11, 1958)

When using chemical substances in the preparation of drugs, in addition to their therapeutical effect, their toxic action on the organism and the tissues, respectively, needs to be thoroughly known. The ratio of the concentrations expressing the therapeutical and toxic actions permits to judge the applicability of the drug. The toxic effect substances exert on tissue cultures is commonly measured in hanging-drop or roller-type cultures [1—6]. Exact determination of tissue toxicity is to-day an indispensable necessity in studying or testing virus-chemotherapeutical agents.

The present work is an attempt to elaborate with the use of our semimicro tissue culture method a tissue toxicity test more precise and less intricate than the tests currently used.

### Materials and methods

The equipment used in measuring tissue toxicity and the method applied in preparing tissue cultures were those described in an earlier paper [7].

Crystal violet, brilliant green, and sublimate, were the chemical substances we tested for their capacity to stimulate tissue growth.

The toxins of *Bacillus oedematiens* (Oe. Sz. 4 Budapest), *B. perfringens* (S, 107, N.C.T.G. + 6125), and *Vibrion septique* (VS. 54. N.C.T.G.) — all products of the *Institute of Human Vaccine Production and Research, Budapest* — were examined for their action on tissue cultures.

### Experimental

1. *Design of the tissue toxicity test and evaluation of the results obtained with it.* The essential features of our method for preparing tissue cultures were the following. Four chicken-heart fragments, each measuring about 1 mm in diameter, derived from a 12 to 13-day-old embryonated egg, were placed side by side on a thin plasma coagulum smeared on the wall of 2 ml phials. The dilutions of the substances to be tested were prepared in a buffered salt solution of our own composition, of which a quantity of 0.5 ml was meas-

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ured to each tissue culture. Growth of the chick-heart fragments from the rotated cultures was read on the 3rd, the 6th, and the 9th day. This was done as follows. Using an ocular micrometer, the fibroblastic growth increments from the edge of the explants were measured radially, and the arithmetical mean of the values so obtained was regarded as the radius of a circle, the area of which was capable of characterising total fibroblastic growth. The details of these computations and of the plotting of the multiplication

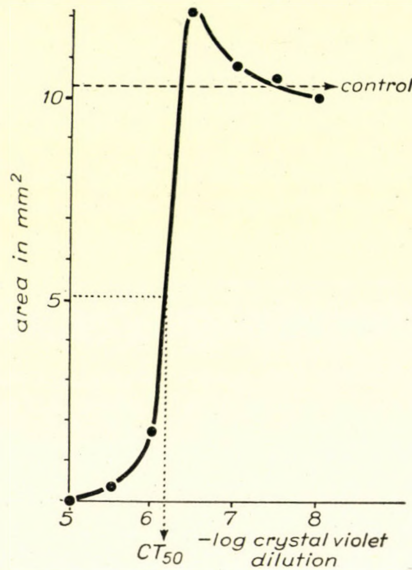


*Fig. 1.* Toxic action of crystal violet on chicken-heart fibroblasts

curve have been described in a previous communication [7]. Projecting onto the ordinate the peaks of the curves plotted from the data obtained at different intervals, we arrived at the maximal multiplication values. These were the data needed for plotting the toxicity curve.

*Fig. 1* shows the toxicity curve for crystal violet. On the abscissa the various dilutions of the substance, on the ordinate the values for maximum growth are shown. In addition to the data so represented, the graph indicates the maximum multiplication of the control tissues, the culture fluid of which was devoid of the substance to be tested.

It can be seen that in certain dilutions the growth increments were greater in relation to the control tissues. It appears that in certain concentrations the action inhibiting tissue growth is followed by a growth-stimulating action. More or less of such increase in growth could be observed in every one of the substances studied.

It seems expedient to express the tissue toxicity of the substances as follows. In our experiments we calculated those dilutions of the substances, where the tissues showed a 50 per cent decrease in growth in relation to the control tissues [cytotoxic action ($CT_{50}$)]. This value was most easily determined graphically, as is shown in *Fig. 1.*

In routine tests it was sufficient to include 10 tubes (containing 40 tissue explants) for each dilution of the substance under testing. *Fig. 1* shows that the toxicity curve is a steeply ascending one and that, therefore, the deviation of the value expressing 50 per cent growth inhibition in this phase, is very slight. Calculated from 20 determinations, the standard deviation of the $CT_{50}$ log values for crystal violet was about $\pm 0.1$ log. In experiments made under similar conditions the standard deviation values do not exceed $\pm 0.2$ log.

In order to avoid the detoxicating action of egg white, which might interfere with the results, the dilutions of the substance to be tested should be preferably prepared in a buffered physiological solution containing no egg white. Although in some of our experiments 0.5 to 1.0 per cent egg white added to the culture fluid did not alter the experimental values obtained in solutions with no egg white in them, still if in subsequent experiments we wished to use a culture fluid of different composition, we would do better by determining the measure of toxicity under these conditions as well.

2. *Determination of the tissue toxicity of some chemical substances.* Determined on the principles described above, the toxicity of crystal violet, brilliant green, and sublimate, was found to be $10^{-6.2}$, $10^{-7.2}$ and $10^{-6.0}$, respectively. That of some additional substances will be dealt with in a forthcoming paper.

3. *Action of toxin and antitoxin on fibroblasts.* Several authors have given attention to the action bacterial toxins and antitoxins exert on tissues [8—9]. Upon the request of Drs. R. BACKHAUSZ and I. VETŐ (of the *Institute of Human Vaccine Production and Research, Budapest)* a few experiments were carried out by us with gas gangrene bacillus toxins prepared by them. The purified toxins of *Bacillus perfringens, B. oedematiens,* and *Vibrion septique* were subjected to examinations for their action on chicken-heart fibroblasts. Graphical representation yielded roughly similar curves for all three of them. *Fig. 2,* for example, illustrates the effect of the *B. oedematiens* toxin. It is remarkable that in certain dilutions this toxin exerted a very marked growth-stimulating action on the tissues.

The $CT_{50}$ values found for the individual toxins were, *B. oedematiens,* $10^{-4.7}$; *B. perfringens,* $10^{-5.7}$; *Vibrion septique,* $10^{-3.4}$.

With a view to establishing how far the growth-inhibiting action was due to the specific action of the toxin, in a number of experiments purified antitoxin was added to *B. oedematiens* toxin. Only in a dilution of $10^{-3}$ did the antitoxin not inhibit tissue growth; in one of $10^{-2}$ it produced slight

growth inhibition. The toxin was used in the experiment in a $10^{-3}$ dilution, which completely suppressed fibroblastic multiplication. Of this toxin dilution equal volumes were added to the antitoxin dilutions. This action

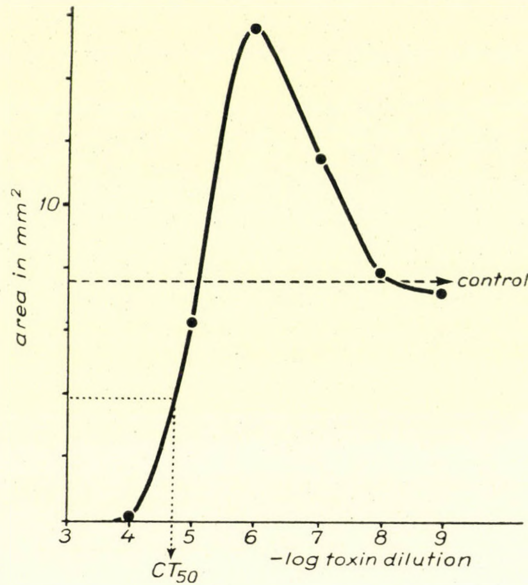


*Fig. 2.* Toxic action of *B. oedematiens* toxin on chicken-heart fibroblasts

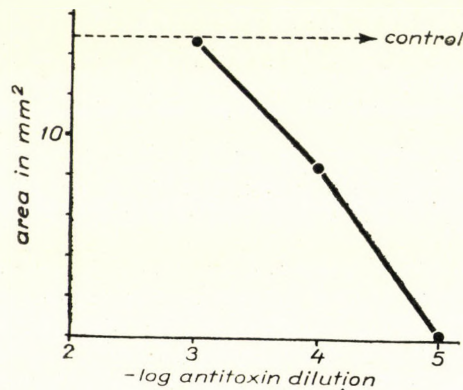


*Fig. 3.* Joint action of *B. oedematiens* toxin-antitoxin on chicken-heart fibroblasts

of the toxin was neutralized by the antitoxin partially in a dilution of $10^{-4}$, and completely in one of $10^{-3}$ *(Fig. 3)*.

These experimental data appear to suggest the elaboration of a toxin—antitoxin titration procedure ; an effort which of course would require further experimentation.

It seems safe to conclude that the method described enables us to ascertain with greater exactness the toxic effect of certain chemical substances on tissues, and that this supplies a more dependable basis on which to assay the applicability of those substances for therapeutical purposes.

## Summary

The quantitative semi-micro tissue culture method elaborated by us has proved suitable for quantitative determination of the tissue toxicity caused by some chemical substances (crystal violet, brilliant green, mercuric chloride) and by the toxins of *B. oedematiens*, *B. perfringens* and *Vibrion septique.*

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# SOME BIOLOGICAL PROPERTIES OF A HUNGARIAN STRAIN OF THE TICKBORNE ENCEPHALITIS VIRUS

## ADAPTATION OF VIRUS TO THE EMBRYONATED EGG AND CHICK EMBRYONIC TISSUE CULTURES

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(Received July 10, 1958)

In the last ten years our knowledge on the viruses of tickborne encephalitis has been widened by a number of interesting discoveries. Human cases earlier described only for the Soviet Union [1] have been revealed in Czechoslovakia [2], Hungary [3], Poland [4], Austria [5] and Yougoslavia [6]. The virus strains isolated in the above countries during the last ten years are closely related to each other as well as to the viruses of Russian spring-summer encephalitis and louping ill. The latter has also proved pathogenic for man [7]. In spite of the great similarity, the variability in the clinical picture and outcome of tickborne encephalitis in different areas makes probable the existence of biological differences between individual strains of the virus.

The present report deals with the adaptation of the Hungarian strain $KEm_1$ to embryonated egg and tissue cultures (TC), and some properties of the egg- and tissue-lines. After 40 passages in TC one of the lines proved to be nonpathogenic for the mouse brain (MB).

### Materials and methods

*Virus.* The $KEm_1$ strain of tickborne encephalitis was used throughout. This was recovered from ticks collected in an endemic area of Hungary in 1952 [8]. The strain was isolated and has been maintained in this laboratory by serial intracerebral inoculation into mice. The virus as used in the adaptation experiments had been passed 70 times in mice. The mouse-adapted virus is pathogenic also for the Syrian hamster and the sheep, as well as for suckling mice, but does not cause illness in the rabbit and the white rat.

*Inoculation of mice.* Mice showing symptoms characteristic of experimental tickborne encephalitis were killed between the 5th and 8th days after inoculation. The brain was ground in a mortar, diluted tenfold in saline and centrifuged with 1500 rpm for 5 min. The supernatants were appropriately diluted with saline and allowed to stand in melting ice until inoculated intracerebrally, 0.03 ml per mouse. In quantitative experiments ten mice were given the same dilution of virus.

*Embryonated eggs.* The age of the embryos used for the allantoic inoculation was 10 or 11 days, whereas for amniotic and yolk sac inoculations 6—8 day-old embryonated eggs were used. The individual dose was generally 0.1 ml.

Allantoic and yolk sac inoculations were made as described by BEVERIDGE and BURNET [9]. As amniotic technique, TAKÁTSY's [10] modification was used. Moving of the embryo was controlled just before the inoculation and thereafter, at least once a day, until the tenth day of incubation. Embryos appearing dead and the survivors at 20 days of age were examined directly. Passage materials were harvested and prepared immediately when death of the embryo had been observed. The yolk membrane was ground in a mortar, diluted tenfold with saline and centrifuged before used as inoculum. The amniotic and allantoic fluids were not

diluted. No antibiotics were used. In quantitative experiments 10 eggs were given the same dilution of virus or serum-virus mixture.

*Tissue cultures*

(a) *Maitland-type cultures* [11] *of the chick embryo.* Torsos of 6—8 day-old embryos or leg muscles of 9 or 10-day-old ones were chopped into fragments approximately 1 cmm. About 20 mg of fragments were placed in an Erlenmeyer flask, inoculated with 0.5 ml viral suspension and allowed to stand at room temperature for 20 min. Then 4.5 ml of a medium composed of equal volumes of Simms $X_6$ solution and bovine amniotic fluid were added and the cultures were incubated at 37° C for 24 or 48 hr before the undiluted culture fluids were used to make subpassages.

(b) *Human embryonic skin-muscle cultures* were prepared as described by ENDERS *et al.* [12]. Four- to five-day-old cultures with abundant marginal growth were infected with virus. The growth medium in each tube was replaced by 0.1 ml viral suspension and the tubes were kept horizontally at room temperature for 20 min. Then 0.9 ml of a mixture consisting of equal volumes of Simms $X_6$ solution and bovine amniotic fluid was added and the tubes were incubated at 37° C as long as needed.

*Antisera*

*Rabbit sera.* The MB-line of $KEm_1$ strain had been passed 70—75 times in MB, the TC-line 70 times in MB and subsequently 60 times in chick embryonic TC before used for hyperimmunization. Rabbits weighing 2.5 kg each, were given four intraperitoneal injections of virus. The interval between two injections was generally 4 days. Five rabbits were immunized with the MB-line, and 5 with the TC-line. Tenfold dilution of MB and ground tissue diluted tenfold in its own nutrient fluid, respectively, were used as inocula in doses of 2.0 ml. The rabbits were killed by bleeding on the 10th day after the last injection. Control sera were prepared by appropriately treating rabbits with normal tissue suspension.

*Human sera.* Serum No. 637 was the convalescent serum obtained from a patient naturally infected by tickborne encephalitis virus in Hungary. Sera 696 and 721 were convalescent specimens from patients accidentally infected in this laboratory by the Czechoslovak strain Ři [13] and the $KEm_1$ strain [14] respectively.

All the sera were inactivated at 56° C for 30 min. before used.

*Neutralization tests.* Equal volumes of undiluted serum and varying dilutions of virus were mixed and incubated at 37° C for 1 hr before inoculation.

*Interference experiments.* The TC-line of virus had been passed in chick embryo TC 40—50 times before use in the interference experiments. The culture fluid was harvested and inoculated into the right hemisphere of 4-week-old mice, 0.03 ml per mouse. Twentyfour or 48 hrs later the mice were challenged by injecting varying dilutions of the MB-line virus into the left hemisphere.

$LD_{50}$ was calculated by the method of REED *and* MUENCH [15].

## Results

*Adaptation of the virus to the chick embryo.* Chick embryos were inoculated into the allantoic, amniotic and yolk sac, alternatively (see Materials and methods). After 3—6 days' re-incubation the embryo was moving slower and soon died. The most conspicuous features in the embryo were petechiae in the epidermis, most frequently on the abdominal and dorsal regions and on the head, as well as in the feather follicles of the older embryos. If latency had been long, gelatinous-oedematous degeneration of the subcutaneous connective tissue was regularly observed. The oedema was often so enormous that the embryo appeared as spherical *(Fig. 1)*. In such embryos the epidermis was separated from the musculature by a colourless, almost quite clear, highly viscous fluid with high protein content. A marked pattern of veins was seen, especially on the head and pericardium as well as on the dorsal

and abdominal regions. A few of the most oedematous embryos had survived the 21st day of incubation and hatched out. The virus in the oedematous substance was easily demonstrable by inoculating mice intracerebrally.

*Fig. 1.* Seventeen-day-old chick embryo, dead on the 9th day following inoculation into the yolk sac with the $KEm_1$ strain

The pathological changes observed in the chick embryo were neutralized by homologous hyperimmune and convalescent sera, as seen in ***Table I.*** The first 3 sera in the Table had originated from convalescent patients, the last 3 were hyperimmune sera prepared in rabbits. The sera were tested against a virus suspension prepared from MB.

**Table I**

*Neutralization of the $KEm_1$ strain MB-line with human and rabbit antisera*

| Experiment | Serum | | Neutralization index as tested in | | |
|---|---|---|---|---|---|
| | N° | Origin | MB | yolk sac | allantoic sac |
| 1 | 637 | Human | 1000 | 500 | n. t. |
| 2 | 696 | Human | >500 | 600 | n. t. |
| 3 | 721 | Human | 1000 | 1000 | n .t. |
| 4 | 777 | Rabbit | 160 | 100 | 80 |
| 5 | 697 | Rabbit | 200 | 100 | 100 |
| 6 | 799 | Rabbit | 130 | 100 | 100 |

n. t. = not tested.

***The mouse virulence of the KEm$_1$ strain during its adaptation to the chick embryo.*** The KEm$_1$ strain as used in the adaptation experiments had been passed 70 times intracerebrally in mice. Three hundredth ml of the initial MB contained $10^7$ mouse $LD_{50}$.

(a) *Attempt to adapt the MB strain to the allantoic sac.* The virulence of the virus for the allantoic sac remained low through the 21 allantoic passages of this experiment ; its mouse virulence showed any change. After 9, 10 and 16

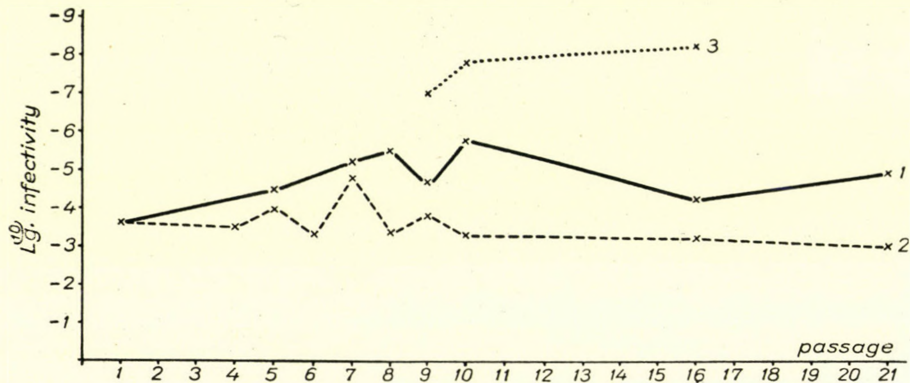


*Fig. 2.* Infectivity of KEm$_1$ strain for eggs and mice after varying number of passages in the allantoic cavity

*Explanations:*
1 : allantoic fluid titrated in the allantoic cavity
2 : allantoic fluid titrated in mice
3 : allantoic fluid titrated in the yolk sac

allantoic passages, however, the virus proved markedly more virulent for the yolk sac than the MB virus in the initial inoculum *(Fig. 2)*.

(b) *Adaptation of the virus to the yolk sac.* In the first few passages the multiplication of the MB virus was weak, as tested in the MB and in the yolk sac. Later, however, its virulence for the yolk sac gradually increased whereas its mouse virulence and virulence for the allantoic cavity remained practically at the initial level *(Fig. 3)*. Thus, serial passages in the yolk sac resulted in a line undistinguishable from that obtained by allantoic passages.

*The growth curve in TC of the mouse-adapted virus.* The growth curve of virus in chick embryo TC has been determined in 8 experiments. In the first 5 experiments the medium and the ground tissue were titrated separately while in the last 3 experiments the tissue was mixed with its own medium and the total virus content of the culture was determined. The titrations were made by inoculating mice intracerebrally. A mixture of 3 individual cultures was titrated in each case. The results are presented in *Fig. 4* and *5*.

The course of each of the curves is essentially the same. The titre of the virus was reduced by 1—4 log units in 2 to 3 hours' incubation. The decline was more marked in the medium than in the tissue. The curves began to rise after 5 or 6 hours and reached the highest levels during the 2nd day of incubation. In glass-stoppered flasks the high levels remained unchanged through the 3rd and 4th days. In wool-stoppered flasks, however, the pH turned into

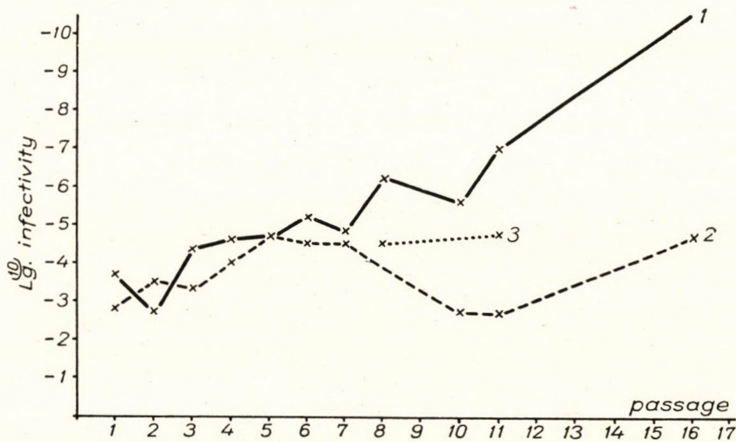


*Fig. 3.* Infectivity of $KEm_1$ strain for eggs and mice after a varying number of passages in the yolk sac

*Explanations:*

1 : yolk membrane titrated in the yolk sac
2 : yolk membrane titrated in mice
3 : yolk membrane titrated in the allantoic cavity

alkaline gradually from the 48th hr of incubation. This was followed by loss in infectivity.

Two attempts were made to determine the growth curve in human embryonic TC of the MB virus. A drop of about 1 log at the 2nd hr of incubation and a slight rise thereafter was observed, but no evidence of viral multiplication has been established.

*Dissociation of the mouse virulence from the egg virulence of the MB virus while passed in chick embryonic TC. Fig. 6* presents the results of two out of the numerous experiments in which the mouse and egg virulences of the MB line were investigated at different phases of its adaptation to chick embryo TC. The virus was transferred on every workday. The undiluted fluid was used as inoculum.

In one of these experiments the initial MB was as infective for the MB as for the embryonated egg. The virus harvested from the 10th passage,

however, was markedly more virulent for the egg than for the mouse. Thereafter the mouse virulence gradually declined while the egg virulence remained at the initial level. The difference between the egg and mouse titres was 1.9 log at the 10th, 4.0 log at the 29th and 6.2 log at the 45th TC passage. The

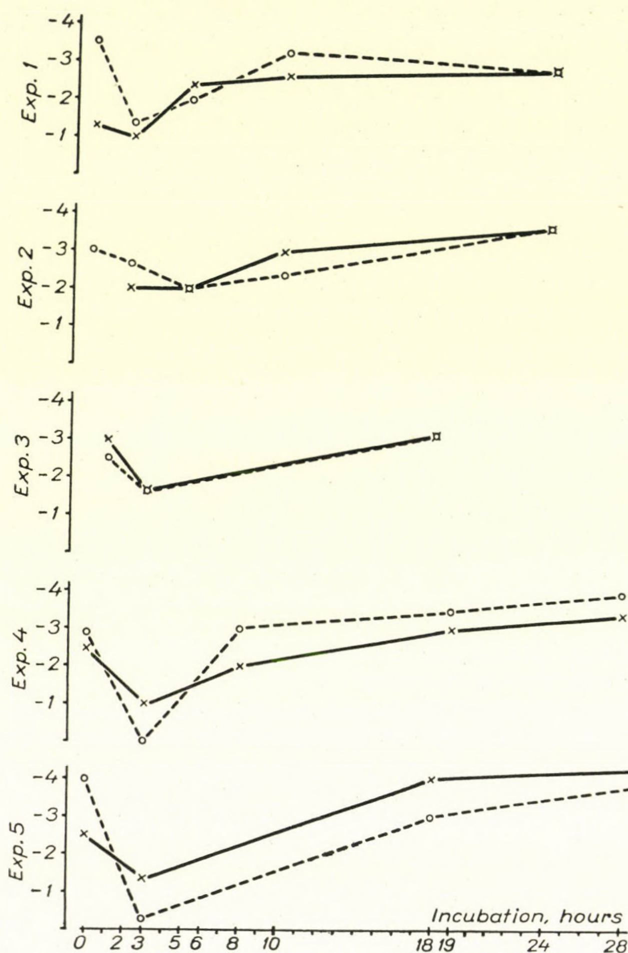


*Fig. 4.* Growth curves of the MB-line of $KEm_1$ virus in chick embryonic tissue cultures.
x———x ground tissue o---------o medium

results of Experiment II were essentially similar but the initial inoculum in this experiment was more infective for eggs than for mice. Accordingly, the differences at later passages were also greater. After 40 passages no pathogenicity for mice could be detected in spite of the $10^{-9}$ titre of the virus in the embryonated egg.

*Neutralization tests. Table II* shows that the anti-TC-line rabbit sera markedly neutralized the MB-line as tested in mice and by inoculation into the yolk sac. It is to be noted that the rabbits were immunized with viral preparations non-pathogenic for mice.

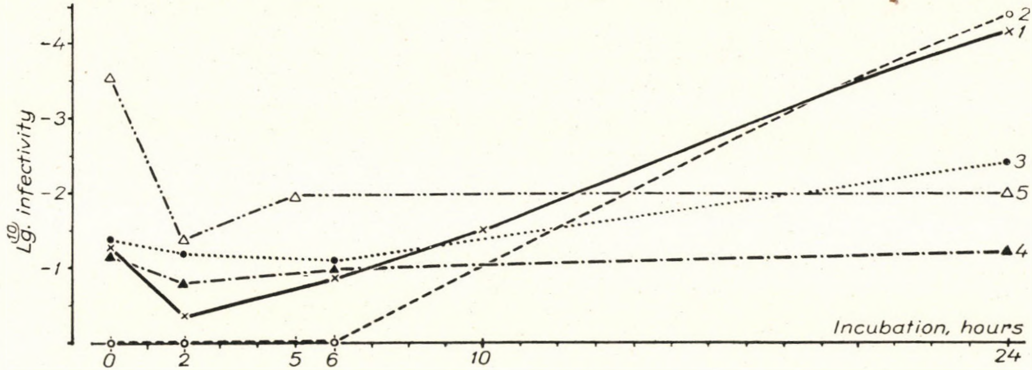


*Fig. 5.* Growth curves of the MB-line of $KEm_1$ virus in chick embryonic tissue cultures

*Explanations;*

1 : Experiment 6 2 : Experiment 7
3 : Experiment 8 4 : Experiment 9
5 : Experiment 10

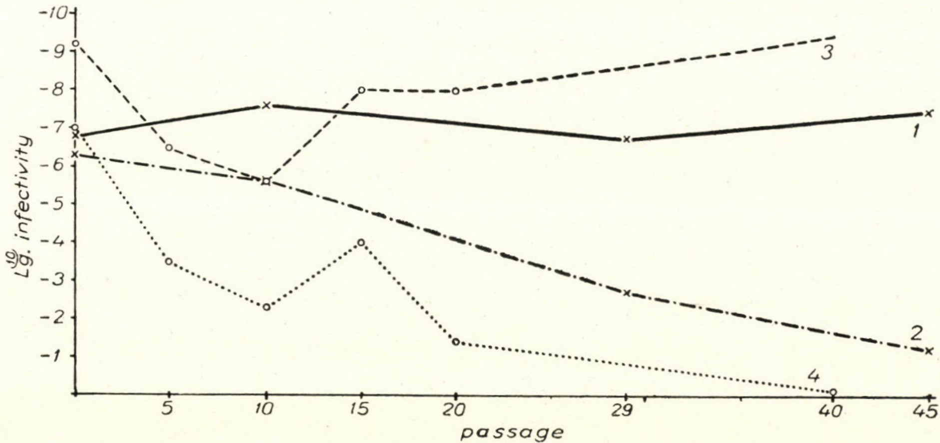


*Fig. 6.* Mouse and egg infectivity of $KEm_1$ strain, after a varying number of passages in chick embryonic tissue cultures

*Explanations;*

1 : Experiment 1 titrated in eggs 2 : titrated in mice
3 : Experiment 2 titrated in eggs 4 : titrated in mice

*Interference of the TC-line with the MB-line.* The interference experiments were performed as described above. The TC-line with no measurable pathogenicity for mice was used as interfering virus. As shown in *Table III*, the

**Table II**

*Neutralization of the MB-line of the $KEm_1$ virus with anti-TC-line rabbit sera*

| Experiment | Serum | Virus | Animal | Titre of virus | Neutralization index |
|---|---|---|---|---|---|
| 1 | TC 326 | MB-line | Chick embryo* | 4.2+ | 130 |
| | Normal rabbit | | | 6.3 | 1 |
| 2 | TC 775 | MB-line | Mouse** | 4.1 | 130 |
| | TC 776 | | | 4.2 | 100 |
| | TC 778 | | | 4.0 | 160 |
| | Normal rabbit | | | 6.3 | 1 |

* = yolk sac inoculation
** = i. cer. inoculation
+ = negative log

**Table III**

*Interference in mice between the TC and MB lines of the $KEm_1$ strain*

| Experiment | Interfering virus | | Interval hour | Challenge virus | | Mortality | Probability of the differences* |
|---|---|---|---|---|---|---|---|
| | Designation | N° of passages | | Designation | Dosage | | |
| 1 | TC line | 39 | 24 | MB line | 320 $ID_{50}$ | 0/19** | 0.001 |
| | — | — | | | | 9/12 | |
| 2 | TC line | 51 | | | | 11/23 | >20*** |
| | — | — | | | | 9/12 | |
| 3 | TC line | 52 | 48 | MB line | 1 : 200 dil. mouse brain | 0/24 | 0.004 |
| | — | — | | | | 5/6 | |
| 4 | TC line | 52 | 48 | MB line | 1 : 2000 dil. mouse brain | 1/16 | 2.75 |
| | — | — | | | | 3/5 | |

* = The calculation of the P values was made by the direct method of FISHER [34].
** = The numerator represents the number of dead animals, the denominator the number of those inoculated.
*** $\chi^2 = 1.40$.

**interference in Experiments 1 and 3 was statistically highly significant and the difference between the interference and control groups in Experiment 4 might be regarded significant too, in spite of the low number of animals. The death rate in the control group of Experiment 2 was also higher than in the interference group but the difference was not significant.**

## Discussion

In the experiments presented in this report, the $KEm_1$ strain appeared partly similar to, partly different from, the strains of tickborne encephalitis virus examined by other authors.

As to the chick embryo infected by the $KEm_1$ strain, the most striking pathological feature was the enormous subcutaneous oedema. It is not quite clear whether other strains of virus cause a similar phenomenon. EDWARD [16] observed gelatinous-oedematous degeneration in chick embryos infected with the louping ill virus but unfortunately, no photographs are presented in his studies on the subject. ANJAPARIDZE [17] mentions only that the embryos which had been infected with the virus of Russian spring-summer encephalitis, often were found swollen and yellow. SLONIM [18] and BEDNAR *and* SLONIM [19] found the histological changes induced by the Czechoslovak virus to resemble those caused by the viruses of louping ill and Russian spring-summer encephalitis. According to these authors, the jaundice might be explained by parenchymatous lesions in the liver, but the genesis of the other changes is uncertain because of the asphyxia *ante mortem* and subsequent autolysis. PINTÉR and BÉLÁDI [20] found the Czechoslovak virus able to kill the chick embryo, but did not register oedema [21].

VESENJAK-ZMIJANAC [22] inoculated chick embryos with Slovenian and Austrian strains of the virus. She often found the embryos swollen and yellow but never oedematous [23]. Neither did ILENKO [24] observe oedema while adapting the virus of bi-phasic meningoencephalitis to the chick embryo.

As to the viral origin of the subcutaneous oedema, BEVERIDGE *and* BURNET [9], as well as we ourselves occasionally found similar lesions in embryos inoculated with saline in the summer. MITSCHERLICH *and* BRATKE [25] brought about similar phenomena by administering histamine or by inducing histamine production. The dilatation of the blood vessels and the exudation in the $KEm_1$ infected embryos might have been induced by the liberation of histamine, or histamine-like substances. Moreover, histamine, being able to constrict the portal vessels, might have been responsible for the enormous ascites and the pattern of veins resembling *caput medusae,* a feature often

observed by us on the abdominal wall. The viral origin of the oedematous changes in our virus-infected embryos has been proved by the fact that they were neutralized by homologous antisera.

In our experiments the $KEm_1$ strain multiplied in chick embryo TC without being cytopathogenic. Of the other tickborne viruses, the louping ill virus was propagated by RIVERS *and* WARD [26], the virus of Russian spring-summer encephalitis by CHUMAKOV [27], in chick embryonic TC. The former virus was found to be cytopathogenic for HeLa cell cultures by OKER-BLOM [28], the latter virus for human embryonic fibroblasts by ZASUKHINA *and* LEVKOVITCH [29]. In chick embryonic fibroblasts the Czechoslovak Ři strain was not cytopathogenic as reported in the experiments of PINTÉR *and* BÉLÁDI [20] whereas DANEŠ [30] found the Hanzal strain cytopathogenic.

It is well-known that some viruses, when growing in a new host, may lose their pathogenicity for the old host without changing the antigenicity. Such "attenuated" strains are widely used for preparing vaccines against smallpox, rabies and yellow fever over the world. Large-scale experimental vaccinations with attenuated strains of the influenza and poliomyelitis viruses have been performed in several countries.

According to SMORODINTSEV *and* KRIVISKY [31], the encephalitis viruses show very little variability. This view has been supported by the experimental studies demonstrating that the viruses of Russian spring-summer encephalitis [17], Czechoslovak tick encephalitis [20], and bi-phasic meningoencephalitis [24] can all be maintained through serial passage in eggs without any loss in their pathogenicity for the mouse. In our own experiments the egg-lines of the $KEm_1$ strain retained their initial pathogenicity for mice and the ability to multiply in the allantoic sac, while acquiring a high virulence for the yolk sac.

A Czechoslovak strain of the tickborne encephalitis virus when propagated in Maitland-type cultures of the chick embryo showed no dissociation of mouse and egg virulence even after 69 passages [20].

Our finding that the $KEm_1$ strain had lost its mouse pathogenicity after 40 passages in TC, is a unique example of the variability of a tickborne encephalitis virus. This might be explained by supposing that this strain was more variable than the strains examined by other authors. However, we used a different technique by making daily passages with heavy inocula just like SABIN [32] had done when he succeeded in rendering all the three types of poliomyelitis virus non-pathogenic for Cynomolgus monkeys.

It has to be noted that the TC-line of the $KEm_1$ strain did not regain its mouse pathogenicity on 10 blind passages in the mouse.

Among the viruses causing encephalitis in man, that of the St. Louis encephalitis has been investigated for its growth curve in TC by SHEN *et al.* [33].

A common feature of this curve and the growth curve of the $KEm_1$ strain is the eclipse phase at the first few hours of growth.

## Summary

(1) The mouse-adapted line of the Hungarian $KEm_1$ strain of tickborne encephalitis virus was found to produce characteristic lesions in the chick embryo. These could be neutralized by homologous antisera.

(2) The virus was easily adaptable to the embryonated egg. The egg-adapted lines retained their full pathogenicity for mice through 20 yolk sac passages.

(3) The virus was serially passed in Maitland-type cultures of the chick embryo.

(4) The virus, when passed in tissue culture, gradually lost its ability to kill the mouse.

(5) In tests carried out in the mouse as well as in the embryonated egg, the hyperimmune sera prepared in rabbits against the tissue culture line of virus was able to neutralize the mouse-adapted strain.

(6) The tissue culture line which was non-pathogenic for the mouse, still interfered with the mouse-killing effect of the mouse-adapted line.

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# ÜBER DIE MIT PILOCARPIN UND KALTWASSER HERBEIGEFÜHRTE »ANAPHYLAXIEÄHNLICHE« REAKTION

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(Eingegangen am 21. August 1958)

Anläßlich unserer Versuche zur experimentellen Herbeiführung der Glomerulonephritis [4] waren an einem Teil der Versuchstiere klinische Symptome und pathologisch-anatomische Veränderungen aufgetreten, die in hohem Maße dem anaphylaktischen Schock glichen. Vorliegende Arbeit befaßt sich mit diesen Beobachtungen.

## Eigene Untersuchungen

1. *Versuche an Kaninchen.* Bei der ersten Versuchsserie verwendeten wir 35 Kaninchen im Gewicht von 2500—3500 g, die an zwei aufeinanderfolgenden Tagen i. v. mit je 2 ml normalem Pferdeserum sensibilisiert wurden. Nach 3 Wochen erhielten die Tiere täglich subkutan 0.01 g Pilocarpin und wurden anschließend in kaltem Wasser gebadet. Das Baden der Kaninchen geschah in der Weise, daß erst ihre Behaarung an der Kreuzgegend abgeschnitten und dann die Tiere im Kaninchenkäfig in ein mit kaltem Wasser (von etwa 10° C) gefülltes Becken gestellt wurden. An den ersten beiden Tagen badeten wir die Tiere 3 Minuten, am dritten und vierten Tage 5 Minuten und an den folgenden Tagen 8 Minuten. Bei 15 der 35 Kaninchen trat akute und subakute Glomerulonephritis auf. An 8 Tieren beobachteten wir im Laufe der Behandlung keine wesentlichen Veränderungen, während bei 12 Kaninchen anaphylaxieähnliche Symptome in Erscheinung traten. Mit den Tieren der ersten beiden Gruppen, bei denen glomerulonephritisartige bzw. keine Veränderungen zu beobachten waren, wollen wir uns in dieser Mitteilung nicht beschäftigen.

Von den 35 Kaninchen waren demnach 12 binnen 1—4 Tagen zugrunde gegangen. Bei 6 dieser Tiere trat unmittelbar nach der 1—3. Behandlung (an einem nach der 1., an vier nach der 2. und an einem nach der 3. Behandlung) hochgradige Atemnot auf, dann sprangen sie im Käfig aufgeregt im Kreise herum und wiesen anaphylaxieähnliche Krampfsymptome auf. Schließlich gingen sie, Schreie ausstoßend, innerhalb von 10—30 Minuten zugrunde. Bei den anderen 6 Kaninchen trat unmittelbar nach der 1—4. Behandlung

(bei einem nach der 1., bei zwei nach der 2., bei einem nach der 3. und bei zwei nach der 4. Behandlung) starke Dyspnoe auf, und die Tiere verendeten unter diesen Symptomen binnen 3—4 Stunden. Es sei bemerkt, daß jene Tiere, an denen diese Symptome nach dem 2—4. Versuch zu beobachten waren, nach den vorangegangenen Behandlungen keinerlei Symptome gezeigt hatten.

*Sektionsbefunde.* Bei der Sektion sämtlicher 12 Kaninchen fiel auf, daß die rechte Herzkammer stark erweitert, ferner die Herzmuskulatur sehr schlaff war und aus den Gefäßen überall flüssiges Blut strömte. Beide Lungen waren stark gebläht, fühlten sich wie ein Daunenkissen an und füllten in allen Fällen die Brusthöhle aus *(Abb. 1)*. Bei 2 Tieren waren an der Lungenoberfläche auch punktartige Blutungen zu sehen, während an 2 anderen Kaninchen auf einer linsengroßen Region der Jejunumschleimhaut eine dunkelrote Verfärbung nachgewiesen werden konnte. An den anderen Organen waren makroskopische Veränderungen nicht wahrnehmbar.

*Histopathologische Veränderungen.* In Übereinstimmung mit dem Sektionsbefund wurde in den Lungen auch histologisch Emphysem festgestellt, das hier und da von herdartigem Ödem begleitet war *(Abb. 2)*. Stellenweise enthielt die Lunge Blutungen. In 2 Fällen sahen wir Blutungen und Nekrose am Epithel der Dünndarmschleimhaut. In der Leber waren an einigen Stellen Stauung und punktartige Blutungen anwesend. Die Milzkapsel war dünn, die Follikel waren kaum erkennbar. Die Sinus waren weit, ihre Endothelauskleidung gequollen, in ihrem Lumen Hämosiderin nachweisbar. In den Glomeruli von 6 Kaninchen war die Basalmembran verdickt.

2. *Versuche an Meerschweinchen.* Bei der zweiten Versuchsreihe verwendeten wir 10 Meerschweinchen im Gewicht von 600—650 g. Die Tiere wurden an 2 aufeinanderfolgenden Tagen mit je 0.2 ml subkutan verabreichtem Pferdeserum sensibilisiert, und 3 Wochen später injizierten wir ihnen subkutan 2 mg Pilocarpin, wonach wir die Tiere in ein mit kaltem Wasser angefülltes Becken warfen, in dem sie 3 Minuten blieben. Nach der 1. Behandlung traten noch keinerlei Symptome in Erscheinung, aber etwa 15 Minuten nach der 2. Behandlung war das Fell sämtlicher 10 Tiere, als wir sie aus dem Wasser herausnahmen, gesträubt, und sie zitterten stark am ganzen Körper. Sieben Tiere gingen innerhalb von 4—5 Stunden unter diesen Symptomen zugrunde, während das Zittern bei 3 Tieren binnen 6—8 Stunden verschwand. Diese 3 Meerschweinchen behandelten wir am 3. Tage auf übliche Weise, worauf etwa 15 Minuten nach der Behandlung wieder Tremor auftrat, ihr Fell gesträubt war und sie nach 3—4 Stunden verendeten.

*Sektionsbefunde.* Bei 3 der 10 Meerschweinchen war die rechte Herzkammer erweitert, bei 5 Lungenerweiterung, bei 1 Lungenerweiterung und Ödem, bei 1 Blutung in der Lunge, bei 1 Blutungen an der Magenschleimhaut und bei 1 anderen Blutungen an der Dünndarmschleimhaut zu beobachten.

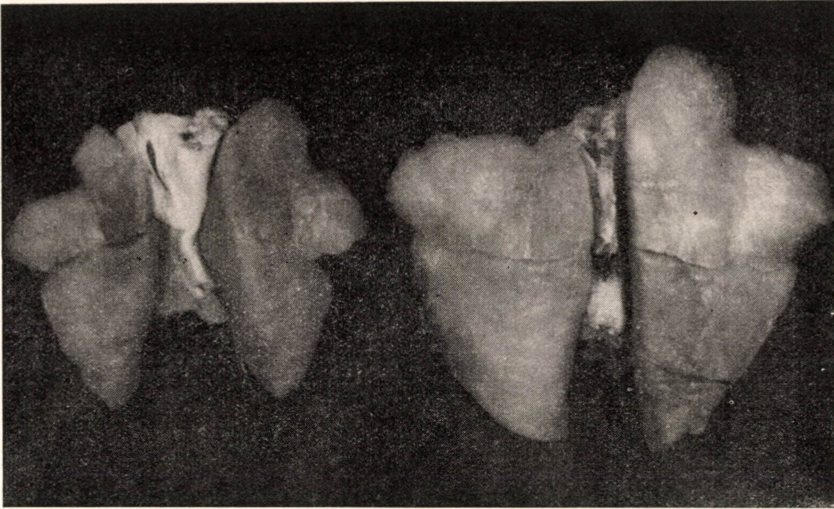

*Abb. 1.* Links, Lunge eines Kontrollkaninchens. Rechts, stark geblähte Lunge, an deren Rand emphysematöse Blasen zu erkennen sind

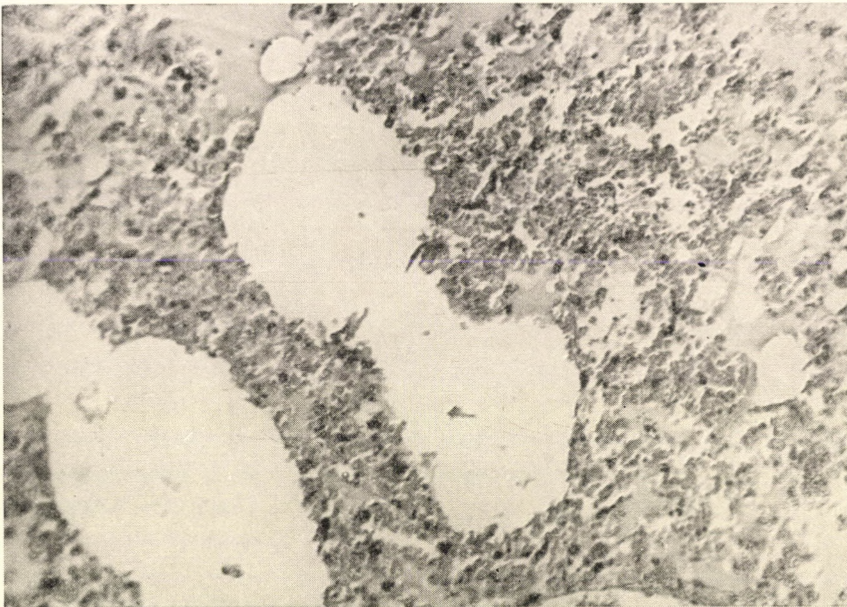

*Abb. 2.* Neben den emphysematösen Lungengebieten Ödem und Blutungen in den Alveolen (Hämatoxylin-Eosinfärbung)

**Tabelle I**

*Befunde nach Sensibilisierung + Pilocarpingabe + Baden im Meerschweinchenversuch*

| Nr. der Tiere | Behandlungsdauer | Sektionsbefund | Histologischer Befund |
|---|---|---|---|
| I | 2 | Dilatation der rechten Herzkammer. Lungenemphysem | Lungenemphysem |
| II | 3 | Lungenemphysem und Ödem | Periarterielles Ödem, mononukleäre Infiltration in der Lunge. Emphysem |
| III | 2 | Lungenemphysem | Periarterielles Ödem in der Lunge. Emphysem |
| IV | 2 | Dilatation der rechten Herzkammer. Lungenemphysem. Punktartige Blutungen an der Dünndarmschleimhaut | Lungenemphysem. Blutungen an der Dünndarmschleimhaut |
| V | 3 | Punktartige Blutungen und Ödem in der Lunge. Punktartige Blutungen im Magen | Blutung und Ödem in der Lunge. Vakuolöse Degeneration der Leber. Blutungen an der Magenschleimhaut |
| VI | 2 | ∅ | Stauung, vakuolöse Degeneration in der Leber |
| VII | 2 | ∅ | Ausgedehnte vakuolöse Degeneration in der Leber |
| VIII | 2 | Dilatation der rechten Herzkammer. Lungenemphysem | Periarterielles Ödem, mononukleäre Infiltration in der Lunge. Emphysem. Vakuolöse Degeneration in der Leber |
| IX | 3 | ∅ | Vakuolöse Degeneration in der Leber |
| X | 2 | ∅ | ∅ |

Die Sektions- und histologischen Veränderungen sind in *Tabelle I* zusammengefaßt.

*Histopathologische Veränderungen.* Den makroskopischen Veränderungen entsprechend sind in der Lunge Emphysem *(Abb. 3)*, Ödem und Blutungen nachweisbar. Stellenweise ist das Lumen der Bronchien sternförmig eingeengt, in ihrer Umgebung sieht man mononukleäre Infiltration *(Abb. 4)*, anderswo

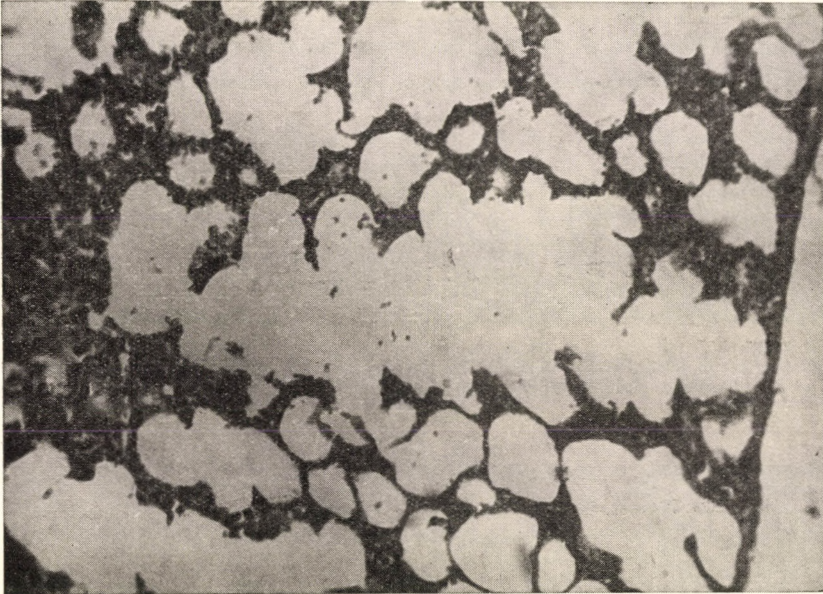

*Abb. 3.* Ruptur der Alveolen in einer Meerschweinchenlunge (Hämatoxylin-Eosinfärbung)

ist das peribronchiale Bindegewebe ödematös aufgelockert. Hier und da ist die Wand einiger kleinerer Zweige der Arteria pulmonalis ödematös geworden, und die periarteriellen Alveolen sind mit einer homogen gefärbten Substanz angefüllt *(Abb. 5)*.

In einigen Fällen ist die vakuolöse Degeneration der Leber sowie Blutung an der Magen- bzw. Dünndarmschleimhaut zu beobachten. In der Milz ist die Struktur der Follikel kaum erkennbar, die Sinus sind weit, ihre Wand gedunsen, im Lumen und in der Wand befinden sich hämosiderinhaltige Phagozyten.

3. *Versuche an Ratten.* In der dritten Versuchsserie sensibilisierten wir 14 Albinoratten im Gewicht von 200—250 g mit 0,1 ml Pferdeserum, die wir an zwei aufeinanderfolgenden Tagen subkutan injizierten. Nach Ablauf von 3 Wochen erhielten die Tiere täglich subkutan 0,5 mg Pilocarpin und wurden dann in ein mit kaltem Wasser gefülltes Becken geworfen, wo sie

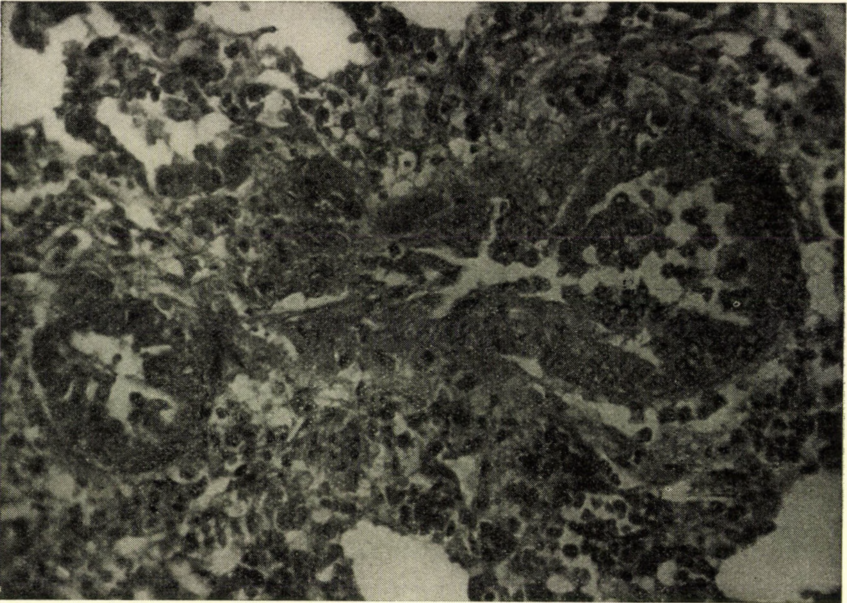

*Abb. 4.* Stellenweise sternförmig eingeengtes Lumen der Bronchien, im Umkreis der Lumina mononukleäre Infiltration (Hämatoxylin-Eosinfärbung)

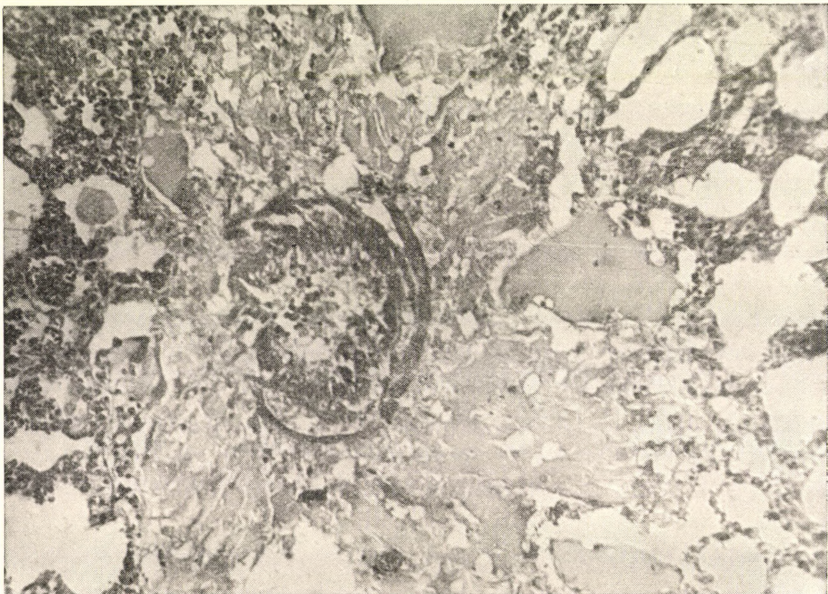

*Abb. 5.* Die Wand der kleineren Zweige der Arteria pulmonalis ist stellenweise ödematös aufgelockert, die benachbarten Alveolen enthalten eine homogen gefärbte Substanz (Hämatoxylin-Eosinfärbung)

nur 3 Minuten blieben, da sie 5 Minuten langes Baden sehr schlecht tolerierten und die vier Extremitäten nach dem Bade ganz steif wurden. Die Behandlung wurde von den Ratten gut vertragen, dem anaphylaktischen Schock gleichende klinische Symptome haben wir an ihnen nicht beobachtet. Nach 16 Behandlungen wurden die Tiere getötet ; die Sektion und histologische Untersuchung ergaben keinerlei pathologische Veränderungen.

4. *Versuche zur Abwehr des anaphylaxieähnlichen Schocks*

(a) 10 Kaninchen von 2500—3500 g Gewicht wurden 3 Wochen nach der Sensibilisierung mit i. v. gegebenem Evipan narkotisiert. Als die Tiere bereits schliefen, injizierten wir ihnen subkutan 0.01 g Pilocarpin und badeten sie auf übliche Weise. Während des Badens vertieften wir die Narkose mit Äther. An 1 Kaninchen traten nach der 1. Behandlung, an 3 Tieren nach der 3. Behandlung nach dem Erwachen aus der Narkose anaphylaxieähnliche Symptome auf, und sie gingen innerhalb von 1½—2 Stunden zugrunde. Bei diesen 4 Tieren fanden wir anläßlich der Sektion und histologischen Untersuchung den im 1. Punkt beschriebenen ähnliche Veränderungen. An den restlichen 6 Tieren waren weder anaphylaktische Symptome noch pathologisch-anatomische Veränderungen festzustellen. Mit Narkose lassen sich also die anaphylaktischen Krämpfe nicht abwehren.

(b) Im folgenden versuchten wir die Schockabwehr, indem wir Atropinum sulfuricum verabfolgten.

Zwanzig 2500—3500 g schwere Kaninchen wurden mit 2×2 ml Pferdeserum sensibilisiert. Nach 3 Wochen injizierten wir subkutan 2 mg Atropin und führten die übliche Behandlung — Verabreichung von 0.01 g Pilocarpin und Baden — am folgenden Tage durch, gaben jedoch den Tieren jeweils 1 Stunde vorher subkutan nochmals 2 mg Atropin. Anaphylaxieähnliche Erscheinungen wurden an keinem Tier beobachtet, ebensowenig sahen wir nach der im Anschluß an 15—20 Behandlungen erfolgten Tötung der Tiere die im 1. Punkt beschriebenen pathologisch-anatomischen und histopathologischen Veränderungen.

5. *Kontrolluntersuchungen*

(a) Zehn 2500—3500 g schwere Kaninchen wurden nach der Sensibilisierung mit subkutan injiziertem Pilocarpin behandelt. An diesen Tieren waren weder anaphylaxieähnliche klinische Symptome noch histopathologische Veränderungen festzustellen.

(b) Zehn Kaninchen im Gewicht von 2500—3500 g wurden sensibilisiert und 3 Wochen später auf übliche Weise in kaltem Wasser gebadet. Pilocarpin wurde diesen Tieren nicht verabreicht. Auch an diesen Kaninchen sahen wir

weder anaphylaxieähnliche klinische Symptome noch bei der Sektion und histologischen Untersuchung wesentliche Veränderungen.

(c) Zehn Kaninchen im Gewicht von 2500—3500 g behandelten wir ohne Sensibilisierung mit Pilocarpin und Kaltwasserbad. Anaphylaxieähnlicher Schock war an den Tieren nicht zu beobachten, auch fanden wir bei der Sektion und histologischen Untersuchung keine entsprechenden Veränderungen.

(d) Von zwölf 600—650 g schweren Meerschweinchen gaben wir nach der Sensibilisierung 4 nur Pilocarpin, 4 wurden nur in kaltem Wasser gebadet, während wir 4 Tieren ohne Sensibilisierung Pilocarpin verabreichten und sie in kaltem Wasser badeten. Anaphylaxieähnliche klinische Symptome traten an keinem Tier auf, auch waren keine pathologisch-anatomischen und histopathologischen Veränderungen anwesend.

## Besprechung

Wie die Untersuchungen ergaben, sind bei Kaninchen nach Sensibilisierung mit normalem Pferdeserum, wenn ihnen anschließend Pilocarpin eingespritzt wird und sie in kaltem Wasser gebadet werden, nach 1—4 Behandlungen dem anaphylaktischen Schock ähnliche klinische Symptome sowie die entsprechenden pathologisch-anatomischen und histopathologischen Veränderungen zu beobachten. Erhielten die sensibilisierten Tiere nur Pilocarpin oder wurden sie nur gebadet, so kam es niemals zu einem anaphylaxieähnlichen Schock. Ebensowenig traten anaphylaktische Veränderungen in Erscheinung, wenn die Behandlung der Kaninchen ohne vorherige Sensibilisierung erfolgte. Der auf die beschriebene Weise herbeigeführte Schock ließ sich durch Narkotisierung der Tiere nicht abwehren, während er nach Vorbehandlung mit Atropin niemals auftrat, d. h. das verabreichte Atropin wehrte die Schockentwicklung ab. Nach Wiederholung der Versuche an Meerschweinchen und Ratten stellten wir fest, daß an Meerschweinchen die von DOERR [7] nach prolongiertem Schock beschriebenen Symptome auftraten und bei der Sektion sowie histologischen Untersuchung die von HARANGHY [10] beobachteten anaphylaktischen Veränderungen anwesend waren. Bei Ratten wurden die dem anaphylaktischen Schock gleichenden Symptome und pathologisch-anatomischen Veränderungen nicht wahrgenommen, was sich wahrscheinlich auf die aus der Literatur bekannte Tatsache zurückführen läßt, daß Anaphylaxie an Tieren verschiedener Art nicht in gleicher Weise hervorgerufen werden kann. Mäuse, Ratten und Ziesel verhalten sich verhältnismäßig refraktär. Darauf dürfte es beruhen, daß es mit unserem Verfahren nicht gelang, an Ratten Schock zustande zu bringen.

Die Frage, nach welchem Mechanismus der vorstehend beschriebene anaphylaktische Schock entsteht, läßt sich schwer beantworten. Nach SILBER

[16] deutet die Möglichkeit der Übertragung des anaphylaktischen Zustandes von einem Tier auf das andere darauf hin, daß die anaphylaktische Reaktion eng mit den Antikörpern zusammenhängt, die nach der Sensibilisierung des Tieres entstehen. Bei unseren Versuchen haben wir den Kaninchen und Meerschweinchen nach der Sensibilisierung Pilocarpin verabreicht. Laut BELÁK, SÁGHY, CSERESZNYÉS [4] sowie ILLÉNYI und BORSÁK [11] erhöht sich auf Wirkung von Pilocarpin die Antikörperproduktion. BELÁK und Mitarbeiter [3] stellten weiterhin fest, daß es sich bei den anläßlich der Immunisierung im Organismus entstehenden Antikörpern um parasympathikoerge Substanzen handelt. GORECZKY und LUDÁNY [9] wiesen nach, daß die auf das vegetative Nervensystem wirkenden Substanzen den Opsonin-, Agglutinin- und Präzipitintiter verändern. Nach ARISTOWSKIJ und Mitarbeitern [1] beruht die Entwicklung der anaphylaktischen Erscheinungen auf der Freisetzung von Azetylcholin und histaminartigen Substanzen, die sich als Resultat der in den Zellen vor sich gehenden Antigen-Antikörperreaktion im Organismus vermehren. Aus den Arbeiten von MASSINO [13], PENKOWSKAJA, TOLPJEGINA, UNDRIKOW und HOMJAKOW [14] wissen wir, daß die Allergene die in den Geweben anwesenden cholinergen Synapsen reizen und die Produktion der entsprechenden neuralen Mediatoren bewirken. Nach KANAREWSKAJA [14] kann man den Organismus auch über das Nervensystem gegen Antigene sensibilisieren. Die Autorin sensibilisierte Hunde durch Läsion des Hypothalamuszentrums gegen Pferdeserum. FILIPP und SZENTIVÁNYI [8] heben ferner die Innervationsstörung der Gefäße bei der Entstehung der hyperergischen Reaktionen hervor. SÜMEGI, GORECZKY und RÓTH [17] vermochten nach experimenteller Bleivergiftung zugleich mit immunbiologischen durch histologische Untersuchung auch neurale Veränderungen nachzuweisen.

WENT und LISSÁK [18] beobachteten, daß der Herzmuskel eine cholinähnliche Substanz sezerniert, wenn man das Herz des sensibilisierten Tieres mit dem entsprechenden Antigen durchströmen läßt. Alle diese Angaben sprechen dafür, daß dem verabreichten Pilocarpin und der Sensibilisierung im Zustandekommen der anaphylaktischen Erscheinungen unbedingt eine Rolle zufiel. Daneben kommt aber auch dem Kaltwasserbad bei der Auslösung der Reaktion Bedeutung zu, da diese an den sensibilisierten Tieren nur auf Wirkung von Pilocarpin nicht auftrat.

Im Zusammenhang mit den Untersuchungen über die Hemmung des anaphylaktischen Schocks muß erwähnt werden, daß über die Narkose gegensätzliche Angaben vorliegen. So wies BEZREDKA [5] nach, daß Meerschweinchen in der Narkose die Verabreichung der anaphylaxieauslösenden Dosis symptomfrei überstehen. Die Antigen-Antikörperreaktion findet auch in diesem Falle statt, löst aber keinen Schock aus. ROSENAU und ANDERSON [15] vermochten BEZREDKAS Ergebnis nicht zu bestätigen, während es AUER und LEWIS [2] bestärkten. Nach KESZTYÜS und Mitarbeitern [12] sei im Falle bereits vor-

handener Sensibilisierung die Narkose ohne wesentlichen Einfluß auf die lokalen und generalisierten anaphylaktischen Manifestationen. Durch die Dauernarkose mit Phenyläthylbarbitursäure wird die Vermehrung der Antikörper im Blut verlangsamt, d. h. gehemmt. Laut DOERR [7] wird der anaphylaktische Schock in der Narkose nicht absolut, sondern nur relativ abgewehrt, weil der letale Schock auch in der Narkose eintreten könne. Auch unsere Versuche ergaben, daß der anaphylaktische Schock mit den angewandten Narkotika nicht verhindert werden konnte.

Einige Autoren empfehlen Atropin zur Abwehr des anaphylaktischen Schocks. Deshalb hatten auch wir dieses Mittel angewandt. Nach DANIELOPOLU [6] übt Atropin seine Wirkung auf zwei verschiedenen Wegen aus: einerseits lähmt es den azetylcholinspaltenden Effekt der Cholinesterase (azetylcholinolytische Wirkung), anderseits hemmt es den parasympathomimetischen Azetylcholineffekt, indem es die Zellen Azetylcholin gegenüber unempfindlich macht. Bei erhöhter Atropindosis kommen beide Funktionen stärker zur Geltung. Wird aber eine zu geringe Atropinmenge verabfolgt, so steht die Azetylcholinwirkung im Vordegrund, und es kommt zum Schock. Im Zusammenhang mit unseren Versuchen muß ferner berücksichtigt werden, daß Atropin den Pilocarpineffekt hemmt, so daß die Abwehr des anaphylaktischen Schocks auch auf diesem Wege erfolgen konnte.

## Zusammenfassung

Nach 1—4maliger Behandlung von mit normalem Pferdeserum sensibilisierten Kaninchen, mit Pilocarpin und anschließendem Baden der Tiere in kaltem Wasser entwickelte sich klinisch sowie pathologisch-anatomisch ein anaphylaxieähnlicher Schock. Wurden die sensibilisierten Tiere nur mit Pilocarpin behandelt oder nur gebadet oder der Versuch ohne Sensibilisierung durchgeführt, so waren ähnliche Symptome und pathologisch-anatomische Veränderungen niemals zu beobachten. Der anaphylaxieähnliche Schock ließ sich durch Vorbehandlung mit Atropin abwehren, mit Evipan-Äthernarkose jedoch nicht. Mit derselben Methode konnte bei Meerschweinchen klinisch prolongierter Schock hervorgerufen werden, und pathologisch-anatomisch entstanden in diesem Fall dem anaphylaktischen Schock entsprechende Veränderungen. Bei Ratten ließ sich ein anaphylaxieähnlicher Schock nach diesem Verfahren nicht auslösen.

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# THE EFFECT OF METHYLENE BLUE ON THE IRON SENSITIVITY OF STREPTOMYCES RIMOSUS FERMENTATIONS

By

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(Received September 8, 1958)

In previous communications [1, 2] we have discussed the sensitivity of *Streptomyces rimosus* fermentations to iron and reported that iron was strongly toxic when the culture medium contained highly unsaturated oils. The catalytic action of iron results in the production of increased amounts of peroxide compunds in such oils and these compunds are directly responsible for the toxic effect. It is well-known that methylene blue with its antioxidative properties is capable of inhibiting toxic peroxide activities. For this reason we investigated the effect of methylene blue on the production of oxytetracycline in the presence of iron.

## Materials and methods

The *Streptomyces rimosus* strain BS-21 was used. Its maintenance and experimental conditions have been described in detail [2].

The experiments were carried out in 500 ml Erlenmeyer flasks, using 100 ml of medium at 28° C and a rotary shaking machine (300 r.p.m. ⌀ 2 cm).

The culture medium was as follows. Soybean meal, defatted, 3 per cent ; potato starch, 0.5 per cent ; corn steep liquor (50 per cent solids), 0,1 per cent ; NaCl, 0.3 per cent ; $CaCO_3$, 0.5 per cent ; sunflower or palm oil, 1 per cent. pH before sterilization : 7.0.

The given quantities of methylene blue and iron (in the form of ferrous sulphate) were added to the medium before sterilization, or in some cases after it, under sterile conditions. Inoculation was made with 1 ml of the suspension prepared on the shaking machine.

The subsequent oxytetracycline content of the fermentation was estimated by means of the *B. subtilis* strain ATCC 6633 [3].

## Results

The results of the experiments on the culture medium containing sunflower oil are shown in *Fig. 1.* Quantities of iron higher than 40 $\mu$g/ml rapidly reduced oxytetracycline production to 200 $\mu$g/ml. 10 $\mu$g/ml of methylene blue had hardly any protective effect, whereas in the presence of 30 $\mu$g/ml production was unaffected even by an iron concentration of 300 $\mu$g/ml. Curve No. 4 illustrates the remarkable phenomenon that while the addition of 90 $\mu$g/ml methylene blue resulted in a 70 per cent inhibition of tetracycline

production, this inhibition was compensated in the presence of certain amounts of iron.

If methylene blue was added 48 hours after inoculating the fermentation, it had practically no effect. In other experiments methylene blue was added when fermentation was started and the iron was added later. In these series methylene blue afforded no protection if the iron was administered more than 48 hours after fermentation had started.

In view of the fact that 30 $\mu$g/ml of methylene blue protected the production against even 300 $\mu$g/ml of iron, we investigated the effect of methylene blue in the presence of palm oil. As shown in *Fig. 2,* the partial inhibition of production which in our previous report had been termed "direct" inhibition, did not occur in the presence of methylene blue.

Informative studies on several redox dyes showed that none of them had an inhibitory action comparable to that of methylene blue, although some protection was afforded by neutral red, dichlorophenolindophenol and safranine. Basic fuchsin and methyl violet *(Table I)* were ineffective. Certain dyes inhibited production.

**Table I**

*Protective action of different dyes*

| Dye | — | 120 $\mu$g Fe/ml |
|---|---|---|
| | Oxytetracycline $\mu$g/ml | |
| — | 1700 | 250 |
| Neutral red 30 $\mu$g/ml | 1820 | 740 |
| Dichlorophenolindophenol 30 $\mu$g/ml | 1480 | 510 |
| Safranine 30 $\mu$g/ml | 650 | 350 |
| Basic fuchsin 30 $\mu$g/ml | 1600 | 250 |
| Methyl violet 30 $\mu$g/ml | 1400 | 250 |

## Discussion

The general use of metallic fermentors and the usual addition of inexpensive industrial by-products to the culture media make it desirable to work out methods affording protection against heavy metals, especially against contamination of the fermentation with iron. In a previous communication it was reported that the toxic action of iron on *Streptomyces rimosus* fermentation was due largely to the catalytic formation of peroxide compounds from the oils in the culture medium. The use of oils with a low iodine number, for example of palm oil, was found to afford protection against that effect. Moreover, the use of palm oil resulted in a partial inhibition, which we sug-

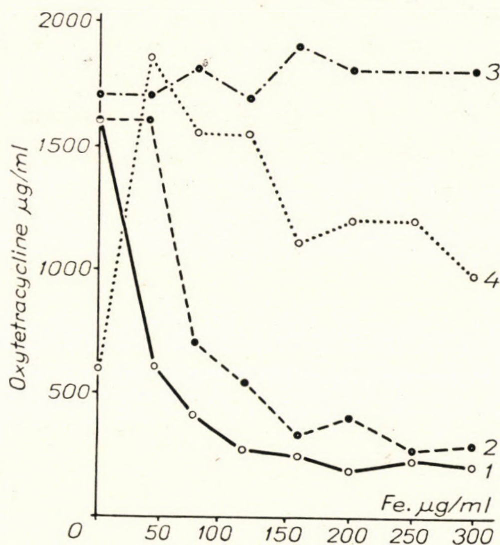


*Fig. 1.* Protective action of methylene blue in culture medium containing sunflower oil

*Signs:*

1. Control
2. 10 µg of methylene blue per ml
3. 30 µg of methylene blue per ml
4. 90 µg of methylene blue per ml

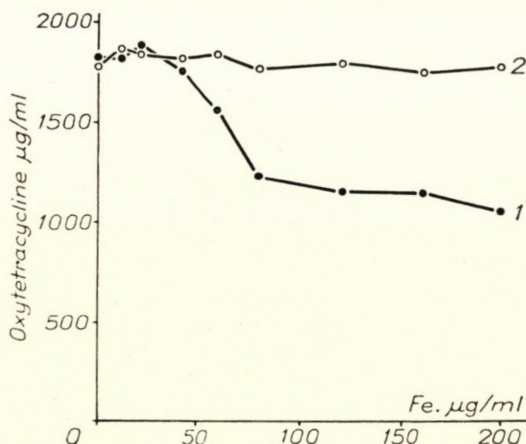


*Fig. 2.* Protective action of methylene blue in culture medium containing palm oil

*Signs:*

1. Control
2. 30 µg of methylene blue per ml

gested to be due to a "direct" action of iron on the metabolism of the microorganism. In the experiments described in the present report, methylene blue has been observed to protect against both kinds of toxic iron action. This represents a new, and in certain respects more extensive method for counteracting the effect of iron. Similar concentrations of methylene blue had been applied in penicillin production [4], with a subsequent rise in the yield. The mechanism of this formerly unexplained phenomenon seems to have been elucidated by the present experiments.

The data reported above do not shed light on the mode of action of methylene blue. According to experiments with different redox dyes, the redox potential values by themselves offer no explanation and the methylene blue effect appears to be specific. Methylene blue may be surmised to block the toxic action of oil peroxides by accelerating their breakdown. Should this be true, the same mechanism might explain inhibition of the "direct" toxic action. Experiments with various dosages, however, contradict this hypothesis. We still adhere to our earlier opinion since, in addition to the present experiments, some others involving the use of a wide variety of culture media have indicated that the upper limit of the yield is determined during the first 30 to 40 hours of growth.

## Summary

The toxic action of iron in the presence of unsaturated oils on *Streptomyces rimosus* fermentation has been observed to be blocked by the addition of 30 $\mu$g/ml of methylene blue to the culture medium.

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# THE SIGNIFICANCE OF THE INOCULATION INTERVAL IN VACCINATION WITH POLYVALENT DYSENTERY AND COMBINED TYPHOID-DYSENTERY-TETANUS VACCINES

By

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(Received October 2, 1958)

The starting point of our present investigations was Glenny's observation [1] that a second antigenic stimulus employed at a proper time greatly increases the antitoxin titre against diphtheria toxoid. According to Schmidt [2], the best time to make the second inoculation is when the stimulative activity of the first antigenic injection has just ceased. Erdős [3] found that of the diphtheria-tetanus-pertussis vaccinations carried out at intervals of 4 to 27 weeks those performed at the extreme 27-week intervals yielded the best results with respect to tetanus and diphtheria antitoxin production.

As regards enteric vaccines, Schütze [4] reported that in his model experiments with *S. typhi murium* vaccine the prolongation of the interval between inoculations enhanced the immunity of vaccinated mice, though significant differences were not noted. Glukovskaya [5] found that in man vaccination with typhoid-paratyphoid A—B vaccines was more effective when the inoculation interval was prolonged.

We have investigated the influence of the interval between inoculations on the immune effect of the single components contained in a polyvalent dysentery vaccine and in combined typhoid-dysentery-tetanus vaccines (in the following, Ty-Dys-Te vaccine). As the first step, model experiments were made in mice.

## Materials and methods

*Preparation and titration of enteric antigens.* The antigens were prepared from the strains *S. typhi* 2 ; *Sh. flexneri* 1b "19" ; *Sh. flexneri* 2a "Zamárdi" ; *Sh. flexneri* 3 "31342" ; and *Sh. sonnei* "Kiss". The antigens prepared according to Boivin *and* Mesrobeanu [6] from deep cultures were purified by dialysis.

The antigenicity of the extracts was determined in active mouse protection tests related to a standard antigen, supplemented by informative mouse toxicity tests [7]. In the light of recent observations [8], the standard composition of the combined vaccines was ensured on the basis of haemagglutination inhibition and the potency of the extracts had been expressed in haemagglutination inhibition units (HIU).

*Preparation and assay of tetanus antigen.* Tetanus anatoxin was prepared by formaldehyde and heat treatment of the sterile filtrate of fluid *Cl. tetani* cultures. The concentrated

and purified tetanus antigen was prepared from the crude anatoxin by treatment with trichloracetic acid, according to SURJÁN, RICHTER and RÉTHY [17].

The value of the antigen was determined by the binding test (1 B.U. = 1 Lf).

*Adsorbent.* In the model experiments on mice and in the polyvalent dysentery vaccine studies, the antigens were adsorbed to a precipitate prepared in the nascent state from 2 per cent alum. As previous investigations had shown the effectiveness of $Al(OH)_3$ [10], with the combined vaccine, the C gamma gel of WILSTÄTTER, as modified by RÉTHY [9], was used.

*Polyvalent dysentery vaccine.* This contained *Sh. flexneri* 1b, 2a and *Sh. sonnei* antigens adsorbed to a precipitate prepared from 2 per cent alum. The dose was 2 times 0.5 ml.

*The Typhoid-Dysentery-Tetanus combined vaccine* contained trichloracetic acid extracts of *S. typhi*, *Sh. flexneri* 2a, 3 and *Sh. sonnei*, adsorbed to the preformed $Al(OH)_3$ gel mentioned above, as well as 10 BU of tetanus toxoid per ml. The dose was 2 times 0.5 ml.

*Groups inoculated*, Males aged 18 to 24 years were inoculated with the polyvalent dysentery vaccine and adolescent boys and girls (equal numbers in each group) aged 12 to 13 and 15 to 18, respectively, were used in the combined vaccine studies. The test subjects were selected at random. There were groups of 50 to 100 subjects each.

*Active mouse protection.* In the model experiments mice were immunized subcutaneously with the constant minimum immunizing dose of the Boivin antigen. Two weeks after the last immunization, the animals were challenged with a 24-hour peptone water culture of the homologous strain diluted in 5 per cent mucin. The size of the infective doses was determined in simultaneous virulence test in non-immunized animals. Observation lasted 3 days after infection.

*Immune response to enteric antigens.* The sera obtained before and 2 weeks after the inoculations were deep-freezed. Before study, equal volumes of the sera from the same group were pooled. The passive mouse protection test was carried out at 3 levels, in groups of 4 to 6 mice, which were immunized intraperitoneally with 0.05—0.005—0.0005 ml of both the pre- and postinoculation sera and were infected intraperitoneally with a single dilution of 24-hour peptone water culture of the homologous strains diluted in 5 per cent mucin. Previous virulence tests indicated this dilution to be 100 to 1000 $LD_{50}$. To determine the infecting dose precisely, control mice were inoculated with the infecting dose chosen in simultaneous tests.

*Immune response to tetanus anatoxin* was studied in individual tests. Subjects showing a serum antitoxin titre of 0.001 IU/ml or higher after the first inoculation as well as persons who may have been inoculated against tetanus, were excluded from evaluation.

The immune responses to the tetanus anatoxin were determined in the usual way, at the L+/2000 level.

*Statistical analysis.* The LD and $ED_{50}$ values were determined from the ratio of surviving mice to those succumbed, according to KÄRBER [11]. In the case of enteric antigens, significance was determined by the $\chi^2$ test,

The results for tetanus were evaluated by comparing the mean group values by STUDENT's *t* test [12].

## Results

Our primary aim was to determine the influence on the antibacterial immunity of the interval between inoculations with enteric antigens in general and with the Shigella antigens in particular. It was obvious that protection experiments are exclusively suitable for this purpose. For this reason mice were immunized actively as a prelude to the main trials.

The protracted stimulation caused by adsorbed antigens may conceal eventual differences and for this reason non-adsorbed Boivin antigen representing a threshold stimulus was used in the model experiments.

*Table I*, showing the results of the experiments with *Sh. flexneri* 2a type antigen, makes it clear that the difference between the 1 and 2 week

**Table I**

*The effect of the inoculation interval on immunity in mice*
(Boivin extract of *Sh. flexneri* 2a type)

| Interval between inoculations (weeks) | Number of inoculations | Total amount of antigen inoculated ml | Value of immunity, expressed in $LD_{50}$ of infective doses |
|---|---|---|---|
| 1 | 2 | 0.0001 | 5 000 |
| 2 | 2 | 0.000J | 50 000 |
| 4 | 2 | 0.0001 | 100 000 |
| ∅ | 1 | 0.0001 | 700 |
| ∅ | 1 | 0.0003 | 1 000 |

and the 1 and 4 week intervals was significant, whereas that between the 2 and 4 week intervals was not ; there was merely a tendency in favour of the longer interval. Similar results were obtained for the adsorbed extract.

These preliminary model experiments had encouraged us to start human trials, on the one hand, and had made it possible to establish, even though only tentatively, the lower limit of the interval to be 2 weeks.

The experiments on human subjects were introduced by inoculations with the polyvalent dysentery vaccine.

Males aged 18 to 24 years were inoculated at intervals of 2, 4 and 8 weeks. Pools of 40 to 50 sera from the single groups were subjected to the passive mouse protection test. The protective titres were followed up for 6 months in the 4-weeks' interval series. The protective value of the convalescent sera tested was of aid in appraising the absolute value of the protective titres.

**Table II**

*The effect of the inoculation interval on immunity in man*
Adsorbed polyvalent dysentery vaccine
(Test strain : *Sh. flexneri* 2a ; 250 $LD_{50}$)

| Interval between inoculations (weeks) | Blood sampling at | Mean immune value of sera, ml | Index (relative potency) |
|---|---|---|---|
| 2 | 4 weeks | 0.1 | 1 |
| 4 | 4 weeks | 0.007 | 14.3 |
| 8 | 4 weeks | 0.004 | 25 |
| 4 | 3 months | 0.0075 | 13.3 |
| 4 | 6 months | 0.037 | 2.9 |
| Convalescent | 0 months | 0.008 | 12.5 |
| sera | 3 months | 0.0625 | 1.6 |

*Table II* shows the protection afforded against one component, *Sh. flexneri* 2a. The immune response was significantly increased in intensity when the interval had been prolonged from 2 weeks to 4 and 8, respectively. The difference in increase between the 4 and 8 week series was not significant. Inoculations made at 2 week intervals yielded titres which were significantly lower than the convalescent serum, while the 4 and 8 week intervals afforded equipotent protection.

After inoculation at 4 resp. 8 week intervals the protective titres were comparable to that of the convalescent serum. The potency of sera of persons who had been inoculated at 4 week intervals was 3 months later still equivalent to that of convalescent serum and after 6 months still as high as that found 3 months after natural infection.

Similar results have been obtained for the other components *(Sh. flexneri* 1b, *Sh. sonnei).*

Subsequently, we combined the dysentery antigens with typhoid and tetanus antigens and exchanged the adsorbent. In this way a vaccine of sufficient activity in man was obtained [13]. This vaccine, too, had to be tested for the effect of the interval between inoculations on the immunogenicity of the single components. It was particularly interesting to determine how the prolongation of the inoculation interval would affect the response to the tetanus component under the preponderance of enteric antigens.

The experiments were carried out in the towns of Eger, in adolescent subjects aged 12 to 13 years, and Pécs, in subjects 15 to 18 years of age. For technical reasons the intervals could not be prolonged beyond 6 weeks. The first group was therefore inoculated at intervals of 4 and 6 weeks ; the second, of 3 and 6 weeks.

The immune response to the tetanus component was studied in the first group. Practically identical results were achieved in a group of subjects between 20 and 24 years of age.

The enteric immune effect, as evaluated by the passive mouse protection test, is illustrated in *Table III.*

*Table III* shows the data for two representative Shigella strains : *Sh. flexneri* 2a and *Sh. sonnei.* In the Pécs group of subjects (aged 15 to 18 years) there are no data for the typhoid component, because this represented here a booster inoculation.

As compared to the basic (preinoculative) ones, the increase in the immune titres was significant in every group, irrespective of the length of the interval. A prolongation of the interval caused the significance to be more marked.

There were, however, differences according to the length of the intervals. In the group inoculated in intervals of 4 and 6 weeks, the titre to *Sh. sonnei* increased significantly in favour of the longer interval, while the titre to *Sh.*

**Table III**

*The effect of prolongation of the inoculation interval on the immune response to the typhoid-dysentery-tetanus vaccine*

| Locality | Interval (weeks) | Strains | Immune value of sera | | Statistical data | |
|---|---|---|---|---|---|---|
| | | | Basic ED 50, ml | Immune ED50 ml | difference between basic and immune $\chi^2$ | difference between 4 and 6 weeks $\chi^2$ |
| EGER | 4 | *Sh. flexneri* 2a | 0.028 | 0.0009 | 8.16 | 1.54 (0.2) |
| | 6 | | 0.05 | 0.0005 | 13.50 | |
| | 4 | *Sh. sonnei* | 0.05 | 0.0016 | 10.44 | 3.12 (0.05) |
| | 6 | | 0.05 | 0.0005 | 20.74 | |
| | 4 | *S. typhi* | 0.032 | 0.001 | 9.11 | 0.11 (0.7) |
| | 6 | | 0.069 | 0.001 | 14.1 | |
| PÉCS | 3 | *Sh. flexneri* 2a | 0.05 | 0.0009 | 10.74 | 5.55 (0.02) |
| | 6 | | 0.05 | 0.0005 | 31.34 | |
| | 3 | *Sh. sonnei* | 0.05 | 0.0016 | 11.37 | 3.22 (0.05) |
| | 6 | | 0.05 | 0.0005 | 20.74 | |

**Table IV**

*The effect of the interval as studied with enteric antigens combined with tetanus anatoxin*

I. Adolescents
[Immunization : 2×0.5 ml (2×5 B. U.)]

| Vaccine | Inoculation interval, weeks | Number of tests (n) | Distribution of immunity I. U./ml | | | | | | m | $\sigma^2$ |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | < 0.01 | 0.011—0.05 | 0.051—0.1 | 0.11—0.5 | 0.51—1.0 | > 1 | | |
| P 14 Ty-DYS-TE | 4 | 35 | — | 4 | 3 | 4 | 4 | 20 | 2.98 | 5.42 |
| P 14 TY-DYS-TE | 6 | 25 | — | — | — | 1 | 4 | 20 | 4.1 | 3.1 |
| P 40 TY-DYS-TE | 4 | 30 | — | 1 | 1 | 1 | 6 | 21 | 3.65 | 4.68 |
| P 40 TY-DYS-TE | 6 | 25 | — | — | — | 1 | 2 | 22 | 4.66 | 2.14 |

**P 14 = "P" 4 — 6 week ≅ 0.01**
**P 40 = "P" 4 — 6 weeks ≅ 0.01**

*flexneri* 2a showed merely a tendency to increase. In the case of *S. typhi* the difference of the two intervals was statistically insignificant.

In the Pécs group of subjects (aged from 15 to 18 years) significant differences were obtained for *Sh. sonnei* and *Sh. flexneri* alike. In this group the interval between inoculations was either 3 weeks or 6 weeks and this greater difference in the length of the intervals may explain the marked difference in response.

The response to the tetanus component was illustrated by the groups immunized with two Ty-Dys-Te vaccines designated P14, resp. P40. The two vaccines differed only in the quantity of adsorbent they contained.

Two important conclusions may be drawn from the data in *Table IV*. First, both vaccines produced significantly higher mean antitoxin titres when inoculated at 6 week intervals than with the 4 week interval. The other, apparently not negligible difference was the elimination of the group of low immunity.

## Discussion

The significant rise caused in the antitoxic titre by the use of a second antigenic stimulus at an appropriate time (GLENNY's secondary stimulus) has discredited the single inoculation-depot principle in the case of vaccines inducing antitoxic immunity. When starting our investigations, it was thought that this immunological rule might apply also to antibacterial vaccines. However, the procedures used to-day for studying antibacterial immunity have considerabe sources of error which interfere with evaluation, and for this reason it was difficult to obtain convincing evidence.

In preliminary mouse experiments adsorbent-free Shigella antigen of threshold activity was used for studying the role of the inoculation interval. It was found that the results achieved by inoculations at one week intervals were much inferior to those obtained on vaccination in intervals of several weeks. The results suggested that the length of the interval may play a role also in the immunization of man with enteric antigens. Moreover, the mouse technique employed appeared to be suitable for the measurement of differences in immunity, though it obviously did not demonstrate minute differences. Another evidence obtained in the mouse experiments was that the statistical difference between the 2 and the 4 week interval was not marked. This fact indicates that there undoubtedly exists a lower limit, *i. e.* an interval, by the prolongation of which immunity can be increased up to a certain level. Beyond this, however, the response is not any more enhanced by a further lengthening of the interval.

Essentially the same results were obtained with the polyvalent dysentery vaccine and with the Shigella components of the combined Ty-Dys-Te vaccine. The difference in response was less convincing between the 4 and 6 week

intervals, than in the case of longer ones, (*i. e.* between 2—8, resp. 3—6 weeks). It should, however, be borne in mind that owing to the wide range of error inherent in the assay of the immune response to enteric components, only great differences may be relied upon.

The fact that the increase of the interval had no influence on the response to the typhoid component, had apparently two reasons. (1) In Hungary numerous individuals are inoculated against typhoid fever every year. Although from the present study we excluded all subjects suspected of having been immunized against typhoid fever, there were no means to ascertain this in every case, so that in some instances the typhoid antigen may have acted as a booster stimulus. This makes it clear why sometimes the interval, or even the repetition of inoculations, had so little, if any effect. (2) A single inoculation with adsorbed typhoid vaccine is well-known to induce sufficient immunity by itself, so that a second inoculation or a change in the interval has very little influence on the immune response [14].

The sensitivity of the methods for assaying immunity to tetanus undoubtedly contributed to the fact that quite small differences in the length of the interval resulted in appreciable changes in the immune response and there occurred a significant difference between the 4-week and 6-week intervals. In another study we examined the immune effect of tetanus toxoid in combination with typhoid, paratyphoid, Shigella and cholera antigens, and compared the 4, 8 and 12 week intervals. It was found that the 8 and 12 week intervals did not differ significantly, just as had been the case with the enteric components *(Table V)*. Convincing evidence has then been obtained that the well-known dependence on the interval of the immunogenicity of tetanus toxoid is unaffected in vaccines composed preponderantly of enteric antigens. Also, as already mentioned, the effect of the prolongation of the interval between inoculations manifests itself not only with a rise of the mean titre, but also with an elimination of groups with low immunity.

Finally, a prolongation of the interval between inoculations affects also the persistency of immunity. Although we possess exact data only for inoculations with polyvalent dysentery vaccine made at 4 week intervals, it seems that the increase in the immunity titre resulting from the prolongation of the interval means at the same time a reduced rate of decrease in immunity. It may therefore be assumed that a prolongation of the interval not only increases the protection afforded by the Ty-Dys-Te vaccine, but, as a result of this, also makes immunity more persistent. Comparative studies with convalescent sera indicate that the immunity achieved by polyvalent dysentery inoculations at intervals of 4 weeks is comparable both in intensity and in duration to the humoral immunity resulting from natural dysentery infection. Our earlier investigations [15] showed the latter to last about ½ year and similar persistency may be attained by immunization, too. However, further investi-

**Table V**

*Interval studies with tetanus anatoxin combined with enteric antigens*
II. Adults

[Immunization : 2×0.5 ml (2×5 B. U.)]

| Vaccine | Inoculation interval weeks | Number of tests (n) | Distribution of immunity I. U./ml | | | | | | m | $\sigma^2$ |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | < 0,01 | 0.011— | 0,051— | 0.11— | 0.51 | > 1.0 | | |
| P 7 TY-PATY-DYS-CHO-TE | 4 | 62 | 5 | 6 | 16 | 7 | 11 | 17 | 1.53 | 6.75 |
| P 7 TY-PATY-DYS-CHO-TE | 8 | 77 | — | 3 | 9 | 4 | 9 | 52 | 3.5 | 4.72 |
| P 7 TY-PATY-DYS-CHO-TE | 12 | 87 | — | — | 3 | 10 | 13 | 61 | 3.7 | 4.35 |

"P" 4 weeks/ 8 weeks $< 0.001$
"P" 4 weeks/12 weeks $< 0.001$
"P" 4 weeks/12 weeks $\cong 0.2$

gations involving epidemiological studies are required to elucidate this problem.

In our experience [16], a single inoculation suffices to induce immunity to typhoid fever for at least two years.

The influence of the inoculation interval on the persistency of tetanus immunity is well-known and has been extensively investigated. There is no reason to doubt that the significant increase in the immunization response to Ty-Dys-Te vaccine inoculated at intervals of 6 weeks at the same time indicates an increase in persistency.

Both the evidence obtained and the considerations discussed indicate that the inoculations with the Ty-Dys-Te vaccine should be made in intervals of 6 weeks, because this will considerably enhance the response to the Shigella and tetanus components and, as suggested by the example of the dysentery vaccine, it may increase also the persistency of immunity.

Data for the booster inoculation and the size of the second immunizing dose will have to be determined by future investigations.

## Summary

(i) In active immunization of mice with non-adsorbed Shigella Boivin extracts, if the second stimulus had been administered after an interval of 2 weeks, the response was signifcantly more marked than with an interval of 1 week, and practically corresponded to the mmunity attained by making the inoculations in intervals of 4 weeks.

(ii) In man, immunization with a polyvalent dysentery vaccine adsorbed to alum precipitate, administered at intervals of 4 weeks, caused the mouse protection titre to rise significantly more than after inoculations made at intervals of two weeks. The protective value of the serum did not increase significantly when the length of the interval was increased from 4 to 8 weeks.

(iii) Prolongation of the inoculation interval from 3 to 6 weeks significantly increased the immunogenic capacity of Shigella antigens contained in combined vaccines adsorbed to $Al(OH)_3$ gel, but prolongation of the interval from 4 to 6 weeks had no such marked effect. The changes of the inoculation interval did not influence the immunogenic effect of the typhoid component.

(iv) The tetanus component of the combined typhoid-dysentery-tetanus vaccine produced a significantly higher mean titre when the inoculation interval had been increased from 4 to 6 or 8 weeks. At the same time, the group showing low immunity became eliminated.

(v) The persistency of immunity has been analysed in the light of the evidence obtained.

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# STUDIES ON THE SPOROCIDAL ACTIVITY OF SOME DISINFECTANTS

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(Received December 4, 1958)

Although testing of disinfectants includes the determination of their sporocidal activity, the more exact laboratory control of these substances makes necessary the revision of the present methods and the application of tests more suitable for practical purposes.

The present work has been undertaken to review by up to date methods the sporocidal activity of some commonly used disinfectants and to consider the employment of the most efficient preparations for practical use.

## Materials and methods

The tests applied were based on STUART's constant boiling hydrochloric acid method [1], differing somewhat from the original procedure mainly in the culture media and in the technique employed.

*Test organisms and preparation of test spores.* According to STUART, any spore-bearing organism can be used. In the present experiments one aerobic organism, *B. anthracis*, and eight different anaerobes, namely *Cl. bifermentans*, *Cl. histolyticum*, *Cl. sordellii*, *Cl. perfringens*, *Cl. oedematiens*, *Cl. sporogenes*, *Cl. septicum* and *Cl. tetani* were used. All strains were of the stock culture collection of this Institute, with the exception of *Cl. tetani*, which was kindly provided by DR. I. VITÉZ, *Institute of Hygiene, University of Budapest.*

*Clostridium* strains were inoculated into several tubes containing Holman's cooked meat medium, while *B. anthracis* was cultivated in tubes containing soil extract broth. The tubes were incubated for 3 days at 37° C, then spore formation and purity of the cultures were controlled by staining according to Gram. The tubes containing the anthrax cultures were then mixed. Similarly, mixtures were made from the cultures for each anaerobic organism by taking 5 ml samples from the supernatants of the Holman media. Silk loops were then placed into the cultures and were allowed to stand for one hour. For the purpose of keeping standard conditions, all contaminated loops to be used in the whole series of experiments were prepared from one culture suspension of the strain under investigation, using 5 loops for each 5 ml of the culture. The silk loops were then transferred from the spore suspension with flamed wire hooks into sterile Petri dishes that had been matted with sheets of filter paper, and were allowed to dry for 3 days at room temperature. The silk loop carriers prepared this way were used for determining the resistance of spores to hydrochloric acid, to boiling in water and to various disinfectants.

*Preparation of silk loops.* From surgical silk No. 3, loops were prepared by wrapping the silk around a pencil three times, slipping the coil so formed off the end of the pencil and holding it with the fingers while passing the ends of the thread through the coil and knotting them. The ends of the thread were sheared off within 1 mm to the coil. This procedure provided an overall length of approximately 8 cm of silk thread, that could be conveniently transferred under aseptic conditions by a wire hook. The loops were then defatted in ether and sterilized in the autoclave. The contamination of the loops with spores was performed as described above.

*Culture media.* In testing the resistance of *Clostridia*, thioglycollate medium was used : $K_2HPO_4$ 2.5 g, NaCl 5 g, agar 0.5 g, sodium thioglycollate 0.5 g, yeast extract 10 ml. Witte peptone 20 g, glucose 0.5 g, dist. water 1000 ml, methylene blue (0.2 per cent) 1 ml, pH 7.2 ; the medium was distributed in tubes at 15 ml amounts and sterilized by steaming. For experiments with the constant boiling HCl method, 20 ml of *N* NaOH was added to 1000 ml of thioglycollate medium.

For obtaining spore suspension from the anaerobes, Holman's well-known cooked medium was used.

Soil extract broth served for obtaining spore suspension from *B. anthracis* and in most cases for testing the resistance of anthrax spores. In examining these spores against mercurial compounds, however, thioglycollate medium was applied. The composition of soil extract broth was as follows. 450 g of garden soil was extracted overnight in 1000 ml of distilled water, then filtered several times through filter paper. The pH of the extract was about 5.2. The extract was then brought to volume and after adding 5 g Liebig extract, 10 g Richter peptone and 5 g NaCl, it was adjusted to pH 6.9. The medium was dispensed in tubes at 10 ml amounts and sterilized. Before use, 10 per cent of ox serum was added to the tubes and the necessary sterility test was performed.

*Constant boiling HCl test.* The test serves for determining whether the spores applied give reliable practical results in testing disinfectants. The spores to be used should resist the HCl for at least 5 minutes. Preparation of constant boiling HCl was performed as described by STUART [1]. The concentration of the distilled fraction of HCl was 20.2 per cent ; this was tubed at 10 ml amounts. As a control, untreated silk loops contaminated with the corresponding spores were placed in thioglycollate medium or in soil extract broth. The tubes containing the HCl were placed in a water bath at 20° C, then five contaminated loops were transferred into each tube. The tubes were vigorously shaken and after 5, 10, 15 and 20 minutes individual loops were withdrawn from the HCl and transferred into the corresponding medium. The media were incubated for 1 week at 37° C, then from the tubes with no visible growth the loops were removed under sterile conditions and transferred into fresh tubes of media. These subcultures were read after a reincubation for 1 week at 37° C. Two loops were used for each exposure time and the whole experiment was made in triplicate.

*Resistance to water at 100° C.* The factors responsible for the resistance of a spore may be of intracellular and extracellular origin [2]. The hereditary properties of the species, the specific gravity and growth temperature of the spore constitute the intracellular factors. As to the extracellular factors, our knowledge is limited mainly to the thermal conditions. Examining resistance to boiling seemed therefore necessary. Three of each series of loops contaminated with various *Clostridia* and with *B. anthracis* were transferred into tubes containing 10 ml of saline. The tubes were placed in a water bath, which was then heated by Bunsen flames. After the saline inside the tubes had come to the boil, the tubes were removed from the water bath at intervals of 2.5, 5, 7.5, 10, 20, 30, and 40 minutes. After standing for 10 minutes at room temperature, the loops were transferred into the corresponding medium (thioglycollate medium for the anaerobes and soil extract broth for the anthrax bacillus). Each exposition time was tested with 3 individual silk loop carriers. The media were incubated for 1 week at 37° C.

*Table I* shows the surviving of various spores in constant HCl and in boiling water. Results are given as extreme values representing the minimum period of exposure time necessary for killing all spores, and, on the other hand, the maximum period of exposure unsatisfactory for the destruction of spores. It is clear from *Table I* that *Cl. perfringens* and *B. anthracis* were killed in HCl within 5 minutes, therefore neither of them could be used as an adequate test organism. It was remarkable that in comparison to others, the spores of these two organisms were the least resistant to boiling. For showing the difference between resistance to disinfectants of spores of *Cl. perfringens* and *B. anthracis* and of other spores, these two organisms were included in the examinations.

*Concentration of disinfectants and method of testing resistance.* The sporocidal activity of six disinfectants was examined. Each disinfectant was tested in the highest concentration still applicable in general practice, as follows. Five per cent formalin, made from commercial formalin containing 36.7 per cent formaldehyde ; 5 per cent phenol solution containing 12 per cent NaCl ; 1 per cent Sterogenol (hexadecilpyridinium bromide) ; 0.1 per cent Famosept (Merfen, Ryfen, phenyl mercuric borate) ; 2 per cent $HgCl_2$ ; 2 per cent Neomagnol (sodium p-toluene sulfonchloramide). To 100 ml of Neomagnol solution 4 ml of 8 per cent HCl was added. The disinfectants were pipetted at 10 ml amounts into tubes covered with metal caps. Into each tube 5 contaminated silk loops were placed. For each exposure time 3 parallel testings were performed and every experiment was made in triplicate. For all disinfectants the same exposure times were kept, namely 10, 30 and 60 minutes and 2, 4, 6, 8 and 24 hours.

**Table I**

*Resistance of various spores to constant HCl and to boiling*

| Spores | Constant HCl (20.2%) | | Water at 100° C | |
|---|---|---|---|---|
| | maximum ineffective | minimum effective | maximum ineffective | minimum effective |
| | exposure time in minutes | | | |
| *Cl. bifermentans* | 15 | 20 | 30 | 40 |
| *Cl. histolyticum* | 10 | 15 | 30 | 40 |
| *Cl. sordellii* | 5 | 10 | 20 | 30 |
| *Cl. perfringens* | — | $<5$ | 2½ | 7½ |
| *Cl. oedematiens* | 10 | 15 | 30 | 40 |
| *Cl. sporogenes* | 5 | 10 | 7½ | 10 |
| *Cl. septicum* | 5 | 10 | 10 | 20 |
| *Cl. tetani* | 5 | 10 | 7½ | 10 |
| *B. anthracis* | — | $<5$ | — | $<5$ |

The tubes were placed in a water bath at 20° C. After the exposure times indicated, the loops were transferred into thioglycollate medium or into soil extract broth. The tubes were incubated at 37° C for 1 week, then from those showing no growth the loops were transferred into fresh medium and reincubated for 1 week. The amount of disinfectant remaining in the silk loops after the second subculture was estimated to be lower than the bacteriostatic concentration. Thus the result given by the second subculture indicates that the concentration of disinfectant satisfactory for killing spores in the test may be regarded as adequate for practical application, provided that the corresponding exposure time can be assured in the latter.

## Results

The sporocidal activity of the six disinfectants is summarized in *Table II*. Results are given as extreme values representing the minimum period of exposure necessary for killing the spores (—), and the maximum period of exposure unsatisfactory for the destruction of spores (+).

Five per cent formalin killed various spores at 20° C within 6 to 24 hours. The spores of *Cl. perfringens* and *B. anthracis* were destroyed in 4 and 2 hours, respectively. Considering the prescription of the testing method, however, no practical conclusion can be drawn from the latter results.

Two per cent Neomagnol killed the dried spores of all *Clostridia* within 8 hours. Spores of *Cl. perfringens* and of *B. anthracis* were destroyed within the shortest exposure time (2 hours). *Cl. tetani* was killed also within 2 hours by Neomagnol, although its spores resisted to the constant boiling hydrochloric acid for 5 minutes.

Sterogenol in 1 per cent concentration and also 2 per cent $HgCl_2$ and 0.1 per cent Famosept had no effect against any of the spores.

**Table II**

*Resistance of various spores to disinfectants*

| Spores | 5% formalin | | 5%phenol + 12%NaCl | | 1% Sterogenol | | 0,1% Famosept | | 2% $HgCl_2$ | | 2% Neomagnol | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | + | — | + | — | + | — | + | — | + | — | + | — |
| *Cl. bifermentans* | 8 | 24 | 24 | — | 24 | — | 24 | — | 24 | — | 6 | 8 |
| *Cl. histolyticum* | 6 | 8 | 24 | — | 24 | — | 24 | — | 24 | — | 6 | 8 |
| *Cl. sordellii* | 6 | 8 | 24 | — | 24 | — | 24 | — | 24 | — | 2 | 4 |
| *Cl. perfringens* | 2 | 4 | 24 | — | 24 | — | 24 | — | 24 | — | 1 | 2 |
| *Cl. oedematiens* | 4 | 6 | 24 | — | 24 | — | 24 | — | 24 | — | 6 | 8 |
| *Cl. sporogenes* | 6 | 8 | 4 | 6 | 24 | — | 24 | — | 24 | — | 6 | 8 |
| *Cl. septicum* | 6 | 8 | 24 | — | 24 | — | 24 | — | 24 | — | 4 | 6 |
| *Cl. tetani* | 4 | 6 | 24 | — | 24 | — | 24 | — | 24 | — | 1 | 2 |
| *B. anthracis* | 1 | 2 | 8 | 24 | 24 | — | 24 | — | 24 | — | 0.17 | 0.5 |

Key : + = maximum ineffective exposure time in hours
— = minimum effective exposure time in hours

No sporocidal activity of 5 per cent phenol was observed and its activity was not considerably increased by the addition of 12 per cent NaCl. The anthrax spores were killed by phenol plus 12 per cent NaCl within 8 to 24 hours at 20° C. A selective sporocidal effect was observed on the dried spores of *Cl. sporogenes,* which were destroyed by this solution within 6 hours.

## Discussion

According to Patterson, a disinfectant is a substance that, generally on inanimate objects, destroys vegetative forms of bacteria [3]. This definition does not require the destruction of bacterial spores, of *Mycobacterium tuberculosis* and of viruses. Testing of the sporocidal activity of disinfectants, however, is indicated by the following points. 1. The practical disinfecting value can reliably be interpreted only by taking into consideration several different properties of a given disinfectant. The laboratory control of disinfectants should include the investigation of sporocidal activity, as a correct estimation of their general disinfecting properties can be obtained this way. 2. The disinfectants examined in this study are widely used in surgical, epidemiological and bacteriological practice. The importance of their sporocidal activity, however, is different according to the range of application. In epidemiological practice a disinfectant is seldom required to be sporocidal (*e. g.* for disinfecting objects contaminated with anthrax spores). In the laboratory it is sometimes also necessary to destroy pathogenic spores with disinfectants. In surgery the importance of sporocidal activity is obvious.

From the results of the present experiments it is clear that among the disinfectants tested only 5 per cent formalin and 2 per cent Neomagnol are effective sporocidal agents. The aqueous solution of formaldehyde is bactericidal, sporocidal and viricidal, and it can be regarded as a substance approaching the ideal disinfectant [4]. Because of its unpleasant pungent smell and its slow reaction rate it is thrown into the background by the other disinfectants ; its combination with other disinfectants, however, is widely used, for disinfecting surgical instruments.

The sporocidal activity of organic chlorine compounds is well-known from the literature [5]. The reaction rate of Neomagnol is higher than that of formalin. Its application is limited by its corrosive property, its sensitivity to changes in concentration and pH, and also by the fact that its effect is greatly influenced by the presence of organic matter.

In the literature some controversies can be found as to the sporocidal activity of quaternary ammonium compounds. According to SPAULDING, benzalkonium chloride killed spores on a contaminated blade within 18 hours [6]. This finding has not been confirmed ; KLARMANN and WRIGHT added a neutralizing agent to the culture medium and observed the growth of tetanus spores even after 48 hours' exposure [7]. NUNGESTER also found that the effect of detergents is chiefly static and not sporocidal [8].

The results obtained in this study did not confirm our earlier finding that 0.1 per cent Sterogenol kills the spores of *B. subtilis* [9]. This was due to the fact that in the earlier experiments yeast extract broth without serum has been used as a culture medium and the cultures were incubated only for 6 days. In the present examinations, the 10 per cent serum content of the medium sufficed to neutralize Sterogenol, and further, the second subculture assured a dilution of the disinfectant not any more static.

The inactivity of $HgCl_2$ on spores is well-known from the literature [10]. The controversies as to the sporocidal activity of organic mercurials can be interpreted as follows. False results have been obtained by neglecting the use of neutralizing media, *e. g.* in our earlier examinations, Famosept (Merfen) had been found sporocidal [11]. No false results will be obtained with thioglycollate medium, which inactivates mercurials and thus eliminates their strong static activity on spores. The other reason of the false results was that numerous authors were using *Cl. tetani* as the test organism. It must be realized that many strains of this species do not form spores ; neither of the 17 *Cl. tetani* strains of the stock culture collection of this Institute is spore-forming (a spore-forming *Cl. tetani* strain has been obtained from the *Institute of Hygiene of the University)*. Thus Famosept in the concentration applied is highly static, but not sporocidal for aerobic and anaerobic spores.

It is known that phenol, due to its solubility in lipoids, penetrates into the cells and damages the protoplasm of bacteria [12]. The disinfecting action

of a phenol solution can be increased by the addition of NaCl, as the latter causes salting out of phenol, *i. e.* decreases its solubility in water and so increases its distribution quotient in favour of the lipoids. Nevertheless, the sporocidal activity of 5 per cent phenol plus 12 per cent NaCl proved to be weak.

According to the present findings, among the disinfectants available in Hungary for epidemiological and bacteriological practice, only two can be regarded as effective sporocidal agents.

## Summary

The sporocidal activity of some disinfectants available in Hungary has been determined.

1. Sterogenol, Famosept and mercuric chloride were observed to exert no sporocidal activity within 24 hours at 20° C.

2. A solution containing 5 per cent phenol and 12 per cent NaCl had a selective sporocidal activity against *Cl. sporogenes* and *B. anthracis*, but was inactive at 20° C against spores of other organisms.

3. Neomagnol in 2 per cent and formalin in 5 per cent concentration were definitely sporocidal. The effect of Neomagnol needs further examination as it seems to be dependent on the pH of the solution.

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# THE INFLUENCE OF CHANGES IN TEMPERATURE AND CONCENTRATION ON THE BACTERICIDAL AND SPOROCIDAL ACTIVITY OF FORMALIN

By

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(Received December 4, 1958)

The authors' earlier studies on disinfectants commonly used in Hungary have been focussed on the relationship between the temperature and the time needed for effective disinfection [1], and on the relationship between the concentration of a disinfectant and the time of exposure necessary for effective disinfection [2]. The test organism used was *S. typhi*. The results of these experiments corresponded to earlier findings known from the literature. Two characteristic indices have been determined by the authors, namely the temperature and concentration coefficients of disinfectants. As to earlier disinfectants, these values corresponded to data of the literature, while the validity of coefficients determined for new Hungarian disinfectants has been confirmed by practical application [3].

In an earlier series of experiments, of the disinfectants tested only Neomagnol (sodium p-toluene sulfonchloramide) and formalin were found sporocidal within 24 hours [4]. As the pH of the Neomagnol solution exerted a considerable influence on the bactericidal and sporocidal activity, this required further investigations. In the present study therefore only formalin has been used for determining the concentration and temperature coefficients previously established concerning *S. typhi* also for dried spores.

## Materials and methods

*The influence of temperature on the activity of formalin.* According to CHICK's examinations, the following relationship exists between temperature and disinfection

$$\frac{k'}{k} = \Theta^{(T'-T)}$$

where $k'$ and $k$ are the velocity constants of the reaction at temperatures $T'$ and $T$, respectively [5]. Taking the reciprocals of the exposure time instead of $k'$ and $k$, the formula will be expressed as

$$\frac{t}{t'} = \Theta^{(T'-T)}$$

By taking the logarithms of the equation and reducing, a linear regression regarding the logarithms of the exposure time will be obtained :

$$\lg t = a - T \lg \Theta$$

where $a = \lg t' + T' \lg \Theta$ is constant, $T$ is the temperature and $- \lg \Theta$ stands for the slope of the linear regression. The temperature coefficient characteristic of a given disinfectant, $\Theta$, is generally determined for 10° C. The closer the value of $\Theta$ to 1, the better applicable the disinfectant.

*Method for determining the temperature coefficient.* Silk loops were contaminated with spores of *Cl. sordellii* as described elsewhere [4]. Five contaminated loops were placed in each of a series of tubes containing 10 ml of 5 per cent formalin. Experiments were carried out at temperatures of 20°, 25° and 30° C. The loops were removed from the disinfectant at intervals of 10 minutes from the 150th to the 250th minute of exposure at 20° C ; at intervals of 10 minutes from the 60th to the 170th minute of exposure at 25° C ; at intervals of 5 minutes from the 25th to the 60th minute of exposure at 30° C. For each temperature and exposure time 10 parallel experiments were done. After the indicated exposure times the loops were transferred into tubes containing thioglycollate medium. The tubes were vigorously shaken and incubated for 24 hours at 37° C. The loops were then transferred into fresh thioglycollate media and reincubated for 1 week. The loops from the tubes showing no growth after 1 week were transferred into fresh media which were finally read after another week of incubation. As a viability control, before the experiments some of the contaminated loops to be used were placed in culture media. Every experiment was carried out in triplicate. The results were evaluated statistically.

*Influence of concentration on the activity of formalin.* According to the work of CHICK and WATSON [6], the relationship between the concentration and the exposure time for vegetative bacteria of a disinfectant can be expressed by the formula

$$t C^n = a$$

where $t$ is the time of exposure necessary for disinfection, $C$ is the concentration, $n$ and $a$ are constants. This means that the effective exposure time is not determined simply by the concentration, but is inversely proportional to the $n^{\text{th}}$ power of the latter. The value of $n$ varies with each disinfectant. By taking the logarithms of the equation and solving it for $\lg t$, a linear regression will be obtained as follows.

$$\lg t = \lg a - n \lg C$$

*Method for determining the concentration coefficient.* Silk loops contaminated with the dried spores of *Cl. sordellii* were used. Formalin was tested in three various concentrations, the concentration being serially increased at a 1.4 fold rate. The loops were removed at 10 minutes of intervals from the various concentrations of formalin as follows. 7 per cent : from the 140th to the 200th minute of exposure ; 9.8 per cent : from the 90th to the 180th minute of exposure ; 13.7 per cent : from the 30th to the 120th minute of exposure. The loops were treated subsequently as at the determination of the temperature coefficient. The results were evaluated statistically.

## Results

*Table I* shows the results of earlier experiments regarding the exposure times destroying *S. typhi* for 1 : 40 diluted formalin at temperatures of 20°, 25° and 35° C (±0.1° C) [1].

The data obtained at the same temperatures were very near to one another. The effective exposure times, however, markedly decreased with increasing the temperature. The data thus corresponded to the relationship indicated above.

**Table I**

*The activity of formalin diluted 1 : 140 against S. typhi at different temperatures*
Effective exposure times in minutes

| 20° | 25° | 30° | 35° |
|---|---|---|---|
| 60 | 50 | 25 | 15 |
| 60 | 50 | 25 | 15 |
| 60 | 50 | 25 | 15 |
| 60 | 50 | 25 | 15 |
| 60 | 40 | 25 | 15 |
| 60 | 40 | 20 | 15 |
| 60 | 40 | 20 | 15 |
| 60 | 40 | 20 | 15 |
| 60 | 35 | 20 | 15 |
| 50 | 35 | 20 | 15 |
| geometric means | | | |
| 59.2 | 41.7 | 22.4 | 15.0 |

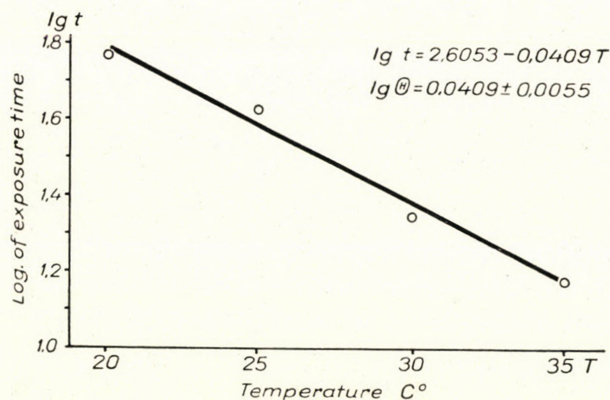


*Fig. 1.* Relationship between the effective exposure time of disinfection and the temperature. *S. typhi;* Formalin 1 : 140

*Fig. 1* shows the mean values obtained at various temperatures and the straight line of the regression equation, the slope of which is — lg $\Theta$. The regression was calculated by taking into consideration all data, and linearity was tested with the $F$ test, using the analysis of variance. The value of temperature coefficient $\Theta$ was 2.6 for 10° C. Of the disinfectants examined by the authors, the temperature coefficient of formalin stands closes to 1.

*Table II* shows the results of two experiments with the dried spores of *Cl. sordellii,* using 5 per cent formalin at 20°, 25° and 30° C. For destroying the spores, formalin had to be used in a higher concentration

**Table II**

*The activity of formalin diluted 1 : 20 against Cl. sordellii at different temperatures*

| Experiment 1 Effective exposure times in minutes | | | Experiment 2 Effective exposure times in minutes | | |
|---|---|---|---|---|---|
| 20° | 25° | 30° | 20° | 25° | 30° |
| 240 | 100 | 55 | 250 | 120 | 55 |
| 230 | 100 | 50 | 240 | 110 | 50 |
| 220 | 90 | 50 | 210 | 110 | 45 |
| 200 | 90 | 50 | 210 | 100 | 40 |
| 200 | 90 | 40 | 210 | 100 | 40 |
| 180 | 90 | 40 | 210 | 100 | 40 |
| 180 | 90 | 40 | 210 | 90 | 35 |
| 180 | 90 | 35 | 200 | 90 | 35 |
| 180 | 90 | 35 | 200 | 90 | 35 |
| 160 | 90 | 30 | 160 | 80 | 35 |
| geometric means | | | geometric means | | |
| 194.1 | 91.2 | 41.7 | 208.0 | 98.0 | 40.3 |

(1 : 20) than for killing *S. typhi*, and even with this increased concentration, considerably longer periods of exposure were required. By increasing the temperature, the effective exposure times were gradually decreasing. Although the decrease was comparatively higher than that with *S. typhi*, the nature of the relationship between temperature and exposure time was the same as that observed with the latter vegetative organism.

In *Fig. 2* the results of the two experiments are presented as mean values for the various temperatures and the corresponding straight lines of regression equations. The linearity of the regression could be statistically confirmed in both cases. The results of the two experiments showed some deviation ; the two values for $\lg \Theta$, however, were within the fiducial limits. The value of the temperature coefficient of formalin for spores was about twice of that for *S. typhi ;* recalculated for 10° C it was 4.6 in the first experiment and 5.2 in the second. Thus, for 10° C rise in temperature the effective exposure period decreased about 5 times, or, in other words, the effect of formalin increased 5 times. As it was shown, the activity of formalin against *S. typhi* increased at a considerably slower rate with the rise in temperature.

In the earlier experiments the $\lg t = \lg a - n \lg C$ formula could be confirmed for the vegetative test organism. The data for formalin of these earlier experiments are detailed in *Table III*.

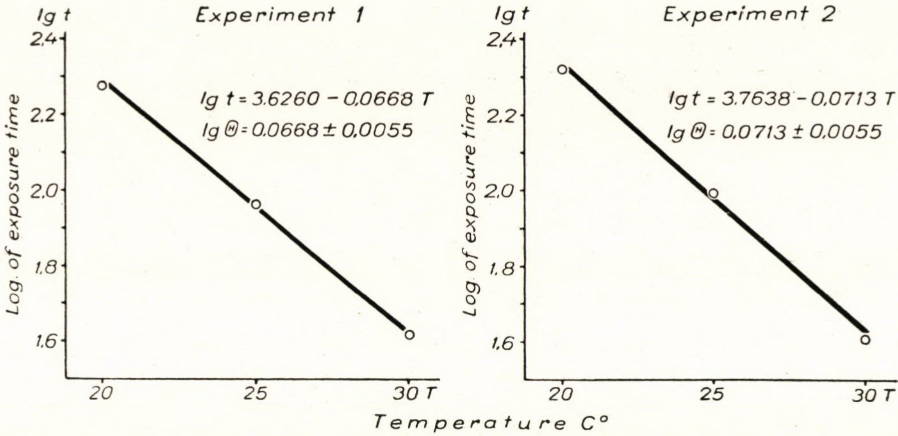


*Fig. 2.* **Relationship between the effective exposure time of disinfection and the temperature.**
***Cl. sordellii;*** **Formalin 1 : 20**

**Table III**

*The activity of various concentrations of formalin against S. typhi at 20° C*

**Effective exposure times in minutes**

| 1:216 | 1:144 | 1:96 |
|---|---|---|
| 75 | 35 | 22.5 |
| 60 | 30 | 20.0 |
| 55 | 30 | 20.0 |
| 55 | 30 | 20.0 |
| 45 | 30 | 20.0 |
| 45 | 25 | 17.5 |
| 45 | 25 | 17.5 |
| 45 | 25 | 17.5 |
| 45 | 25 | 17.5 |
| 40 | 20 | 15.0 |
| | geometric means | |
| 50.0 | 27.3 | 18.6 |

The effective exposure time decreased considerably with increasing the concentration of the disinfectant.

*Fig. 3* shows the results of these experiments. Using logarithms, statistically proved linear regressions were obtained, which approximated the experimental mean values. Of the disinfectants examined in that previous study, formalin exhibited the lowest concentration coefficient, in other words, by

changes in the concentration it was in the case of formalin that the course of disinfection was the least affected. This, naturally, is another advantageous property of formalin, in addition to its temperature coefficient approaching 1.

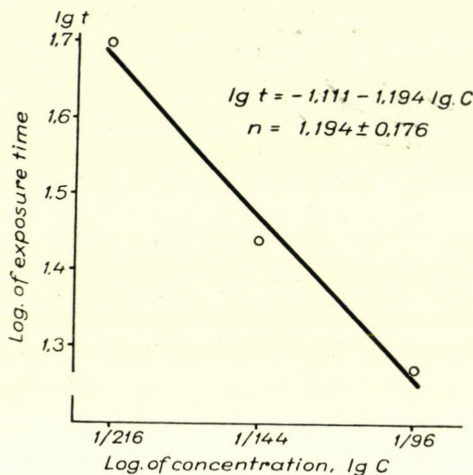


*Fig. 3.* Relationship between the effective exposure time of disinfection and the concentration of formalin. *S. typhi;* Temperature 20° C

The effective exposure times against *Cl. sordellii* obtained in two experiments with geometrically increasing concentrations of formalin are shown in *Table IV.*

The mean values presented in *Table IV* clearly show that the relation obtained is not conformable to the formula of CHICK and WATSON. If this formula were valid for dried spores, the effective exposure times would show a proportional decrease.

The linear regression between the concentrations and the logarithms of exposure times could not be proved statistically. *Fig. 4* shows the mean of the logarithms of the exposure times plotted against the logarithms of concentration. It is clear that the effective exposure times were more decreased at a higher concentration than at a lower one. No linear regression was obtained by analysing the united results of the two experiments. A linear regression was, however, obtained by calculating from the exposure times and from the logarithms of the concentrations. This calculation is presented in detail. At first, the averages and standard deviations are shown in *Table V* and *Fig. 5.*

From *Fig. 5* it can be concluded that no correlation exists between the mean of exposure times and their standard deviation and, also, that the standard deviations do not differ significantly from one another. Thus the analysis of variance may be performed.

**Table IV**

*The activity of various concentrations of formalin against Cl. sordellii at 20° C*

| Experiment 1 Effective exposure times in minutes | | | Experiment 2 Effective exposure times in minutes | | |
|---|---|---|---|---|---|
| 7.0% | 9.8% | 13.7% | 7.0% | 9.8% | 13.7% |
| 220 | 190 | 110 | 220 | 160 | 110 |
| 220 | 180 | 110 | 220 | 160 | 90 |
| 210 | 170 | 110 | 210 | 150 | 80 |
| 210 | 160 | 110 | 200 | 140 | 70 |
| 210 | 160 | 110 | 190 | 140 | 70 |
| 200 | 150 | 100 | 190 | 140 | 70 |
| 200 | 150 | 100 | 180 | 130 | 70 |
| 190 | 150 | 90 | 180 | 130 | 70 |
| 190 | 150 | 80 | 180 | 120 | 70 |
| 190 | 120 | 80 | 170 | 110 | 60 |
| geometric means | | | geometric means | | |
| 203.2 | 157.4 | 98.9 | 193.6 | 137.1 | 75.3 |

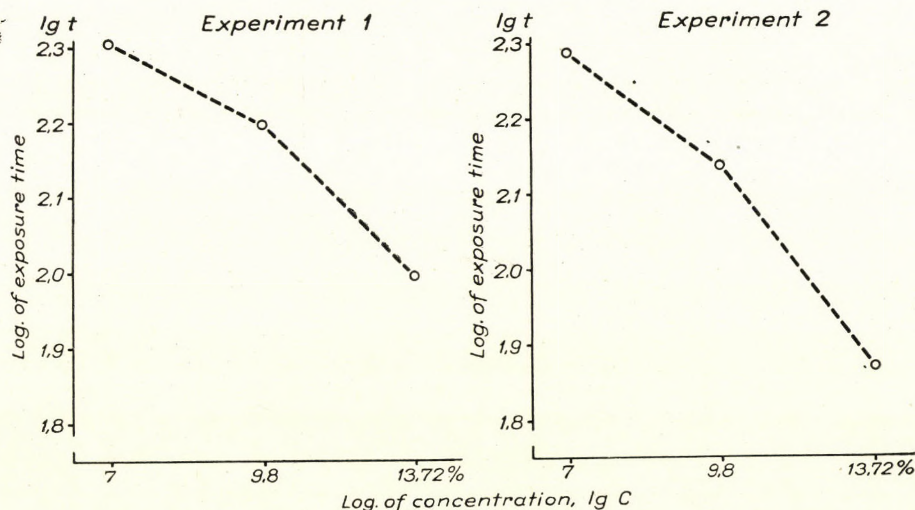


*Fig. 4.* Relationship between the effective exposure time of disinfection and the concentration of formalin. *Cl. sordellii;* Temperature 20° C

According to the analysis of variance, as shown in *Table VI,* the assumption of a linear regression would be justified. Concerning the logarithms of

**Table V**

*The activity of various concentrations of formalin against Cl. sordellii at 20° C*

Average exposure times and their standard deviation for the various concentrations

| Concentration | | Experiment 1 | | Experiment 2 | |
|---|---|---|---|---|---|
| % | lg | average exposure time in minutes | standard deviation in minutes | average exposure time in minutes | standard deviation in minutes |
| 7.0 | 0.84 | 204 | 11.7 | 194 | 17.8 |
| 9.8 | 0.99 | 158 | 19.3 | 138 | 16.2 |
| 13.7 | 1.14 | 100 | 12.5 | 76 | 14.3 |
| | $S_{xx} = 0.450$ | $S_{xy} = 156.0$ | | $S_{xy} = 177.0$ | |

**Table VI**

*Analysis of variance for the data of Table IV*

Experiment 1

| Nature of variation | d. f. | Sum of square | Mean square |
|---|---|---|---|
| Regression | 1 | 54 080 | |
| Deviations from regression | 1 | 240 | 240 |
| Between log-concentrations | 2 | 54 320 | |
| Within log-concentrations | 27 | 6 000 | 222.2 |
| Total | 29 | 60 320 | |

$F = 1.08$
$F_{0\cdot2}(1;27) = 1.73$

Experiment 2

| Nature of variation | d. f. | Sum of square | Mean square |
|---|---|---|---|
| Regression | 1 | 69 620 | |
| Deviations from regression | 1 | 60 | 60 |
| Between log-concentrations | 2 | 69 680 | |
| Within log-concentrations | 27 | 7 040 | 260.7 |
| Total | 29 | 76 720 | |

$F = 4.35$
$F_{0\cdot2}(27;1) = 15.2$

the exposure times, significant $F$ values were obtained in both experiments, showing that the regression was not a linear one.

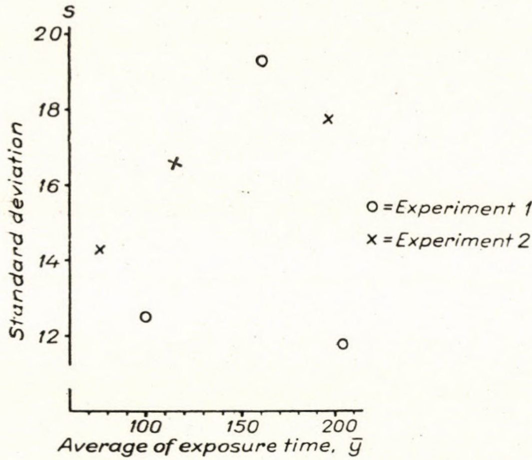


*Fig. 5.* Standard deviations for the average of exposure times

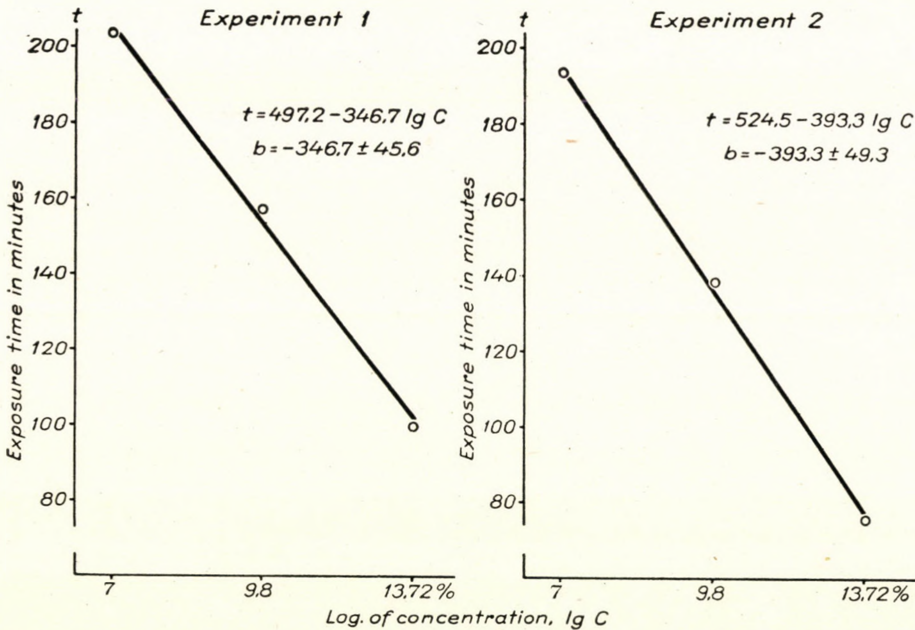


*Fig. 6.* Relationship between the effective exposure time of disinfection and the concentration of formalin. *Cl. sordellii;* Temperature 20° C

Calculating from the exposure times and from the log-concentrations, log-linear regressions were obtained as seen in *Fig. 6*, which shows the equation of linear regression and the fiducial limits of the regression coefficients.

## Discussion

The results of the present experiments confirmed Chick *and* Watson's relation for the influence of temperature on the sporocidal activity of formalin. The temperature coefficient of formalin was shown to be somewhat higher for spores of *Cl. sordellii* than for *S. typhi.*

A surprising result was obtained as to the action of various concentrations of formalin against *Cl. sordellii,* namely that the well-known and verified relation concerning the disinfection of vegetative forms of organisms was not valid for dried spores. As for the latter, instead of Chick *and* Watson's relation, a simple log-linear relation was found to be valid. This corresponds to the Weber—Fechner law, a principle well-known in biology, according to which to increase the effect in arithmetical progression, the stimulus has to be increased in geometrical progression. It should be stressed that it would be early to draw any final conclusions from the present investigations. For completely elucidating the question, the exposure times should be examined for a wider variation of concentrations.

## Summary

The relationship between the temperature of formalin on the one hand, and its concentration, bactericidal, and sporocidal activity on the other, has been investigated and compared.

1. The sporocidal activity of formalin has been found to change with variations in the temperature according to a relation generally valid for all vegetative organisms. The value of the temperature coefficient of formalin for spores of *Cl. sordellii* was twice that found for *S. typhi.* Accordingly, the sporocidal activity of formalin is affected by the temperature more than its bactericidal activity.

2. In examining the effect of various concentrations of formalin, the Chick—Watson relation has not been found valid for dried spores. Instead of this, a simple log-linear regression has been observed. This means that on increasing the concentration in geometrical progression, the exposure time will be decreasing arithmetically.

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# A WATER-BORNE OUTBREAK OF ENTERITIS ASSOCIATED WITH ESCHERICHIA COLI SEROTYPE 124 : 72 : 32

By

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(Received December 10, 1958)

In 1944 and 1945, some *E. coli* strains showing antigenic relationship to *Sh. dysenteriae 3* were isolated in a U. S. Army laboratory in Italy by EWING from cases of enteritis and from food handlers [1]. Three of the strains were studied by WHEELER and STUART who found that two of them had *O* antigens identical with the *O* antigen of strain *Large-Sachs* Q 771 (now designated as *Sh. dysenteriae 3*). Biochemically, these cultures were slow-lactose-fermenting typical *E. coli* strains and both contained a „Vi-like" antigen [2].

In 1949, HOBBS, THOMAS and TAYLOR described a school-outbreak of food poisoning caused by an organism called "paracolon 411" [3]. The midday meal of the children included cold salmon. About 14—24 hours after eating the midday meal, the children complained of headache and malaise followed by diarrhoea and vomiting ; in some cases pyrexia to 40° C was present, and persisted for a day or two. Recovery followed generally after 48 hours of illness. From the faeces of the patients slow-lactose-fermenting typical *E. coli* strains were isolated on desoxycholate-citrate agar. The isolated strains were serologically identical. The organism was agglutinated at titres of 1:40—1:80 by six of the sera from seven patients ; the same titres were given, however, by 9 out of 12 control sera. The pathogenicity of "paracolon 411" was proved by human volunteer experiments and by an accidental laboratory infection. One of the authors (J. TAYLOR) isolated similar strains from an outbreak of food poisoning in a prisoner-of-war camp in Leicestershire and from two sporadic cases.

According to EWING, the strains isolated by himself in Italy, some other cultures collected from England, Brasil and the United States and also the strains of HOBBS *et al.* belonged to *E. coli O* group 124 [1].

In 1957, KÉTYI, KNEFFEL *and* DOMJÁN in Hungary reported a water-borne epidemic of enteritis caused by an organism belonging to *E. coli O* group 124 [4]. The patients developed a mild enteritis with pyrexia up to 38.2° C. Serological examinations showed that the O antigen of the standard *E. coli* O : 124 strain, of *Sh. dysenteriae 3* and of a strain from the outbreak

were identical. In examining sera from the patients, the causative organism was agglutinated at a titre of 1:10 by three sera, at titres of 1:20—1:40 by two sera and only one of the sera showed a titre of 1:160.

In 1958, RÉDEY and CSIZMAZIA isolated a number of *E. coli* O group 124 strains from a mass outbreak of water-borne enteritis in Veszprém, Hungary [5].

EWING (1956) reported that all *E. coli* O:124 strains so far examined contained K antigen 72 in addition to the O antigen. Antigen K:72 belongs to the B antigens and, according to an earlier designation, it corresponds to B:17. Strains belonging to group O:124, K:72 can be subdivided by H antigens into three serotypes : H:19, H:30, H:32 and into non-flagellar variants [6].

## Materials and methods

The *E. coli* O : 124 strains examined in the present study were isolated on desoxycholate-citrate agar in the *Public Health Laboratory of Eger.* Other bacteriological examinations were performed in the *Department of Bacteriology, State Institute of Hygiene.*

Biochemical reactions were carried out as described in the International Bulletin of Bacteriological Nomenclature and Taxonomy [7], the serological examinations were made according to KAUFFMANN [8]. In the antigenic analysis, EWING's strain 227 was used, which had been kindly supplied by DR. I. KÉTYI, *Institute of Microbiology, Medical University of Pécs.* The agglutinin titre of the sera from patients was determined by haemagglutination test. Sheep erythrocytes were treated with the supernatant of the boiled suspension of *E. coli* O : 124 strains 227 and 916, using NETER's method [9]. Erythrocytes treated with the two different strains showed the same titre to all the sera tested. In *E. coli* O : 124 serum and in O : 124, K : 72 serum they were agglutinated at titres of 1 : 1024 and 1 : 4096, respectively. As no pure K : 72 serum could be produced, it seemed questionable whether the erythrocytes were sensitive to K agglutinins. Experience, however, has shown that, in the procedure used, K antigens are generally adsorbed by red blood cells. The sera from patients were inactivated at 56° C for 30 minutes. Dilution of sera and determination of haemagglutination titres were performed by TAKÁTSY's spiral-loop method [10]. Sera containing agglutinins for the erythrocytes themselves were absorbed by normal sheep erythrocytes. Antibiotic sensitivity testing was carried out using "Biotest" paper discs, manufactured by the *Institute for Serobacterial Production and Research "Humán".*

## Record of the outbreak

Most of the infections occurred in a holiday-camp for children, in Parádsasvár, county Heves. From July 3 to July 14, 1958, 108 out of 200 children were affected (54.0 per cent). Between July 5 and July 17, 36 out of 62 members of the camp-staff were reported ill. The first group of children left the camp on July 14, and two days later a new group of 200 children arrived. Of the second group, 53 (26.5 per cent) children were affected. During this period 50 cases occurred in the village Parádsasvár (443 inhabitants) and 8 cases were reported from the neighbouring villages. Thus the total number of reported cases was 255.

The infections occurring in the two groups of children allowed a good estimation of the incubation period of the disease. As shown by *Fig. 1,*

3 to 4 days elapsed from the arrival of the second group of children until the onset of symptoms. The symptoms persisted for 3 to 4 days in both groups. The patients complained of nausea, lack of appetite and diarrhoea. The temperature was slightly above normal in most cases ; some of the patients, however, had a temperature of 39—40° C. Most patients had dyspeptic, putrescent watery stools. Dysenteric, mucous stools and rectal tenesm also occurred in some patients, together with numerous abortive cases. From the

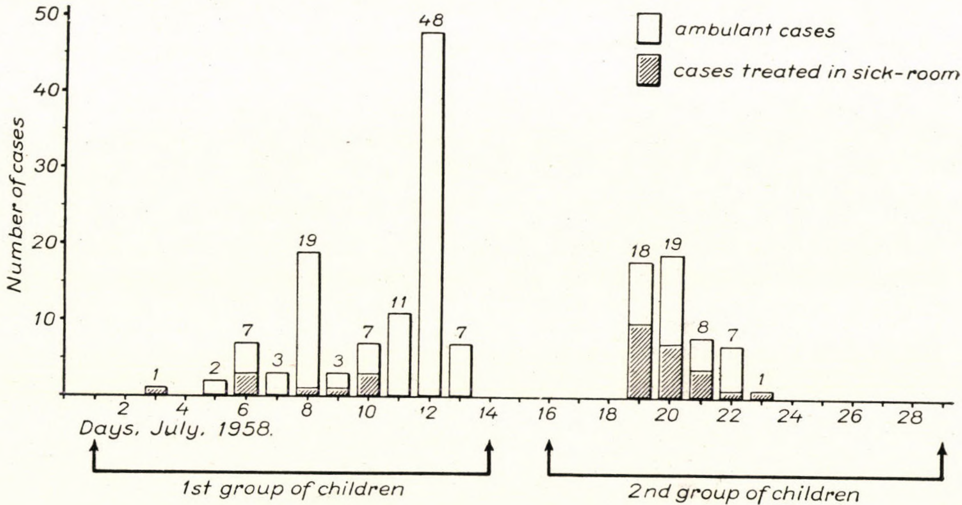


*Fig. 1.* Incidence of enteritis among children

first group only a few, from the second group nearly half of the affected children were treated in the sick-room. Thus, as a whole, the outbreak can be regarded as a relatively mild one.

The source of infection was traced by Dr. L. Kubinyi, *Department of Water Hygiene, State Institute of Hygiene.* The sewage conduit from the camp was defective at a point 5 meters from the well of a mineral spring. Thus sewage penetrated through the soil into the mineral water. The causative agent was isolated from the contents of a breakpressure-reservoir situated near the point of the defect. The sulphurous-carbonic water of the mineral spring was usually consumed by the staff of the camp and by inhabitants of the village. No further cases occurred among the children after they had been forbidden to drink the polluted mineral water. Similarly, after closing the well and ceasing to bottle the water, no further cases were reported from the village. The carrier causing the first pollution of the well with *E. coli* O:124 could not be detected.

The mild infection was treated by prescribing saline purgatives, activated charcoal and sulfonamides. This treatment was effective although neither of the strains were *in vitro* sensitive to sulfonamides.

## Bacteriology

From 255 cases, 107 samples of faeces were examined. *E. coli* O:124 strains were isolated from 19 patients ; as from some cases the organism was isolated on repeated occasions, altogether 24 strains were available for study. On desoxycholate-citrate agar the organism produced fairly well growing, non-lactose-fermenting, shigellaform colonies. On Russel agar it grew with red butt and slight gas production. The biochemical reactions given by these strains were like those given by slow-lactose-fermenting typical *E. coli* strains.

*Table I* presents a comparison of the biochemical behaviour of *E. coli* O group 124 strains described by different workers. It is seen that in this respect there is no important difference between the strains from various sources.

The living cultures of the 24 isolated strains were agglutinated by serum O:124, K:72 prepared with the living culture of EWING's strain 227. The living cultures of the strains under investigation were inagglutinable or slightly agglutinable in dilutions 1:10 or 1:20 of the pure O serum prepared with strain 227 heated for 2 ½ hours at 100° C. Heating the suspensions of the strains for 1 hour at 100° C rendered them agglutinable to the end titre in pure O serum.

**Table II**

*Analysis of* O *and* K *antigens of strain* 916

| Antigens | OK serum, strain 227 | | OK serum, strain 916 | | O serum strain 227, unabsorbed |
|---|---|---|---|---|---|
| | Unabsorbed | Absorbed by strain 916 (living) | Unabsorbed | Absorbed by strain 227 (living) | |
| 227 living | 1 280 | 0 | 640 | 0 | 0* |
| 916 living | 640 | 0 | 640 | 0 | 0* |
| 227 1 hr. 100° C | 10 240 | 0 | 10 240 | 0 | 10 240 |
| 916 1 hr. 100° C | 10 240 | 0 | 10 240 | 0 | 10 240 |

* Slight agglutination at dilutions 1 : 20 and 1 : 40.

Cross absorption tests were carried out with strain 916, which had been isolated from a patient in the course of the present outbreak. *Table II* shows the cross absorption test for strains 227 and 916. The complete antigenic structure of strain 227 was O:124, K:72, H:32. For cross absorption, two types

**Table I**

*Biochemical behaviour of E. coli* O : 124 *strains according to various authors*

| Authors | Number of strains | Glucose | Adonitol | Arabinose | Dulcitol | Inositol | Lactose | Maltose | Mannitol | Rhamnose | Raffinose | Sucrose | Salicin | Sorbitol | Xylose | $H_2S$ | Gelatin | Indole | Urea | Methyl red | Voges-Proskauer | KCN | Amm. citrate | Malonate | Phenylalanine |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| WHEELER and STUART | 2 | ++ | — | + | + | . | $+^{2-28}$ | $+^{2-28}$ | + | + | . | — | — | $+^{2-28}$ | + | . | . | + | — | . | — | . | — | . | . |
| HOBBS *et al.* | 14 | ++ | — | + | $+^{1-13}$ | — | $+^{4-21}$ | + | + | — | . | — | — | + | + | — | — | + | — | . | . | . | — | . | . |
| KÉTYI *et al.* | 7 | ++ | — | + | $+^{1-9}$ | — | $+^{2}$ | + | + | + | — | — | — | + | + | — | — | + | — | + | — | — | — | . | . |
| RÉDEY and CSIZMAZIA | 50 | ++ | — | + | $+^{3-5}$ | — | $+^{5-11}$ | + | + | × | . | — | — | + | + | — | — | + | — | + | — | . | — | . | . |
| Present | 24 | ++ | — | + | + | — | $+^{3-7}$ | + | + | × | × | — | — | + | + | — | — | + | — | + | — | — | — | — | — |

++ acid and gas within 24 hrs.
\+ fermentation within 24 hrs. or positive reaction
$+^{3-7}$ fermentation after 3—7 days
× late and weak reaction
— negative

of antigen suspensions were prepared from both strains : living and heated for 1 hour at 100° C. OK sera were prepared with living suspensions of strains 227 and 916. A pure O serum was prepared with strain 227 heated for 2½ hours at 100° C. According to *Table II*, both antigens were agglutinated practically to the same titre in both sera for the living strains. Cross absorption of these sera with the living strains caused a total exhaustion of agglutinins. Due to the inhibitory action of K antigens, the living cultures were practically inagglutinable in pure O serum. The suspensions became O agglutinable after heating at 100° C for 1 hour.

H antigen suspensions of the 24 strains from the outbreak uniformly showed agglutination to the end titre in an absorbed H serum for strain 227. For preparing H antigen suspensions, it was necessary to make subcultures in semisolid agar. Consequently, the antigenic formula of the strains from the outbreak can be characterized according to the *E. coli* antigenic schema as 124:72:32, where the number before the first colon means the O antigen, number 72 shows the K antigen (designated earlier as B:17) and, finally, number 32 refers to the H antigen.

The strains from the outbreak were uniformly sensitive to chlortetracycline, oxytetracycline, chloramphenicol, streptomycin, neomycin and polymyxin B, moderately resistant to erythromycin and resistant to penicillin and to sulfonamides.

**Table III**

*Titres of sera from various sources against E. coli antigens* O : 124 *and* K : 72 *adsorbed on red blood cells*

| Group of sera | Total number of sera | Number of sera giving end titres of | | | | | Mean titre* |
|---|---|---|---|---|---|---|---|
| | | 4 | 8 | 16 | 32 | 64 | |
| Patients from the outbreak | 25 | 2 | 8 | 7 | 4 | 4 | 15.8 |
| Other patients (Widal sera) | 35 | 3 | 7 | 11 | 7 | 7 | 18.7 |
| Healthy individuals (Wassermann sera) | 67 | 15 | 21 | 16 | 10 | 5 | 11.6 |

* mean titre $= \sqrt[N]{d_1^{n_1} \, d_2^{n_2} \ldots d_n^{n_n}}$
$d_1, d_2 \ldots d_n$ = reciprocals of serum dilutions
$n_1, n_2 \ldots n_n$ = number of sera giving the corresponding titre
$N$ = total number of sera

*Table III* presents the agglutinin titres of sera from patients against *E. coli* O:124 and K:72 antigens adsorbed on red blood cells. As a control,

sera from other patients (Widal sera) and sera from normal persons (Wassermann sera) were tested. Sera agglutinating normal sheep erythrocytes were often met with : 5 out of 25 sera from the outbreak, 19 out of 54 Widal sera and 41 out of 108 Wassermann sera behaved in this manner. Such sera from the outbreak absorbed by normal erythrocytes gave specific agglutination. Control sera with agglutinins for normal red blood cells have not been included in the Table. *Table III* shows in each examination group the number of sera giving various end titres. Considering the mean of the titres, it is clear that there was no significant difference between the sera of the three groups.

## Discussion

The epidemic described in the present paper was within a short period the third water-borne outbreak in Hungary, whose seriously suspected causative agent has been identified as an organism belonging to *E. coli* O group 124. The pathogenic role of *E. coli* O:124 bacteria seems now to be confirmed by different observers, as in the outbreaks the faeces from the patients contain the organism in large numbers but no other known pathogenic bacteria. The possible pathogenicity of *E. coli* O:124 has been shown also by our recent isolations of this organism from cases of sporadic enteritis.

In the present investigations, only 24 out of 107 faeces were positive for *E. coli* O:124 ; this may have been due to the fact that in the first days of the outbreak the *Public Health Laboratory of Eger* had no specific antiserum and had observed the causative agent only by its cultural and biochemical properties.

The agglutinin response in patients suffering from *E. coli* O:124 enteritis is poor, similarly to that in infants infected with pathogenic *E. coli* strains. Considering the short course and the localization of the infection, the poor agglutinin response is not surprising.

The observations of Hobbs *et al.*, of Kétyi *et al.* and of our own have shed some light on the clinical course of the disease due to *E. coli* O:124. The difference found between the incubation periods may be explained by a variation in the infective doses.

It has to be pointed out that *E. coli* O:124, unlike *E. coli* serotypes associated with epidemic infantile enteritis, was uniformly pathogenic to infants, children and adults. The clinical course of this type of enteritis and and the ability of the causative agent to produce mass infections makes it necessary to subject the reconvalescent carriers to the same preventive measures as are usual for the control of dysentery and salmonellosis.

## Summary

A water-borne outbreak affecting 255 persons has been described. According to the antigenic analysis, the causative agent isolated from the faeces of the patients corresponded to EWING's strain 227. Within a short period the present epidemic was the third water-borne outbreak of enteritis in Hungary associated with *E. coli O* : 124. The nature of the infection caused by this organism makes it necessary to subject the convalescent carriers to the same preventive measures as are usual for the control of dysentery and salmonellosis.

*Acknowledgement.* The authors wish to thank DR. G. SERESS, medical officer of health, for collecting the epidemiological data and providing the diagram.

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# CYTOMORPHOLOGICAL CHANGES IN HUMAN EMBRYONIC KIDNEY TISSUE CULTURES INFECTED WITH ADENOVIRUS TYPE 5 STRAINS

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(Received December 22, 1958)

Since the initial work of Rowe *et al.* [1] published in 1953, several authors have investigated the degenerative changes induced by different types of adenovirus in various tissue cultures. The cytopathic changes in individual cells were also studied.

Rowe *et al.* [1] studied the cytopathogenic effect of adenoviruses in human embryonic tracheal cells, whereas subsequent investigators used mainly HeLa [2, 3, 4] and monkey kidney cell cultures [4, 5]. The effect of adenoviruses on Detroit 6 [6], human embryonic lung [2] and rabbit kidney cultures [7] has been also studied. Type and history of the strains in these studies were also different. Accordingly, more or less different changes as caused by adenoviruses have been described. Nevertheless, irrespective of strain and host, the primary effects and the most prominent changes were always localized in the cell's nucleus.

This report describes the alterations in human embryonic kidney cells infected with adenovirus type 5.

## Materials and methods

*Virus strains.* Four strains of type 5 adenovirus were used in the experiments. One of them was kindly supplied by Dr. U. Krech *(Switzerland)*, another by Dr. I. Béládi *(Szeged)*. Two strains were recovered by us from tonsils [8]. The seed virus was produced in human amniotic cultures and titrated in human embryonic kidney cell cultures. A single inoculum in the main experiments contained $10^4$ $TCID_{50}$ of virus.

*Tissue cultures.* The human amniotic cell cultures were prepared as described earlier [9, 10], but in several cases a Hungarian preparation of elastase was used instead of trypsin, as recommended by Füzi *et al.* [11]. In preparing nutrient fluids, a 5 per cent stock solution of lactalbumine hydrolysate (LAH) dissolved in Hanks' solution was used. The growth medium for human amniotic cells consisted of 20 per cent human serum, 10 per cent LAH stock solution and 70 per cent Hanks' solution.

The kidney cell cultures were prepared by trypsinization from the kidneys of 3—5 month old human embryos. The growth medium contained 20 per cent human serum, 8 per cent LAH stock solution and 72 per cent Hanks' solution. The cell suspension was distributed in tubes, 1 ml in each. A $18 \times 5$ mm coverslip was placed in each tube to obtain monolayer cultures suitable for staining and microscopical examination. The maintenance solution for both types of culture consisted of 5 per cent rabbit serum, 5 per cent LAH stock solution and 90 per cent Hanks' solution. The cultures were incubated at 37° C.

*Method of following the cellular changes.* Once in every 8—12 hours until the 120th hour after infection the coverslip preparations were removed from a few culture tubes were always examined in parallel. The coverslip cultures were rinsed several times with saline and, after 5 to 8 minutes' fixation in methanol, stained with Giemsa's solution. The working dilution of the stain was prepared by making up 2 drops of the stock solution to 1 ml with distilled water. The latter was boiled and cooled before use. The exact length of the staining period, varying usually between 40 and 80 minutes, was established in each case by controlling the preparations under the microscope while staining. Preparations removed 80 hours or more after infection were stained with dilute Giemsa's solution.

## Results

The intranuclear changes in human embryonic kidney cells following infection with $10^4$ $TCID_{50}$ of adenovirus type 5 were variable. The presence or absence of some kinds of changes seemed to be dependent upon the length of the interval between infection and examination, suggesting a sequence of these changes progressing with time. In the case of other intranuclear alterations, we could not observe any progress with time. For convenience of description, the sequence of changes, as thought to be progressing with time, has been divided into 5 stages. It is to be emphasized that the onset of the same type of alteration in the individual cells was not synchronous. A variety of altered cell types were present in most of the infected cultures studied, and several intact cells were still present on the 5th day after infection. In the following, we characterize the five stages of degeneration by the changes most frequently observed in the given periods.

*Stage I.* From 16 to 30 hours after infection an intranuclear eosinophilic granulation, the most predominant early change, became apparent and made the altered cells easily distinguishable from the control cells *(Fig. 1).* Three different patterns of eosinophilic granulation were observed.

(a) Diffuse intranuclear granulation was the most frequent feature *(Fig. 2).* Rarefied or clear zones beneath the nuclear membrane and round the nucleoli were often observed. The nucleoli could easily be distinguished from the rest of the nuclear substance.

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*Plate 1 and 2.* The photographs represent human embryonic kidney tissue cultures, particularly the cells' nuclei, stained with Giemsa's solution. Infection with adenovirus type 5. Magnification, about 1600 ×.

*Fig. 1.* Normal nucleus. Nucleoli are clearly visible.

*Fig. 2.* Nucleus 38 hours after infection. Eosinophilic diffuse granulation. Note rarefied zones round the nucleoli.

*Fig. 3.* Intranuclear round aggregates of eosinophilic granules, 38 hours after infection. Nucleoli (N) are distinguishable.

*Fig. 4.* Round eosinophilic aggregates with circular structure, 60 hours after infection. Nucleoli (N) are distinguishable.

*Fig. 5.* Bundle-like eosinophilic granulation, 44 hours after infection. Nucleoli can be distinguished.

*Fig. 6.* Eosinophilic (E) and basophilic (B) aggregates at the centre of the nucleus.

*Fig. 7.* Eosinophilic central cluster with granular structure, 60 hours after infection. Distinguishable nucleoli (N).

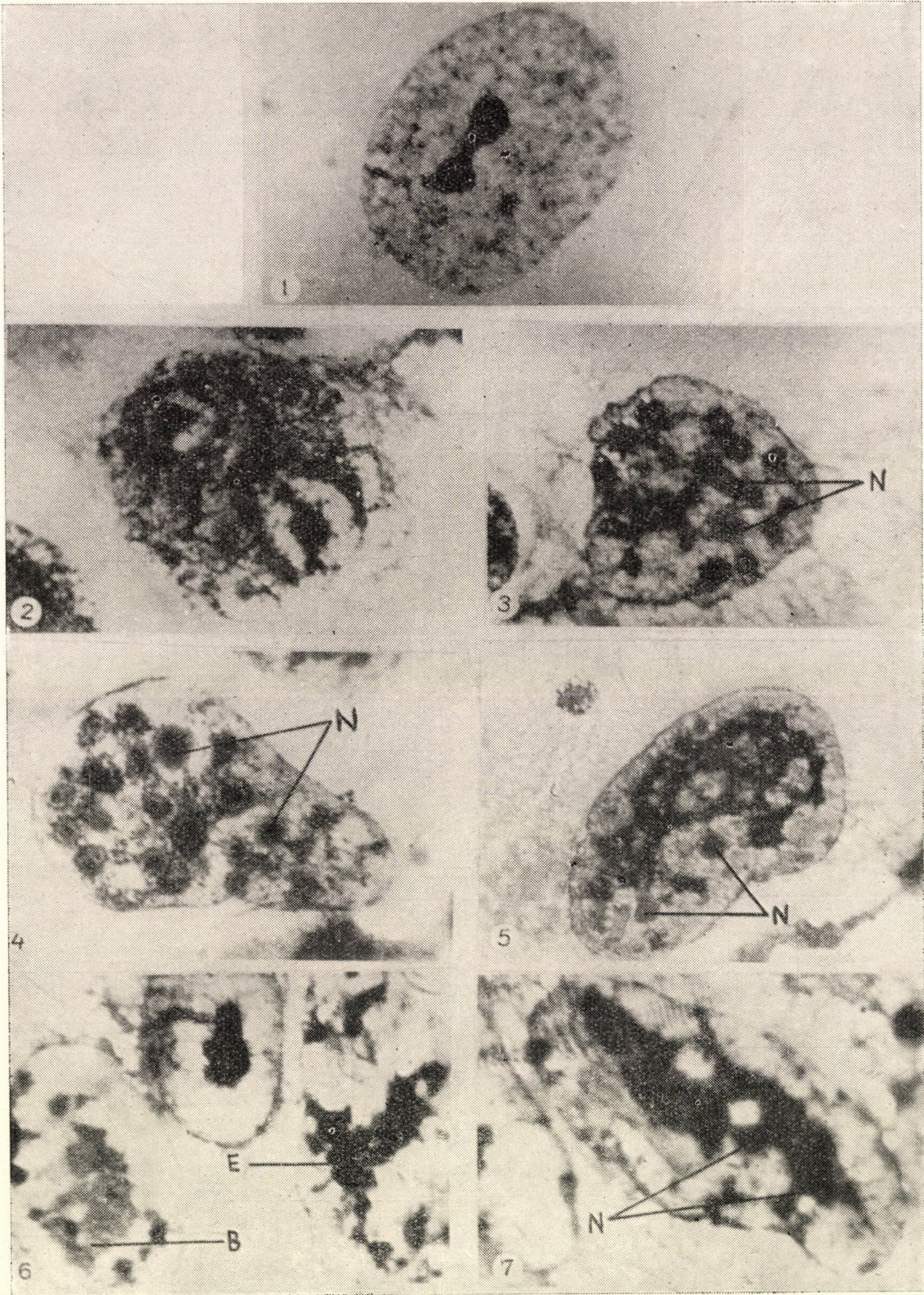



*Plate 1*

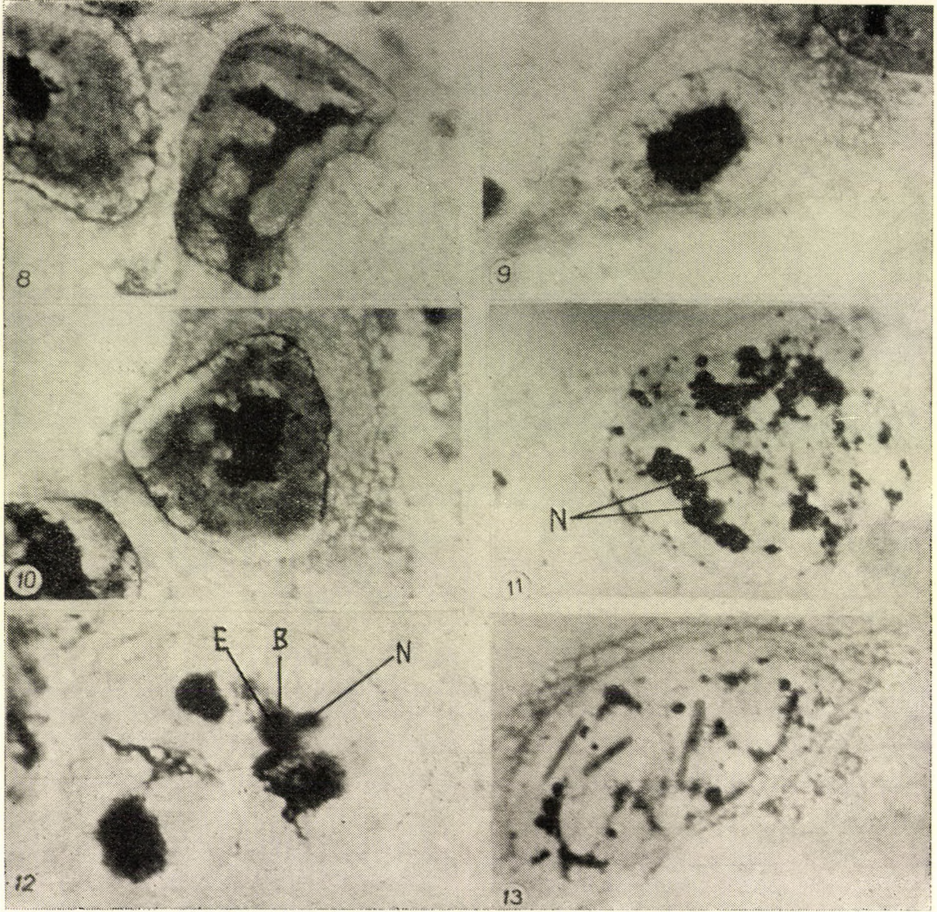



*Plate 2*

*Fig. 8.* Eosinophilic central cluster in which granular structure is not visible. Nucleolus is hardly distinguishable. 60 hours after infection.

*Fig. 9.* Cell 60 hours after infection. Cytoplasm, nucleus and intranuclear eosinophilic central mass are clearly visible. Nucleolus is not visible.

*Fig. 10.* Basophilic central mass. The rest of the nucleus appears glassy. Rarefied zone beneath the nuclear membrane. Nucleolus is not visible. 60 hours after infection.

*Fig. 11.* Dark-staining, homogeneous eosinophilic bodies larger than the granules in the above Figs. Persisting nucleoli (N). 38 hours after infection.

*Fig. 12.* Intranuclear eosinophilic bodies (E) larger than those in Fig. 11, surrounded by a basophilic ring. Nucleolus (N) can be recognized. 60 hours after infection.

*Fig. 13.* Rod-shaped crystal-like intranuclear bodies, 60 hours after infection.

(b) In some of the cells the eosinophilic granules formed round or oval aggregates. The rest of the nucleus was either rarefied or diffusely granulated *(Fig. 3)*.

(c) Small groups of eosinophilic granules were often surrounded by a clear zone and a granular outer ring *(Fig. 4)*.

*Stage II.* From 30 to 45 hours after infection the appearance of clusters probably deriving from the diffuse granulation of Stage I was the most predominant feature. The granules in the clusters were still well visible *(Fig. 5)*. Most of the clusters were eosinophilic, only few were observed to turn basophilic *(Fig. 6)*. The nucleoli were well-differentiable.

*Stage III.* From 40 to 50 hours after infection the granules were gathering in the centre of the nucleus to form there oblong clusters or bundles. In some of the nuclei the clusters were still granulated and the nucleus could be distinguished *(Fig. 7)*, in others neither granular structure nor distinct nucleoli were observable *(Fig. 8)*. Eosinophilic staining was still predominant.

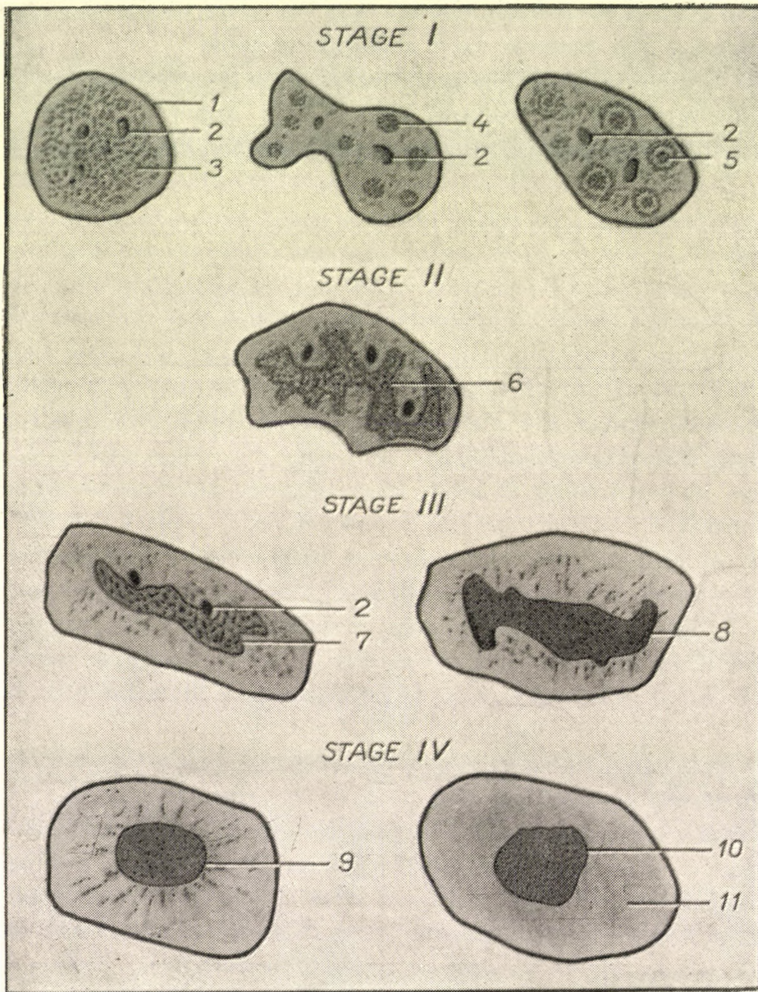
*Stage IV.* From 45 to 60 hours after infection the majority of the cells was already affected. The nucleoli could not be distinguished. The nuclear substance formed a single round central mass, which was more often intensely eosinophilic than basophilic. The intranuclear background was almost clear or some faint structure of compartments radiating out from central mass was observable. Later the background became pink and glassy and the central mass basophilic *(Fig. 10)*.

*Stage V.* From 60 to 75 hours after infection no granulation could any more be observed and a decrease in the size of the cytoplasm became obvious. (In the cytoplasm only vacuola and some heterogeneity had previously been visible). The whole cell began to shrink and basophilic staining became marked. Finally, the intensely basophilic cells rounded up and lost their structure. From that time on, more and more cells detached from the coverslip and, on the 5th day after infection, only few cells, most of them rounded, basophilic and structureless, were seen.

The first 4 stages of the hypothetical sequence of changes are represented diagrammatically in *Text-Fig. 1.*

In some preparations we observed certain other changes which could not be inserted into the hypothetical sequence of changes as outlined above. In a few of the preparations removed after the 30th hour, besides the predominant occurrence of the granular type of degeneration, nuclei containing 20 to 30, apparently structureless, strongly eosinophilic bodies, were also observed *(Fig. 11)*. Other nuclei, after the 50th hour, contained 2 or 3 larger eosinophilic bodies, each surrounded by a basophilic ring *(Fig. 12)*. Finally, about the 60th hour after infection, we observed nuclei containing 2 or 3 rod-shaped bodies which often seemed crystalline in outline. In addition, the same nuclei showed one of the more frequent types of degeneration *(Fig. 13)*.

Nearly all the affected cells appeared to be enlarged and many of them also deformed. Comparative data on the size of normal and virus-infected nuclei are presented in *Table I*. Since the figures represent only one dimension, *viz.* the longest diameters of the nuclei, the difference, as shown in *Table I*, reflects the increase in nuclear size only approximately.




*Text-fig. 1.* Diagrammatic representation of human embryonic kidney cell nuclei infected with adenovirus type 5. 1 = nuclear membrane, 2 = nucleolus, 3 = eosinophilic diffuse granulation, 4 = aggregate of eosinophilic granules, 5 = eosinophilic aggregate with circular structure, 6 = eosinophilic granular cluster, 7 = central cluster with eosinophilic granulation, 8 = eosinophilic structureless central cluster, 9 = eosinophilic central mass, 10 = basophilic central mass, 11 = glassy background.

**Table I**

*Frequency distribution in per cent of the longer diameters of control nuclei and those infected with adenovirus type 5*

| Nuclei | Diameter in $\mu$ | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 7—8 | 9—10 | 11—12 | 13—14 | 15—16 | 17—18 | 19—20 | 21—22 | 23—24 | 25—26 | 27—28 | 29—30 |
| Control | 5* | 15 | 16 | 17 | 18 | 16 | 8 | 4 | 1 | — | — | — |
| Infected | — | 1 | 2 | 10 | 17 | 18 | 22 | 11 | 8 | 7 | 2 | 2 |

* = Per cent of diameters measured.

It is seen that about 80 per cent of the nuclear diameters fell between 9 and 18 $\mu$ in the group of the normal cells. The same proportion of the virus-infected cells showing degenerative changes measured between 13 and 22 $\mu$ in diameter.

## Discussion

It is difficult to establish which of the above-described degenerative changes were connected with the viral synthesis and which were merely manifestations of non-specific cell damage and abnormal metabolite activities of the virus-infected cell. The view that the nuclear granules are in relation with the viral synthesis, is supported by the observation that the eosinophilic granulation became later basophilic. According to BOYER *et al.* [3], this sequence suggests the initial laying down of a basic protein, with the late addition of acid components, of which DNA appears to constitute an important part. The accumulation of DNA, on the other hand, may be a manifestation of the intranuclear viral synthesis.

Findings by electron microscopy also support this view [12, 13, 14]. In cells infected with adenovirus a great number of intranuclear virus-like bodies showing crystal-like arrangement were found.

## Summary

The changes occurring in human embryonic kidney tissue cultures after infection with type 5 adenovirus have been investigated. The early changes were localized in the nucleus. The first intranuclear change was an eosinophilic granulation, either diffuse or forming aggregates. The latter were often surrounded by a clear zone and a granulated outer ring. The nuclei of the affected cells were enlarged. Through several intermediate stages the intranuclear eosinophilic granulation progressed to a basophilic central mass. Finally, the cytoplasm decreased in size and the shrunken, rounded cells detached from the glass. Less frequently the pattern of nuclear degeneration was different. Some nuclei contained 20 to 30 minute, while others 2 or 3 larger, eosinophilic bodies each surrounded by a basophilic ring, or 2 or 3 rod-shaped, crystal-like inclusion bodies.

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A kiadásért felel az Akadémiai Kiadó igazgatója Műszaki felelős : Farkas Sándor
A kézirat nyomdába érkezett: 1959. III. 18. — Terjedelem: 8 (A/5) ív, 45 ábra

Akadémiai Nyomda Budapest, V. Gerlóczy u. — 48460/59 — Felelős vezető: Bernát György

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# STUDIES ON THE IMMUNE EFFECT OF SHIGELLA AND TETANUS ANTIGENS COMBINED WITH "BOOSTER" ANTIGENS

By

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(Received October 2, 1958)

The immune effect of simultaneously administered antigens has been discussed in the literature. Some authors write about an adjuvant, others about an antagonistic action. Barr *and* Llewellyn-Jones [1, 2, 3] found that an interference in the immune effects of associated antigens resulted mainly when one of the antigens acted as a booster stimulus. The immune effect of the booster antigen interferes with that of the antigen acting as the basic immunisant and the latter is considerably less effective than if administered alone.

Chen *et al.* [4] fully confirmed the results of Barr *and* Llewellyn-Jones in that they found that the immune effect of antigens administered together with the booster diphtheria antigen was much weaker in infants possessing a basic immunity against diphtheria than in those without such basic immunity.

These observations are of paramount importance, especially in areas where vaccinations are carried out along broad lines. Under such conditions one or another of the vaccines used almost inevitably contains a component or components acting as a booster stimulus. The phenomenon observed by Barr *et al.* may thus seriously interfere with the efficiency of a vaccination campaign ; some individuals may remain unprotected or will develop insufficient immunity.

Vaccinations are widely performed in Hungary so that there are hardly any subjects who would not have been inoculated against diphtheria, tetanus, typhoid fever or pertussis. The problem raised by the report of Barr *and* Llewellyn-Jones is therefore of great significance in this country. This has made us to study the phenomenon in subjects immunized earlier with diphtheria, tetanus, pertussis and/or typhoid vaccines, investigating the response to such combined vaccines in which one or more components acted as booster antigens.

## Materials and methods

(i) *Antigens.*

1. Tetanus and diphtheria anatoxins were purified by the trichloracetic acid method of SURJÁN, RICHTER and RÉTHY [5] to a grade of 300 B. U./mg PN and 900 to 1.100 Lf/mg PN, respectively. The value of anatoxins is given in Lf for diphtheria and in B. U. (binding unit ; 1 B. U. is practically equivalent to 1 Lf) for tetanus.

2. The pertussis antigen was a bacillary pertussis vaccine ; its microbial count was determined on the basis of the N. I. H. standard [6].

3. Enteric antigens. (*a*) Enteric antigens were producted by mass cultivation in fermentors, from the following strains : *Salmonella typhi* Ty. 2. ; *Shigella flexneri* 2 a "Zamárdi" ; *Shigella flexneri* 3 "31342" ; *Shigella sonnei* "Kiss".

(*b*) At the end of culturing, extract antigen was prepared from the centrifuged bacterial mass by the method of BOIVIN and MESROBEANU [7]. After dialysis, physiological correction and filtration for sterility, the antigens were assayed by the haemagglutination inhibiton (HI) test developed by RAUSS and KÉTYI [8], against a standard antigen. The antigenic value of the vaccine is given in HIU (haemagglutination inhibition units) in *Table I.*

(ii) *Vaccines and technique of vaccination.*

The composition of the vaccines used in the present and the previous immunizations is shown in *Table I.* Preparation of the TY—DYS—TE vaccines has been described [9]. If two doses were administered, the interval between the vaccinations was 4 weeks.

The vaccines used are denoted by the following abbreviations. Precipitated typhoid vaccine, TY ; polyvalent dysentery vaccine, DYS ; precipitated diphtheria anatoxin, DI ; combined diphtheria-tetanus-pertussis vaccine, DI—TE—PE ; combined diphtheria-tetanus-pertussis-dysentery vaccine, DI-TE-PE-DYS ; combined typhoid-tetanus vaccine, TY-TE ; combined typhoid-dysentery-tetanus vaccine, TY-DYS-TE.

**Table I**

*Composition and dose of the vaccines used in the investigations*

| Vaccine | Dose | Quantity of antigen /ml | | | | | | | Adsorbent, ml |
|---|---|---|---|---|---|---|---|---|---|
| | | Lf | B. U. | $10^9$ germs | HIU | | | | |
| | | Di | Te | Pe | Fl. 2a | Fl. 3 | So. | Ty | |
| Ty | 1 × 1 ml | — | — | — | — | — | — | 8 | 2 per cent alum |
| DYS | 2 × 0.5 ml | — | — | — | 10 | 5 | 10 | — | 4.5 mg $Al(OH)_3$ |
| DI | 2 × 1 ml | 30 | — | — | — | — | — | — | 1 mg $Al^{+++}(PO_4)$ |
| DI—TE—PE | 2 × 0.5 ml | 30 | 10 | 45 | — | — | — | — | 4.5 mg $Al(OH)_3$ |
| TY—TE | 2 × 1 ml | — | 5 | — | — | — | — | 4 | 2 per cent alum |
| DI—TE—PE—DYS | 2× 0.5 ml | 30 | 10 | 45 | 10 | 5 | 10 | — | 4.5 mg $Al(OH)_3$ |
| TY—DYS—TE 1. | 2 × 0,5 ml | — | 10 | — | 10 | 5 | 10 | 8 | 4.5 mg $Al(OH)_3$ |
| TY—DYS—TE 2. | 2 × 0.5 ml | — | 10 | — | 10 | 5 | 10 | 8 | 1.5 mg $Al(OH)_3$ |

(iii) *Immunizations.*

1. Organization. After suitable randomisation, the subjects to be inoculated (who were of the same age) were so grouped that their sex distribution, health conditions and social conditions be closely similar.

2. Blood samples taken 1 week before and 2 weeks after immunization were tested as described below.

(iv) *Control of the immune response and evaluation of the results.*

1. The immune response to enteric antigens was determined by the intraperitonial passive mouse protection test, at 3 levels (0.05, 0.005 and 0.0005 ml). The animals were infected intraperitoneally with 24-hour cultures of the test strains, using suspensions prepared with 5 per cent mucin and containing 100 to 1000 $LD_{50}$. Observation lasted 3 days.

Immunity to enteric components was determined on the basis of the results of the passive mouse protection tests and on that of the $ED_{50}$ ratio of the basic and immune sera, as computed according to KÄRBER. This indicated also the rise in titre. The significance of differences or homogeneity was determined by the $\chi^2$ test.

2. Control of immunity to diphtheria. The diphtheria antitoxin titres of the sera obtained before and after inoculations were determined by the rabbit-intracutaneous technique of JENSEN [10], at the Lr/3000 level.

3. Control of immunity to tetanus. The serum samples were assayed individually, at the L/2000 level, in albino mice weighing 16 to 18 g.

4. The immune responses to diphtheria and tetanus antigens were expressed by arithmetic means. The differences were analysed by Student's method [11].

## Results

*Immune effect of Shigella antigens.* In the first step, the immune response to the TY-DYS-TE vaccine was determined in subjects aged 12 to 13 years.

Two vaccines, identical in composition but prepared from different antigen batches at different points of time, were used. Correspondingly, two groups containing roughly the same number of subjects were formed and each of these were divided into two subgroups. Immunization was carried out after suitable randomisation.

In the first subgroups the patients had been inoculated 2 times with 1.0 ml of TY-TE vaccine one year earlier and showed a tetanus immunity of at least 0.05 I. U./ml.

The members of the second subgroup had never been immunized against either typhoid fever or tetanus, as far as it could be jugded from their history and the records. Tests made prior to the present immunization showed none of these subjects to have titres against tetanus reaching 0.001 I. U./ml.

It is clear from *Table II* that in the first groups the repeated two inoculations with tetanus anatoxin resulted in a mean titre of 12.4 I. U./ml, as compared to 13.8 I. U./ml for the other group. When tetanus anatoxin was used for inducing basic immunity, the titres averaged 2.8 and 3.2 I. U./ml, respectively. The difference in immune effect between the booster and the basic immunization was significant.

It is noteworthy that in the first subgroups, in which the typhoid vaccine acted as a booster antigen, the anti-typhoid mouse protection titre showed no rise, as compared to that of the group immunized basically.

As far as the Shigella antigens are concerned, statistical analysis showed no differences even when the Shigella antigens had been used in combination with booster antigens. Similar results have been obtained by the $\chi^2$ test in homogeneity studies.

**Table II**

*Immune response to Shigella antigens combined with booster antigens*

| Vaccine | Booster antigen | Sera | Titres of sera | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | *Sh. flexneri* 2a | | *Sh. flexneri* 3 | | *Sh. sonnei* | | *S. typhi* | | Tetanus |
| | | | ED 50 ml | x | ED 50 ml | x | ED 50 ml | x | ED 50 ml | x | I. U./ml |
| P 40 | typhoid, | basic | 0.005 | 5 | 0.05 | 10 | 0.028 | 10 | 0.1 | 5 | 12.4 |
| TY— | tetanus | immune | 0.0009 | | 0.005 | | 0.005 | | 0.016 | | |
| DYS— | — | basic | 0.005 | 10 | 0.016 | 10 | 0.028 | 17 | 0.1 | 5 | 2.8 |
| TE | | immune | 0.0005 | | 0.0016 | | 0.0016 | | 0.016 | | |
| 1—3 | typhoid, | basic | 0.005 | 10 | 0.05 | 5 | 0.05 | 31 | 0.1 | 5 | 13.8 |
| TY— | tetanus | immune | 0.0005 | | 0.009 | | 0.0016 | | 0.016 | | |
| DYS— | — | basic | 0.016 | 10 | 0.09 | 6 | 0.1 | 11 | 0.09 | 6 | 4.2 |
| TE | | immune | 0.0016 | | 0.0016 | | 0.009 | | 0.016 | | |

x rise of passive mouse protection titres.

*Table III* shows the results of immunization with DI-TE-PE-DYS vaccine. These children had been immunized against diphtheria 4—5 years, with DI-TE-PE 3—4 years, previously. The children in the first group showed an immunity of 0.03 A. U./ml or higher against diphtheria, those in the second group had in addition tetanus antitoxin levels of 0.05 I. U./ml or higher. To each group a random collective immunized with DYS vaccine served as control.

The data in *Table III* show that the subjects with basic immunity to diphtheria responded to the two inoculations with the DI-TE-PE-DYS vaccine with an average diphtheria immunity of 8.4 A. U./ml. This is equivalent to what may be expected when the diphtheria vaccine acts as a booster antigen. The tetanus immune response averaged 1.7 I. U./ml and none of the children tested showed titres lower than the threshold level of 0.05 I. U./ml.

In regard to the immune effect of the Shigella component, *Sh. sonnei* was used as the representative agent. It was found that, statistically, the immune effect of the *Sh. sonnei* component of the DYS vaccine in the control group was equivalent to that produced by the same component of the DI-TE-PE-DYS vaccine given to the members of the "diphtheria booster" group.

The immune response to the *Sh. sonnei* component, as used in combination with the booster antigens DI-TE-PE, appears to be inferior to that

**Table III**

*Immune response to Sh. sonnei antigen (I)*
(Children aged 3 to 6 years)

| Vaccine, dose | Booster antigens | Basic immunization, before years | Sera | Serum titres | | | |
|---|---|---|---|---|---|---|---|
| | | | | *Sh. sonnei* ED 50 ml | x | Di AU/ml | Te IU/ml |
| DI—TE—PE—DYS | diphtheria | 5—6 | basic | 0.1 | 20 | 8.4 | 1.7 |
| 2 × 0.5 ml | | | immune | 0.005 | | | |
| DYS | | | basic | 0.05 | 18 | — | — |
| 2 × 0.5 ml | — | — | immune | 0.0028 | | | |
| DI—TE—PE—DYS | diphtheria tetanus | 2—4 | basic | 0.09 | 5 | 6.9 | 8.7 |
| 2 × 0.5 ml | pertussis | | immune | 0.016 | | | |
| DYS | — | — | basic | 0.05 | 10 | — | — |
| 2 × 0.5 ml | | | immune | 0.005 | | | |

x = rise of passive mouse protection titre.

obtained by the use of the control DYS vaccine. However, the difference is not significant statistically and is within the limits of error of the assay.

In this group the average immunity produced by the booster inoculation with diphtheria and tetanus antigens was comparable to that produced by the usual booster inoculation.

The data in *Table IV* show the results of immunizations of infants and young children, by means of different vaccines and different techniques. The random groups embraced 19 to 26 subjects. Within three groups the booster antigens were DI, TE and PE. As controls, children possessing no basic immunity were inoculated with the DI-TE-PE-DYS vaccine ; another control group was immunized with DYS vaccine.

Immunization was carried out in the following ways.

(i) Children with basic DI, TE, PE immunity were immunized with a single dose of 0.5 ml of DI-TE-PE-DYS vaccine. The control group was immunized with DYS vaccine in the same way.

(ii) Children with basic DI, TE, PE immunity were inoculated with two doses of 0.5 ml each of the DI-TE-PE-DYS vaccine. The controls were subjects with basic immunity induced by two 0.5 ml doses of DI-TE-PE-DYS vaccine, as well as subjects inoculated twice with 0.5 ml of DYS vaccine.

**Table IV**

*Immune response to Sh. sonnei antigen (II)*
(Children aged 6 to 30 months)

| Vaccine, dose | Booster antigens | Basic immunization, before months | Sera | Serum titres | | | |
|---|---|---|---|---|---|---|---|
| | | | | *Sh. sonnei* | | Di | Te |
| | | | | ED 50 ml | x | AU/ml | IU/ml |
| DI—TE—PE—DYS 1 × 0.5 ml | diphtheria, tetanus, pertussis | 12 | basic | 0.09 | 5 | 6.3 | 7.2 |
| | | | immune | 0.016 | | | |
| DYS 1 × 0.5 ml | — | — | basic | 0.1 | 10 | — | — |
| | | | immune | 0.009 | | | |
| DI—TE—PE—DYS 2 × 0.5 ml | diphtheria, tetanus, pertussis | 12 | basic | 0.05 | 3 | 12.1 | 11.3 |
| | | | immune | 0.016 | | | |
| DI—TE—PE—DYS 1 × 0.5 ml DYS 1 × 0.5 ml | diphtheria, tetanus, pertussis | 12 | basic | 0.09 | 5 | 6.3 | 7.2 |
| | | | immune | 0.016 | | | |
| DI—TE—PE—DYS 2 × 0.5 ml | — | — | basic | 0.05 | 5 | 1.4 | 2.1 |
| | | | immune | 0.009 | | | |
| DYS 2 × 0.5 ml | — | — | basic | 0.1 | 6 | — | — |
| | | | immune | 0.016 | | | |

x rise of passive mouse protection titre.

(ii)1. Considering the phenomenon described by BARR *and* LLEWELLYN-JONES, a further group was also set up from the beginning. Subjects with basic DI, TE, PE immunity were given a booster DI, TE, PE inoculation by administering 0.5 ml of DI-TE-PE-DYS vaccine and, to complete an eventually poor dysentery immunity resulting from the BARR effect, one 0.5 ml dose of DYS vaccine was administered one month later. The controls to this group were those mentioned under the former paragraphs.

The results were as follows.

(i) The infants with basic DI, TE, PE immunity responded to one inoculation with DI-TE-PE-DYS vaccine by an average diphtheria and tetanus immunity corresponding to the booster inoculation. The *Sh. sonnei* antigen was less potent than the antigen of the DYS vaccine used as the control. The difference in titre, however, was not significant statistically.

(ii) After inoculation with two 0.5 ml does of the DI-TE-PE-DYS vaccine, the group possessing basic immunity to DI, TE, PE showed a considerably higher diphtheria and tetanus antitoxin immunity than either the former group or the controls inoculated with the DI-TE-PE-DYS vaccine. In the latter group diphtheria and tetanus immunity was in each member higher than the threshold value.

The single groups did not considerably differ in their immune response to *Sh. sonnei* antigen. Statistical analysis revealed a homogeneity of the immunity of the different groups. Also, the immune response to subsequent inoculations with DI-TE-PE-DYS and DYS vaccines was the same as that found in the other groups.

*Immune response to tetanus antigen*

The immune effect of tetanus anatoxin was studied in two main groups, using TY-DYS-TE vaccines with equivalent antigen content. The only difference between the two kinds of vaccines was exclusively in the concentration of gel. Within the two main groups, one subgroup each had been immunized with TY vaccine, one and two years before the present immunization. The single dose of typhoid antigen was equivalent to 8 HIU.

When evaluating the immune response to tetanus, we left out the subjects who had been inoculated against tetanus and those whose basic serum contained 0.001 I. U./ml or more antitoxin.

In the investigations, random groups of subjects from 12 to 13 years of age were immunized with two doses of 0.5 ml each of the TY-DYS-TE vaccine. The immune effect of the tetanus component was determined by the individual assay of blood samples taken from immunized subjects.

**Table V**

*Immune response to Tetanus anatoxin*

| Vaccine | Booster antigen | Distribution of tetanus immunity I. U./ml | | | | | | Arithmetic mean ($\overline{m}$) of tetanus titres | $\sigma^2$ |
|---|---|---|---|---|---|---|---|---|---|
| | | 0·01 | 0·011—0·05 | 0·051—0·1 | 0·11—0·5 | 0·51—1·0 | 1·0 | | |
| TY—DYS—TE | typhoid | — | — | 5 | 10 | 23 | 42 | 2.52 | 4.85 |
| + 4.5 mg $Al(OH)_3$ | — | — | 4 | 3 | 4 | 4 | 20 | 2.98 | 5.42 |
| TY—DYS—TE | typhoid | — | — | 2 | 3 | 5 | 65 | 4.4 | 2.45 |
| + 1.5 mg $Al(OH)_3$ | — | — | 1 | 1 | 1 | 6 | 21 | 3.65 | 4.65 |

The data in *Table V* show that the immune effect of the vaccine containing gel in a higher concentration was weaker than that of the vaccine

containing less adsorbent. However, there was no marked difference between average immunity and individual distribution of immunity in the groups previously inoculated with TY. Statistical analysis by the Student method showed the difference between the means to be statistically not significant.

## Discussion

Studies have shown that the immune effect of Shigella antigens is not lower when they are administered together with one or more such antigens to which the organism has already "learnt" to produce immune bodies. The differences found were statistically not significant. However, the immune response was more marked in a few cases, in which the Shigella antigens were administered without booster antigens. This occurred in the investigations shown in *Table III*; the immune response to *Sh. sonnei* of subjects immunized with the DI-TE-PE vaccine 2 to 4 years earlier was only about half the *Sh. sonnei* immunity of the control group. The same was the case when the infants immunized 1 year before with DI-TE-PE vaccine were given just one dose of the DI-TE-PE-DYS vaccine *(Table IV)*. In this latter group the immune effect was about half of that found in the control group inoculated with the DYS vaccine.

It is again to be emphasized that these differences were statistically not significant and they were probably due to the limits of error of the assay. The lower immune effect of the Shigella component in the DI-TE-PE-DYS vaccine cannot be ascribed to a competitive action of the other antigens in the vaccine, either. In subjects previously not inoculated there was namely no difference between the immune response to the Shigella components of the DI-TE-PE-DYS vaccine and those of the single DYS vaccine *(Table IV)*.

The fact that, as determined by the passive mouse protection test, two inoculations with the DYS vaccine produced a lower immunity than the single inoculation is another evidence suggesting the limits of error of the assay to be responsible for the difference.

The basic immunity induced by tetanus anatoxin was studied by the use of the TY-DYS-TE vaccine ; no difference either in the mean titre or in the distribution of immunity was found between the controls and the group in which the typhoid components of the vaccine used acted as a second booster inoculation.

Thus, our human studies have been unable to confirm the existence of the phenomenon described by BARR *and* LLEWELLYN-JONES. Our results were at variance also with those reported by CHEN *et al.* On the other hand, we could confirm the results obtained by HEGYESI *et al.* [5a], who studied the immune effect of tetanus anatoxin under conditions similar to those employed by us. We believe this to be due to the fact that the vaccine used

by BARR *and* LLEWELLYN-JONES, as well as by CHEN *et al.* were different from those used in our trials.

In our opinion, the "Barr effect" may reliably be avoided by carefully determining the quantitative and qualitative composition of the antigens injected and ensuring the maximum immune effect of the antigens. In earlier investigations [12] we have shown that the maximum immune effect of the antigen can be ensured by using the correct proportion of antigen and adsorbent. This will apparently guarantee a suitable immune effect not only with the basic immunization, but also when combined antigens are administered to induce immunity in an organism, in which one, or even three, antigens already act as boosters.

## Summary

(i) The Barr effect has been studied in children immunized with typhoid-dysentery-tetanus and diphtheria-tetanus-pertussis-dysentery vaccines after previous inoculation with typhoid-tetanus and diphtheria-tetanus-pertussis vaccines.

(ii) The immune effect of the booster Shigella antigens was not weaker in the children possessing basic immunity to typhoid, tetanus, or to diphtheria, tetanus and pertussis than in children not immunized previously.

(iii) The immune response to the typhoid antigen combined with tetanus toxoid in the presence of basic immunity to typhoid was not statistically different from that given by subjects previously not immunized against typhoid.

(iv) In our opinion, the vaccine used by us, containing a correct proportion of antigen and adsorbent, ensures maximum immune response to the antigen. This might have been the cause that the Barr phenomenon did not appear under the experimental conditions employed.

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# WIRKUNG DER ANTIBIOTIKA AUF SALMONELLA TYPHI IN DER MENSCHLICHEN GALLE

Von

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(Eingegangen am 14. Oktober 1958)

Im Hinblick auf die große hygienische Bedeutung des Typhusbazillen-Dauerausscheidertums haben sich viele Autoren mit diesem Problem beschäftigt, ohne daß es bisher gelungen wäre, die Frage vollständig zu lösen. Die früheren immunologischen und therapeutischen Versuche zur Behebung des Typhusbazillen-Dauerausscheidertums sind ergebnislos verlaufen. Die Bazillenfreiheit der Dauerausscheider zu einem gewissen Prozentsatz ist nur nach zwei Methoden zu erzielen.

1. Durch die Cholezystektomie (natürlich nur bei jenen Patienten, die die Erreger mit der Galle ausscheiden). In den zusammenfassenden Arbeiten über dieses Thema sowie in den über eine größere Zahl von Fällen referierenden neueren Publikationen variiert der Prozentsatz der geheilten Fälle zwischen 58—100% und macht meistens ungefähr 80% aus. Die in Ungarn erzielten Resultate [6] erreichen die untere Grenze dieser Zahlen.

2. Mit antibiotischer Behandlung. Nachfolgend fassen wir die bisherigen einschlägigen Literaturangaben tabellarisch zusammen *(S. Tab. I)*. In *Tabelle I* wurden nur solche Kranken berücksichtigt, die mit der Galle die Bazillen ausscheiden, und auch von diesen nur jene, bei denen mindestens einige Monate hindurch Nachuntersuchungen vorgenommen wurden. In *Tabelle I* sind auch die nicht näher gekennzeichneten, nur als Dauerausscheider erwähnten Fälle zu finden. Die Typhus- und Paratyphusbazillendauerausscheider sind in *Tabelle I* gemeinsam angeführt.

**Tabelle I**

| Verwendetes Mittel | Anzahl der untersuchten Fälle | Geheilte Fälle** | |
|---|---|---|---|
| | | Anzahl | % |
| Chloramphenicol | 50 | 1 (?) | 2,0 (?) |
| Streptomycin | ? | ∅ | ∅ |
| Tetracycline | 27 | 7 (?) | 25,9 (?) |
| Penicillin | 114 | 28 | 24,6 |
| Tetrajodphenolphthalein | 79 | 9 | 11,4 |

* Gegenwärtige Anschrift: *Pharmakologisches Institut der Medizinischen Universität*, Budapest.

** Die einschlägige Literatur stellt Verf. gern zur Verfügung.

Wie aus *Tabelle I* ersichtlich, vermag man mit Chloramphenicol und Streptomycin kaum Heilung zu erzielen, und die Nachuntersuchung von 6 der 7 mit Tetracycline behandelten Fälle kann nicht als zuverlässig angesehen werden. Die besten Resultate ergab Penicillin. Es kann nur eine der Ursachen für die Wirksamkeit von Penicillin sein, daß die Autoren — im Gegensatz zu den anderen Antibiotika — davon erheblich größere Mengen anwandten als die therapeutisch üblichen ; auf eine andere Ursache werden wir in vorliegender Arbeit hinweisen. Von den mit Penicillin arbeitenden Autoren wandten GERMER [4] und VAICHULIS [8] auch Tetrajodphenolphthalein an ; vielleicht sind ihre günstigen Ergebnisse diesem Mittel zuzuschreiben. — In *Tabelle I* sind zu Vergleichszwecken die nur mit Tetrajodphenolphthalein erfolgreich behandelten Fälle gesondert angegeben.

Aus obiger Zusammenstellung geht hervor, daß es — wenn auch die Angaben hier und da Widersprüche zeigen — möglich ist, mit chemotherapeutischer Behandlung eine gewisse Sanierung zu erzielen. Wahrscheinlich deshalb nur eine beschränkte, weil bei der überwiegenden Mehrzahl der Dauerausscheider Gallensteine, eine mehr oder minder schwere Gallenblasenentzündung und gegebenenfalls auch andere Veränderungen anwesend sind, die den Bazillen den vom Blutstrom bzw. von der Galle her wirkenden antibakteriellen Mitteln gegenüber Schutz bieten können. Die Behandlung derartiger Fälle muß naturgemäß ergebnislos bleiben. Demgegenüber kommt chronische Gallenausscheidung auch bei Personen vor, bei denen die Gallenblasenwand intakt oder kein Stein anwesend ist, und deren Gallenblase sich cholezystographisch füllt. Vergleichen wir das Verhältnis solcher in der Literatur erwähnten Fälle (10—20%) mit dem Prozentsatz der Fälle, wo die Chemotherapie Heilung herbeiführte, so finden wir eine ausgesprochene Ähnlichkeit. Daraus kann geschlossen werden, daß die chemotherapeutische Behandlung in jenen wenigen Fällen erfolgreich ist, in denen chronische pathologische Veränderungen nicht oder nur in geringem Maße vorhanden sind und es daher nur einer Sterilisierung der Gallenflüssigkeit bedarf. Diese Auffassung betont auf Grund seiner eigenen Untersuchungen auch VAICHULIS [8], daß nämlich bei funktionierender Gallenblase eine chemotherapeutische Behandlung angewandt werden könne, bei Anwesenheit von Steinen und sich nicht füllender Blase aber der Herd entfernt werden müsse.

In Betracht kommt die antibiotische Behandlung auch unmittelbar nach der Cholezystektomie zu ihrer Unterstützung, bzw. in Fällen, wo die früher durchgeführte Operation unwirksam geblieben ist. In beiden Fällen wurden Resultate mit Penicillin, Terramycin, ja auch mit Chloramphenicol erzielt, doch erscheint nicht ausgeschlossen, daß die Heilung zum Teil als Spätfolge der Operation betrachtet werden kann. In den oberen Gallenwegen können natürlich schon von vornherein Herde anwesend sein, in diesen Fällen bleibt die chemotherapeutische Behandlung wahrscheinlich wirkungslos.

Aus den obigen Ausführungen ergibt sich die Frage, warum die Bazillendauerausscheider mit noch funktionierender Gallenblase nur mit Penicillin, mit den anderen Antibiotika aber nicht geheilt werden können. Anderseits kommen postoperativ sämtliche Antibiotika in Frage (soweit man aus den wenigen untersuchten Fällen schließen darf). Wir nahmen an, daß die verschiedenen Antibiotika in der Galle unterschiedlich wirken, und entschlossen uns daher, diese Frage eingehender zu untersuchen. Über die Wirkung der Antibiotika gegen Typhusbazillen in der menschlichen und tierischen Galle stehen keinerlei Literaturangaben zur Verfügung.

## Material und Methoden

Die Wirkung der Antibiotika wurde parallel in der Galle bzw. in Bouillon in zweifachen Verdünnungsserien untersucht. Mit Rücksicht darauf, daß die bakterizide Wirkung ermittelt werden sollte, stellten wir die hemmende Konzentration durch Abimpfung von jeder Epruvette mit einer Öse nach 24- bzw. 48stündiger Inkubation fest. Dies entspricht ungefähr den Verhältnissen *in vivo*, da die Konzentration in der Gallenblase nach einmaliger Antibiotikumgabe 12—24 Stunden unverändert bleibt [10, 11, 12] und bei täglich mehrmaliger Verabreichung ein konstanter Spiegel in der Lebergalle aufrechterhalten werden kann. Die als Subkulturen verwendeten bouillonhaltigen Röhrchen beobachteten wir 24 Stunden, oft aber drei Tage lang. Die Subkultur von dem bakteriziden Effekt nicht mehr zeigenden Röhrchen wurde in allen Fällen auf Agar ausgebreitet, und die gewachsenen Kolonien wurden agglutiniert; es wurden nur jene Versuche verwertet, bei denen nach der Agglutination Typhusbazillen gewachsen waren und sich die entsprechenden Proben (aus der Galle und Bouillon) als steril erwiesen. Die bakteriziden Konzentrationen waren erfahrungsgemäß bei 48stündiger Inkubation besser reproduzierbar als bei 24stündiger, weshalb wir in *Tabelle II* die ersteren angegeben haben.

Die mit den verschiedenen Antibiotika gewonnenen bakteriziden Konzentrationen sind in unseren Versuchen höher als die in der Literatur im allgemeinen angegebenen Werte, was darauf zurückgeführt werden kann, daß letztere gewöhnlich nur die bakteriostatische Konzentration darstellen.

Die Röhrchen der Verdünnungsreihen enthielten bei unseren Versuchen 2 oder 1 ml, weil bei Anwendung einer größeren Menge die einzelnen Gallenproben nicht ausgereicht hätten. Das benutzte »kleine Inokulum« bestand aus jeweils 1 Tropfen der Verdünnung 1 : 500 000 einer 24stündigen Bouillonkultur, das »große Inokulum« aus je 1 Tropfen der Verdünnung 1 : 100. In einigen Fällen verwendeten wir auch ein größeres Inokulum. Wir benutzten *S. typhi*-Stämme mit der internationalen Bezeichnung »Vi 965« und »Ty 2« sowie in einigen Versuchen einen von einem Bazillenausscheider isolierten Laboratoriumsstamm (»624 A«, besonders in Versuchen mit Chloramphenicol). Zum Teil wurden die Untersuchungen mit mehreren Stämmen durchgeführt.

Bei den anaeroben Versuchen wurde steriles Paraffinöl auf die in den Röhrchen der Verdünnungsreihe befindliche Flüssigkeit gegossen, nachdem letztere erst aufgekocht und dann geimpft wurde.

Von den bei unseren Versuchen verwendeten, mit Hilfe der Duodenalsonde gewonnenen Gallen erhielten wir die A-Galle direkt, die B-Galle nach Einführung von Magnesiumsulphat. Die von Lebenden stammende Lebergalle gewannen wir durch das Kehrsche Rohr, die Blasengalle durch Punktion der resezierten Gallenblasen und die »Prosekturgalle« durch Aufschneiden der von Leichen stammenden Gallenblasen. Die Gewinnung der entsprechenden Galle stieß oft auf Schwierigkeiten, was die Ursache dafür ist, daß unsere Untersuchungen hier und da nicht vollzählig sind. Die tierischen Gallen wurden mittels Blasenpunktion gewonnen.

Das pH wurde mit der elektrischen Apparatur Typ Cambridge, die Viskosität mit dem Ostwaldschen Viskosimeter, die Trockensubstanz durch Trocknung bei 105° C, der Bilirubingehalt auf Grund der van der Bergschen Reaktion bestimmt.

## Ergebnisse

Wir haben die bakterizide Wirkung der Antibiotika auf *S. typhi* in den auf verschiedene Weise erhaltenen menschlichen Gallen untersucht und verglichen diese zum Teil mit den in der Rindergalle gewonnenen Angaben. Die Mehrzahl der Untersuchungsergebnisse ist in *Tabelle II* zusammengefaßt. Die Gallen verwendeten wir teils nativ, teils nach vorheriger Sterilisierung mit Hitze. Die in *Tabelle II* angegebenen Zahlen entsprechen jener niedrigsten Antibiotikumkonzentration in den Verdünnungsreihen, aus der — nach 48stündigen Inkubation — keine *S. typhi* mehr wuchsen. In Klammern sind die Ergebnisse der parallel vorgenommenen Bouillonversuche angeführt. Das pH der Bouillon stellten wir in einem Teil der Fälle — in Analogie zu den alkalisch reagierenden Gallen — auf 8,5 ein ; später benutzten wir nur Bouillon mit pH 7,2, weil der Terramycineffekt bei einigen der diesbezüglichen Kontrollversuchen, der der drei anderen Antibiotika aber bei den zwei verschiedenen pH immer identisch war. Wurden drei oder weniger Paralleluntersuchungen durchgeführt, so haben wir die Resultate in den Tabellen einzeln angeführt, bei mehr als drei Versuchen jedoch die Durchschnittswerte errechnet und die Anzahl der vorgenommenen Untersuchungen in Kursivschrift angegeben. Jede einzelne Untersuchung wurde mit einer jeweils anderen Gallenprobe vorgenommen, nur im Falle der mit der Duodenalsonde gewonnenen B-Galle bzw. der aus der Prosektur stammenden aufgekochten Galle benutzten wir in etwa der Hälfte der Fälle ein von mehreren Personen stammendes vermischtes Material.

Aus den Angaben der *Tabelle II* können wir verschiedene Schlüsse ziehen. Erstens hat Terramycin in sämtlichen untersuchten Gallenarten eine geringere Hemmungswirkung ausgeübt als in Bouillon. Ein anderes Bild zeigte die Chloramphenicol- und Streptomycinwirkung. Die bakterizide Konzentration beider Antibiotika war in der Rindergalle und »dünnen« menschlichen (d. h. Leber- sowie Duodenal-A- und B-) Galle praktisch dieselbe wie in Bouillon. Demgegenüber wirkten Chloramphenicol und Streptomycin in der konzentrierten Leichengalle 10—20mal schwächer, und der Effekt von Chloramphenicol war in der chirurgischen Blasengalle im Vergleich zu der Wirkung in Bouillon etwas herabgesetzt. Wieder anders verhielt es sich mit Penicillin, das in allen Gallen ebenso oder etwa doppelt so stark wirkte wie in Bouillon. Seine bakterizide Konzentration betrug auch in den konzentrierten Gallen einige E/ml, während die der anderen Antibiotika einige 100 $\mu$g/ml ausmachte.

Im Zusammenhang mit obigen Versuchen ergibt sich die Frage, ob die schwächere Wirkung der Antibiotika in einigen Gallen nicht auf deren Hitzesterilisierung zurückgeführt werden muß. Bei der Untersuchung der vorher als steril festgestellten Gallenproben sowie der durch den Seitz-Filter filtrier-

ten bzw. mit Quarzlicht sterilisierten Duodenalgalle fanden wir indessen, daß die Wirkung von Penicillin und Chloramphenicol mit den in der aufgekochten Galle festgestellten Resultaten praktisch übereinstimmte.

Tabelle II

*Bakterizide Wirkung der Antibiotika auf S. typhi in Galle und Bouillon (letzterer Wert in Klammern) in μg/ml, unter Verwendung des »kleinen Inokulums«*

| Antibiotikum | Rindergalle aus der Blase, nativ | Ursprung und Sterilisierung der menschlichen Gallen | | | |
|---|---|---|---|---|---|
| | | Kehrsches Rohr, Lebergalle, gekocht | Duodenal-B-Galle, aufgekocht | Von Cholezystektomie stammende native Galle | Prosekturgalle, aufgekocht |
| Penicillin—G E/ml | 3,1 (6,2) | 3,1 (3,1) | 2,8 (6,7)<br>*6 Fälle* | 3,1 (6,2) | 5,3 (6,8)<br>*6 Fälle* |
| Streptomycin | — | 12,5 (25,0) | 10,1 (10,1)<br>*4 Fälle* | | 250,0 (12,7)<br>*5 Fälle* |
| Razämisches Chloramphenicol | 10,9 (8,1)<br>*5 Fälle* | 3,1 (6,2) | 3,1 (12,5)<br>6,2 (12,5)<br>6,2 (6,2) | 20,1 (13,2)<br>*9 Fälle* | 120,0 (10,6)<br>*15 Fälle* |
| Terramycin | 12,5 (6,2) | 25,0 (6,2)<br>12,5 (6,2) | 50,0 (3,1)<br>50,0 (6,2) | 100,0 (3,1)<br>50,0 (3,1) | 165,0 (11,1)<br>*5 Fälle* |

*Bemerkung:* Ein Teil der als »aufgekocht« bezeichneten Materialien wurde nicht durch Kochen, sondern fraktioniert sterilisiert. Weitere Erklärungen vgl. im Text.

Da Chloramphenicol als spezifische Heilmittel der Typhus-Krankheit betrachtet wird, schien es interessant, näher zu untersuchen, warum dieses Antibiotikum in der konzentrierten Galle schwächer wirkte, da diese Tatsache eine Erklärung für die Wirkungslosigkeit von Chloramphenicol bei Dauerausscheidertum bieten könnte. Die nicht entsprechende Vermischung der »dicken« Galle bei der Herstellung der Verdünnungsreihe konnte nicht die Ursache der verminderten Wirkung sein, weil sich die bakterizide Konzentration nicht veränderte, als wir die nötige Chloramphenicol separat in jedes einzelne Röhrchen gaben. Das Phänomen muß also mit der Konzentrierung der Galle zusammenhängen. Um diese Frage klarzustellen, haben wir einige physikalische Eigenschaften der verschiedenen Gallen gemessen und die gewonnenen Werte mit jener bakteriziden Konzentration verglichen, die Chloramphenicol in den gleichen Gallenproben zeigte. Die Wirkung von Chloramphenicol drückten wir mit dem sog. C-Quotienten aus, worunter wir das Resultat eines Bruches verstehen, bei der die bakterizide Chloramphenicolkonzentration in der Galle durch die bakterizide Chloramphenicolkonzentration in Bouillon dividiert wird. Die Ergebnisse dieser Untersuchun-

gen sind in *Tabelle III* zusammengefaßt, der entnommen werden kann, daß der C-Quotient mit dem Trockensubstanzgehalt der Gallen parallel, zu ihrem pH jedoch im umgekehrten Verhältnis ansteigt, d. h. die Chloramphenicolwirkung abnimmt. In den tierischen Gallen fanden wir denselben Effekt.

**Tabelle III**

*Angaben über verschiedene Gallen*

| Ursprung der Galle | Anzahl der untersuchten Fälle | pH | Relative Viskosität | Trockensubstanzgehalt % | Bilirubin mg% | »Chloramphenicol-Quotient« |
|---|---|---|---|---|---|---|
| Rinderblasengalle ....... | 4 | 8,47 | 1,82 | 8,27 | 12,5 | 1 |
| Hundeblasengalle ....... | 1 | 8,5 | | | | |
| Katzenblasengalle ...... | 3 | 6,36 | 3,51 | | | 8* |
| *Menschliche* | | | | | | |
| Lebergalle ........... | 1 | 8,40 | | 2,9 | | 0,5 |
| Duod.-A-Galle ........ | 4 | 8,07 | 1,27* | 3,4 | 16,0* | 0,62 |
| Duod.-B-Galle ........ | 15 | 7,31 | 1,55 | 5,64 | 171 | 1,12 |
| Cholezystektomiegalle | 13 | 7,64 | 3,85* | 11,08 | 600 | 1,57 |
| Prosekturgalle ........ | 19 | 6,55 | hoch, nicht meßbar | 14,09 | 550 | 6,33 |

*Bemerkungen :* Die Zahlen stellen Mittelwerte dar. Die mit * bezeichneten Bestimmungen wurden aus weniger Proben vorgenommen, als die »Anzahl der untersuchten Fälle« ausmacht. Die Bestimmungen wurden in nativer Galle, die des C-Quotienten in nativer bzw. gekochter Galle vorgenommen.

Bisher hatten wir das »kleine Inokulum« benutzt, das je ml Galle nur einer Ausgangsbazillenzahl von einigen 1000 entspricht, während bei der Sterilisierung der Gallenwege auch die Vernichtung einer viel größeren Bazillenmenge nötig sein kann. Deshalb führten wir auch Versuche mit einem 5000mal größeren Inokulum durch. Die Resultate stimmten mit den bei Benutzung des »kleinen Inokulums« ermittelten Ergebnissen im wesentlichen überein, doch wirkte nur eine um ein bis zwei Verdünnungen größere Antibiotikumkonzentration hemmend. Die bakterizide Penicillinkonzentration war unter diesen Umständen in der Duodenal-B-Galle 3—6, in der Prosekturgalle 10—20 E/ml. Benutzten wir dagegen ein durch Zentrifugieren eingedicktes Inokulum, so daß die Ausgangsbakterienzahl der Epruwetten mit der einer 24stündigen Bouillonkultur übereinstimmte, so wurden die Bazillen nur von 100—200 E/ml Penicillin getötet. Dasselbe Ergebnis beobachteten wir, wenn wir eine Galle verwendeten, die vorher mit *S. typhi* beimpft und 24 Stunden bei 37° C inkubiert worden war. Das Untersuchungsmaterial der letzten beiden Versuchsserien war Duodenal-B-Galle. Die bakterizid wirkende Penicillinkonzentration ist bei Anwendung derart großer Inokuli deshalb beachtens-

wert, weil sich nach unseren Bakterienzählungen *S. typhi* in vitro in den verschiedenen menschlichen Gallen in 24 Stunden ungefähr im gleichen Ausmaß vermehren wie in Bouillon.

Im Hinblick darauf, daß in den Gallenwegen anaerobe Verhältnisse herrschen, untersuchten wir, ob obige Resultate nicht ausschließlich bei aeroben Bedingungen gelten. In einigen Fällen erwies sich die bakterizide Chloramphenicolkonzentration bei den anaerob eingestellten Verdünnungsreihen in der Prosekturgalle mit der in der Kontrollserien festgestellten identisch.

Endlich gaben wir in einigen Versuchen Penicillin zu der von Bazillenträgern gewonnenen Duodenalgalle. In einigen Fällen wurden die Typhusbazillen von 100 oder weniger E/ml Penicillin vernichtet, während 100 E/ml in anderen Fällen unwirksam blieben.

## Besprechung

Wenn wir feststellen wollen, welches der untersuchten Antibiotika zur Sterilisierung der Galle von Dauerausscheidern in Betracht kommt, so müssen wir die in vitro bakteriziden Konzentrationen mit den beim Menschen erreichbaren Gallenkonzentrationen vergleichen. Über die Gallenausscheidung der Antibiotika stehen viele Angaben zur Verfügung ; im folgenden beziehen wir uns nur auf diejenigen, welche den nach einmaliger Verabreichung einer der therapeutischen nahekommenden Dosis entstehenden Gallenspiegel betreffen, und zwar geben wir immer die erzielte maximale Konzentration an. Da weder mit der Einkonzentration der Antibiotika in der Blase noch mit ihrer Sekretion durch die Blasenwand aus dem Blutstrom gerechnet werden kann (Zaslow), unterscheiden sich die Blasen- und Lebergallenkonzentrationen nicht wesentlich voneinander. Nach peroraler Verabfolgung von 4 g Chloramphenicol (einer sehr großen Dosis!) vermochte Woodward [9] 27 $\mu$g/ml in der Blasengalle nachzuweisen ; Glazko [5] fand nach 1 g 1,0—2,8 $\mu$g/ml in der Lebergalle. Diese Werte sind niedriger als die unserseits in der Leichengalle wirksam gefundenen 60 bzw. in verdünnter Galle bakterizid wirkenden 3—6 $\mu$g/ml. (Die Angaben in *Tabelle II* entsprechen dem Zweifachen dieser Werte, weil sie sich auf razämisches Chloramphenicol beziehen.) — Ähnlich verhält es sich bei Streptomycin ; nach i. m. Einspritzung von 100 000 E betrug der erreichte Blasengallenspiegel 0,8—3,9 E/ml [10], während die Lebergalle nach i. m. Verabreichung von 500 000 E 10 E/ml enthielt [1], die bakteriziden Konzentrationen indessen 250 bzw. 10—25 E/ml betrugen. — Nach peroraler Darreichung von 250 mg Terramycin waren in der Blase 0,9—6,1 [12], in der Lebergalle 4.5 $\mu$g/ml anwesend [3], während nach unseren Untersuchungen 50—165 bzw. 12—50 $\mu$g/ml erforderlich wären. —

Demgegenüber ergaben 250 000 E Penicillin nach i. m. Einspritzung eine Blasenkonzentration von 8—25 E/ml bzw. eine Lebergallenkonzentration von 5—7 E/ml, während wir 2,0—5,3 E/ml als bakterizide Konzentrationen feststellten.

Nach obigen Ausführungen sind also die in der Gallenblase anwesenden Chloramphenicol-, Streptomycin- und Terramycinkonzentrationen keinesfalls imstande, bakterizide Wirkung auszuüben. Es wird daher verständlich, daß es den meisten Autoren nicht gelungen ist, die Dauerausscheider von ihren Bazillen zu befreien. Die in der Lebergalle festgestellten Chloramphenicol- und Streptomycinkonzentrationen kommen den bei unseren Versuchen als nötig erscheinenden nahe, so daß diese zur postcholezystektomischen Behandlung in Frage kommen, vor allem wenn man sie längere Zeit verabreicht und so ein höherer Gallenspiegel als der obige erzielt wird. Mit Penicillin aber vermag man sowohl in der Blasen- wie Lebergalle mit der therapeutischen Dosis einen höheren Spiegel herbeizuführen, als man davon in vitro zur Hemmung in der Galle benötigt. Damit lassen sich die verhältnismäßig günstigen Resultate bei der Behandlung der Bazillendauerausscheider mit Penicillin erklären. Wir gelangen daher zu dem Schluß, daß zur direkten Behandlung der Dauerausscheider nur Penicillin in Frage kommen kann. Außerdem Penicillin das am wenigsten toxische Antibiotikum ist und daher seine Dosis auf das Vielfache erhöht werden kann.

Die obigen Überlegungen gründeten sich auf die bakteriziden Konzentrationen, die mit dem »kleinen Inokulum« gewonnen wurden. Indessen waren nur sehr große Penicillinmengen imstande, die in der Galle bereits vermehrten Typhusbazillen zu vernichten. Dies bedeutet, daß der Gebrauch von Penicillin auch nur dann versprechend ist, wenn wir den Bazillengehalt der Galle choleretisch herabzusetzen vermögen.

Naturgemäß kann die Anwendung von Penicillin nur bei jenen Dauerausscheidern in Betracht kommen, in deren Gallenblase keine oder nur geringe chronische pathologische Veränderungen anwesend sind. Laut ZASLOW sind zwar die Antibiotika auch im Inhalt nicht funktionierender Gallenblasen nachweisbar, doch erscheint die Sterilisierung der Gallenflüssigkeit nicht ausreichend, wenn pathologische Veränderungen vorliegen. Dies geht aus der Tatsache hervor, daß die Duodenalgalle im Laufe der Penicillin-, ja auch der Chloramphenicolbehandlung nach mehreren Autoren negativ geworden war, die Kranken aber nach Abschluß der Kur weiterhin Bazillen ausschieden. In diesen Fällen kann demnach ausschließlich die Herdresektion — in der Mehrzahl der Fälle die Cholezystektomie, seltener die Entfernung des Choledochussteines, die Eröffnung des Abszesses — zum Erfolg führen. Nach der Cholezystektomie erscheint die antibiotische Behandlung zur Vernichtung der in den oberen Gallenwegen, im Darmtrakt oder anderswo im Organismus vorübergehend anwesenden Typhusbazillen sowie zur Verhin-

derung der Entstehung neuer Herde theoretisch als zweckmäßig. Wenn derartige Herde von vornherein anwesend waren, so wird diese Behandlung naturgemäß nicht erfolgreich sein.

## Zusammenfassung

Die bakterizide Wirkung von Antibiotika auf *Salmonella typhi* wurde in der auf verschiedene Weise erhaltenen menschlichen Galle untersucht. Ein Vergleich der *in vitro* bakteriziden Werte mit den von mehreren Autoren mitgeteilten Gallenkonzentrationen dieser Mittel ergibt, daß zur Sterilisierung der Gallenflüssigkeit *in vivo* nur Penicillin in Frage kommt, aber auch dieses wahrscheinlich nur in Kombination mit Gallentreibung. Zu günstigen Resultaten kann die Penicillintherapie nur bei Bazillendauerausscheidern führen, bei denen keine oder nur geringe chronische Gallenblasenveränderungen anwesend sind.

*Dankbezeigungen.* Den Leitern und Mitarbeitern des Mikrobiologischen Institutes der Medizinischen Universität Budapest sowie der Station für Gesundheitswesen und Epidemiologie, Budapest, welche die Durchführung dieser Arbeit ermöglichten und förderten, sowie dem Wissenschaftlichen Ausschuß für das Gesundheitswesen Ungarns sei für ihre Unterstützung der Dank des Verfassers auch an dieser Stelle ausgesprochen.

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# STUDIES ON THE OCCURRENCE IN HUMAN SERA OF COMPLEMENT FIXING ANTIBODIES TO CONTAGIOUS CANINE HEPATITIS VIRUS

By

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(Received November 13, 1958)

Canine hepatitis as an infectious disease was first described by RUBARTH [1] in 1947. Since then the disease has been proved to be spread among dogs all over the world [2—7]. Its occurrence in Hungary was reported by KAPP [8] and OLÁH [9].

According to our present knowledge, beside the dog, the fox is the only animal susceptible to contagious canine hepatitis (HCC) virus. As demonstrated by SIEDENTOPF *and* CARLSON [3], the fox responds to the infection with encephalitis instead of hepatitis.

DELAGE *and* MARTIN [10] reported in 1955 that a great proportion of the monkey sera examined by them contained complement fixing (CF) antibodies to HCC virus. The same authors obtained similar results when testing 1000 specimens of human sera : 22 per cent of the sera proved to contain antibodies.

LAFRANCHI *and* DE JACO [11] found high CF titres to HCC virus in the convalescent sera of two patients presenting a high temperature and neurogenic symptoms. A dog was inoculated with the blood of one of the patients and killed 30 days later. The dog's liver and spleen were markedly swollen but no virus could be isolated.

Based upon epidemiological and clinical studies, MARTIN *and* GORET [12] suppose some relation to exist between HCC virus and the viruses of human hepatitis and poliomyelitis, and believe that HCC virus might be pathogenic for man.

To obtain more data on human relations of the virus, in the present work CF tests were carried out in human sera using HCC viral suspension as antigen.

## Materials and methods

RUBARTH's method for the CF test was slightly modified. Antigen was prepared from the liver of 2—3 months old dogs infected artificially with HCC virus and bled out during the terminal agony. The liver was ground and diluted with saline to make a 20 per cent suspension. After centrifugation with 5000 rpm for 15 min. the supernatant was filtered through Seitz EK pad to eliminate the eventual anticomplementary activity before used as

antigen. Control antigens were prepared in the same manner from the liver of normal dogs or of dogs infected with canine distemper virus. Fresh or, in a few cases, lyophilized complement was used. Sensitized erythrocytes were prepared by mixing a 2.5 per cent suspension of sheep erythrocytes with an equal volume of haemolysin in a sufficient concentration to cause complete haemolysis in the presence of an appropriate amount of complement.

Human and animal sera were inactivated at 56° C for 30 minutes before tested in 3 tubes. Tubes 1, 2 and 3 contained 0.2, 0.1 and 0.05 ml serum, respectively, made up to 0.5 ml with saline ; 0.5 ml antigen and 0.5 ml complement. The tubes were incubated at 37° C for 30 minutes and sensitized erythrocytes were then added, 1 ml in each tube. After a second incubation period of 30 minutes at 37° C the test was read and allowed to stand at room temperature for 2 or 3 hours when reading was repeated. The test was regarded as positive if fixation was complete in at least the first tube. In the Tables, incomplete lysis is signed as doubtful and complete lysis as negative.

## Experimental

Serum specimens from 308 healthy persons of various age were tested for CF antibodies to HCC virus. The results are summarized in *Table I.* Seventytwo sera (23 per cent) gave a positive reaction.

**Table I**

*Complement fixation tests to contagious canine hepatitis virus in* 308 *normal human sera*

| No. of sera tested | Positive | Doubtful | Negative | Positive per cent |
|---|---|---|---|---|
| 308 | 72 | 7 | 229 | 23 |

The specificity of the test was controlled by testing 6 out of the positive sera with an antigen prepared from normal dog livers and another from livers of dogs infected with canine distemper virus. None of the sera reacted with the control antigens.

The proportion of individuals giving a positive reaction was, to some extent, higher in the group of adult persons than among 8 to 10 year-old children, but the difference was not significant. A more definite difference was established between the communities tested. The frequency rate of positive persons was particularly high, 41 per cent, in a closed community counting 82 members. Unfortunately, we were not able to obtain any information on epidemics experienced by the community before the serum samples were withdrawn.

One of the possible explanations of the occurrence in human sera of CF antibodies to HCC virus might be if the virus were capable of infecting man, causing either clinical symptoms or latent infection. This seems, however, to be contradicted by the CF tests performed with the sera of the 6 employees of this laboratory. Though all the 6 persons used to take care of the sick animals and carried out necropsies without any special precaution, none of them had CP antibodies in their sera.

It seemed therefore more probable that the presence of CF antibody to HCC virus in human sera was due to an antigenic relation of HCC virus to another virus capable of infecting man. The viruses (A and B) of human hepatitis having been especially suspected, serum samples of 145 patients with hepatitis were tested for CF antibody to HCC virus. We attempted to differentiate cases of serum hepatitis from those of infectious hepatitis by the anamnestic data. The hepatitis sera were withdrawn 1 to 8 weeks after the onset of illness. The results are presented in *Table II*.

**Table II**

*Contagious canine hepatitis virus complement fixation tests with human hepatitis sera*

| Disease | No. of sera tested | Positive | Doubtful | Negative | Positive per cent |
|---|---|---|---|---|---|
| Infectious hepatitis | 106 | 29 | 5 | 72 | 27 |
| Serum hepatitis | 39 | 5 | 0 | 34 | 12 |

Out of the 106 patients with the diagnosis of infectious hepatitis, 29 (27 per cent) gave a positive test, a slightly higher proportion than that found for normal sera *(Table I)*. It is to be noted that neither a rise nor a drop in the CF titres was observed when the same patients were examined repeatedly during the illness and convalescence.

The proportion of positive persons in the group of patients with serum hepatitis was even lower than in the group of healthy persons *(Table I)* but the difference was not significant.

It can thus be concluded that the positive CF tests to HCC virus in human sera cannot be attributed to previous infection with hepatitis virus A or B.

LAFRANCHI's two cases [11] mentioned above called our attention to a possible relation of the occurrence of HCC antibodies to some neurovirus infection. We tested, therefore, 23 serum samples of poliomyelitis convalescents. As shown in *Table III*, the occurrence of the CF antibodies was about the same as in the group of healthy persons.

**Table III**

*Contagious canine hepatitis virus complement fixation tests with human poliomyelitis convalescent sera*

| No. of sera tested | Positive | Doubtful | Negative | Positive per cent |
|---|---|---|---|---|
| 23 | 6 | 2 | 15 | 26 |

CF tests were carried out also in anti-influenza A and B hyperimmune ferret sera. Antibodies were not demonstrated.

## Discussion

We have confirmed the data of DELAGE *and* MARTIN [10] on the occurrence of CF antibodies to HCC virus in a great proportion of normal human sera. CF antibody was demonstrated in 72 (23 per cent) out of the 308 normal sera tested. The frequency occurrence of CF antibodies in adult sera was somewhat higher than that in children's sera, but the number of children's sera was too low to draw any conclusion.

The difference among the communities studied by us seems to be more pregnant. In one of the communities antibody was demonstrated in 41 per cent of the sera in contrast to the 23 per cent average for the other groups. The difference might be attributed to a previous epidemic experienced by the group showing the higher rate of positive sera. Unfortunately, we have no information on this subject.

MARTIN *and* GORET [12] have supposed man to be susceptible to HCC virus. The fact that, in spite of the continuous exposition to infection, none of the six employees of our laboratory had CF antibodies, does not support this conception.

It seems more likely that the CF titres in human sera are due to infections with a virus antigenically related to HCC virus. Our results suggest the hypothetical virus to be not identical with the viruses causing hepatitis in man. Its relation to the poliomyelitis and influenza viruses is also unlikely.

## Summary

1. Complement fixing antibodies specific for contagious canine hepatitis virus were demonstrated in 23 per cent of 308 normal human sera tested.

2. The proportion of persons with complement fixing antibodies in one of the communities examined was definitely higher than in the others.

3. Since complement fixing antibody could not be demonstrated in the sera of six persons being in continuous contact with the virus, we suppose that man is not susceptible to contagious canine hepatitis virus.

4. The quantitative occurrence of complement fixing antibodies in human hepatitis sera was approximately the same as in the group of normal human sera.

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# THE UTILIZATION OF MALONATE BY CANDIDA ALBICANS*

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(Received November 29, 1958)

The role of malonic acid as a nutrient for various groups of organism has been described in previous papers [1—3].

The examinations have shown that malonic acid may serve as an adequate source of carbon and energy and have called attention to the process of its breakdown and to its role in the intermediate metabolism.

Malonic acid may be produced from several substances in the metabolic process [4—8]. The breakdown of malonic acid is possible in two different ways, namely to acetic acid by direct decarboxylation through the action of bacteria and bacterial enzymes [9—15], and by an oxidative process preceding the formation of acetic acid [16—18].

The formation of succinic and acetoacetic acid has been observed in living animals and the same type of reaction has been shown to occur on the action of enzyme preparations [19, 20].

A number of observations have been made as to the stimulating activity of malonic acid on the intermediate metabolism of algae [21], of plant [22—24] and animal tissues [25—28]. Fungi were also shown to be capable of assimilating this substance [1]. In the breakdown of malonic acid by *Botryotinia fuckeliana,* the probable intermediates were acetic acid and succinic acid, while oxalic acid proved to be the end product [2]. The assimilation of malonic acid by pathogenic fungi has also been reported [3]. In the present report the assimilation of malonate is discussed.

## Materials and methods

In the investigations *Candida albicans* strain 85/957—OKI, isolated from pathological material was used. In systematic characters it corresponded to a strain obtained from the *C. B. S. Yeast Division*, *Delft*, *Holland.*

The growth from some Sabouraud glucose-agar slants was suspended, washed three times and used for inoculating 4 l of the fluid medium described elsewhere [3]. The cultures were incubated at room temperature.

After 1 or 2 weeks of incubation the culture was centrifuged, washed three times and after determining the wet weight, it was suspended in the corresponding fluid medium and manometric measurements were performed by the conventional technique. Production of $CO_2$ was calculated from the results of parallel experiments with and without KOH [29].

* Partly presented at the 4*th International Congress of Biochemistry*, *Vienna*, September, 1958.

Each experiment was carried out in 2.5 ml volume at 26° C, continuously shaking the suspension at a rate of 120 per minute. The cells were suspended in malonate nutrient medium, in a M/15 phosphate buffer of pH 6.0 and in nutrient medium containing no malonate and alkali. The addition of malonic acid and the inhibitors* was performed by dissolving them in M/15 phosphate buffer of pH 6.0. Malonic acid and the inhibitors were dissolved in such concentrations that adding a 0.5 ml sample of the solution to the culture provided the concentration indicated in the Figures.

For subculturing the organism, malonic acid-free medium was employed. The nutrient role of the inoculum will be discussed later.

## Experimental

The general process of malonic acid utilization was investigated by WARBURG's method.

*Fig. 1* shows that an approximately linear oxygen uptake immediately folloved the addition of malonic acid. As the endogenous respiration was 4 $\mu$l in 150 minutes, this was omitted from the Figure.

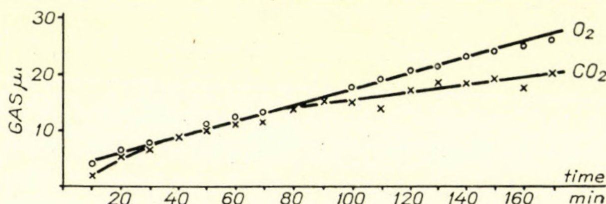


*Fig. 1.* Respiration of *Candida albicans* in malonate medium (0.5% malonic acid, 0.4% NaOH, 0.5% $(NH_4)_2SO_4$, 0.1% $KH_2PO_4$, 0.07% $MgSO_4 \cdot 7\ H_2O$, pH 6.0). Values correspond to 10 mg dry weight

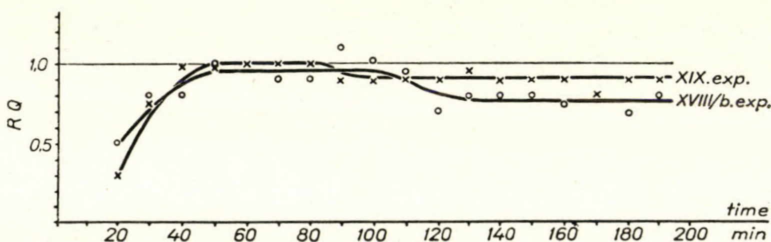


*Fig. 2.* The respiratory quotient of *Candida albicans* plotted against time. Malonate medium : Exp. XIX : pH 6.0, Exp. XVIII/b : pH 5.0

The curve for the aerobic decarboxylation differed from that for the oxygen uptake only in a lag period at the beginning and in its descending final section.

To demonstrate the lag period of $CO_2$ production clearly, the respiratory quotient plotted against time is shown in *Fig. 2.* Every point of the curve represents the R. Q. values summarized for the time indicated. It will be

* Explanation to the abbreviation of inhibitors : MJA = monoiodoacetate, DNP = = 2,4 dinitrophenol, SA = sulphanylamide, KCN = potassium cyanide, NaF = sodium uoride, $K_{III}$ = Komplexon III(disodium ethylendiamine tetraacetate), As = sodium arsenate $Na_2HAsO_4$).

observed that the respiratory quotient was well under 1 during the first 10 or 20 minutes of the experiment ; it reached 1 approximately at the 30th minute ; it stayed at this value until the 60th to 70th minute ; finally it again decreased under 1.

The action of enzyme inhibitors on the oxidation of malonate is shown in *Figs. 3* and *4*.

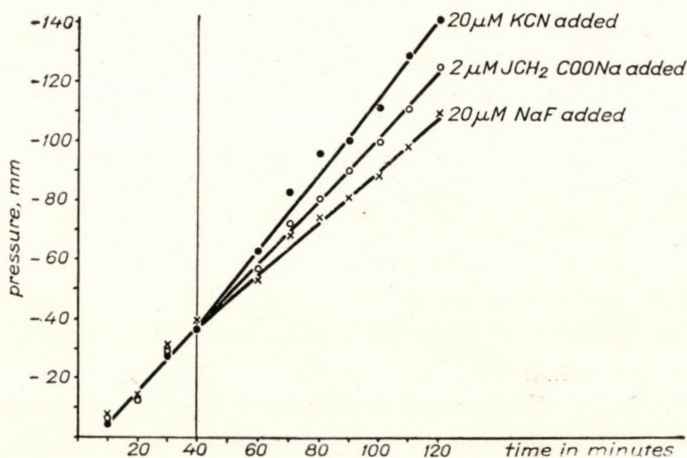


*Fig. 3.* Oxygen uptake in malonate medium by *Candida albicans* in the presence of inhibitors. To Warburg vessels containing 2 ml medium, 0.2 ml 0.1 *M* KCN, 0.2 ml 0.1 *M* NaF or 0.2 ml 0.01 *M* MJA were added at the time indicated by the vertical line. Values correspond to 60 mg dry weight

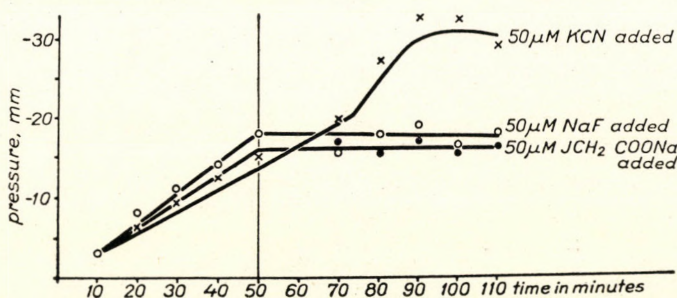


*Fig. 4.* Oxygen uptake in malonate-phosphate buffer by *Candida albicans* in the presence of inhibitors. To Warburg vessels containing 2 ml *M*/15, pH 6.0 phosphate buffer containing 0.5% malonic acid, 0.5 ml 0.1 *M* KCN, 0.5 ml 0.1 *M* NaF or 0.5 ml 0.1 *M* MJA were added. Values correspond to 15 mg dry weight

The cells which had been treated as described above were suspended in malonate-nutrient-medium *(Fig. 3)* and in phosphate buffer solution *(Fig. 4)*. The inhibitors were added as indicated by the vertical line, after

an incubation of 40 or 50 minutes. In the experiments shown in *Fig. 3* the final concentration of the inhibitors was $0.99 \times 10^{-3}$ *M* for MJA, $9.9 \times 10^{-3}$ *M* for KCN and $9.9 \times 10^{-3}$ *M* for NaF. The corresponding concentrations in the experiments shown in *Fig. 4* were $20 \times 10^{-3}$ *M*, $20 \times 10^{-3}$ *M* and $20 \times 10^{-3}$ *M*. According to *Fig. 1* the graph representing oxygen uptake is linear within 150 minutes of incubation. Thus the direction of the control graphs in *Figs. 3* and *4* were determined by preincubation in malonic acid medium containing no inhibitors for 40 and 50 minutes, respectively. The changes produced by the inhibitors in the respiration were therefore evaluated by comparing the curve obtained with the prolongation of the straight line of the control.

The Figures show that 2 $\mu M$ MJA and 20 $\mu M$ KCN were ineffective, while 20 $\mu M$ NaF slightly inhibited the oxidation of malonic acid in nutrient medium. In phosphate buffer containing malonic acid no further oxygen uptake was observed after the addition of 50 $\mu M$ MJA or 50 $\mu M$ NaF; 50 $\mu M$ KCN acted at the beginning as a stimulant, while after 30 minutes it exerted an inhibitory action. The curves for aerobic $CO_2$ production in the presence of these inhibitors were similar to those for the $O_2$ uptake.

The action of other inhibitors on the respiration of *Candida albicans* in phosphate buffer containing malonate is shown in *Table I.*

It is clear that the inhibitors listed did not block oxygen uptake immediately, nor did they completely inhibit the respiration throughout the experiment. Production of $CO_2$ was, however, completely inhibited by these substances.

**Table I**

*Oxygen uptake in malonate-phosphate buffer by Candida albicans in the presence of inhibitors*

| Inhibitors conc. $10^{-3}$ M | $O_2$ consumption $\mu l$ | | | | |
|---|---|---|---|---|---|
| | 10′ | 20′ | 30′ | 40′ | 50′ |
| None | 2.5 | 5.5 | 8.5 | 11.5 | 14.0 |
| DNP 0.2 | 2.5 | 5.0 | 6.5 | 8.0 | 8.0 |
| $NaN_3$ 20.0 | 2.5 | 4.5 | 5.5 | 7.0 | 7.5 |
| $K_{III}$ 10.0 | 1.0 | 2.0 | 2.5 | 3.5 | 3.5 |
| As 8.0 | 2.5 | 4.5 | 6.0 | 7.5 | 7.5 |
| SA 1.0 | 2.0 | 4.5 | 6.5 | 8.0 | 9.0 |

Concentration of inhibitors means the end-concentration in 2.5 ml malonate-phosphate buffer of pH 6.0. Addition of inhibitors occurred at 0 minute. Values correspond to 20 mg dry weight.

As to anaerobic $CO_2$ production, in phosphate buffer containing malonic acid practically no $CO_2$ was formed even after 60 minutes' incubation in

nitrogen atmosphere. No production of $CO_2$ was induced by adding to this system 0.01 *M* methylene blue as a hydrogen acceptor.

The oxidation of malonate was increased by yeast extract to 125 per cent.

## Discussion

The present investigations show the primary role of oxidation over decarboxylation in the breakdown of malonic acid by *Candida albicans*. This finding corresponds to that of LINEWEAVER [16], KARLSON [17] and WOLFE *and* RITTENBERG [18], who in the breakdown of malonic acid reported an oxidation process to precede the formation of acetic acid. The oxidation of malonate by nephrectomized rats was shown by WICK, WOLFE *and* NAKADA [30]. It should be noted that the views stating the primary role of decarboxylation are not uniform as to the end-product of this process. According to several authors the end-product corresponds to acetic acid [9—15], while others have shown it to be a substance related to propionic acid [14, 31]. The condensation of acetic acid into aceto-acetic acid following the formation of the former [19] and the production of succinic acid [20] have also been described.

From the data obtained in this study the primary role of oxidation may be presumed, as (i) compared to the $O_2$ uptake the beginning of $CO_2$ production is delayed ; (ii) at start the R. Q. curve is well under 1 ; (iii) decarboxylation fails to take place under anaerobic conditions ; (iv) the addition of oxidation inhibitors stop the production of $CO_2$ as well, while $O_2$ uptake is not inhibited by substances paralysing decarboxylation.

In a previous experiment with *Botryotinia fuckeliana* the author demonstrated the primary role of decarboxylation, as this reaction was anaerobic and it remained so after homogenization in spite of the lost oxidative activity.

Although drawing final conclusions on the basis of the present investigations would not be justified, some observations regarding the breakdown of malonic acid will be discussed. From the chemical structure of malonic acid it follows that oxidation affects the hydrogen atoms of the methylene group, whose reactivity is well known [32]. The R. Q. value remaining approximately at 1 for a long period shows the probable intermediate role of glyoxylic acid. The formation of an organic acid as an end-product instead of $CO_2$ and $O_2$ is obvious, since in contrast to the values found, in case of perfect oxidation the R. Q. would be 1.5. In accordance with this, from 10 day cultures an organic acid with low $R_f$ value could be isolated by paper chromatography.*

* Unpublished data.

The results obtained by the inhibitors suggest the role of various enzymes. This, however, could only be confirmed by experiments with cell-free extracts of the organisms.

The presence of small quantities of malonic acid in the inoculum causes the alteration of the results, *e. g.* in using malonic acid as an inhibitor to the dehydrogenase enzymes of *Candida albicans* cultured on malt or molasses agar.* Some vegetables as beets and germinating barley contain considerable quantities of malonic acid. Malt and molasses agar probably also contain some malonic acid, thus organisms cultivated on these media may, to a degree, be trained to this substance.

From the present investigations a further conclusion can be drawn. In the supposition that the role of malonic acid in the organism is restricted to an inhibitory action on enzymes, it has been employed for inhibiting the reactions of living cells or enzyme preparations. Our results make it clear that, considering the nutrient role of malonic acid, this conception has to be reviewed. Malonic acid should be used only in definite cases with adequate controls, and the results should be evaluated with caution.

## Summary

In the utilization of malonate by *Candida albicans*, oxidation has been shown to play a primary role over decarboxylation. In the first period of respiration in malonic acid medium a delay was found to occur in the production of $CO_2$, as compared to the $O_2$ uptake. Some substances inhibited simultaneously both $O_2$ uptake and $CO_2$ production, while others, confirming the independence of oxidation, inhibited the production of $CO_2$ earlier. The secondary role of $CO_2$ production was proved by its dependence on the inhibition of $O_2$ uptake. The lack of $CO_2$ production under anaerobic conditions also confirmed the primary metabolic role of oxidation.

From the values of the respiratory quotient, it has been concluded to the intermediate role of glyoxylic acid and the existence of a further oxidated end-product.

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# DATA ON THE SIGNIFICANCE OF STANDARDIZATION IN DETERMINING O-ANTISTREPTOLYSIN TITRE

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(Received December 4, 1958)

The significance of antistreptolysin determination in the laboratory diagnosis of rheumatic fever and other streptococcal diseases was first emphasized by Todd [1] in the thirties. Later, when repeating Todd's experiments, Hodge and Swift [2] found that reproducible results could be obtained only under carefully established conditions, as both the active (reduced) and the inactive (oxidized) forms of O-streptolysin (OSL) were able to bind O-antistreptolysin (ASO), while the haemolytic activity was only associated with the reduced form. Thus, when like in Todd's first experiments ASO titre was simply determined as the highest dilution of serum inhibiting haemolytic activity of a given amount of OSL, remarkable irregularities were caused by the fact that different OSL preparations contained oxidized and reduced OSL in different proportions.

Since 1945, the *State Seruminstitut, Copenhagen,* has been producing an international standard ASO preparation according to the principles of Ipsen [3]. By the use of this preparation, a direct comparison of the titres of the standard ASO and the test sera was made possible, avoiding inconsequencies possibly resulting from the actual composition characteristics of the OSL preparation employed.

Though the use of ASO standard had been strongly recommended by Ipsen [3] and further emphasized also by Köhler [6, 7] and Hedberg [8], examinations not using the above standard have still been published [4, 5]. Several authors from different laboratories using the same method have found ASO standardization to be of prominent importance. The present work was carried out to establish whether or not the use of standard ASO allows a comparative evaluation of the results obtained by different methods.

## Materials and methods

*Sera.* 100 random, fresh, inactivated serum specimens from the routine material of the *Research Laboratory of the National Institute of Rheumatism and Medical Hydrology* were examined.

*Streptolysin.* Unconcentrated OSL preparations (batch Nos. 6, 7 and 8), prepared according to Kalbak [9] at the *Research Institute for Vaccine Production*, were used.

To characterize OSL preparations, their minimum haemolytic dose (MHD) and the combining unit (CU) were determined. MHD represented an amount of OSL that haemolyzed

**Table I**

| Values obtained by | | Differences in titre grades | Incidence |
|---|---|---|---|
| tube | micro | | |
| method | | | |
| 50 | 84 | +3 | 1 |
| 60 | 42 | —2 | 3 |
| | 60 | 0 | 3 |
| | 70 | +1 | 1 |
| 70 | 42 | —3 | 2 |
| | 84 | +1 | 2 |
| 84 | 70 | —1 | 1 |
| | 84 | 0 | 4 |
| | 100 | +1 | 1 |
| | 120 | +2 | 1 |
| | 143 | +3 | 1 |
| 100 | 84 | —1 | 6 |
| | 100 | 0 | 2 |
| | 170 | +3 | 1 |
| 120 | 50 | —5 | 1 |
| | 84 | —2 | 2 |
| | 120 | 0 | 4 |
| 143 | 70 | —4 | 1 |
| | 84 | —3 | 1 |
| | 120 | —1 | 3 |
| | 143 | 0 | 2 |
| | 170 | +1 | 2 |
| 170 | 100 | —3 | 2 |
| | 120 | —2 | 2 |
| | 143 | —1 | 2 |
| | 170 | 0 | 2 |
| | 200 | +1 | 9 |
| 200 | 100 | —4 | 1 |
| | 140 | —2 | 2 |
| | 170 | —1 | 7 |
| | 200 | 0 | 3 |
| | 240 | +1 | 1 |
| | 336 | +3 | 1 |
| 240 | 170 | —2 | 1 |
| | 200 | —1 | 2 |
| | 240 | 0 | 2 |
| | 286 | +1 | 1 |
| | 336 | +2 | 1 |
| 286 | 170 | —3 | 2 |
| | 200 | —2 | 3 |
| | 240 | —1 | 3 |
| 336 | 336 | 0 | 1 |
| 400 | 336 | —1 | 2 |
| 672 | 400 | —3 | 1 |
| | 560 | —1 | 1 |
| 1120 | 560 | —4 | 1 |
| 1344 | 1344 | 0 | 1 |
| 1600 | 960 | —3 | 1 |
| Total | | | 100 |

0.5 ml 2 per cent sheep erythrocyte suspension in 1.5 ml final volume. CU corresponded to an amount of OSL that neutralized completely a single international unit of ASO. For our preparations used in the present experiment, the following values have been found : OSL batch number 6, one CU corresponded to 15 MHD ; batch No. 7, one CU contained 6 MHD ; batch No. 8, one CU contained 4 MHD.

*ASO Standard.* Standard ASO prepared by the *Research Institute for Vaccine Production* (batch No. 4) equivalent to the international Danish ASO standard was used throughout.

*Methods of titration.* At the Department for Serology of the *Research Institute for Vaccine Production* the tube method as modified by BÖSZÖRMÉNYI [10] was used. The final volume was 2 ml containing 1 ml serum dilution, 0.5 ml OSL solution and 0.5 ml 2 per cent washed sheep erythrocyte suspension, the latter being added after 15 minutes of incubation. At the *Research Laboratory of the National Institute of Rheumatism and Medical Hydrology* the micromethod of BOZSÓKY [11] was used. Titrations were performed with the micro-titration device described by TAKÁTSY[12]. The final volume was 0.1 ml consisting of 0.05 ml serum dilution, 0.025 ml of OSL solution and 0.025 ml of 2 per cent washed sheep erythrocyte suspension, the latter being added after 15 minutes of incubation.

In both the BÖSZÖRMÉNYI [10] and the BOZSÓKY [11] method parallel with the test serum titrations a dilution series of ASO standard was also prepared and OSL and sheep erythrocytes added.

The actual titre was calculated by comparing the titre observed in the patient serum with the titre of the ASO standard, the latter being used as a factor.

## Results

The results obtained by titrating the 100 serum samples according to the two different titration methods are presented in *Table I*. Comparison of the two methods was made on the basis of differences observed in the titre. The unit of difference was the quotient of the dilution series ($\sqrt[4]{2}$). A titre difference amounting to 4 units equalled quotient 2, thus half resp. double values. There were only four cases when the difference exceeded 3 units, while 83 out of the 100 differences were within the range $\frac{1}{\sqrt{2}}$ to $\sqrt{2}$.

The absolute titre values were mostly low, indicating a moderate incidence of acute streptococcal infection among the patients examined.

In *Table II* we present the distribution of differences obtained by comparing the results of micro-titrations to those of tube titrations. It is to be seen that the most frequent (27) difference was one unit to the advantage of the tube method. When computing the mean of the differences, the differences of the same value were grouped and weighted by their respective frequencies. As the weighted sum of differences was found to be 97 downwards (lower values by the micromethod) and 35 upwards, these figures yield an average difference of $\frac{35-97}{100} = -0.62$ unit.

When arbitrarily taking the average result yielded by the tube method as 100, that of the micromethod is $\frac{100}{\left(\sqrt[4]{2}\right)^{0.62}} = 89.8$ (where $\frac{1}{\sqrt[4]{2}}$ represents one unit difference downwards), i. e. titrations carried out by the micromethod generally yield 10.2 per cent lower values.

**Table II**

| Differences in titre grades x | Incidences observed m' | | Normal distribution m | | $\frac{(m'-m)^2}{m}$ |
|---|---|---|---|---|---|
| — 5 ........... | 1 | | 0.81 | | |
| — 4 ........... | 3 | 13 | 2.90 | 12.18 | 0.055 |
| — 3 ........... | 9 | | 8.47 | | |
| — 2 ........... | 13 | | 17.08 | | 0.975 |
| — 1 ........... | 27 | | 23.69 | | 0.462 |
| 0 ........... | 22 | | 22.66 | | 0.019 |
| 1 ........... | 19 | | 14.95 | | 1.097 |
| 2 ........... | 2 | 6 | 6.78 | 9.44 | 1.254 |
| 3 ........... | 4 | | 2.66 | | |
| Total ........... | 100 | | 100 00 | | $\chi^2_{[3]} = 3.862$ |

Normal difference in titre grades $\bar{x} = -0.62$
Standard deviation = 1.613

P = 29 per cent

The distribution of differences of the results obtained by the two methods was in fair agreement with the normal distribution curve. In the third column of *Table II* we present the calculated incidences of normal distribution at the given mean and spread.

The comparison of the normal and the actual distribution was made by the $\chi^2$ test [13]. Results are given in the fourth column of *Table II*. Differences in the results obtained by the micromethod and the tube-method could thus been regarded as being in good agreement within the limits of sampling error.

As to the differences in the results obtained by the micromethod and the tube-method, the examination of significance was also made [13]. We recall here that the average difference was 10.2 as expressed in per cent and −0.62 in titre grades. Significance was calculated by the formula,

$$t_{[99]} = \frac{\bar{x}}{S}\sqrt{N} = \frac{0.62}{1.613}\sqrt{100} = 3.84$$

$$P < 0.1\%$$

According to this result, the very moderate difference (10.2 per cent) between the values obtained by the two different techniques may be regarded as statistically significant. Consequently, the accuracy of titres observed

by either method was very satisfactory within the limits of sampling error. In this particular case the significance of the 10 per cent difference, being practically unimportant, proves the good agreement and exactness of both methods.

## Discussion

The results of ASO determinations performed in different laboratories by two different methods exhibited a statistically significant deviation of 10.2 per cent. This represented a fair parallelism, as in serological tests using twofold dilution series clinical laboratories usually consider a one-step deviation as a value within acceptable limits of error. KALBAK [9], when working with standard ASO under standardized conditions, found a $\pm$ 20 per cent deviation in parallel experiments and gave this value as the sensitivity limit of ASO determination. As a conclusion, the good agreement of our results obtained in different laboratories and by different methods may be ascribed to the use of a common ASO standard as a factor [3, 10]. The most important advantage of this method seems to be its independence of the actual composition characteristics of the OSL preparation used.

Incompatibilities in the results obtained in different laboratories not using standard ASO preparations [5] and determining the appropriate amount of OSL only by titrating its haemolytic activity, may readily be explained by the reasons presented above.

## Summary

Sera of 100 random patients have been titrated for their O-antistreptolysin content. Titrations were carried out in two different laboratories by two different methods, both making use of the same standard ASO preparation as a factor. Though the Streptolysin-O preparations corresponding to one minimal haemolytic dose represented different numbers of combining units, a deviation of only 10.2 per cent was found in the parallel results if the appropriate amount of Streptolysin-O was determined by its standard O-antistreptolysin-binding capacity.

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# NOCARDIA UNIFORMIS, A NEW SPECIES FROM SOLONETZ SOIL

By

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(Received December 19, 1958)

In examining the microflora of the degraded solonchak-solonetz soil occurring in extensive areas of the Hortobágy steppe, Eastern Hungary, some strains of a new species belonging to the genus *Nocardia* [7] were isolated. These organisms occurred exclusively in the deep region of the columnar $B_1$ horizon of the soil. In the annual dynamic variation the maximum accumulation of soluble salts has been observed in this same region. In connection with this, the strains of the new species proved to be highly salt-tolerant organisms.

## Materials and methods

Cultural properties were examined by using the following media. 1. Glucose-asparagine agar. 2. Synthetic agar. 3. Fluid synthetic medium. 4. Fluid glucose-$KNO_3$ medium. 5. Peptone-glucose agar. 6. Starch agar. 7. Peptone-meat-extract agar. Media 1 to 7 were prepared as described by LINDENBEIN [4]. 8. Glucose-tryptone agar of BURCKHOLDER *et al.* [2]. 9. Peptone-glycerol agar. 10. Potato agar. 11. Carrot agar. Media 9 to 11 were prepared according to BALDACCI *et al.* [1]. Utilization of carbon sources was examined by using the synthetic basal media described by PRIDHAM *and* GOTTLIEB [6] and by MCCLUNG [5]. Acid-fastness was demonstrated according to GORDON *and* SMIDT [3].

## Nocardia uniformis nov. spec.

*Morphology.* At the beginning of cultivation, the filaments of the substrate mycelium rapidly break up into rods and less frequently into coccoid bodies. The size of these forms is 0.7—1.1 $\mu$ by 1.1—4.0 $\mu$. In old cultures swollen, club or bottle shaped forms appear. The hyphae of the slightly developed aerial mycelium are straight or waved, nonseptate and contain oval oidiospores. The mycelium is Gram positive and is not acid-fast.

*Cultural properties.* On any of the agar media tested, an uniform type of colonies is formed. The strains give non-butyrous colonies growing into the agar, with moderately striated dull surface covered with slightly developed white powder-like aerial mycelium. The colour of the colonies is a constant yellowish orange, it never turns into red or yellow; no soluble pigment is produced. In fluid synthetic media a surface pellicle resembling agar colonies

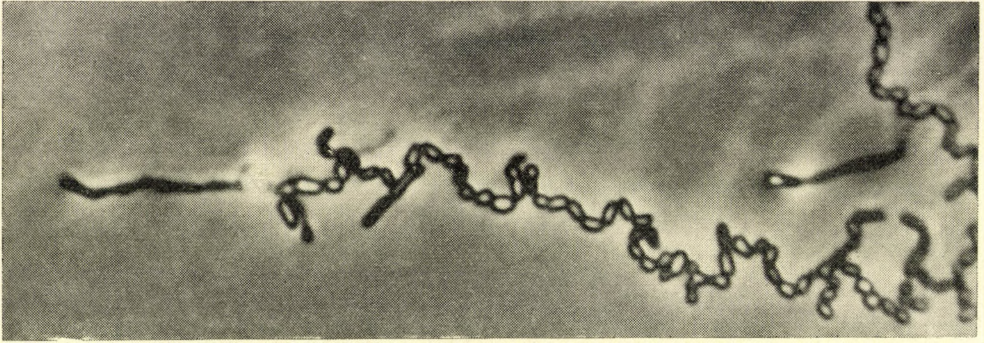

*Fig. 1.* Vegetative mycelium breaking up into rods and coccoid bodies. From a wet-chamber culture, 4 days, glucose-asparagine agar. Photo : Objective, Ph HI 90 ; ocular, Project 6, 3 : 1

*Fig. 2.* Club shaped forms from a 9 day old wet-chamber culture. Photo : as indicated above

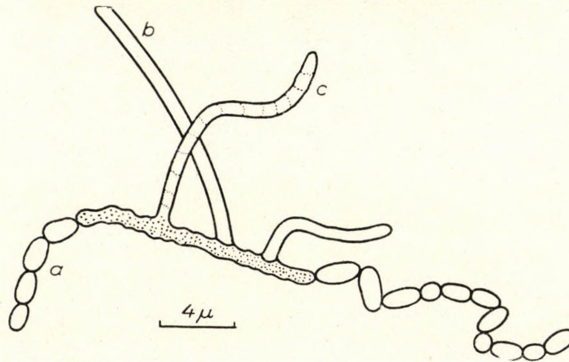


*Fig. 3.* Schematical representation showing breaking up of the substrate mycelium (a), the filaments of the aerial mycelium (b) and formation of cylindrical oidiospores (c)

**Table I**

*Utilization of carbon sources by N. uniformis*

| | Pridham and Gottlieb's synthetic agar medium, N source: $(NH_4)_2 SO_4$ | | | McClung's synthetic medium N source: $NaNO_3$ | |
|---|---|---|---|---|---|
| | | | | fluid medium | agar slant medium |
| Strains | B—X/a | B—X/b | B—X/c | B—X/a | B—X/a |
| l-arabinose | — | — | — | — | — |
| d-xylose | — | (—) | — | — | — |
| d-galactose | — | — | — | — | — |
| d-glucose | + | + | + | + | + |
| d-fructose | + | + | + | + | + |
| d-mannose | — | (—) | — | — | (—) |
| l-sorbose | — | — | — | — | — |
| cellobiose | + | + | + | + | + |
| lactose | (—) | (—) | — | — | — |
| maltose | (—) | — | — | (—) | — |
| sucrose | — | — | — | — | — |
| raffinose | — | — | — | — | — |
| l-rhamnose | — | — | — | — | — |
| melicitose | — | — | — | — | — |
| dulcitol | — | — | — | — | — |
| glycerol | + | + | + | + | + |
| d-mannitol | + | + | + | + | + |
| d-sorbitol | (—) | (+) | (—) | (—) | (—) |
| adonitol | — | — | — | — | — |
| salicin | — | — | — | — | — |
| mesoinositol | + | + | + | + | + |
| dextrin | — | — | — | — | — |
| inulin | — | — | — | — | (—) |
| starch | — | — | — | — | — |
| glycogen | — | — | — | — | — |
| sodium acetate | (—) | (—) | — | — | — |
| sodium citrate | — | — | — | — | — |
| sodium formiate | — | — | — | — | — |
| sodium oxalate | — | — | — | — | — |
| sodium tartrate | — | — | — | — | — |
| paraffin | . | . | . | — | . |
| control without C | — | — | — | — | — |

Key: + = good growth, definite utilization — = no utilization
(+) = poor growth, doubtful utilization . = not examined
(—) = very poor growth

is formed. In fluid glucose-$KNO_3$ medium the cultures give a yellowish orange deposit.

*Physiological properties.* No liquefaction of gelatin, peptonization and coagulation of milk, hydrolysis of starch, inverting of sucrose or decomposition of cellulose. Utilization of paraffin slight or none ; no decomposition of fats. Nitrates rapidly reduced to nitrites. No haemolysin, $H_2S$, antibiotics or bacteriolytic substances produced. Optimum temperature of incubation, 14—37° C ; optimum pH, 6.0—10.0. Growth not inhibited by 30 per cent $Na_2SO_4 \cdot 10\ H_2O$, 30 per cent $MgSO_4 \cdot 7\ H_2O$, 10—11 per cent NaCl and 12 per cent $NaNO_3$. The utilization of various substances as source of carbon is shown in *Table I*, which shows that both important basal media give an uniform spectrum of utilization.

## Discussion

The most characteristic property of *N. uniformis* is its uniform appearance on a variety of media. It markedly differs from other *Nocardia* species in its unusually poor physiological activity. In contrast to the others, *N. uniformis* does not utilizes uch important sources of carbon as mannose, dextrin and inulin [5]. In the genus *Nocardia* the most limited spectrum of carbon source utilization is shown by this species. This spectrum has been stable on cultivation. As, according to recent investigations [8], the utilization of various carbon sources is the chief differential characteristic of *Actinomycetaceae*, the most important properties of the new species can be summarized as follows. (i) Characteristic and very limited spectrum of carbon source utilization. (ii) Extra site of occurrence. (iii) Strikingly constant cultural behaviour.

*Note.* The strains of *N. uniformis* are maintained in the culture collection of this laboratory.

## Summary

A new species of *Nocardia* occurring in the deep region of the $B_1$ horizon of solonchak-solonetz soil has been described.

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# SOME EPIDEMIOLOGICAL CHARACTERISTICS OF VIRAL HEPATITIS IN HUNGARY

By

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(Received December 29, 1958)

Although viral hepatitis (in the following, hepatitis) has been notifiable in Hungary since 1950, reliable epidemiological data are available only since 1952. In the present report 87 000 cases of hepatitis notified during the six-year period, 1952 to 1957, are analysed.

*Table I* shows that, like in most countries [1], in Hungary the number of notified cases was increasing from year to year during that period. However, the earlier the data the less reliable they are. The increase in the incidence cannot therefore be regarded as caused exclusively by a wider spread of the disease.

**Table I**

*Notified cases of, and deaths by, hepatitis, 1952—1957*

| Year | Cases in | | | Deaths in | | |
|---|---|---|---|---|---|---|
| | Budapest | Country | Hungary | Budapest | Country | Hungary |
| 1952. | 2402 | 4849 | 7251 | 8 | 61 | 69 |
| 1953. | 3590 | 9219 | 12809 | 47 | 122 | 169 |
| 1954. | 3532 | 9323 | 12855 | 54 | 113 | 167 |
| 1955. | 4289 | 11294 | 15583 | 38 | 114 | 152 |
| 1956. | 5197 | 14418 | 19615 | 51 | 118 | 169 |
| 1957. | 3922 | 15208 | 19130 | 39 | 99 | 138 |

In each of the years 1956 and 1957 the number of notified cases approximated 20 000 ; of these, 5000 and 4000, respectively, occurred in Budapest. It is to be noted, however, that intensive investigations in some areas revealed at least as many cases to have escaped notice as those that had been recognized [2]. Supposing that this is a general rule, *the yearly incidence of hepatitis in Hungary, including recognized and unrecognized cases, might be estimated to 40 000.*

The figures to be quoted in the following represent, or have been calculated from, the notified cases only.

*Table II* contains some summarizing ratios.

**Table II**

*Hepatitis morbidity, mortality (per 100 000 inhabitants) and case fatality (per cent), 1952—1957*

| Designation | 1952 | 1953 | 1954 | 1955 | 1956 | 1957 | 1952—57 average |
|---|---|---|---|---|---|---|---|
| Budapest: | | | | | | | |
| Morbidity rate ... | 138.0 | 201.1 | 193.0 | 228.7 | 279.4 | 210.9 | 209.1 |
| Mortality rate ... | 0.5 | 2.6 | 2.9 | 2.0 | 2.7 | 2.1 | 2.2 |
| Case fatality ..... | 0.3 | 1.3 | 1.5 | 0.9 | 1.0 | 1.0 | 1.0 |
| Country: | | | | | | | |
| Morbidity rate ... | 62.5 | 118.2 | 188.6 | 142.2 | 181.2 | 191.5 | 136.1 |
| Mortality rate ... | 0.8 | 1.6 | 1.4 | 1.4 | 1.5 | 1.2 | 1.3 |
| Case fatality ..... | 1.2 | 1.3 | 1.2 | 1.0 | 0.9 | 0.6 | 1.0 |
| Hungary: | | | | | | | |
| Morbidity rate ... | 76.3 | 133.6 | 132.7 | 158.9 | 199.5 | 194.9 | 149.8 |
| Mortality rate ... | 0.7 | 1.8 | 1.7 | 1.6 | 1.7 | 1.4 | 1.5 |
| Case fatality ..... | 1.0 | 1.3 | 1.3 | 1.0 | 0.9 | 0.7 | 1.0 |

The average morbidity rate for Hungary in the six-year period was 150 per 100 000 ; it was markedly higher for Budapest (209) than for the country (136). In each of the six years, morbidity for the capital was higher than that for the country, but the difference was diminishing from year to year. In 1952, the former was more than twice higher than the latter, whereas in 1957 the difference did not exceed 9 per cent. The decrease in the difference might be ascribed to the improving accuracy of reporting cases in the country.

The average hepatitis mortality rate was 1.5 per 100 000 for Hungary ; it was also higher for the capital (2.2) than for the country (1.3).

The *average case fatality was equally 1.0 per cent* for Budapest, the country and the whole of Hungary. Nevertheless, case fatality for the country trended downwards while that for Budapest did not change remarkably. The decreasing fatality in the country, that can be attributed to an improvement in reporting cases rather than to the improvement of therapeutical measures, resulted in a remarkable difference in case fatality between Budapest (1.0) and the country (0.6) in 1957.

The monthly incidence of hepatitis in Hungary, in Budapest and in the country from 1950 to 1957 is illustrated in *Fig. 1.*

The seasonal trend seems to be characteristic. The Budapest curve and the country curve run nearly parallel during the first 9 months of each year and the curves reach or approximate their peak in October. In the last two months, however, the two curves diverge from each other. This is illus-

trated in *Fig 2*, presenting the average seasonal trend for the six-year period, 1952 to 1957.

*Fig. 2* shows that hepatitis incidence, both in the capital and in the country, was lowest during the second quarter of the year. Both curves

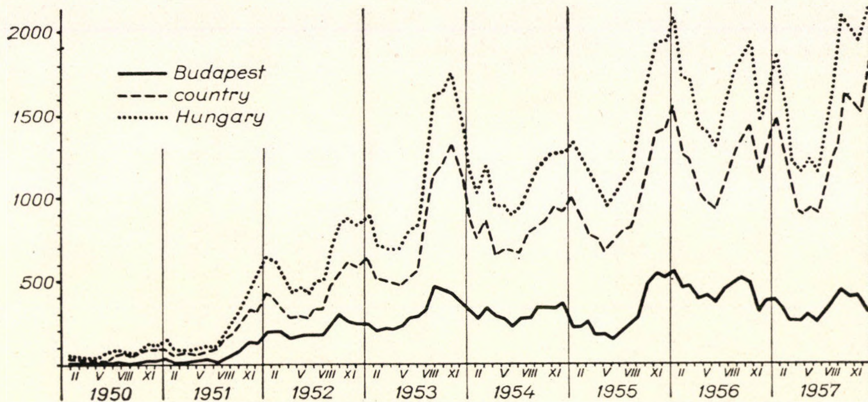


*Fig. 1.* Incidence of hepatitis by month in Hungary, 1950—1957

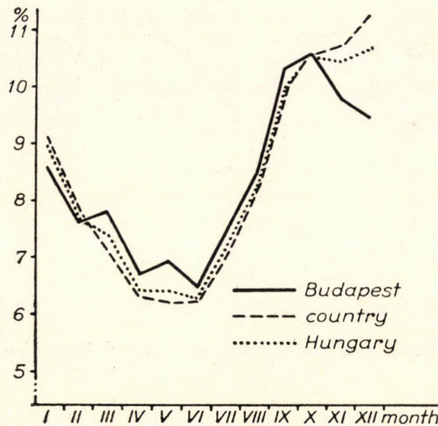


*Fig. 2.* Incidence of hepatitis by month. Per cent of the yearly incidence, 1952—1957

begin to rise in July and rise until in October they have reached an approximately equal value. Thereafter the Budapest curve trends downwards while the country curve, though moderately, rises further, even in December. The seasonal curve for Hungary, being determined mainly by the country curve, also has its peak in December.

The seasonal trend of hepatitis in Budapest resembles the well-known trend of the infectious diseases spread by the enteric route. The shift of the

peak to October can be explained by the long incubation period of hepatitis. The October cases must have been infected in August or September when enteric infections are commonest.

As to the country curve, the highest incidence of hepatitis in December indicates that infection had occurred most frequently on October and November, *i. e.* in a season when the incidence of enteric diseases is strongly declining. Our data do not allow further conclusions.

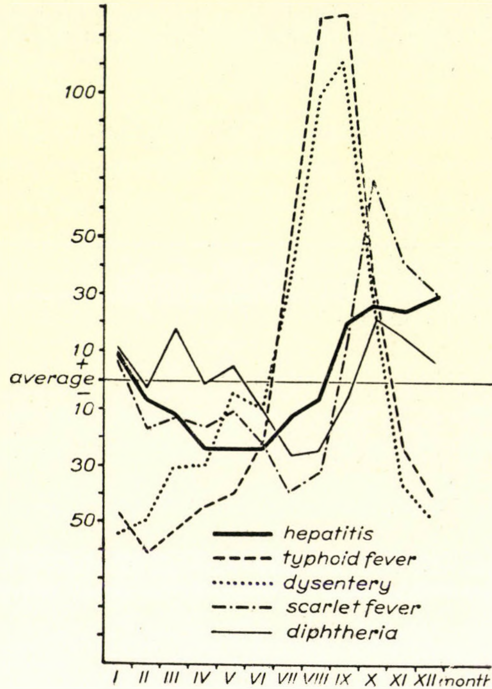


*Fig. 3.* Average seasonal fluctuation of 5 infectious diseases, 1952—1957

Several authors observed the peak of the hepatitis curve to shift to November or December, or even to January [2]. The phenomenon was explained as follows. After the school year has started, the chance of infection is great and the number of susceptible children is high. One month or two months have to pass until as large a proportion of children will have been infected and acquired immunity as sufficient to incidence [3]. This assumption is supported by the age distribution of the cases under discussion as presented below.

In *Fig. 3* the seasonal trend of hepatitis is compared with those of two enteric diseases (typhoid fever and dysentery) and two infectious diseases mainly transmitted by droplets (scarlet fever and diphtheria). The rise of

the hepatitis curve lags one or two months behind those of enteric diseases and is still rising in the last two months of the year when even the curves of the two airborne diseases show a marked decline.

The age distribution is presented in *Fig. 4* and, in detail, *in Table III*.

*Fig. 4* shows that in the six-year period, 1952 to 1957, *children aged 3 to 10 years displayed a considerably higher morbidity rate than the younger and older age groups.*

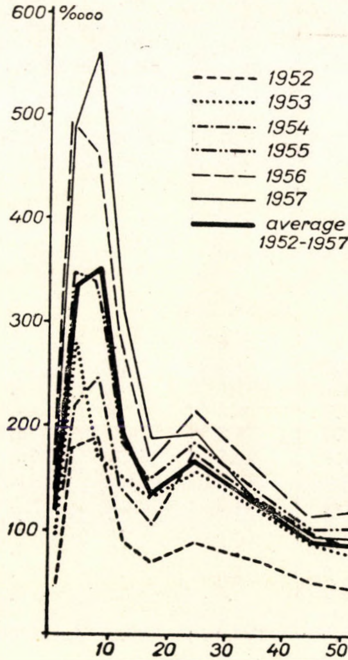


*Fig. 4.* Age specific hepatitis morbidity rates, 1952—1957

According to the data presented in *Table III*, 43.4 per cent of the patients were under 15 years of age. To obtain more precise information, age specific morbidity rates were calculated. The case incidence for 100 000 children between 3 and 6 years of age was 490 in 1956, and 488 in 1957; the corresponding figures for 7 to 10 year-old children were 463 and 560 respectively. Beyond these age groups the age specific morbidity rate shows a gradual decline disturbed only by a slight rise at 20—29 years of age. It can be concluded, therefore, that in the period in question hepatitis occurred most frequently in the age group of 3 to 10 years, *i. e.* among children of the kindergarten age and those in the lower forms of primary school.

The age specific morbidity rate, as calculated for each year of age under 15 and illustrated in *Fig. 5*, allows more detailed analysis. The curve

**Table III**

*Age distribution of hepatitis,* 1952—57

| Age group years | 1952. | 1953. | 1954. | 1955. | 1956. | 1957. | 1952—1957 total |
|---|---|---|---|---|---|---|---|
| | | | A. Number of notified cases | | | | |
| 0— 2 ....... | 231 | 502 | 646 | 800 | 956 | 740 | 3875 |
| 3— 6 ....... | 1132 | 1851 | 1485 | 2387 | 3428 | 3520 | 13803 |
| 7—10 ....... | 1159 | 1810 | 1484 | 2094 | 2936 | 3690 | 13173 |
| 11—14 ....... | 527 | 1023 | 845 | 1154 | 1671 | 1845 | 7065 |
| 15—19 ....... | 511 | 978 | 838 | 1076 | 1184 | 1337 | 5924 |
| 20—29 ....... | 1381 | 2452 | 2671 | 2850 | 3178 | 2749 | 15281 |
| 30—39 ....... | 906 | 1607 | 1731 | 1789 | 2173 | 1857 | 10063 |
| 40—49 ....... | 690 | 1208 | 1443 | 1432 | 1497 | 1219 | 7489 |
| 50—59 ....... | 438 | 830 | 931 | 1201 | 1416 | 1105 | 5921 |
| 60—69 ....... | 179 | 371 | 518 | 560 | 732 | 668 | 3082 |
| 70 ........... | 62 | 134 | 160 | 202 | 308 | 257 | 1123 |
| unknown ..... | 35 | 43 | 103 | 38 | 136 | 143 | 498 |
| Total ........ | 7251 | 12809 | 12855 | 15583 | 19615 | 19130 | 87243 |
| | | | B. Per cent | | | | |
| 0— 2 ....... | 3.2 | 3.9 | 5.0 | 5.1 | 4.9 | 3.9 | 4.4 |
| 3— 6 ....... | 15.6 | 14.4 | 11.5 | 15.3 | 17.2 | 18.4 | 15.8 |
| 7—10 ....... | 16.0 | 14.1 | 11.5 | 14 3 | 15.0 | 19.3 | 15.1 |
| 11—14 ....... | 7.3 | 8.0 | 6.6 | 7.4 | 8.5 | 9.6 | 8.1 |
| 15—19 ....... | 7.0 | 7.6 | 6.5 | 6.9 | 6.1 | 7.0 | 6.8 |
| 20—29 ....... | 19.0 | 19.1 | 20.8 | 18.5 | 16.2 | 14.4 | 17.5 |
| 30—39 ....... | 12.5 | 12.5 | 13.5 | 11.5 | 11.1 | 9.7 | 11.5 |
| 40—49 ....... | 9.5 | 9.4 | 11.2 | 9.2 | 7.7 | 6.4 | 8.6 |
| 50—59 ....... | 6.0 | 6.8 | 7.3 | 7.7 | 7.2 | 5.8 | 6.8 |
| 60—69 ....... | 2.5 | 2.9 | 4.0 | 3.6 | 3.8 | 3.5 | 3.5 |
| 70 ........... | 0.9 | 1.0 | 1.3 | 1.3 | 1.6 | 1.3 | 1.3 |
| unknown ..... | 0.5 | 0.3 | 0.8 | 0.3 | 0.7 | 0.7 | 0.6 |
| Total ........ | 100.0 | 100.0 | 100.0 | 100.0 | 100.0 | 100.0 | 100.0 |
| | | | C. Age specific morbidity rate per 100 000 | | | | |
| 0— 2 ....... | 43.8 | 93.9 | 115.6 | 135.9 | 161.0 | 132.1 | 115.2 |
| 3— 6 ....... | 177.1 | 276.9 | 215.7 | 344.5 | 490.1 | 488.0 | 335.8 |
| 7—10 ....... | 187.9 | 297.0 | 243.3 | 337.9 | 463.0 | 559.7 | 351.3 |
| 11—14 ....... | 89.0 | 171.4 | 139.8 | 188.5 | 274.2 | 310.1 | 195.7 |
| 15—19 ....... | 69.1 | 133.5 | 114.9 | 147.2 | 164.9 | 187.7 | 135.7 |
| 20—29 ....... | 88.7 | 158.5 | 173.5 | 186.4 | 213.4 | 190.2 | 167.8 |
| 30—39 ....... | 73.4 | 129.7 | 139.4 | 141.0 | 164.4 | 134.6 | 131.0 |
| 40—49 ....... | 50.4 | 87.1 | 102.9 | 102.3 | 110.7 | 95.7 | 91.4 |
| 50—59 ....... | 40.5 | 74.9 | 82.3 | 104.5 | 121.7 | 93.7 | 86.9 |
| 60 ........... | 21.1 | 43.4 | 57.2 | 62.7 | 83.3 | 72.2 | 57.4 |
| Total ........ | 76.3 | 133.6 | 132.7 | 158.9 | 199.5 | 194.9 | 149.8 |

representing the average yearly attack rates for the period 1952 to 1957, appears regular in shape, showing a minimum at one year of age, then a rather steep rise up to 6 years and a gradual decline thereafter. Both the

ascending and descending parts of the curve are approximately straight : *the curve has no secondary peaks at the age groups involved in the official vaccination programme.* In Hungary, vaccination of the following age groups was compulsory during the given period. (i) Infants under one year of age were vaccinated with combined diphtheria-pertussis-tetanus vaccine ; (ii) one-

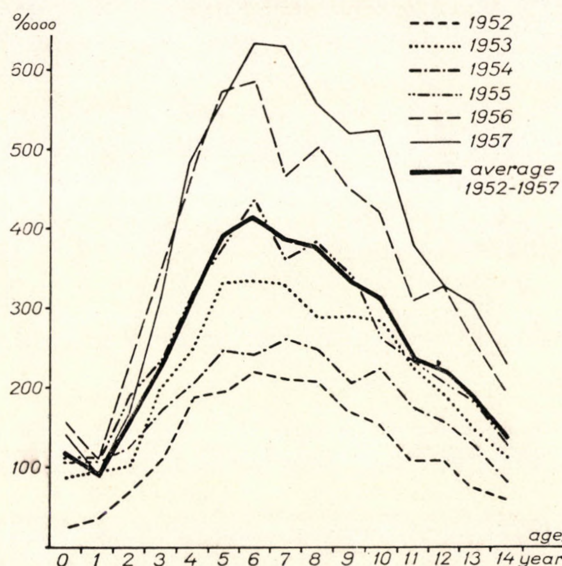


*Fig. 5.* Age specific hepatitis morbidity rates for children 0 to 14 years of age, 1952—1957

year-old children with the same vaccine and against smallpox ; (iii) six- or seven-year-old children in the first form of primary school were revaccinated with diphtheria-pertussis-tetanus vaccine ; (iv) children aged 12 or 13 years in the sixth form of primary school were vaccinated with a combined typhoid-tetanus vaccine. The lack of any irregularity in the hepatitis curve at the age groups listed above suggests that *mass vaccination performed either intramuscularly or subcutaneously or by scarification had no part in the transmission of the disease.*

The age distribution of hepatitis is similar to that of the infectious fevers generally termed "children's diseases". In these the relative resistance of adults can be explained by an acquired immunity induced by a previous attack or latent infection. A similar assumption concerning hepatitis cannot be proved until an appropriate laboratory procedure for testing immunity will have been available.

The monthly incidence of hepatitis in different age groups is illustrated in *Fig. 6.* The curves for Budapest and those for the country have been drawn separately.

Among the curves illustrating incidence in the country, the curves for the 3 to 14 year-old children show a very definite seasonal trend, in contrast to the curves related to the groups under 3 and over 30 years of age showing no seasonal fluctuation. It might be supposed that the groups uninfluenced by the season are mainly affected by serum hepatitis. The incidence of serum hepatitis was found to be independent of season by several authors. The

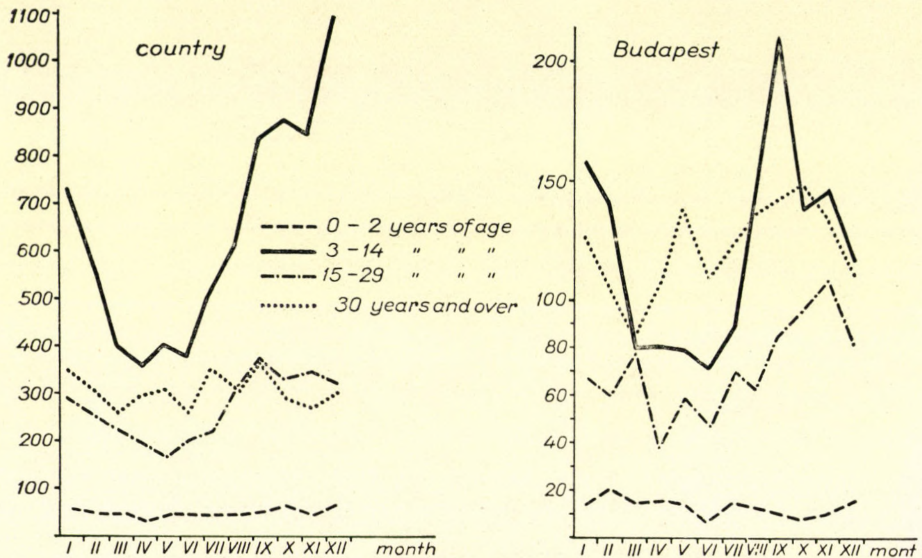


*Fig. 6.* Incidence of hepatitis by age and month, 1957

characteristic seasonal trend for the groups from 3 to 14 years of age makes it probable that in these groups, although other routes of transmission are also possible, *the enteric route is predominant*. The characteristic seasonal trend of hepatitis in the country (see *Fig. 2*) can be ascribed exclusively to the latter age groups.

The Budapest curves right in *Fig. 6* hardly differ from the corresponding country curves. The only remarkable difference is that the age groups from 3 to 14 years show the highest case incidence in September instead of December, thus the predominance of enteric infections seems to be more definite.

Case fatality by age is illustrated in *Fig. 7*. The average case fatality for the six-year period was 1.0 per cent, as mentioned above; it was about 10 times higher, 9.9 per cent, for infants under one year of age, and also high, 2.5 per cent, for the group of one and two year-old children. For the following age groups, case fatality was markedly lower, showing a minimum (0.1 per cent) between 11 and 14 years. In older age, case fatality is again higher, attaining 3.6 per cent between 60 and 69 years of age and 6.8 per

cent over 70. Thus, case fatality runs approximately parallel with the general mortality rate.

On the other hand, there is some *seasonal variance in case fatality*. *Fig. 8* illustrates the average monthly fatality rates for the six-year period,

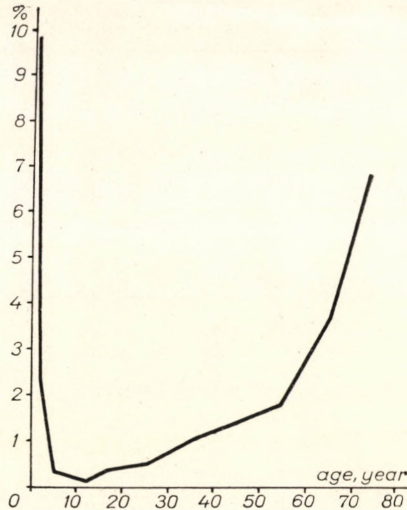


*Fig. 7.* Hepatitis case fatality by age, 1952—1957

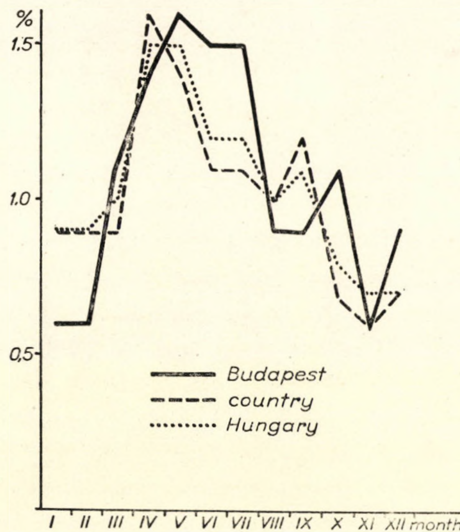


*Fig. 8.* Hepatitis case fatality by month, 1952—1957

1952 to 1957. Case fatality was lowest at the end of the year (0.6 per cent) and highest (1.6) in the spring, about 3 times as high as in December. This is due to the fact that in the spring the attack rate for the age groups dis-

playing the lowest case fatality is low, thus, a comparatively high proportion of the cases belongs to the youngest and oldest age groups showing higher case fatality.

**Table IV**

*Incidence of hepatitis among the medical staff, 1956 and 1957*

| Designation | 1956. | 1957. |
|---|---|---|
| Total number of physicians and chemists | 16 381 | 17 189 |
| Incidence | 78 | 55 |
| Morbidity per 100 000 | 476.2 | 324.8 |
| Number of medical employees | 30 962 | 34 206 |
| Incidence | 305 | 219 |
| Morbidity per 100 000 | 985.1 | 639.1 |
| Morbidity per 100 000 inhabitants over 20 year of age | 141.5 | 119.6 |

**Table V**

*Hospitalized cases of hepatitis, per cent*

| Year | Hospitalized cases, per cent | | |
|---|---|---|---|
| | Budapest | Country | Hungary |
| 1952 | 66.1 | 73.0 | 70.7 |
| 1953 | 72.6 | 69.1 | 70.1 |
| 1954 | 82.8 | 76.5 | 78.3 |
| 1955 | 81.2 | 74.4 | 78.4 |
| 1956 | 84.6 | 75.9 | 78.2 |
| 1957 | 90.2 | 72.7 | 76.3 |

It has been demonstrated that viral hepatitis is more frequent among the medical staff than in any other group of the population. *Table IV* presents the cases recorded among the medical staff in 1956 and 1957. In 1956 the general morbidity rate for adult persons (over 20 years of age) was 141 per 100 000, whereas 476 for physicians and chemists and 985 for the ungraduated medical staff. The same rates for 1957 were 120, 325 and 639, respectively. Thus, *the morbidity rate for physicians and chemists exceeds round three times, that for the ungraduated medical staff more than six times the general attack rate for adults.* The data available did not allow to separate the incidence among physicians from that among chemists. Nevertheless, physicians are thought to be definitely more frequently affected by hepatitis than chemists.

Some information on the hospitalization of patients with hepatitis is presented in *Table V*.

The general rate of hospitalization varied between 70 and 80 per cent during the six-year period. This rate was neither increasing nor decreasing significantly in the country, but showed a marked increase, from 66 per cent to 90 per cent, in Budapest.

## Summary

In Hungary viral hepatitis became notifiable as late as 1950. In 1956 and 1957 the yearly number of reported cases came close to 20 000. During the six-year period, 1952 to 1957, the attack rate, as calculated from the reported cases, was markedly higher for Budapest than for the country. The seasonal trend for Budapest and for the country was approximately the same, except for the early winter months. In Budapest after an October peak morbidity began to decline while in the country a moderate rise up to December was observed. The seasonal fluctuation for hepatitis was less marked than for other enteric infectious diseases. Most cases occurred among childern of the kindergarten age (3—6 years) and school children of the first four forms of primary school (7—10 years of age) ; the age specific attack rate shows a gradual decline thereafter. The rise and drop of the age distribution curve for the given period were continuous, showing no secondary peaks at the age groups involved in the compulsory vaccination programme. The age distribution of the cases was similar to the age distribution characteristic of infectious diseases of which the low incidence among adults can be explained by the immunity induced by a previous infection. Seasonal fluctuation in morbidity was most marked for the age groups between 3 and 14 years, whereas no such fluctuation was apparent under 3 and over 30 years of age. It is supposed that a high proportion of cases in the last two groups was due to serum hepatitis showing no characteristic seasonal trend. During the six-year period, 1952 to 1957, the attack rate for the medical staff was about 6 times higher than the general rate for the adult population. The annual case fatality attained about 1 per cent. Case fatality was about three times higher in the spring than early in winter and ran parallel with the general mortality curve, being highest among the youngest and oldest subjects.

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# PREPARATION OF TRICHOTHECIN BY FERMENTATION

By

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(Received January 14, 1959)

Brian *and* Hemming [1] were the first to devote attention to the antifungal action of *Trichothecium roseum.* The pure crystalline active substance was later isolated from surface cultures of the mould by Freeman *and* Morrison [5], and given the name trichothecin. Subsequently, they reported in detail their method of preparing the new antibiotic in submerged cultures, and the results of their studies concerned with yield, purification, and biological properties. They obtained generally 40 to 50, exceptionally 115 $\mu$g/ml yields of the active substance ; the crystalline trichothecin yields varied between 60 and 70 per cent [6, 7]. In 1950 and 1951, reports were published on the chemical properties of the antibiotic [8] and its production in soil [9]. In 1955, Freeman [4] prepared trichothecin by fermentation in a medium composed of ammonium tartarate, unpurified glucose, corn steep liquor, and inorganic salts, obtaining a maximum yield of 200 $\mu$g/ml after 90 hr incubation at 25° C. He also studied the substance for its toxicity and its activity against fungi pathogenic to man.

Phytopathology is the field in which the most appreciable practical results have been achieved with trichothecin. In Freeman *and* Morrison's spectrum [6] there figures only a single phytopathogenic fungus *(Botrytis allii),* but in the work of Darpoux *et al.* [2] it was shown as early as 1951 that the culture filtrate of *T. roseum* can be employed with success in the control of wheat smut *(Tilletia foetida).* In 1952, the same authors [3] found that this culture filtrate as well as trichothecin extract of 25 $\mu$g/ml concentration were both preventive and therapeutic agents against powdery mildew in barley. Finally, they established that *in vitro* the growth of about five important fungi was inhibited by trichothecin concentrations of 10—100 $\mu$g/ml, while at 100 $\mu$g/ml it reduced *Phoma lingam* incidence in rape from 88 to 10—14 per cent. As regards Hungary, *in vitro* tests in progress in the *Research Institute of Plant Protection* have shown trichothecin concentrations of 1—38 $\mu$g/ml to inhibit the growth of 19 phytopathogenic fungi [10]. In concentrations of less than 100 $\mu$g/ml the active substance was toxic neither to the germinating seed nor to the green parts of different plants [10, 11].

On the evidence of field experiments, spraying the flowers of sour cherry trees with trichotechin of 50 $\mu$g/ml reduced *Monilia cinerea* infestation by 70 to 95 per cent [11].

In all experiments touched upon in the foregoing, trichothecin prepared in surface cultures was used. But this mode of preparing the active substance is uneconomic ; first, the yield is too low, generally not more than 40 to 60 $\mu$g/ml ; secondly, the incubation time is too long, taking 25 to 30 days. A rising demand for this substance in ever wider field experiments on plant protection, and in attempts at using it to other ends, seemed to justify a search for some cheaper method of the preparation of trichothecin.

## Materials and methods

In each of a number of 500-ml Erlenmeyer flasks 100-ml amounts of culture fluid were inoculated with a known number of conidia. Rotated at 320 r. p. m., the cultures were incubated usually for 160 hr. From the 64th hour onward, samples were taken at 24-hour intervals to read the yields. Generally, the maximum yields were reached between the 88th and 112th hour, and were followed by slightly decreasing production.

Using 24-hour shaken inoculum, cultures were grown in 10-litre glass fermentors of 5-litre working volume or in 40-litre fermentors of iron ; in the former at 320 r. p. m., with air at 1 vol/vol/min., at 27° C. The maximum yields generally showed after 80 to 90 hr incubation.

In the bioassay for trichothecin production elaborated by FREEMAN *and* MORRISON [6], cylinder plates layered with *Penicillium digitatum* are used. Finding that readings are not possible until after three days' incubation at 25° C, and then not dependable, these authors themselves prefer the more cumbersome, but exact, *Penicillium digitatum* spore germination test.

In our investigations agar plates each provided with six cups were layered with *Saccharomyces carlsbergensis.* The inoculated layer containing 6—8 $\times$ $10^6$ cells per ml was found to be the most favourable, permitting readings to about 10 per cent accuracy after incubation for 24 hr at 26° C. With a view to attaing highest possible accuracy, on each plate a standard solution of 30 $\mu$g/ml concentration was measured into two diagonally opposed cups, and any errors due to differences in the inoculum, the thickness of the layer, the consistency of the agar, etc., were corrected on the basis of the values obtained for the standard zones. (For the crystalline trichothecin from which the standard solution was prepared we are indebted to Dr. G. G. FREEMAN.)

The correlation of the trichothecin concentrations and the diameters of the inhibited zones as observed on the use of the method described, is illustrated in *Fig. 1*.

## Results

Our shaken culture experiments were carried out in two directions : different culture fluids and different strains were studied for their capacity to yield trichothecin, the former in particular reference to the quantitative and qualitative aspects of the carbon and nitrogen sources. In addition, the effect of a number of natural substances and — in view of the intended use of iron fermentors — that of iron, was included in the investigations. Of some 40 culture fluids of different composition the one consisting of commercial saccharose, malt extract, corn steep liquor, and ammonium tartarate, was found to give the highest yield ; generally, 200 to 250 $\mu$g/ml ;

exceptionally, 300 μg/ml. *Table I* summarises the data for 13 such compositions. The presence of 100 μg/ml of ferro ion in the medium, did not influence trichothecin production.

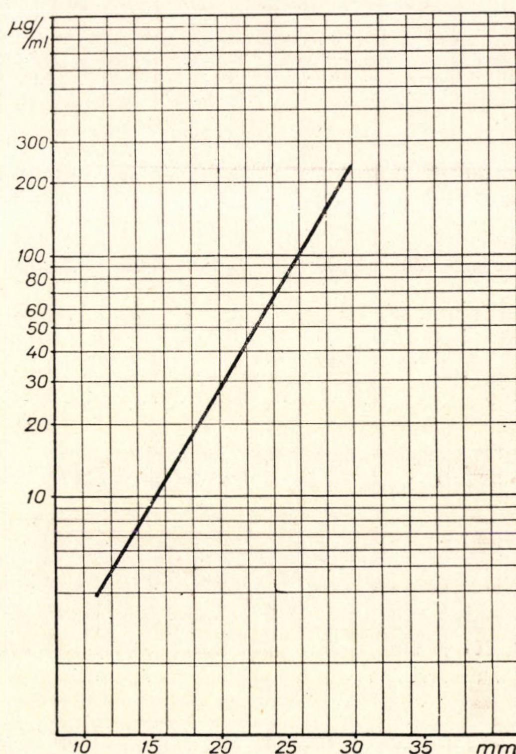


*Fig. 1.* Correlation between trichothecin concentrations and diameters of inhibited zones as observed on agar cup plates layered with *Saccharomyces carlsbergensis*

*T. roseum* strains were cultured from seven different sources, viz. :

| | | |
|---|---|---|
| From a fruit of *Pyrus communis* | — strain marked | III. 12 |
| From a fruit of *Malus pumila* | — ,, ,, | III. 109 |
| From a leaf of *Lilium candidum* | — ,, ,, | III. 181 |
| From a leaf of *Zea mays* | — ,, ,, | III. 188 |
| From a leaf of *Allium cepa* | — ,, ,, | III. 189 |
| From a twig of *Malus pumila* | — ,, ,, | III. 244 |
| From a steam of *Chenopodium sp.* | — ,, ,, | III. 245 |

Of these, the strain labelled III. 189 produced the highest yield : 280 μg/ml. The data for all seven strains are shown in *Fig. 2.*

**Table I**

*Culture fluid components in g/l*

| Serial No. | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Saccharose ..... | | | 30 | | 20 | 50 | | 10 | | | | | |
| Glucose ....... | | | | 5 | 30 | | | | | | | | |
| Lactose ....... | | | | 25 | | | | | | | | | |
| Comm. sugar .. | | 10 | | | | | 10 | | 10 | 10 | 50 | 60 | 10 |
| Starch sugar ... | 50 | | | | | | | | | | | | |
| Malt extract ... | | 50 | | | | | 50 | 50 | 50 | 50 | 50 | | 50 |
| Corn steep liquor ....... | 10 | | | 10 | 10 | 10 | 1 | 3 | 1 | 1 | 1 | 1 | 1 |
| Yeast extract... | | 5 | 20 | 4 | | | 5 | 5 | 5 | | 5 | 5 | 5 |
| Ammonium tartarate........ | 2 | 3 | | 3 | 2 | 2 | 2 | 1 | 2 | 2 | 2 | 2 | 2 |
| $MgSO_4$ ........ | 0.5 | | 0.5 | 0.5 | 0.5 | | | | | | | | (+100 $\mu$g/ml ferro ion) |
| $K_2HPO_4$ ....... | 1 | | 1 | 1 | 1 | 1 | | | | | | | |
| KCl ........... | 0.5 | | 0.5 | 0.5 | 0.5 | 0.5 | | | | | | | |
| $FeSO_4$ ......... | 0.01 | | 0.001 | 0.001 | 0.001 | 0.001 | | | | | | | |
| $NaNO_3$ | | | 3 | | | | | | | | | | |
| Max. yield of active substance in $\mu$g/ml (mean value for 3 tests) ....... | 97 | 200 | 26 | 0 | 82 | 112 | 300 | 136 | 203 | 203 | 270 | 260 | 215 |

Malt agar plates inoculated with the selected *T. roseum* strain were prepared and at once subjected to ultraviolet irradiation at 40 cm for from 1 to 60 minutes, using a "Hanau SU 700" lamp (capacity, $1.49 \times 10^{-2}$ W/sq. cm at 1 m). Treatment for 10 minutes produced no appreciable spore-killing action, while on plates irradiated for 40 minutes growth was no longer perceptible. From 2 to 8 isolated colonies were observed to develop on plates treated for 20 to 30 minutes. Colonial productive capacity was studied in shaken cultures. Only the isolate marked III. 189/c showed overproduction ; all the others produced trichothecin at the same or a slightly lower rate than did the initial culture. In *Fig. 3* we compare the yield of the initial culture with that of the only overproducing isolate.

In relation to the initial culture there was no overproduction of the active substance on agar containing 300 and 500 $\mu$g/ml of trichothecin.

The highest yields of active substance attained in fermentors 120—200 $\mu$g/ml were commonly identical with those obtained in simultaneously grown shaken cultures.

While with FREEMAN *and* MORRISON's [7] method of extracting the substance by shaking with chloroform, only 80 to 90 per cent of the trichothecin was recovered, an almost 100-per cent recovery was attained with tetrachloroethane and dichloroethane. The trichothecin was purified by means

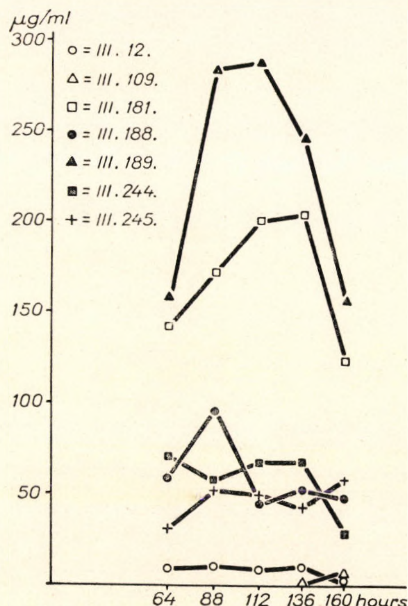


*Fig. 2.* Trichothecin production by *T. roseum* strains isolated from different sources

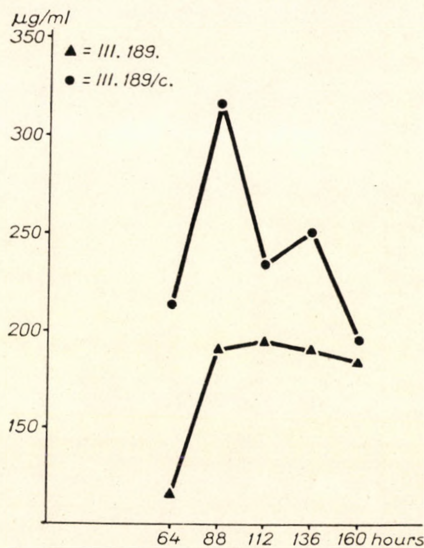


*Fig. 3.* Productive capacity of the initial culture compared with that of an ultraviolet-treated isolate marked III. 189c

of $Al_2O_3$ chromatography and elution with ethy lether followed by recrystallization from normal hexane. About 50 per cent was recovered as the pure crystalline substance. As regards crystalline form, melting point, solubility, and biological activity, the trichotechin prepared by us agreed with the substance supplied by Dr. G. G. FREEMAN.

## Summary

Against 40 to 80 μg/ml of the active substance obtainable in 25 to 30 days in surface cultures, a maximum trichotechin yield of 200 to 250 μg/ml was obtained in 88 to 112 hours by means of fermentation.

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# INCIDENCE OF MICROFILARIA IN MONOLAYER MONKEY KIDNEY CELL CULTURES

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(Received January 30, 1959)

Since the description of the method for large-scale monkey kidney cell culture production by YOUNGNER [1], a remarkable number of monkeys have been used for that purpose in the different laboratories all over the world. Nevertheless, in spite of the very frequent incidence of different *Filaria* in these animals [2—5] we know of only one report that by PRIER [6], concerning the presence of *Microfilaria* in trypsinized monkey kidney cell suspensions stained for microscopic examination. The appearance of *Microfilaria* in tissue cultures has not been reported. On the other hand, several authors have described successful maintenance *in vitro* of different *Schistosoma* in human and different animal sera [7—12]. SENFT *and* WELLER [13] even succeeded in observing growth and regeneration of *Schistosoma mansoni* in tissue culture media, consisting of bovine amniotic fluid Hanks' solution, inactivated horse serum and beef embryo extract.

The present report deals with the observation of unidentified *Microfilaria* in *Cynomolgus* monkey kidney cell cultures.

In the course of poliomyelitis vaccine production in this laboratory, the kidney of a single *Cynomolgus* monkey was trypsinized separately, according to the method of YOUNGNER [1]. At necropsy the animal was found to be moderately infected with parasites *(Oesophagostomum?)* though no signs of *Microfilaria* contamination could be observed in the kidney, neither at the inspection nor at the mincing of the organ. At further proceeding, *i. e.* trypsinization, filtration and centrifugation, the cell suspension appeared entirely normal. In stained preparations (citric acid methyl violet dye), when examined microscopically to determine the cell count, no unusual microscopic elements were found. The cell suspension after appropriate dilution was dispensed to 663 tubes (200 000 cells per tube) and 17 Roux flasks (20 million cells per flask). The initial medium consisted of 88 per cent Hanks' solution, 2 per cent calf serum and 10 per cent of 5 per cent solution of lactalbumin hydrolysate. The cultures were incubated at 37° C for 6 days. At that time both the tube and Roux flask cultures were examined microscopically. Out of the 663 tubes 67 exhibited 1 to 4 vigorously moving *Microfilaria*. The Roux

flasks were all found to contain *Microfilaria*. As these organisms can only be seen under the microscope and as the fluid phase might have washed away the *Microfilaria* from the surface of the cultures, it is very probable that originally more tubes had been contaminated but the parasites were only detected when attached to the cell monolayer (Fig. 1).

Tubes apparently uncontaminated were used for titrating poliomyelitis virus. When reading the results on the 3rd and 6th days of incubation, the titre was found to be in good agreement with that obtained in normal cultures, though in some tubes vigorously moving *Microfilaria* were observed.

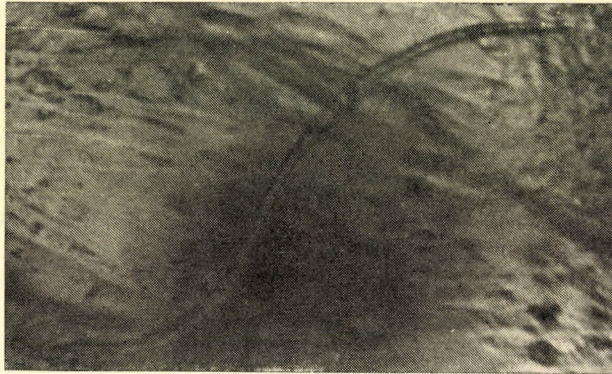

*Fig. 1.* Incidence of *Microfilaria* in monolayer monkey kidney cell culture. Living *Microfilaria* with the cell monolayer in the background. Magnification about 250 times

The 67 primary tube cultures contaminated with *Microfilaria* were reincubated for further observation. On the 7th day the medium was changed to No. 199 synthetic medium [14], and the fluid change was repeated weekly during the period of observation (30 days).

Though the possibility of washing away *Microfilaria* when performing fluid changes could not be excluded, most of the tubes remained contaminated up to the second fluid change (14th day). Nevertheless, at that time only one single parasite was found still moving, while the others were motionless and part of them exhibited signs of autolysis. No morphological changes referring to any development of the surviving *Microfilaria* were observed. At the 30th day of observation no *Microfilaria* could be detected in any culture, and the cell monolayer exhibited signs of degeneration.

The Roux flasks were also observed for thirty days. Fluid change to medium No. 199 was first performed on the 7th day and then repeated weekly. On the 8th day 3 flasks were versenized and the cell suspension was dispensed to test tubes, 200 000 cells into each. To initiate the growth of secondary cultures, the same medium was used as in the primary cultures. After 24 hours extensive outgrowth of cells was observed and in about 1 per

cent of the secondary tubes living *Microfilaria* were found. After 48 hours the cultures rapidly degenerated, in contrast to normal cultures. The experiment was repeated with further 3 flasks on the 11th day. In this case 100 000 cells were transferred into each tube, otherwise the procedure was exactly the same as 3 days earlier. The secondary cultures after extensive initial growth rapidly degenerated up to the 3rd day following versenization. No living *Microfilaria* could be detected in these tubes at daily microscopic examination.

In the residual primary Roux flask cultures the *Microfilaria* lived for about 16 days and after this period they underwent changes similar to those observed in the tube cultures. The cell monolayer showed signs of degeneration on the 28th day.

At the time of the observation of the appearance of *Microfilaria* in the cultures, 5 monkeys of the same lot were still alive. The blood of these animals was examined in respect of *Microfilaria*. Out of the 5 animals, 4 were found to harbour *Microfilaria*, as revealed by the blood samples examined microscopically after acetic acid treatment. When these animals had been killed to remove their kidneys for tissue culture purposes, only moderate parasitic infection was detected. The tissue cultures prepared from these animals were all free of *Microfilaria*.

## Summary

The incidence of live *Microfilaria* in monkey kidney tissue cultures from an animal displaying moderate infestation with the parasite has been described.

The presence of *Microfilaria* in primary tissue cultures did not affect cell growth nor the cytopathogenic effect of poliomyelitis virus.

Secondary cultures prepared by versenization from *Microfilaria*-contaminated Roux flasks degenerated within 3 days though they contained *Microfilaria* only exceptionally.

In the primary cultures maintained by weekly fluid change for 30 days, *Microfilaria* survived for about a fortnight.

*Acknowledgements:* The author is indebted to Dr. L. Nemesséri, *Department of Parasitology, State Institute for Veterinary Hygiene, Budapest,* for his valuable help and criticism, and to E. K. Novák, *Department for Mycology, State Institute of Hygiene, Budapest,* for taking the photograph.

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# NEUE ANGABEN ÜBER DIE VERBREITUNG DER EXTRAPULMONALEN MENSCHLICHEN TUBERKULOSE VOM BOVIN-TYPUS IN UNGARN

Von

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(Eingegangen am 11. März 1959)

Vor einigen Jahren haben wir Einzeilheiten über die Verbreitung der allgemeinen Tuberkulose vom Bovin-Typus in Ungarn mitgeteilt [1]. In vorliegender Arbeit referieren wir ergänzend über das Vorkommen der extrapulmonalen Tuberkulose vom Bovin-Typus in einen umfangreichen Material.

Die ausländische Literatur über die Tuberkulose vom Bovin-Typus läßt sich kaum mehr überblicken. Die aus der Zeit vor 1945 stammenden ungarischen Angaben sind recht ärmlich und beziehen sich auf verhältnismäßig wenige Fälle [2—7]. Später führte die Forschung und diagnostische Arbeit auch auf diesem Gebiet eine Besserung herbei. In den Jahren 1950—51 vermochte man die Rolle des Bovin-Typus schon auf Grund von nahezu 2300 gezüchteten Stämmen festzustellen.

In jener Statistik waren die extrapulmonalen Kranken dem Landesdurchschnitt entsprechend zu 6% repräsentiert. In den letzten Jahren wurde der menschliche Bovintyp von mehreren ungarischen Autoren untersucht [8—14].

## Material und Methoden

Wir untersuchten die aus extrapulmonalen Krankheitsformen stammenden *Mycobacterium tuberculosis* Stämme, die aus Untersuchungsmaterialen, welche man uns aus verschiedenen Teilen des Landes eingeschickt hatte, gezüchtet worden waren. Die Züchtung wurde nach der bei der Routinearbeit üblichen Methode vorgenommen. Insgesamt untersuchten wir 248 Stämme, deren Mehrzahl, nämlich 147 Kulturen, aus den eingesandten Harnproben stammte, während 25 Stämme aus Eiter und die übrigen aus Liquor, weiblichen Genitalien, kranken Gelenken, Lymphknoten und Stuhl gezüchtet wurden.

Die biologischen Eigenschaften der INH-resistenten Bakterien können sich bekanntlich [15] verändern (Kultivierbarkeit, Form der Kolonien, Virulenz usw.) Wir untersuchten daher die Empfindlichkeit unserer Stämme INH, Streptomycin und PAS gegenüber. Zugleich wurde die Katalaseaktivität der INH-resistenten Kulturen bestimmt. Im weiteren befaßten wir uns nur noch mit empfindlichen, katalasepositiven Stämmen, um ihren Typus zu bestimmen.

Unter den verschiedenen Typbestimmungsmethoden wandten wir die Tierimpfung an, weil wir der Meinung sind, dieses Verfahren sei am zuverlässigsten. Obwohl der Wachstumstypus, die Glyzerinophobie, der Glyzerinabbau, der Nikotinsäurenachweis usw. in vielen Fällen gute Orientierung bieten, halten wir doch den Tierversuch vom Gesichtspunkt der Zielsicherheit für vorteilhafter. Für die Impfungen wurden Kaninchen von 2 kg Gewicht benutzt. Statt des klassischen intravenösen Impfverfahrens wandten wir jedoch die Intrakutanmethode an, die sich in unserem Institut gut bewährt hat [16] und die im wesentlichen darauf

beruht, das 0.2 ml Bakteriensuspension (1 Normalöse Kultur in 2 ml physiol. Kochsalzlösung), in die Haut der Kaninchen geimpft, an der Impfstelle einen Prozeß in Gang setzten. *Mycobacterium tuberculosis* vom Human-Typus verursacht nur regressive Veränderungen, während der Bovin-Typus ein sich ausbreitendes torpides Geschwür zustande bringt, welches nicht heilt. Zu Kontrollzwecken wurden auch Suspensionen der Stämme *H*37 *Rv.* und *Ravenel* eingeimpft, um die an der Kaninchenhaut erscheinenden Veränderungen vergleichen bzw. auswerten zu können. (Die Saprophyten rufen nur eine geringe, rasch heilende Schwellung hervor.) Die Feststellung der Lokalreaktionen erfolgte zweiwöchentlich, und 6 Wochen nach der Impfung wurde die Typuseinordnung abgeschlossen.

## Ergebnisse

Es wurde die Typuseinordnung von insgesamt aus 248 extrapulmonalen Fällen stammenden Stämmen vorgenommen, von denen sich 155 Kulturen, d. h. 62,5%, als Human-Typus erwiesen und 88 Stämme, d. h. 35,4%, die Eigenschaften des Bovin-Typs zeigten. Eine doppelte Infektion, d. h. das gemeinsame Vorkommen des Human- und Bovin-Typus, sahen wir in 5 Fällen (2,1%). Bei 11 der 88 Bovin-Stämme handelte es sich um solche von vorübergehenden Charakter und verringerter Virulenz *(Tabelle I)*. Anläßlich

**Tabelle I**

*Aufteilung der Typen der aus extrapulmonalen Erkrankungen isolierten Mycobacterium tuberculosis Stämme*

| Untersuchungsmaterial | Zahl | Human | | Bovin | | Mischtypus (beide) | |
|---|---|---|---|---|---|---|---|
| | | Zahl | % | Zahl | % | Zahl | % |
| Harn | 147 | 103 | 70 | 44 | 30 | | |
| Eiter (extrapulm.) | 25 | 15 | 60 | 10 | 40 | | |
| Weibl. Gen.-Tbc. (Menstr.-Blut, Uterus-Sekret, Abrasions Material) | 12 | 6 | 50 | 6 | 50 | | |
| Andere (Liquor, resezierte Organstückchen usw.) | 64 | 31 | 48 | 28 | 44 | 5 | 8 |
| Insgesamt | 248 | 155 | 62,5 | 88 | 35,4 | 5 | 2,1 |

der vor 8 Jahren durchgeführten Untersuchungen (aus weniger extrapulmonalen Fällen) gewannen wir ein mit dem vorliegenden übereinstimmendes Resultat (36%). Bei den extrapulmonalen Bovin-Erkrankungen in Ungarn ist demnach offenbar seither keine Verschiebung eingetreten.

Nach *Tabelle I* dominieren in unserem Material die aus Urogenital Tuberkulose stammenden *Mycobacterium tuberculosis* Stämme. Von den aus weiblicher Genitaltuberkulose nachgewiesenen 12 Stämmen gehörte auffallenderweise die Hälfte zum Bovin-Typus. Obgleich die Zahl dieser Fälle nicht genügt, um endgültige Schlußfolgerungen zu ziehen, darf doch festgestellt werden, daß die Frauen im allgemeinen, wahrscheinlich im jugendlichen Alter, enteral von der Milch und den Milchprodukten tuberkulöser Kühe

infiziert werden. Diese Feststellung stimmt mit der Auffassung anderer ungarischer Autoren [11, 13] überein, nach deren Ergebnissen die Anzahl der vom Bovin-Typus verursachten Fälle von Urogenital-tuberkulose selbst 33% erreichte. Die hier gegenwärtig eingeleitete systematische Bekämpfung der Rindertuberkulose wird auf diesem Gebiet zweifellos eine Besserung herbeiführen.

## Zusammenfassung

Es werden die Ergebnisse der im Jahre 1958 vorgenommenen Untersuchungen von aus extrapulmonalen Krankheitsformen stammenden *Mycobacterium tuberculosis* Stämmen mitgeteilt sowie Bedeutung und Rolle der Koch-Bakterien vom Bovin-Typus bei diesen Krankheitsformen erörtert. Die Typenbestimmung der 248 Mycobakterienstämme erfolgte durch Tierimpfungen nach der für Kaninchen neuerdings ausgearbeiteten Intrakutanmethode.

Von den untersuchten arzneiempfindlichen, katalaseaktiven Stämmen gehörten 62,5% zum Human- und 35,4% zum Bovin-Typus. Mischpopulationen vom Human- + Bovin-Typus (Doppelinfektion) kamen in 2,1% der Fälle vor. Mycobacterium tuberculosis vom Bovin-Typus spielte in 29% der Fälle von Urogenitaltuberkulose, in 40% der mit eitrigen Prozessen einhergehenden Erkrankungen und schließlich in nahezu 50% der weiblichen Genitaltuberkulosen eine pathogene Rolle.

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# MEGACINOGENY: INDUCIBLE SYNTHESIS OF A NEW IMMUNOSPECIFIC SUBSTANCE

By

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(Received April 14, 1959)

Some strains of *Bacillus megaterium* produce megacin, an antibacterial principle [5, 6] which differs from the antibiotics in several essential points. This principle is the consequence of lethal biosynthesis and its origin is associated with the lysis of the cells [7, 12]. The formation of megacin can be induced by a small dose of ultraviolet light and in this respect it is completely reminiscent of lysogeny. A further characteristic of megacin is its very narrow antibacterial spectrum [8].

It has been found recently [10] that megacin concentrates of high potency can be prepared from fluids of ultraviolet light-induced cultures of the megacinogenic strain 216 grown in defined medium. Megacin was easily concentrated by adsorption and elution on anionic-cellulose, ECTEOLA, prepared according to PETERSEN *and* SOBER [17]. Individual lots of concentrates contained $2-3 \times 10^5$ units/ml megacin, and, as an average, about 3 000 units were found pro $\mu$g nitrogen in the concentrates. These concentrates were successfully used in establishing the specific mode of action of megacin and it has been found [9] to break the osmotic barrier of the cytoplasm membrane. The enzyme-like action of megacin, furthermore the sensitivity of this bacteriocin to commercial trypsin preparation (Difco 1 : 250), suggested that megacin is of protein character. The purpose of the present report is to describe observation which indicate that megacin is an immunospecific protein formed in consequence of the lethal biosynthesis induced by ultraviolet irradiation.

## Materials and methods

The general experimental techniques used were the same as those describes in our previous publications.

*Strains.* For the production of megacin we employed *Bacillus megaterium* 216 [6]. The titration of megacin was carried out with phage-resistant *B. megaterium* strain "Mut-C" [11]. The non-megacinogenic mutant 216 meg$^-$ was isolated from strain 216 [12].

*Megacin concentrates.* These were prepared from ultraviolet light-induced cultures of *B. megaterium* strains 216 in defined medium, as it has been described recently [10]. Concentrates used in these experiments contained $2-4 \times 10^5$ units/ml megacin.

*Culture media.* Horse meat extract agar, containing 1 per cent peptone and 1.5 per cent agar, *YP* (yeast extract peptone) broth and *YDC* (yeast extract plus enzymically digested casein) medium were prepared as it has been described previously [6, 7].

*The titration and unit of megacin.* The technique of titration has been described recently [10]. In principle, the highest dilution which produced inhibition on the surface of indicator plates containing "Mut-C" organisms represents the unit of megacin, *i. e.* the reciprocal value of the titre expresses the number of megacin units present in 1 ml.

*The production of immune sera.* The homologous sera of strains *B. megaterium* 216 and 216meg$^-$ were obtained in accordance with our earlier report on the subject [11] and will be referred to below as antibacterial (AB) sera.

In order to produce megacin antibody, rabbits were injected intravenously with 5 ml doses of the concentrates, twice a week over a period of six weeks. Sera of rabbits immunized with megacin concentrates are designated as anti-megacin (AM) sera.

*Titration of the neutralizing power of megacin antibody.* Amounts of 100 units megacin in 0.5 ml saline were transferred to test tubes to which was added 0.5 ml from the serial dilutions of the serum. For 24 hours the mixtures were kept in the refrigerator and 0.02 ml of each was dropped on the surface of the indicator plate. The highest dilution of serum neutralizing the bactericidal action of 100 units of megacin is taken as the neutralizing titre of anti-megacin serum.

*Precipitation.* To determine the precipitation titre, 0.2 ml amounts of serum diluted with the same quantity of saline were added to 0.2 ml amounts of antigen dilution. For determination of quantitative precipitation, 1 ml amounts of specific anti-megacin serum were transferred to cooled centrifuge tubes and varying quantities of antigen were added. The total volume of the mixture was completed to 4 ml and left standing in a refrigerator for 48 hours. The cold centrifuged precipitate was washed twice with cold saline and digested with sulphuric acid ; the N content was determined by nesslerization.

*Complement fixation test.* Four units of haemolysin and 1.5 units of complement were used. The amounts of serum dilution and that of antigen were 0.25 ml each. Total volume of the system was 1.25 ml. Incubation was at 37° C for 30 minutes.

*Preparation of extracts from non-induced organisms.* A young culture (optical density 0.45) of strain 216 grown in *YDC* medium was centrifuged and the collected organisms were washed with saline. The sediment was resuspended in the same volume of saline as that of the culture used. Crystalline lysozyme (100 *ug*/ml) was added to the suspension, and after lysis of the cells, the solution was cleared by centrifugation.

*Preparation of specific anti-megacin serum (SAM serum).* The sera of rabbits immunized with megacin concentrates contained anti-cellular antibodies beside megacin antibody. Highly specific sera were obtained by removing anticellular antibodies with the extract from non-induced organisms. Five ml of extract obtained as described above from non-induced culture of strain 216 were added to 10 ml anti-megacin serum and kept in a refrigerator for 48 hours. The precipitate formed was removed by centrifugation. The absorbed serum was highly specific for megacin, while it failed to give precipitation or a complement fixation test with the bacterial extract of strain 216.

*Enzymes.* Twice crystallized preparations were used in the experiments. The preparations were kindly supplied by Prof. J. H. NORTHROP *(University of California).* Chymotrypsin and pepsin preparations were salt free, while the trypsin preparation contained 50 per cent $MgSO_4$.

## Results

*Heat and enzymic inactivation of megacin.* Megacin concentrates, after being sterilized by filtration through sintered glass filtre, can be kept in the refrigerator in an unchanged condition for several months.

In the experiments performed with megacin preparations derived from complete culture media, megacin proved to be thermolabile and sensitive to commercial trypsin preparations [6]. It seemed necessary to repeat these experiments with megacin concentrates from defined medium and with crystalline enzyme preparations.

To check the heat lability of megacin, one ml of concentrate was added to 4 ml buffer solution and titrated for megacin. In alkaline medium, megacin was very sensitive to heat. There was a considerable inactivation even at 37° C, when the pH of the medium was 7.5 to 8, or more. On the other hand, in a phosphate buffer of pH 7.1 megacin was not affected at 37° C within 4 hours.

The heat lability of megacin in acid buffers was moderate. There was no appreciable inactivation in glycine-HCl buffer of pH 2 at 37° C after 4

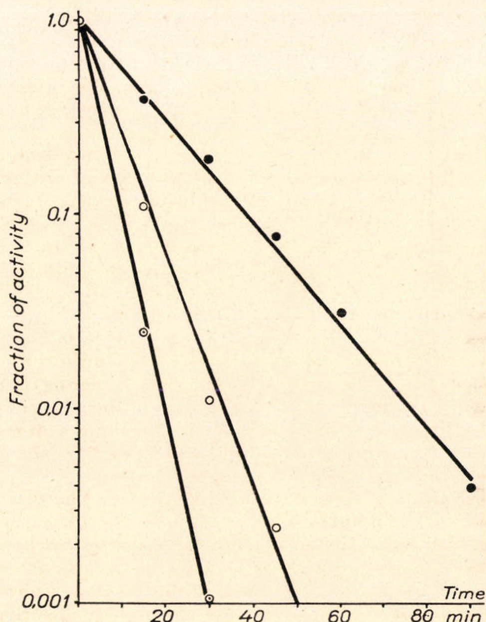



*Fig. 1.* Heat inactivation of megacin in 0.07 *M* phosphate buffer at 80° C
● pH 5.6; ○ pH 7.0; ⊙pH 8.1

hours. *Fig. 1.* gives information concerning the heat inactivation of megacin at 80° C.

In order to study the sensitivity of megacin to enzyme action, equal volumes of concentrate and enzyme solution in buffer were mixed and incubated at 37° C. By crystalline pepsin at acid pH megacin was rapidly inactivated. The maximum of inactivation occurred at pH 2. Even 1 μg/ml of pepsin was capable of inactivating the bactericidal activity of the concentrate within two hours. It should be stressed again that in the absence of enzyme at this acidity no change occurred in the titre of the concentrate.

The protein nature of megacin is also supported by its sensitivity to chymotrypsin. When 100 μg/ml of crystalline chymotrypsin were added to

the concentrate in a phosphate buffer of pH 7.17, a considerable loss in the bactericidal titre resulted. Because of the alkaline sensitivity of megacin, chymotrypsin inactivation could not be studied at optimum pH values. It was striking and contrary to our earlier observation that megacin proved to be resistant to trypsin. It should, however, be noted that twice crystallized trypsin (100 μg/ml) was being used, while in the past a commercial trypsin concentrate, probably contaminated with chymotrypsin, had exclusively been employed. It still remains questionable whether or not megacin is

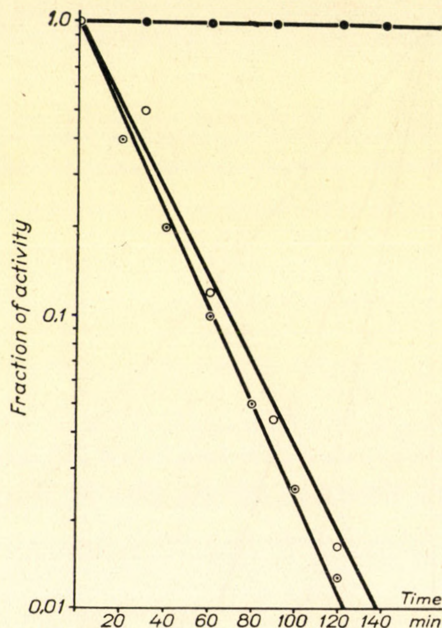



*Fig.* 2. Inactivation of megacin by enzymes at 37° C — ● Trypsin 100 μg/ml, pH 7.17 ○ Chymotrypsin 100 μg/ml, pH 7.17, ⊙ Pepsin 1 μg/ml, pH 2.0

entirely resistant to trypsin, in view of the fact that its activity could not be tested at the optimum alkalinity. *Fig. 2* shows the inactivation of megacin by pepsin and chymotrypsin.

*The antigenic nature of megacin.* In the blood serum of rabbits injected repeatedly with megacin concentrates, increasing amounts of antibody appeared parallel with the progress of immunization. In the early stage of immunization the sera gave precipitation with the concentrates, but they did not precipitate the extracts produced from the cells of the non-induced strain 216 lysed by lysozyme. Serum samples obtained after further inoculations precipitated the concentrates up to a dilution of 1/64, while the cell extracts were precipitated up to dilutions of 1/4 to 1/8.

Several observations showed that the antigenic properties of the concentrates are in the first place due to their megacin content. In this respect the most essential point is that these sera were capable of neutralizing the bactericidal action of megacin. No such effect was displayed by normal rabbit sera or by antibacterial sera of strain 216.

The anti-megacin property of the sera can be estimated with sufficient reliability. The method of assay employed by us involves, however, the error inherent in the twofold serial dilution of serum. The neutralizing capacity of anti-megacin antibody was identical at 37° C, at room temperature, and at ice-box temperature.

**Table I**

*Agglutination and megacin neutralization by anti-megacin and by antibacterial immune sera*

| Serum | Agglutination** | | Neutralization | |
|---|---|---|---|---|
| | Strain | Titre* | Incubation | Titre |
| Antibacterial 216 (rabbit 1.) .......... | 216 | 128 | 24 hr.at 0° | <8 |
| ,, 216 (rabbit 2.) .......... | 216 | 320 | 24 ,, | <8 |
| ,, 216 meg$^-$ | 216 | 500 | 24 ,, | <8 |
| Anti-megacin (rabbit 1.) ............. | 216 | <32 | 24 ,, | 8 000 |
| ,, ( ,, ,,) ............. | 216 meg$^-$ | <32*** | 3 hr.at 0° | 2 000 |
| ,, ( ,, ,,) ............. | | | 24 hr.at 26° | 8 000 |
| ,, (rabbit 2.) ............. | 216 | <32 | 24 hr.at 4° | 10 000 |

* Reciprocal value of titre.
** Agglutination : Incubation for 4 hr. at room temperature and overnight in an ice-box.
*** Anti-megacin serum did not agglutinate suspension of *B. megaterium* strain 216 and 216 meg$^-$. At lower dilutions gradually a lysis of cells appeared.

It is seen from *Table I* that sera of rabbits injected with megacin concentrates are capable of neutralizing the bactericidal action of megacin. Two different rabbit sera neutralized 100 units/ml megacin up to dilutions of 1:8000 — 1 : 10 000. On the other hand, these sera did not agglutinate the suspension on non-induced cells of strain 216 and that of its non-megacinogenic mutant (216 meg$^-$) at 1 : 32 dilution. At lower dilutions lysis of cells appeared gradually. Antibacterial sera, *i. e.* sera of rabbits immunized with cells from non-induced cultures of strain 216 or with suspensions made from the non-megacinogenic mutant (216 meg$^-$), were effective in agglutinating the suspension of strain 216. These sera, however, were not capable of neutralizing the bactricidal action of megacin at a dilution of $^1/_8$.

It is easy to conclude from the above observations that the sera of rabbits injected with megacin concentrate contain an antibody specific to megacin itself. The precipitation of these sera with extracts from non-induced

organisms indicates the presence of antibacterial antibodies beside anti-megacin antibody. As it has been described in the methodological part of this report the antibacterial antibodies were readily removed from the sera with extracts of non-induced bacteria. Specific anti-megacin serum (SAM) was precipitated with megacin lysates or concentrates but did not display any reaction or complement fixation with cell extracts of the non-induced culture of strain 216. The correlation between the precipitable antibody content and the neutralizing capacity of SAM serum is illustrated by quantitative precipitation (see *Table II*).

**Table II**

***Precipitation and neutralization of megacin by specific anti-megacin (SAM) serum***

| Megacin input in units $\times 10^5$ . | 6.3 | 4.6 | 3.1 | 2.3 | 1.53 | 0.76 | 0.15 | 0.00 |
|---|---|---|---|---|---|---|---|---|
| Nitrogen precipitated ($\mu$g) ...... | 87.5 | 96.8 | 111.7 | 109.5 | 112.7 | 62.5 | 28.1 | 0.00 |
| Megacin in supernatant, units $\times 10^5$ | 1.1 | 0.08 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| Neutralization by the supernatant | 0 | 0 | 120 | 240 | 480 | 960 | 1350 | 2700 |

One ml of specific anti-megacin serum was mixed with various amounts of concentrates. The volume of the mixture was made up to 5 ml the tests were made in duplicate. After incubation at 4° C for 48 hr, the precipitate was sedimented and the supernatant removed, assayed for megacin, and for its neutralization effect with 100 units of megacin. Neutralization is expressed by the reciprocal value of the highest dilution of supernatant capable of neutralizing the said amount of megacin. In a control tube not containing megacin, this value was found to be 2700.

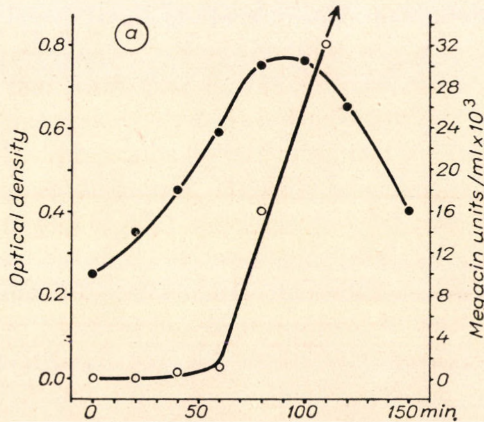
The data of the experiment showed that the amount of the precipitate is parallel either with the megacin content of the supernatants or with the excess neutralizing capacity. The neutralizing antibody is thus most probably identical with the precipitating antibody of the SAM serum.

As indicated by precipitation tests carried out with antibacterial serum and megacin concentrate, the neutralization of megacin is not due to a non-specific absorption of a heterologous precipitate. One ml of antibacterial serum yielded 425 $\mu$g nitrogen if brought together with the optimum amount of megacin concentrate ; however, the megacin content of the supernatant did not diminish appreciably.

SAM serum showed complement fixation, both with crude lysates of induced cells and with megacin concentrates, up to a titre of 1 : 250 dilution in the system. The specificity of this reaction was evidenced by the fact that the same test failed to yield a positive result if carried out with the lysozyme extract of non-induced cells.

*Dynamics of megacin formation.* The ultraviolet irradiation culture of strain 216 reaches its maximum growth after 90 to 110 minutes' reincubation in individual experiments [6]. Alföldi [1] found that the production of

megacin precedes its liberation. He succeeded in detecting intrabacterial megacin in the lysozyme extract of organisms from culture samples taken at 45 min after ultraviolet induction. This observation is in good accordance with the appearance of the first cytological changes occurring in induced cells [7]. In view of the above facts, the question arose whether the appearance and increase of bactericidal power of the induced culture is synchronous with the formation of the immunospecific substance characteristic of lysates or concentrated lysates obtained from cultures irradiated with ultraviolet light.



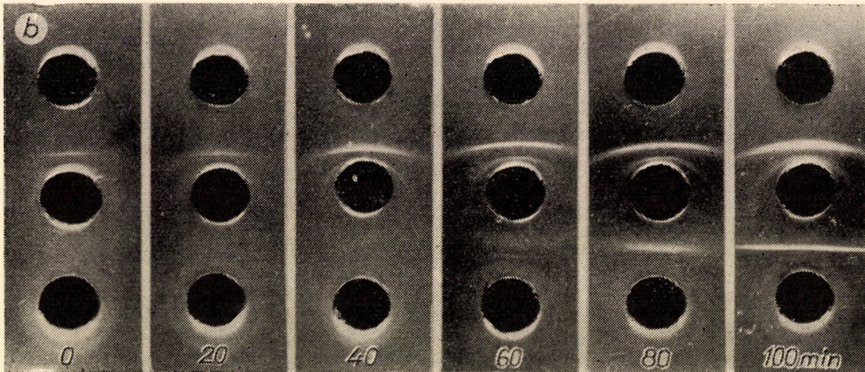




*Fig.* 3*a* and *b*. The course of formation of bactericidal activity and that of the immunospecific substance in ultraviolet irradiated culture of *Bacillus megaterium*, strain 216. The experiment was carried out in YDC medium. U. v. irradiation at 0 minute. The samples were lysed with crystalline lysozyme (10 μg/ml). *a* ●— optical density of culture —○ megacin titre *b*. Immunospecificity of individual samples takes at intervals from culture. The extracts were added into central holes. Upper holes contained antibacterial serum, while the lower holes were filled with specific anti-megacin (SAM) serum

In experiments carried out with ultraviolet-induced cultures of strain 216, aliquots of culture taken at intervals after irradiation and reincubation were lysed with lysozyme. The extracts thus obtained were assayed for megacin and serologically analysed by diffusion in agar gel, using specific anti-megacin and anti-bacterial serum. This latter was produced in rabbits by the injection of a non-induced cell suspension of strain 216. The results of this experiment are demonstrated in *Fig.* 3.

It is seen from *Fig. 3a* that only a small amount of megacin (1200 units/ml) was found in the sample taken at 60 min. Subsequently, a gradual increase of bactericidal titre occurred, up to $64 \times 10^3$ units/ml, the peak value of megacin production. The increase of bactericidal titre went parallel with the appearance of a new immunospecific substance revealed by precipitation with SAM serum (see *Fig. 3b)*. A very faint and thin line is see at about the middle of the distance between the holes containing SAM serum and those with culture extract from the 60 min sample. In fact, similar but even less definite lines appeared with the 20 and 40 min samples. It should, however, be stressed that traces of megacin (100 to 500 units/ml) were found also in these samples. A marked precipitation appeared when the bactericidal titre of the sample was considerable (16 to $32 \times 10^3$ units/ml). This observation clearly shows that the appearance and increase of antibacterial principle in the induced cultures of strain 216 run parallel with the formation of a new immunospecific component.

## Discussion

A considerable number of well-known antibiotics (*e. g.*, gramicidin, gramicidin-S, polymyxin), and some less intensely studied ones, like biocerin [13], circulin [15], have been isolated from *Genus Bacillus* cultures. The lytic principle of *Bacillus cereus* [16] and of *Bacillus terminalis* [4] are associated with the autolysis of cells in the regressive period of growth. Megacin, on the other hand, is produced in exponentially growing cultures under the effect of inducing factors, as a result of lethal biosynthesis [6, 12]. Its characteristic origin and narrow spectrum [8] have encouraged us to classify this antibacterial principle among the bacteriocins.

Our recent observations have yielded further data concerning the characteristics of megacin. Its sensitivity to twice crystallized pepsin and chymotrypsin, furthermore its antigenic effect are fairly good evidences as to the protein nature of megacin. Consequently, megacin displays properties quite unlike the above-mentioned antibiotics. Although the antibiotics of *Genus Bacillus* are of polypeptide character, as far as we know they do not possess any antigenic properties.

The colicins are regarded as the prototypes of bacteriocins, and at least colicin K bears a resemblance to megacin in this respect [3]. Although both colicin and megacin are proteins provided with antigenic properties, they show a striking difference as to their origin. Ultraviolet irradiation does not induce the formation of colicins althought it may somewhat increase their production [2]. KELLENBERGER *and* KELLENBERGER [14] maintain that the cells of colicinogenic strains do not suffer lysis unless they also harbour prophage for simultaneous induction. There is a close connection between the colicin K produced by *Escherichia coli* and the O somatic antigen of the cells [3]. So far, all the studies concerned with megacin point to its being a protein of markedly anionic character, developing apart from the cell material harbouring other kinds of antigen. Megacin appears to be the result of a *de novo* protein synthesis in the cells. Its synthesis is more reminiscent of temperate phages and adaptive enzymes than showing a likeness to the origin of the colicins or antibiotics. In this respect, megacinogeny may prove to be a new, interesting subject in studying the biosynthesis of proteins.

## Summary

Concentrates obtained from cultures in synthetic medium of *Bacillus megaterium*, strain 216, were used for studying the characteristics of megacin. Megacin was found to be very unstable at alkaline reaction, above pH 7.5, whereas no appreciable inactivation was observed between pH 2 and 7 at 37° C in 4 hours. Megacin is a heat labile antibacterial principle rapidly inactivated by crystalline pepsin ; it is sensitive to crystalline chymotrypsin at pH 7.2, but stable in the presence of twice crystallized trypsin (100 *u*g/ml) at pH 7.2.

Rabbits immunized with megacin concentrates developed an antibody which specifically precipitated megacin and neutralized its bactericidal action. Megacin appears to be an immunospecific protein with bactericidal action, the formation of which is the result of a *de novo* protein synthesis in the cells.

*Acknowledgement:* The authors are greatly indebted to Prof. J. H. NORTHROP *(University of California)* for his gift of crystalline enzyme preparations.

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A kiadásért felel: az Akadémiai Kiadó igazgatója Műszaki felelős: Farkas Sándor

A kézirat nyomdába érkezett: 1959. VI. 24. — Terjedelem: 6,75 (A/5) ív, 23 ábra

49132/59 — Akadémiai Nyomda, Budapest — Felelős vezető: Bernát György

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# THE USE OF REDUCING DYES FOR THE RAPID DETERMINATION OF THE DEVELOPMENT OF STREPTOMYCES RIMOSUS IN SUBMERGED CULTURE

By

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(Received November 24, 1958)

Mycelial respiration during the growth in aerated submerged culture of members of the genus *Streptomyces* producing antibiotics is usually determined in the Warburg apparatus [1]. From the changes in mycelial respiration studied in this way it is possible to conclude to the physiological state of the microorganism at a given moment. Using the Warburg apparatus, DOSKOČIL *et. al.*[2] studied the respiration of a strain of *Streptomyces rimosus* under fermentation conditions. The Warburg technique is not suited for rapid determinations of hyphal respiration in fermentations on industrial scale.

At present, the following methods are available for rapid determination of development in submerged culture.

1. The use of reducing dyes indicating the activity of the biological oxidation-reduction enzyme systems of the hyphae.

2. Controlling respiration of the culture by polarography [2, 3].

3. Studying the changes in the oxidation-reduction potential of the culture [4].

Best suited for mass determination appears to be the method mentioned under 1.

An advantageous means by which to measure the activity of redox systems playing a decisive part in hyphal respiration, is the use of 2-3-5-triphenyl tetrazolium chloride (TTC). Using it, FRED *and* KNIGHT [5] determined the dehydrogenase activity of the mycelium of a lower fungus, *Penicillium chrysogenum*, grown in aerated liquid medium, but the results they obtained were not quantitative.

It is not yet known whether during cultivation of Actinomyces it is possible to demonstrate with dye reductions significant changes taking place in the biological oxidation-reduction system of the hyphae, and if so, what correlations there exist between them and mycelial respiration and antibiotic production, respectively.

In the present work, the oxytetracycline-producing *Streptomyces rimosus* strain BS-21 and its variants were studied in aerated submerged culture, with a view to elaborating a simple yet rapid method, with the use of redox

dyes, which would be capable of indicating the changes taking place in the physiological state of the fungus during antibiotic production.

## Materials and methods

*Medium.* The culture medium, designated TFH, consisting of 3% soybean meal, 0.5% potato starch, 0.4% corn steep liquor (50%), 0.2% calcium carbonate, 0.3% sodium chloride, and 0.4% vegetable (sunflower) oil. Before sterilization at 125° C for 30 minutes, the pH was 7.1. This culture medium is essentially the same as the liquid medium composed by HORVÁTH *et al.* [6].

*Strain. Streptomyces rimosus* BS—21, for which we are indebted to the *Research Institute of the Pharmaceutical Industry, Budapest.* A 24—30 hour vegetative inoculum was used in inoculating the culture. The ratio of inoculum to medium was 1 to 10.

*Technical conditions.* Growth of the organism was carried out in 1-cu.m iron fermentation vessels, at 28° C. Paddle stirrers at 190 r. p. m. were used. The medium was aerated with 1 : 1.15 litres of air per min. Samples were taken at 4-hour intervals during the cultivation.

*Respiration.* Mycelial respiration was studied in the Warburg apparatus (Laboratory type, 1958). Instead of a standard buffer, fresh culture medium was employed. Samples of fermentation juice were diluted with it fivefold. During incubation at 28° C, manometer readings were taken every 15 minutes. Respiration rates referred to volume of culture medium were expressed in ml $O_2$/ml/hr.

*Biological oxidation-reduction systems.* Using 2-3-5-triphenyltetrazolium chloride and methylene blue (MB) solutions, the activity of the oxidation-reduction system was determined by the following methods.

1. The TTC method was a modification of that described by CURRIER *and* DAY [7].

One ml of a sterile 2% glucose solution, 1 ml of a buffer ($Na_2HPO_4$) of pH 8, 2 ml of a 0.5% aqueous solution of TTC, and 1 ml of native fermentation juice.

The components were mixed in sterile glass-corked tubes, under vacuum. Following incubation in the dark at 28° C for two hours, the formazan which had formed from TTC was extracted with isopropanol, and made up with the solvent to an end volume of 25 ml. The light-absorbing capacity of the formazan solution (A%) was then measured photometrically (LANGE's photoelectric colorimeter, type IV), applying a green filtre.

2. Determination by the use of MB solution.

*Solution A.* One g of crystalline methylene blue dissolved in 100 ml of absolute alcohol.

*Solution B.* Twenty ml of solution A + 80 ml of sterile distilled water.

*Solution C.* Twenty ml of solution B + 100 ml of sterile distilled water.

Five ml of fermentation juice and one ml of MB solution C were mixed in Thunberg tubes, under vacuum. The time required for complete decoloration of the MB solution was registered with a stop-watch (MB reduction time).

*Oxytetracycline assay.* Oxytetracycline formation was determined at 24 hour intervals during the culture cycle. Titration was performed with the agar diffusion method, using *Bacillus subtilis* ATCC 6633.

The curves obtained in the evaluation of our experimental results were plotted from the mean values for 50 fermentations carried out under identical conditions.

## Results

In our cultures, the production of oxytetracycline generally lasted 120 to 140 hours. The formazan that had formed during this time by reduction from TTC, was found to vary in amount. The MB reduction times likewise differed.

Plotting the values obtained at individual measurement for formazan A% against those for MB reduction time, each of the two resulting curves was the reflection of the other.

With the shortest MB reduction time was formazan formation at its peak. At the initial stage of the fermentation process this took place 20 to 30 hours. At this stage, antibiotic production was still insignificant. In the subsequent stage, the decrease in the complex activities of the biological oxidation-reduction system resulted in an increase in the MB reduction time, proportionally to the decrease in formazan formation. This stage coincided in time with that of intensive oxytetracycline production. At the end of the 120 to 140-hour culture period, the values for the formazan A% and the MB reduction time were found to have settled approximately at the level of the

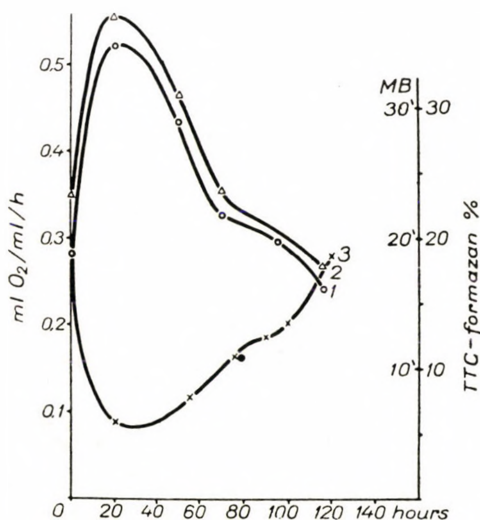


*Fig. 1.* Respiration of *Streptomyces rimosus* strain BS-21 and activity of the biologica oxidation-reduction system at a high rate of production oxytetracycline.
Curves : 1 = TTC → formazan formation, 2 = respiration, 3 = MB reduction time

starting phase of the fermentation. In this period, stagnation of the antibiotic titre was observed.

A comparison of the data for mycelial respiration with those for the activity of the biological oxidation-reduction enzyme system revealed that the rate of respiration was the highest at the time when enzymic activity was the most intense *(Fig. 1)*.

In culture fluids highly contaminated with iron ions (100—150 μg/ml) antibiotic production was low.

A shift from the neutral towards an alkaline pH was observed to take place in the fermentation juice, when such variants of the strain BS-21 were subjected to fermentation as had been derived from serial passages on the identical culture medium and possessed but a reduced capacity to produce oxytetracycline.

For the activity of the biological oxidation-reduction system of the mycelium producing but little oxytetracycline, and for respiration, characteristic curves, again, were obtained.

The initially (in the first 10 to 25 hours) very powerful enzymic activity and respiration soon began to slacken. From the 60th hour onwards, the curves for MB reduction time and formazan A% indicate enzymic activity to have been at its lowest. A certain rate of respiration was observed at the same time *(Fig. 2)*.

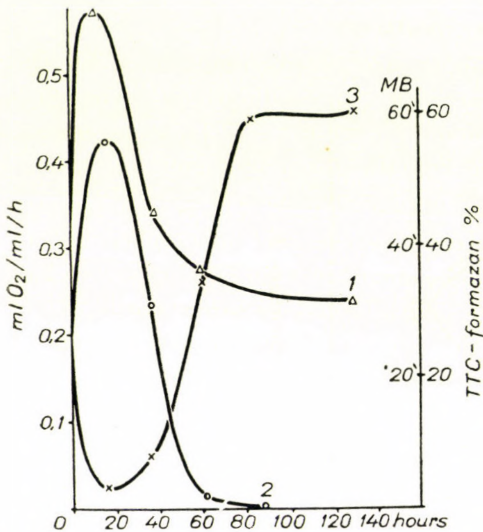


*Fig. 2.* Respiration of *Streptomyces rimosus* strain BS-21 and activity of the biological oxidation-reduction system at a low rate of oxytetracycline production.

Curves : 1 = respiration, 2 = TTC → formazan formation, 3 = MB reduction time

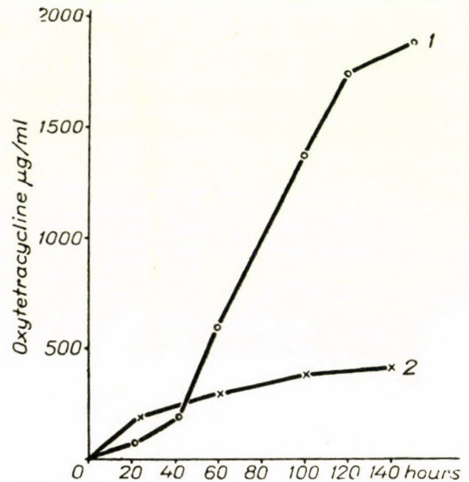


*Fig. 3.* Changes with time in the oxytetracycline production of cultures of *Streptomyces rimosus* strain BS-21.

Curves : 1 = high rate of OTC production, 2 = low rate of oxytetracycline production

As both the oxygen and hydrogen activating systems have a decisive role in cell respiration, we might suppose that this small rate of respiration was caused by the auto-oxidation of the components.

Comparison of the respiration curves for the intensively and the less efficiently producing cultures showed that while the respiration maxima referred to volume of fermentation juice were nearly on the same level, the changes with time in the respiration rate differed from one another. A similar but more particularly distinct difference was noted in the dye-reducing capacity of culture fluids producing oxytetracycline at a satisfactory and a lesser level, respectively.

## Discussion

The agreement observed to exist during cultivation between the values for the conversion of TTC to formazan, or methylene blue to leucomethylene blue, and the respiration rate, seems to indicate that with these colour reactions we can keep under observation some of the enzymes having a part in respiration.

According to data in the literature, TTC and methylene blue are suitable for the demonstration of the presence of anaerobic dehydrogenases [5, 8]. BRODIE *and* GOTS [9], as also NICKERSON *and* MERKEL [10] observed similar colour reactions in the presence of flavoproteins.

The intensity of hyphal respiration is successively determined by the intensity of the respiratory enzymes' activities. Accordingly, it seems permissible to regard the values obtained in the conversion of TTC to formazan, and MB to LMB, as resulting from the activities of a biological oxidation-reduction enzyme system.

It was unnecessary to add some dehydrogenizable metabolite except glucose in the TTC test to bring about the colour reaction, wherefore we had to abstain from identifying the enzymes more closely. In other words, we of necessity neglected investigation into the question of what components of the biological oxidation-reduction system were responsible for the colour reactions.

In connection with the colour reaction, the problem arose that with the MB and TTC tests we do not exclusively measure the intensity of the oxidation-reduction enzymic activity, but establish an adequate correlation between the activity of the respiratory enzymes of the mycelial mass and the reduction time or the production of formazan. The intensity of enzyme activity in iron-contaminated or alkalizing fermentations was found to depend not on the amount of mycelium but the physiological state of the hyphae. A relatively smaller amount of mycelium was still capable of yielding high values for formazan A% or very low ones for MB reduction time.

In iron-contaminated or alkalizing fermentations (up to pH 9,0) the enzymes controllable by colour reactions soon cease to be active probably because they are inhibited in their action, although at the same time respiration is observable.

The toxic role played by iron in *Streptomyces rimosus* fermentation has been pointed out by HORVÁTH *et al.*[6]. In their experience, a high iron content (in excess of 80 $\gamma$/ml) involves the formation of poisonous oil peroxides in the culture medium. The period during which they accumulate (from 0 to 24 hr) coincides with the time of increase in the activity of the biological oxidation-reduction system and the rate of respiration.

Mycelial respiration studied in the Warburg apparatus was read at 15-minute intervals, as long as its intensity did not change. Some data of the curve for respiratory intensity are stated as the arithmetic mean of the three respiration values read at 15-minute intervals.

The data obtained with either dye reductions or respiration measurements may be equally indicative of the initial metabolic processes in the inoculated culture, which become gradually intensified, to decrease after having attained a certain peak. Elevated metabolism is incident to the low oxytetracycline production. According to some data, the metabolic processes slow down when antibiotic production takes place [11]. In fermentations producing the antibiotic at a low level, the decrease in metabolism, which follows its peak, is not accompanied by marked oxytetracycline production.

If the weight of the methylene blue reduced by the culture fluid of equal volume in the unit of time is known, it becomes possible to lend a quantitative interpretation to the concept of MB reduction time introduced in connection with the conversion of MB to LMB.

The methylene blue method controlling the activity of the individual members of the biological oxidation-reduction system, taken in conjunction with the data obtained for respiration and antibiotic yield, presents a rapid means by which to keep under routine observation the development of Streptomycetes in aerated liquid cultures.

The curves for enzymic activity plotted with the aid of redox dyes furnish particularly valuable data if they can be brought in numerical correlation with the changes in the metabolism of the culture.

Experiments are in progress to clear the biochemical and physiological questions that have arisen in the course of the present work.

## Summary

Respiration of the hyphae of the *Streptomyces rimosus* strain BS-21, grown in submerged aerated liquid culture, was studied in the Warburg apparatus, and the activities of an oxidation-reduction enzyme system were investigated, using methylene blue and triphenyl tetrazolium chloride solutions.

For the oxidation-reduction enzymic activity and for respiration, curves were obtained which were typical of fermentations yielding oxytetracycline at a satisfactory rate. With iron-contaminated or markedly alkalizing fermentations, these curves assumed peculiar shapes, running courses different from those of typical fermentations. Such cultures possessed but limited capacity to produce oxytetracycline.

In the authors' view, the colour reactions obtained with methylene blue or triphenyl tetrazolium chloride may be regarded as resulting from the activities of a biological oxidation-reduction system.

Knowing the above relationships, the use of triphenyl tetrazolium chloride and particularly of methylene blue solutions, furnishes the tool for speedy and dependable determinations of the physiological state of *Streptomyces rimosus* organisms grown in submerged culture.

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# A NEW METHOD FOR THE INVESTIGATION OF SH. SONNEI CULTURES

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(Received February 17, 1959)

Morphological investigation of bacterial colonies is an important diagnostic method. To the accepted classical procedures of examination new methods have been added in recent years, requiring the use of a microscope and oblique illumination [2—5, 14]; the latter technique appears to be suitable for the purpose of routine faecal bacteriology [13]. The present report deals with the structure of isolated colonies of *Sh. sonnei*, as observed by the aid of a magnifying-glass under oblique rays of light with varying refraction.

## Materials and methods

*Culture.* Agar plates prepared in various ways were used as culture media, but the best effects have been obtained with agar where broth had been replaced by yeast extract. An adequate technique of inoculation is most important the success of investigation being greatly dependent on the growth of numerous separate, completely isolated colonies in the culture. A suitable technique of spreading should therefore be employed (consecutive smearing of several plates with the same inoculum).

*Colony morphology.* Inoculated culture media are incubated for a day at 37° C and then examined with a hand lens under illumination with oblique rays of light. It is unnecessary to work in a dark room, but direct sunshine must be avoided. We use an electric bulb (40 to 60 W) as the source of light.

While the structure of a colony is investigated, the following points merit attention.

(i) The surface of the vertically held Petri dish should in every position remain parallel with the edge of the table.

(ii) The distance between the culture and the source of light should be such as to permit optimum vision of the structural characteristics of the colony under inspection, and of any discernible contours it may have. In the case of one day old colonies with star of *Sh. sonnei*, this distance is about 8 to 10 cm.

(iii) The degree of refraction should be found at which optimum vision of the pattern formed inside the colony is ensured. If an imaginary straight line drawn from the centre of the source of light to the colony is regarded as the path of light, the optimum ray angle formed with the surface of Petri dish in the case of one day old *Sh. sonnei* colonies will be between 60° and 70°.

Thus the surface of the Petri dish held in the left hand should be kept parallel with the edge of the table, in the vertical plane, and the culture turned to face the observer, the colonies are examined through a hand lens held in the right hand. Somewhat laterally from the source of light, the plate is brought at a distance of 6—12 cm from the lamp and, still in the above position, is moved to and from and also right and left until the structure of the colony becomes clearly visible in all its depth and width.

With the plate in the original position, when the ray, starting from the source of light and passing through the colony to be investigated and the centre of the hand lens, reaches the observer's eye in the imaginary straight line, the colony is not inspected, because in this

position the light is dazzling and reliable data are rarely gained. The structure of the colony should be studied in the light of an oblique ray falling on the surface of the plate ; a complete investigation involves observation of the plate in nine different positions adjusted to the various refraction angles *(Table I)*. The consecutive plate positions are obtained by lateral movement of the medium and the magnifying-glass — in the plane of their normal position — until, one after the other, each of the refraction angles listed in *Table I* has been approximately attained. There is no need for precise determination of the refraction angles, since by reference to the brief descriptions in column 3 of *Table I* the position of the plate is easily recognized. Should the colony contain parts differing in refraction, its material will nevertheless display the cited colours where the various dots, grains, lines, and other phenomena will appear as on a background.

**Table I**

*Nine positions of agar plate in oblique light*

| Plate position No. | Ray of light, degree of refraction | Appearance of optically homogeneous colonies |
|---|---|---|
| 1. | 75 | even brownish colour |
| 2. | 70 | even greyish colour |
| 3. | 65 | translucent, colourless |
| 4. | 60 | even pale blue |
| 5. | 55 | greenish, glossy |
| 6. | 50 | orange, glossy |
| 7. | 45 | glossy red |
| 8. | 40 | whitish, glossy |
| 9. | 30 | colourless, glossy |

*Identification of Shigella cultures* by modern morphological, biochemical, and serological procedures has been reported elsewhere. The other — identified — bacterial strains we received from the *Bacteriological Department* of the *State Institute of Hygiene.*

*Absorbed sera* were prepared by the technique of RAUSS ; their agglutination titre was 1 : 160 to 1 : 320.

*Conjunctival infection* was induced by the methods discussed in earlier works [9—11].

*Stored strains* were kept at temperatures varying between 0 and 10° C.

## Results

By the use of oblique light and a hand lens certain structural peculiarities can be distinguished in separately grown isolated colonies of various intestinal bacteria. In the present report the structure of *Sh. sonnei* cultures will be dealt with.

*Structure of Sh. sonnei colonies.* Colonies of *Sh. sonnei* grown on agar can be divided into three large groups on the basis of their optic structure.

1. Most conspicuous are *the colonies with star,* which have been termed so owing to the visible presence of irregular, star-like, strongly refractive formations in the substance of the less refractive colony *(Fig. 1)*. Such formations can be seen most distinctly in plate positions 1—2 and 5—7, at times equally well in both extreme positions, or only in one. As a rule, some expe-

rience is indispensable to be able to discover the star-like contours in *Sh. sonnei* colonies. At the beginning it is advisable to look for the contours in the coloured phase (plate positions 5—7), farther to the side of the source of light, when the vivid fluorescence of the stars is easily discernible.

The star-like shape is due to the more refractive bundles of unequal width and length being situated radially in the optically homogeneous, less refractive substance. Compared to the diameter of the colony, the bundles may vary between 1 : 10 and 1 : 1 in length, and from hardly noticeable line-like forms to a size corresponding almost to the diameter. They either

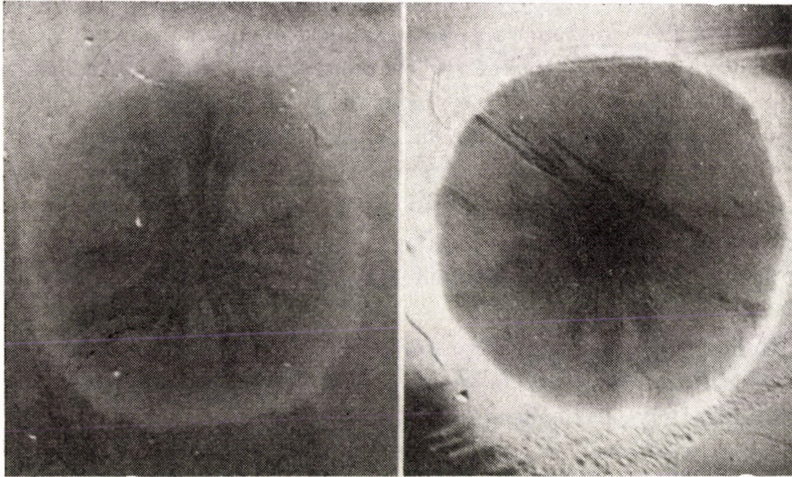

*Fig. 1.* Colonies with star of *Sh. sonnei* magnified twelve to fourteen times (Photographs made by E. K. Novák)

end in a point tapering off towards the periphery of the colony, or in dented or approximately straight lines.

Owing to the extreme variability shown by the radial pattern, irregular shapes occur, differing so much from one another that the resemblance to a star is lost. We have, nevertheless, decided for this term, since star-shaped outlines are encountered in the majority of cultures and in most of the colonies.

Colonies with star are so characteristic of *Sh. sonnei* that on this basis an only colony of this strain can be discovered and isolated in mixed Shigella cultures. In completely isolated colonies, the star-like pattern can be detected already after twenty hours' incubation ; during storage of the culture in a refrigerator or at room temperature the shape of the star may become indistinct or change, but often it is easily distinguished even after fourteen days.

The star-like structure is present in 75 to 100 per cent of the colonies of freshly isolated (1—2 week old) *Sh. sonnei* cultures. While the strain is

stored, the colonies with star diminish in number, and after prolonged storage cannot always be traced in the subcultures *(Table II)*.

In a one-day culture studied under direct light, the colony with star measures 2—3 mm in diameter. It is whitish, sligthly turbid, rounded with regular contours and sharp margins, a moist and shining flat formation with a smooth surface bulging faintly towards the centre around which a radial ribbing may appear in a few days.

2. The second group includes the Sonne colonies where *formations are absent*; these colonies may therefore be regarded as optically homogeneous. Judged by its refractivity, this group may be divided into subgroups.

*a)* Homogeneous, highly refractive colonies. The whole colony consists of a highly refractive substance spreading almost evenly without any contours. Its refraction is similar to that of the star of type 1 colonies. Accordingly, in the first and second positions of the plate, the colony appears as a very turbid, dense brown or grey circular formation. After some experience it can infallibly be differentiated from other homogeneous types. Differentiation is facilitated by slide agglutination (see later) and by subculturing : the subculture of highly refractive colonies invariably contains colonies with star. This type of colony we have been able to observe only as an exception, every time in freshly isolated cultures.

*b)* Homogeneous, moderately refractive colonies. From the preceding subgroup they differ only by being less refractive ; in the first and second plate positions they are consequently found to appear as mildly turbid, brownish or greyish circular formations. Their subcultures do not yield colonies with star. This type of colony is as characteristic of stored cultures as are colonies with star of freshly isolated strains. In cultures of the latter, their number is small, but on storage they occur with increasing frequency ; in some twelve to eighteen months old strains they are found in pure, or nearly pure, cultures *(Table II)*.

*c)* Homogeneous colonies with minimum refractivity. The colonies ranged in this group differ from the above-mentioned two types by their remarkably slight refractivity. Thus, in the first and second plate positions they are almost transparent, and in the seventh position — when the colonies marked type 2b show a bright red colour — 2c colonies fluoresce in a greenish colour. As a result of the latter property, they are easily differentiated from the moderately refractive colonies. This form has been encountered only in small numbers and only in the case of old cultures *(Table II)*.

By study in direct light no difference could be established between one day cultures of colonies belonging to the first and second group ; type 2c alone could be recognized as scarcely turbid, translucent, almost colourless colonies.

**Table II**

*Types of colonies in Sh. sonnei cultures stored for varying periods of time*

| Storage time of strain | Number of strains | Proportional presence in first subculture of colony types | | | | |
|---|---|---|---|---|---|---|
| | | 1 | 2a | 2b | 2c | 3 |
| 1 to 14 days | 3 | +++++ | + | + | — | — |
| | 2 | ++++ | +++ | + | — | — |
| | 16 | +++++ | — | + | — | — |
| | 2 | +++++ | — | +++ | — | — |
| | 1 | ++++ | — | +++ | — | — |
| | 2 | +++ | — | ++++ | — | — |
| 15 to 28 days | 10 | +++++ | — | + | — | — |
| | 1 | +++++ | — | +++ | — | — |
| | 1 | ++++ | — | +++ | — | — |
| | 1 | +++ | — | ++++ | — | — |
| | 2 | + | — | +++++ | — | — |
| 1 to 4 months | 2 | +++++ | — | +++ | — | — |
| | 3 | +++ | — | ++++ | — | — |
| | 4 | +++ | — | +++++ | — | — |
| | 10 | ++ | — | +++++ | — | — |
| | 2 | + | — | +++++ | — | — |
| | 1 | ± | — | +++++ | — | — |
| | 3 | — | — | +++++ | — | — |
| 12 to 18 months | 3 | +++++ | — | ++ | — | — |
| | 1 | ++++ | — | ++ | — | — |
| | 3 | ++++ | — | +++ | — | — |
| | 1 | ++++ | — | ++++ | — | — |
| | 3 | +++ | — | ++++ | — | — |
| | 1 | +++ | — | +++++ | — | — |
| | 2 | ++ | — | +++++ | — | — |
| | 1 | ++ | — | +++++ | + | — |
| | 3 | + | — | +++++ | — | — |
| | 1 | ± | — | +++++ | — | — |
| | 15 | — | — | +++++ | — | — |
| | 5 | — | — | +++++ | + | — |
| | 3 | — | — | +++++ | + | + |

Signs:
+++++ 3/4 to 5/6 part of colonies
++++ 1/2 to 2/3 part of colonies
+++ 1/5 to 1/3 part of colonies
++ 1/20 to 1/6 part of colonies
\+ 1 or 2 colonies in the whole culture
± not recognized with certainty
— not encountered in the whole (distinctly isolated) culture

Under special conditions we have observed mixed forms of 2b and 2c which appeared practically as the negatives of colonies with star : an irregular star-like formation consisting of minimally refractive substance was visible in the centre of the moderately refractive colony.

3. In our material the presence of *colonies with a network pattern* has been rare ; such colonies are covered by a close network composed of more or less delicate and thicker lines whose spaces are filled with minimally refractive substance. In the first and second positions of the plate, this network displays a porcelain white colour and may form rows of rough granules in the glossy zone. This type of colony has been encountered in small numbers and only in subcultures of strains that had been stored for a prolonged period of time. Under direct illumination it presents a characteristic picture : the whitish network is visible on the somewhat irregularly circular flat pin's head size colony with indented margin and uneven surface.

*Structure and fermentative capacity of the colony.* From star-shaped and moderately refractive homogeneous colonies of two *Sh. sonnei* cultures we prepared separate suspensions in physiological saline and then inoculated a sugar series with one drop of each of the fresh suspensions *(Table III).* As to

**Table III**

*Fermentation by types 1 and 2b of Sh. sonnei colonies*

| Substrate | *Sh. sonnei* cultures | | | |
|---|---|---|---|---|
| | No. 24. | | No. 28. | |
| | Colonies | | | |
| | 1 | 2b | 1 | 2b |
| Arabinose ....... | + | + | + | + |
| Xylose .......... | — | — | — | — |
| Rhamnose ...... | + | + | + | + |
| Glucose ......... | + | + | + | + |
| Saccharose ....... | +9 | +9 | +11 | +11 |
| Lactose ......... | +9 | +9 | +13 | +11 |
| Maltose ......... | + | + | + | + |
| Mannitol ........ | + | + | + | + |
| Dulcitol ......... | — | — | — | — |
| Adonitol ........ | — | — | — | — |
| Sorbitol ......... | — | — | — | — |
| Salicin .......... | — | — | — | — |
| Inositol ......... | — | — | — | — |

Signs : + fermentation within 24 hours without any development of gas
+9 fermentation within 9 days without any development of gas
— no fermentation during a thirty day period of observation

fermenting capacity, no significant difference was observed between star-like and homogeneous colonies. Investigation of the fermentative capacity of "network" colonies yielded similar results.

*Type of colony and agglutinability.* Agglutinability of the isolated colonies divided into various groups was studied on slides with absorbed, 1 : 20 dilutions of *S*, phase II, and of *R* antisera *(Table IV)*. As revealed by the results, the connection existing between colony type and agglutinability is so close that the morphology of the colony affords unerring inferences concerning its antigen content. Under the described conditions of study, the refractive capacity of *Sh. sonnei* possessing *S* antigen is unquestionably greater than that of the phase II bacteria. Therefore, the colonies containing *S* antigen alone appear as homogeneous highly refractive formations, the absolutely phase II colonies as homogeneous, moderately or minimally refractive forms. According to agglutination tests, the highly refractive, irregular star-like pattern in the colonies with star consists of *S* individuals, the moderately refractive homogeneous substance of phase II individuals. In network colonies we found phase II and *R* antigens, the latter chiefly when the colonies

**Table IV**

*Antigenic structure of Sh. sonnei colonies varying in optic structure, on the basis of slide agglutination*

| Optic structure of colony | Number of investigated colonies | Age of culture in days* | Results of slide agglutination with absorbed antiserum *Sh. sonnei* | | |
|---|---|---|---|---|---|
| | | | *S.* | Phase II | *R.* |
| With star | 6 | 1 | +++ | ++ | — |
| With star; centre | 8 | 1—7 | +++ | + | — |
| With star; centre | 39 | 1—8 | +++ | — | — |
| With star; marginal parts | 3 | 2—8 | — | +++ | — |
| With star; marginal parts | 3 | 1—8 | + | +++ | — |
| With star; marginal parts | 5 | 1—7 | ++ | +++ | — |
| With star; marginal parts | 1 | 7 | + | — | — |
| With star; marginal parts | 4 | 1 | +++ | +++ | — |
| Highly refractive, homogeneous | 29 | 2 | +++ | — | — |
| Moderately refractive, homogeneous | 41 | 1—8 | — | +++ | — |
| Moderately refractive, centre | 14 | 2 | — | +++ | — |
| Moderately refractive, margin | 3 | 2 | — | +++ | — |
| Slighty refractive, homogeneous | 21 | 1—3 | — | +++ | — |
| Network pattern | 15 | 1 | — | +++ | ++ |
| Network pattern | 14 | 2 | — | +++ | +++ |

* Time from transfer to agar plates

had been left standing for one or two days at room temperature. This type agglutinated spontaneously in physiological saline solution.

*Serial subcultures.* Simultaneous parallel or shortly repeated investigation of the same strain yielded identical results, thus the morphology of the colony was consistent. Next, we studied the changes that colonies of different structure undergo in the course of serial transfer.

(i) Daily serial transfers (on altogether ten occasions) were made of highly refractive homogeneous colonies in which *S* antigen alone was demonstrated by slide agglutination. In the subcultures star-like and highly refractive homogeneous colonies appeared consistently; however, from the fifth subculture on, we also found moderately refractive homogeneous (wholly phase II) colonies, thus pure *S* cultures have not been obtained by serial transfer.

(ii) Ten subcultures were prepared in the same way with the centres of colonies with star. Among the many stared colonies, the development of a small percentage of moderately refractive, homogeneous (phase II) colonies has been noted in every instance.

(iii) The results of serial transfers of marginal parts from colonies with star depended on whether the homogeneous substance of the colony was in fact transferred in an isolated state; in such cases pure phase II cultures were produced already in the first subculture. When the stalk of the star had also found its way into the inoculum, the culture that grew consisted of mixed, 1 and 2b type colonies.

(iv) Whether we used the centre or the margin of colonies for transfer, the subcultures derived from 2b and 2c colonies yielded cultures identical with the colonies that had served as starting point.

(v) Network colonies produced growth of pure cultures containing only network colonies.

If colonies with star are stored at room temperature or in a refrigerator, *S* antigen can be demonstrated in the colony centre for a long time (possibly a fortnight), and type 1 colonies may grow in the subculture even when under direct illumination the original colony corresponds to the *R* form (irregular shape, indented margin, rough surface).

*Test for virulence.* Homogeneous, moderately refractive and colonies with star were removed separately from agar cultures of the same *Sh. sonnei* strain; groups of five guinea pigs were infected on both eyes with types 1 and 2b, respectively. Typical keratoconjunctivitis shigellosa developed in every eye infected with type 1 colonies, while those infected with type 2b all remained intact. As evidenced by conjunctival infection, *Sh. sonnei* cultures consisting of colonies with star are virulent in guinea pigs, whereas cultures consisting of homogeneous, more faintly refractive colonies are avirulent.

During keratoconjunctivitis shigellosa induced by cultures of *Sh. sonnei*, the pathogen grown from the ocular discharge appeared mostly in colonies

with star. In addition, highly, and sometimes moderately, refractive colonies have at times also been encountered ; colonies with phase II antigen can therefore be obtained also directly from the affected organism.

*Use of our method in bacteriological work.* Knowledge of the colony structure allows to isolate the various phases of *Sh. sonnei* cultures and by means of serial subcultures practically pure *S* cultures and pure phase II cultures may be obtained.

In the majority of freshly isolated *Sh. sonnei* cultures, we found colonies with star susceptible to agglutination with homologous antiserum *S, i. e.* stars incorporating antigen *S* seemed to be characteristic of Sonne bacteria. We then proceeded to investigate whether agar cultures of other intestinal bacteria included colonies having the same structure. Among 236 strains belonging to the family of *Enterobacteriaceae (Table V)* only in five *E. coli*

**Table V**

*Number of morphological tests performed with colonies of various instestinal bacteria*

| Type of strain | Cultures stored for | | | | |
|---|---|---|---|---|---|
| | 1 to 14 days | 15 to 18 days | 1 to 4 months | 12 to 18 months | Total |
| *Sh. dysenteriae* 2 | 2 | — | 3 | — | 5 |
| *Sh. flexneri* 1a | — | — | 1 | — | 1 |
| 1b | — | — | — | 2 | 2 |
| 2a | 14 | 15 | 19 | 7 | 55 |
| 3 | 15 | 4 | 6 | 3 | 28 |
| 4a spec. | 2 | 1 | 2 | 2 | 7 |
| 4a gr. | — | 1 | 1 | — | 2 |
| 6 | 1 | — | — | — | 1 |
| X | 4 | — | — | — | 4 |
| Y | — | 2 | 1 | — | 3 |
| *S. typhi* | 9 | — | — | — | 9 |
| *S. paratyphi B* | 1 | — | — | — | 1 |
| *E. coli* | 50 | — | — | — | 50 |
| 055 | 1 | — | — | — | 1 |
| 0111 | 8 | — | — | — | 8 |
| 0124 | — | — | 8 | — | 8 |
| *Proteus* | 8 | — | — | — | 8 |
| *Ps. aeruginosa* | 2 | — | — | — | 2 |
| Lactose-positive not identified bacterium | 38 | — | 2 | 1 | 41 |
| Total | 155 | 23 | 43 | 15 | 236 |

cultures did we find colonies with star which, however, were easily differentiated from *Sh. sonnei* colonies of similar structure. The substance of *E. coli* stars — causing rapid fermentation of lactose — is not susceptible to agglutination with antiserum *Sh. sonnei S*, but the two kinds of star-like colony often manifest morphological differences too.

Thus our method appears suitable for the identification of *Sh. sonnei* colonies. Pin-point amounts of the culture to be investigated (or of suspicious colonies found in elective media smeared with faeces) should be placed on agar plates with due consideration to ensuring the growth of adequately isolated colonies. After one day's incubation, the colonies are studied as explained before, and the colonies with star agglutinated on a slide with *Sh. sonnei S* serum. In the presence of colonies with star agglutinable *S* antiserum, the culture may be ragerded as one of *Sh. sonnei.*

## Discussion

The morphology of *S*, phase II, and *R* forms of *S. sonnei* cultures as seen in direct light is well-known ; however, as early as 1943 RAŠKA stated that changes in a colony's form and its antigen structure do not proceed in parallel [6]. In fact, the *S* and *R* variants of Sonne bacteria or their phases I and II defy unerring differentiation by reliance on current morphological criteria. Contradictory data have been published also on the virulence of *Sh. sonnei* cultures belonging to phase II [7] ; however, on the basis of recent findings this variant may be regarded as avirulent [1, 8, 9, 10, 12].

By means of our method, *Sh. sonnei* cultures possessing various antigens can be easily recognized and differentiated. It thus emerges that a great many of the contradictions found in the literature are presumably due to the authors concerned having worked with phases of inadequate purity. It is also evident that *S* cultures cannot be purified completely because in most cases individuals containing *S* antigen and such as contain phase II antigen occur mixed within a colony. Phase II cultures, on the other hand, can be obtained in an adequately pure condition demonstrating that in the absence of phase I the phase II is avirulent. Virulence in Sonne bacteria may hence be ascribed to the *S* antigen or some other closely related factor.

In the aetiology of bacillary dysentery, the importance of Sonne bacteria has been increasing all over the world, therefore a reliable procedure for their identification may be of interest. We believe our method to be reliable, although not with all kinds of *Sh. sonnei* cultures. Colonies with star develop only in the presence of both phase I and phase II antigens, so that their absence does not yet speak against the diagnosis of *Sh. sonnei*. There are, however, always considerable numbers of colonies with star in freshly isolated cultures, thus our precedure may prove to be a reliable aid in routine bacteriological work.

Positive results not only make it possible to identify Sonne bacteria, but also evidence virulence of the strain under investigation.

Knowledge concerning the connection between morphology and structure of a colony allows an approximate or complete differentiation and isolation of the various phases of *Sh. sonnei* cultures ; this may be of use in vaccine production.

Finally, it must be emphasized that our statements refer exclusively to isolated colonies of entirely independent growth ; in the case of colonies that have developed close to one another or are forming confluent cultures, conditions may be quite different.

## Summary

(i) A new procedure has been described, consisting in studying the morphology of completely isolated bacterial colonies with a hand lens while the colonies under investigation are exposed to rays of light reaching them at various angles of incidence.

(ii) A star-like pattern has been found characteristic of *S* colonies of *Sh. sonnei;* in such colonies the central star-shaped part is agglutinated by *Sh. sonnei S* serum, the marginal part by phase II serum.

(iii) The morphology of colonies formed by *Sh. sonnei* phase II, or the *R* variant has been discussed.

(iv) In conjunctival infection the colonies with star proved to be virulent, while the optically homogeneous, moderately refractive colonies were found to be avirulent.

(v) In freshly isolated *Sh. sonnei* cultures the majority of colonies is stared, hence our method may be employed in routine bacteriological work for the identification of Sonne bacteria and the determination of their virulence.

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# THE OCCURRENCE OF ORGANISMS CAUSING THE BACTERIAL DISEASES OF BEAN IN HUNGARY

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(Received March 14, 1959)

The bacterial diseases of bean have been known in Hungary only symptomatologically. No attempts have so far been made for the isolation and identification of the pathogens. The full knowledge of the part played by bacteria in the diseases of bean is particularly important, as the four species of bacteria responsible for the diseases in other countries cause symptoms which cannot be differentiated from one another. Chiefly on the basis of the German literature, ***Pseudomonas phaseolicola (medicaginis*** **f. sp.** ***phaseolicola)*** and *Xanthomonas phaseoli* have been supposed to be the most common causative agents in Hungary [6, 12]. The purpose of the present work has been the identification of the pathogens and a study of their occurrence in this country.

## Materials and methods

Isolations were performed from yellowing infected seeds, spotted leaves and pods with "grease spot". The pathogenic organism was obtained most easily and in purest culture from the seeds. Prior to cultivation the seeds were rinsed in alcohol and flamed. The green parts of the plants were thoroughly washed in running water.

For isolating and maintaining the cultures, meat-infusion agar was at first used. This was later replaced by glucose-casein-hydrolysate agar, as the former medium damaged *X. phaseoli* var. *fuscans* after 3 to 5 days of incubation. On glucose-casein-hydrolysate agar the cultures remained viable for several months without making subcultures. Parallel with the destruction of the cultures, the colour of meat-infusion agar turned into brown. No brown pigment was formed on glucose-casein-hydrolysate agar. On this medium the growth of *X. phaseoli* was also better and this organism remained viable for a longer period. The growth of *Ps. phaseolicola*, however, was slower on this medium than on meat-infusion agar.

The identification of the organisms was performed by the commonly used methods, employing the usual media. Utilization of carbohydrates was examined in fluid synthetic medium with Andrade indicator; incubation lasted for 25 days. The production of ammonia and $H_2S$ was shown in 1 per cent peptone water. Nitrate reduction was tested in a liquid medium containing $KNO_3$, sugar and salts.

Pathogenicity of the isolated bacteria was tested in green-house by artificial infections. One foliage leaved beans were infected with *C. flaccumfaciens* using HEDGES' needle prick method [5]. For providing a continuous source of infection, a needle was placed in the hair of a brush, which was then dipped into the suspension of the organism. Inoculation of stem by the above method was performed between the cotyledons and the first foliage leaf.

## Results

*Identification and occurrence of pathogenic bacteria.* In describing the various pathogens, only cultural and biochemcial properties differing from those found by previous workers will be mentioned.

*Isolation and identification of Corynebacterium flaccumfaciens.* From infected seeds, the causative agent of bacterial wilt of beans was most frequently isolated. On meat-infusion agar in 2 to 3 days it produced convex, cream-yellow colonies. On subculturing, the pathogenicity of the organism was preserved as long as 2 years. When cultivated for a longer period, it produced two types of colonies. Type A corresponded to the original form, type B was smaller and less creamy in colour. Artificial infections showed that both of the separated colony types retained the original pathogenicity and both of them exhibited an uniform behaviour to phage [8]. The same type of colony variation was observed by HEDGES [5].

The following biochemical reactions were observed. Acid was produced from glucose, galactose, arabinose, xylose, raffinose, sucrose, maltose, mannose, mannitol, fructose, salicin and glycerol. Weak acid was produced from lactose and dextrin after 15 days of incubation. No acid was formed from dulcitol and asparagin. In the literature, the production of acid by the organism in question has been recorded only from glucose, fructose, maltose, lactose, sucrose, galactose and glycerol [10]. The organisms isolated in the present study grew slowly on gelatin ; no liquefaction occurred within 3 weeks. According to HEDGES, *C. flaccumfaciens* slightly liquefied gelatin after 9 weeks [5]. In contrast to HEDGES' finding, instead of clot formation in litmus milk, peptonization began to take place after 5 days of incubation. In some cases HEDGES observed peptonization without clot formation. In litmus milk, due to acid production from glucose, the organism caused pink coloration ; reduction of litmus began on the 7th day. Production of ammonia was observed in most of the experiments ; some cultures produced slight amounts or no ammonia. In other biochemical reactions the cultures behaved as known from the literature.

*The isolation and identification of Xanthomonas phaseoli* var. *fuscans.* This was another frequently occurring organism in Hungary, causing blight of beans. By its brown pigment diffusing into the medium it was easily distinguished from the closely related *X. phaseoli.* Isolations were performed from a variety of leaves, pods and seeds obtained from various parts of the country. The organism was first isolated in 1954. In broth culture it formed a powerful brown pigment within 3 to 4 days. Similarly to *C. flaccumfaciens,* on subculturing for a longer period it produced two types of colonies. In contrast to the original culture, the variant designated B lost the ability of producing gums and it formed smaller colonies brighter yellow in colour.

Infection of the original culture by phage resulted in uniform circular plaques 3 to 5 mm in diameter, while infection of the variant B led to the formation of smaller, irregular, indistinct plaques [7].

Acid was produced from glucose, galactose, arabinose, xylose and sucrose. A weak reaction was observed in the media containing lactose, raffinose, mannose and salicin. No acid was formed from maltose, dextrin, dulcitol, mannitol, glycerol, fructose and asparagin. According to BURKHOLDER, acid was produced from glycerol and mannitol [2].

Formation of ammonia and $H_2S$ in peptone water was more intensive than that observed with *X. phaseoli.*

In other biochemical properties the organism was similar to *X. phaseoli.*

*Isolation and identification of Xanthomonas phaseoli.* The organism was isolated from infected leaves and pods with "grease spots" for the first time in 1957 from material collected in Central Hungary. Three strains showing uniform pathogenic cultural and biochemical properties were examined.

The strains produced acid after 10 days from glucose, galactose, arabinose and sucrose ; after 15 days from lactose, raffinose, maltose, mannose and fructose. No acid was produced from dextrin, dulcitol, mannitol, salicin and asparagin. According to HEDGES [4] and BURKHOLDER [2], the organism produced acid from glycerol.

In contrast to a strain obtained from Germany, the Hungarian strains strongly liquefied gelatin. According to SMITH, gelatin is liquefied by this organism [9].

The results of other examinations corresponded to those described in the literature.

*Isolation and identification of Pseudomonas phaseolicola (medicaginis* f. sp. *phaseolicola).* The first isolation was made in 1957. In contrast with other authors we observed no diffusion into the agar of green fluorescent pigment. According to BURKHOLDER, the lack of pigment formation is characteristic of this organism and it is by this property that it may be differentiated from *Ps. medicaginis* [1].

Acid was produced from glucose, galactose, arabinose, xylose, raffinose, sucrose, mannose and fructose. No acid was produced from lactose, maltose, dextrin, dulcitol, mannitol, glycerol, salicin and asparagin. This result differs from that of STAPP *and* KOTTE whose organism was raffinose-negative [11].

BURKHOLDER [1] stated that gelatin was not liquefied ; according to others [3] and the present investigations, it was slowly liquefied. In contrast to DOWSON's observation, starch was not hydrolysed in the present experiments.

*Artificial infections.* On plants artificially infected with *C. flaccumfaciens*, after 5—7 days a slight mosaic-like spotted yellowing of the topmost leaves was observed. Yellowing among the leaf-veins often shown by plants growing

in the fields was not always observed in plants growing in the green-house. The green-house plants generally exhibited a dull yellowish-green parchment-like necrosis extending over the entire area of the leaf-blades. By infecting older plants of 3—4 foliage leaves, the symptoms developed much later and were slighter. The causative agent was isolated from the infected vascular system.

The cultures of the other organisms were suspended in water and sprayed on the leaves. Symptoms similar to those described above developed after 6—9 days at 28° C. Confirming the pathogenicity of the strains isolated in Hungary, from the artificially infected plants the causative agent was reisolated in every case.

*The occurrence of bacteria pathogenic to beans in Hungary.* Seeds and plants collected from various parts of the country were systematically investigated for 4 years. Altogether 151 strains were isolated and identified. *C. flaccumfaciens* was found to be the organism most widely occurring throughout the country and causing the heaviest damages. According to the examination of seeds, the damage caused by these bacteria is more important than anthracnose due to *Colletotrichum lindemuthianum. C. flaccumfaciens,* the causative agent of wilt, was isolated in 132 out of 151 cases. *X. phaseoli* var. *fuscans* took the second place as to frequency and damage. Although this organism was isolated only in 14 out of 151 cases, it occurred in every region of the country. *X. phaseoli* and *Ps. phaseolicola* were not isolated until the summer of 1957, although on the basis of the literature these organisms have been supposed to be the most important pathogens in Hungary. *X. phaseoli* and *Ps. phaseolicola* were recently isolated from 3 and 2 cases, respectively *(Table I).*

**Table I**

*Distribution of pathogens isolated from infected plants and seeds obtained from various parts of Hungary in 1954—1957*

| Organism | Number | Per cent |
|---|---|---|
| | of isolations | |
| *Corynebacterium flaccumfaciens* ...... | 132 | 87,5 |
| *Xanthomonas phaseoli* var. *fuscans* ... | 14 | 9,0 |
| *Xanthomonas phaseoli* ............... | 3 | 2,0 |
| *Pseudomonas phaseolicola* ............ | 2 | 1,5 |
| Total ......................... | 151 | 100,0 |

It should be noted that the distribution of organisms shown in *Table I* cannot be regarded as permanent, since yearly changes in the weather may

cause the predominance of any of the mentioned pathogens. There is no doubt, however, that during the 5 year period of the present investigations the primary role was played by *C. flaccumfaciens.*

Few data are available as to the degree of damage caused by these bacteria. Two types of damage should be considered : wilt caused by *C. flaccumfaciens* decreases chiefly the yield of seeds, while the disease due to the other bacteria has a comparatively slight influence on seed production, but in consequence of blight it unfavourably affects the market price of green podded beans.

The investigations carried out for years with *C. flaccumfaciens* and *X. phaseoli* var. *fuscans* have shown that the infected plots yielded a 55 to 75 per cent decrease in production as compared to the control. (The data of these experiments will be published later.) Under favourable conditions there may occur a wide spread of the infection : *e. g.* in 1950 and 1951 practically no seeds could be harvested at the Agricultural Experimental Station Kompolt. As to the damage caused in the whole country, no reliable data are known. The extent of the infection is clearly shown by the fact that the presence of the characteristic yellow spots on white bean seeds has been observed in every granary of the villages and farms inspected.

## Discussion

The bacterial diseases of bean are wide-spread all over the world. A number of investigations have been made, especially as to the pathology of halo blight caused by *Ps. phaseolicola* and of common blight caused by *X. phaseoli.* In contrast to data in the literature, in Hungary *C. flaccumfaciens* and *X. phaseoli* var. *fuscans* were the most frequent. The cause of this finding is not quite clear. The special climate of Hungary may be responsible for the frequent occurrence of *C. flaccumfaciens,* since unlike the other pathogens mentioned it does not require high humidity and abundant rain. Bacteria causing local spots can pass through the stomata only with the aid of active water drops. No active water drops are necessary for the spread of wilt, since penetration of the causative agent is possible through the vascular system as far as the seeds. The importance of *C. flaccumfaciens* in Hungary may thus be attributed to the arid climate.

It would be difficult to throw light on the frequent occurrence of *X. phaseoli* var. *fuscans.* This organism may be considered indigenous in Central Europe, as it was first isolated by BURKHOLDER from seeds grown in Switzerland. This supposition seems to be confirmed by the fact that in Europe *X. phaseoli* var. *fuscans* has been isolated exclusively in the U. S. S. R., Switzerland and Yugoslavia.

## Summary

The bacterial diseases of bean have been known in Hungary only symptomatologically. The first evidence of the occurrence in Hungary of *Corynebacterium flaccumfaciens*, *Xanthomonas phaseoli* var. *fuscans*, *Xanthomonas phaseoli* and *Pseudomonas phaseolicola (medicaginis* f. sp. *phaseolicola)* has been presented. According to observations performed from 1954 to 1957, *C. flaccumfaciens* occurred most frequently and caused the most damages. The percentage distribution of the isolated pathogens was 87.5 for *C. flaccumfaciens*, 9.0 for *X. Phaseoli* var. *fuscans*, 2.0 for *X. phaseoli* and 1.5 for *Ps. phaseolicola*. The first two organisms were isolated from every part of the country, while the two others occurred only in the area situated between the Danube and Tisza. The high frequency of *C. flaccumfaciens* may be explained by the special climate of Hungary.

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# ÜBER DIE WIRKUNG DER ULTRAVIOLETT-BESTRAHLUNG AUF DIE STEROIDOXYDATIONS-FÄHIGKEIT VON ASPERGILLUS NIGER*

Von

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(Eingegangen am 27. März 1959)

Arbeiten, welche die Wirkung mutagener Agenzien auf die Steroidoxydationsfähigkeit der Mikroorganismen behandeln würden, sind uns nicht bekannt. Vorliegende Mitteilung berichtet über derartige Untersuchungen. Über den untersuchten Mikroorganismus und seine Oxydationseigenschaften haben wir bereits Angaben veröffentlicht [5] ; es handelt sich um einen ursprünglich zur Zitronensäureproduktion isolierten *Aspergillus niger*-Stamm, der aus Progesteron (I) 11$\alpha$-Oxyprogesteron (II), 11-Epicorticosteron (III) und zwei mehr polare Substanzen (IV, V) bildet *(Abb. 1)*.

## Material und Methoden

Der *Aspergillus niger*-Stamm Nr. 47 wurde auf Bierwürzeschrägagar durch monatliche Abimpfungen aufrechterhalten. Die Ausstreichungen erfolgten ebenfalls auf Bierwürzeagar. Die Sporensuspension für die Impfung bereiteten wir von den Schrägagarkulturen mit 5 ml sterilem destilliertem Wasser. Mit je 1 ml Sporensuspension beimpften wir in 500 ml-Erlenmeyer-Kolben 100 ml Nährboden, die 20 g Malzextrakt und 10 g Pepton in 1 Liter Leitungswasser enthielten ; das pH betrug vor der Sterilisierung 5,5. Sterilisierung : 15 Minuten bei 120° C. Die beimpften Kolben wurden bei 28° C mit 200 Drehungen am Schütteltisch inkubiert. Zu den 48stündigen Kulturen gaben wir sterilerweise 20 mg Progesteron, in 1 ml Aceton gelöst. Nach weiteren 24 bzw. 48 Stunden wurden die Kulturen nach der von PETERSON und Mitarbeitern beschriebenen Methode [4] extrahiert. Der Extrakt wurde eingedampft und am Schleicher—Schüllschen Papier Nr. 2043 a oder b im Propylenglykol-Toluolsystem untersucht [1]. Die Papiere wurden mit phthalsäurigem Paraphenylendiamin entwickelt [2] Die Monosporenisolate isolierten wir mit dem Zeissschen Gleitmikromanipulator. Die Bestrahlung erfolgte mit einer Germicidlampe zu 15 W, die nach dem 99%igen Absterben am Leben gebliebenen Kolonien impften wir auf je 3 Schrägagare. Die Steroidumbildungsfähigkeit des Isolates untersuchten wir in Schüttelkolben, die mit den auf diesen Schrägagaren gewachsenen Kulturen beimpft worden waren.

## Ergebnisse

Vor Einleitung der Mutationsversuche untersuchten wir 400 Monosporenkulturen, ob sie dieselben Oxydationsprodukte liefern. Sämtliche Isolate verhielten sich einheitlich : sie bildeten aus Progesteron die in Abb. 1 gezeigten Substanzen. Sodann führten wir die Bestrahlung der Sporensuspen-

* Diese Arbeit wurde auf dem IV. Biochemischen Weltkongreß (Wien, September 1958) kurz vorgetragen.

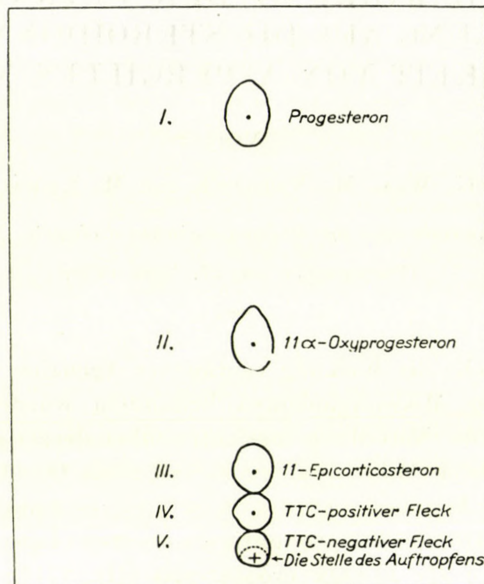


*Abb. 1.* Propylenglykol-Toluol-System. Entwicklungszeit : $4^h$

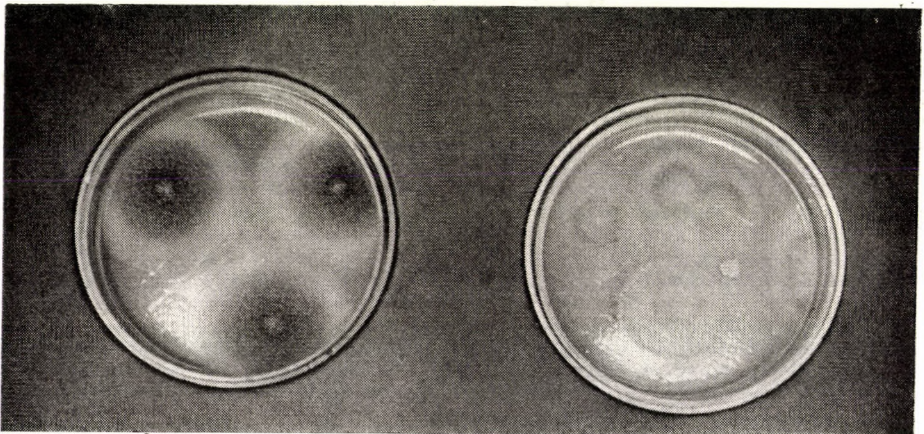

*Abb. 2.* Links : 5tägige Kolonien des Stammes *A. niger* Nr. 47 auf Bierwürzeagar. Rechts : 5tägige Kolonien des Stammes *A. niger* Nr. 22—1 auf Bierwürzeagar

sion des Stammes Nr. 47 mit Ultraviolettlicht durch. Jedes Isolat wurde zweimal untersucht und dieser Parallelversuch in zwei aufeinander folgenden Wochen vorgenommen. Im weiteren beschäftigten wir uns nur mit denjenigen Stämmen, deren Biooxydationseigenschaften bei diesen Versuchen von den

am Originalstamm festgestellten abwichen. Diese Wiederholungen erfolgten indessen nur nach Untersuchung sämtlicher isolierten Stämme. Insgesamt untersuchten wir 186 von nach Ultraviolettbestrahlung gewachsenen Kolonien isolierte Stämme. Unter diesen waren 8, die Progesteron in beiden Parallelversuchen nur zu zwei Oxydationsprodukten, den in *Abb. 1* mit II und V bezeichneten Substanzen, oxydierten. Auffallend war, daß alle diese Varianten viel schlechter sporulierten als der Originalstamm (*Abb. 2*).

Als diese Varianten bei Abschluß der Versuchsreihe nochmals untersucht wurden, gewannen wir z. B. mit der mit 22—12 bezeichneten das Resultat, daß unter den gegebenen Bedingungen wiederum alle vier Oxydationsprodukte nachgewiesen werden konnten. Zugleich sporulierte der Stamm erneut kräftig, zeigte also auch nicht mehr das charakteristische morphologische Bild der Variante (*Abb. 3*).

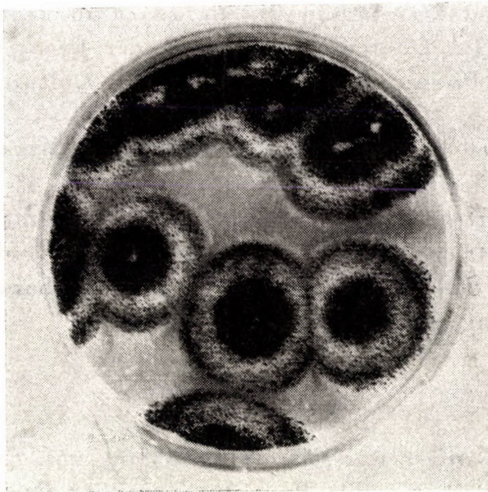

*Abb. 3.* 7tägige Kolonien der Variante *A. niger* 21—12 auf Bierwürzeagar

Von diesen »revariierten« Schrägagarkulturen bereiteten wir eine Sporensuspension und stellten Monosporenkulturen her. Bereits unter den ersten 20 Isolaten fanden wir eine schlecht sporulierende Variante (22—1), die Progesteron nur zur II. und V. Substanz oxydierte, d. h. wieder ein kleineres Oxydationsspektrum aufwies.

Nunmehr untersuchten wir die Stabilität des Isolates 22—1 : die Suspension der einwöchigen Schrägagarkultur wurde ausgebreitet, darin die Anzahl der Kolonien gezählt und die Zahl der gut sporulierenden Typen

bestimmt. Nach erneuter Isolierung einer schlecht sporulierenden Kolonie, die wieder ausgestrichen wurde und so fort, gewannen wir die in *Tabelle I* zusammengestellten Angaben. Die Rückvariation stellt demnach eine ziemlich konstante Zahl dar: das Verhältnis der gut sporulierenden Kolonien beträgt in den Ausstreichungen ungefähr 4%. Wurden die gut sporulierenden Kolonien auf Schrägagar geimpft und in der Schüttelkultur untersucht, so erzeugten sie aus Progesteron immer 4 Oxydationsprodukte.

Suspendierten wir die Sporen von der Schrägagarkultur eines gut sporulierenden Stammes, so erhielten wir eine konzentriertere Sporensuspension als von einer schlecht sporulierenden Kultur. Aus diesem Grunde untersuchten wir die Wirkung der Impfsporenzahl auf die progesteronoxydierende Eigenschaft der Schüttelkulturen. Von dem gut sporulierenden Stamm Nr. 47 beimpften wir mit unverdünnter, 2-, 4- und 10fach verdünnter Suspension, aus der Variante (22—1) aber mit konzentrierter Suspension einige Schüttelkulturen. Die Impfsporenzahl und die in den einzelnen Kulturen nachweisbaren Oxydationsprodukte sind in *Tabelle II* zusammengefaßt. Nach den Ergebnissen ist die Impfsporenzahl innerhalb der Grenzen unserer Untersuchungen auf die Progesteronumgestaltung der Kulturen ohne Wirkung gewesen.

Wir führten auch Kontrollversuche durch, indem wir mit konzentrierter Suspension beimpfte Kulturen von *Aspergillus niger* Nr. 47 vor der Dosierung mit sterilem destilliertem Wasser verdünnten und das umzugestaltende Progesteron den konzentrierten sowie 2-, 4- und 10-fach verdünnten Kulturen zugaben. Wie *Tabelle III* zeigt, hat sich der Oxydationscharakter des Stammes durch die Verdünnung nicht verändert.

## Besprechung

Von *Aspergillus niger* war seit der Mitteilung von FRIED, THOMA *und* PERLMAN bekannt, daß der Pilz Progesteron in $11\alpha$ Position oxydiert [3]. ZAFFARONI und Mitarbeiter teilten mit, mit einem anderen *Aspergillus niger*-Stamm könne Progesteron in Desoxycorticosteron umgewandelt werden [6]. Unser Stamm führt beide Oxydationen auf einmal aus. Wir dachten daher an die Möglichkeit, daß der Stamm nicht einheitlich sei, sondern auch Individuen enthalte, die funktionell den von ZAFFARONI und Mitarbeitern oder PERLMAN und Mitarbeitern beschriebenen Stämmen entsprechen. Die mit den Monosporenkulturen vorgenommenen Versuche schlossen diese Möglichkeit aus.

Nach der Bestrahlung erhielten wir morphologische Varianten, und zwar vom Stamm Nr. 47 eine Variante, die viel schwächer sporulierte. Diese Varianten verfügten auch über veränderte Steroidoxydationsfähigkeit: aus Progesteron bildeten sie hauptsächlich $11\alpha$-Oxyprogesteron und in Spuren die mit V. bezeichnete mehr polare Substanz. Nachdem wir die Möglichkeit aus-

**Tabelle I**

*Die Revariation der Variante 22—1*

| Zeichen der Zerstreuung<br>1. | Anzahl aller ausgewachsenen Kolonien<br>2. | Anzahl der gut sporulierenden Kolonien<br>3. | 3 in Prozentsatz von 2 ausgedrückt<br>4. |
|---|---|---|---|
| 1 | 591 | 24 | 4 |
| 2 | 538 | 39 | 7,2 |
| 3 | 97 | 4 | 4,1 |
| 4 | 63 | 1 | 1,6 |
| 5 | 103 | 0 | 0 |
| 6 | 39 | 3 | 7,7 |
| 7 | 230 | 9 | 3,9 |
| 8 | 77 | 4 | 5,2 |
| 9 | 207 | 5 | 2,4 |
| 10 | 109 | 1 | 0,9 |
| 11 | 66 | 0 | 0 |
| 12 | 110 | 10 | 9 |
| Zusammen : | 2230 | 100 | 4,5 |

**Tabelle II**

*Die Wirkung der Inokulummenge auf die Umwandlung des Progesterons*

| Inokulum | Gewonnene Umwandlungsprodukte | | | |
|---|---|---|---|---|
| $2{,}34 \times 10^7$ *A. niger* 47-Sporen | II. | III. | IV. | V. |
| $1{,}17 \times 10^7$ *A. niger* 47-Sporen | II. | III. | IV. | V. |
| $5{,}8 \times 10^6$ *A. niger* 47-Sporen | II. | III. | IV. | V. |
| $2{,}34 \times 10^6$ *A. niger* 47-Sporen | II. | III. | IV. | V. |
| $5{,}7 \times 10^6$ Variante 22—1 Sporen | II. | | | V. |

**Tabelle III**

*Die Umwandlung von Progesteron durch verschieden verdünnte Kulturen des Aspergillus niger 47 und die Kultur der 22—1 Variante*

| Kultur | Gewonnene Umwandlungsprodukte | | | |
|---|---|---|---|---|
| *A. niger* 47 | II. | III. | IV. | V. |
| *A. niger* 47 ; 2mal verdünnt | II. | III. | IV. | V. |
| *A. niger* 47 ; 4mal verdünnt | II. | III. | IV. | V. |
| *A. niger* 47 ; 10mal verdünnt | II. | III. | IV. | V. |
| Variante 22—1 | II. | | | V. |

geschlossen hatten, daß die veränderte Oxydationseigenschaft der Kulturen die Folge der sich aus dem morphologischen Bild ergebenden niedrigeren Impfsporenzahl oder des schwächeren Wachstums sei, gelangten wir zu dem Schluß, daß die Steroidoxydationsfähigkeit des Stammes durch Ultraviolettbestrahlung verändert werden konnte. Mit mutagenen Agenzien läßt sich demnach die Steroidoxydationsfähigkeit der Mikroorganismen ebenso beeinflussen wie z. B. ihre Antibiotikumproduktion. Im vorliegenden Fall gewannen wir eine Mutante, die quantitativ weniger Oxydationsprodukte mit besserer Ausbeute erzeugte als der ursprüngliche Stamm.

Die Parallelität, mit welcher sich die Sporulierungsfähigkeit des Stammes und die Funktion des steroidoxydierenden Enzyms verändert haben, war überraschend. Die schlecht sporulierende Variante mutierte mit konstanter Geschwindigkeit zu gut sporulierenden Abkömmlingen zurück, und zugleich gewannen diese ihre volle Steroidoxydationsfähigkeit wieder. Es ist jedoch zu bemerken, daß die Sporulation, wie ein Blick auf *Abb. 2* und *3* zeigt, bei den revariierten Kolonien nicht ganz denselben Typus aufweist wie bei dem Originalstamm Nr. 47. Wir besitzen bisweilen keine Angaben über den Punkt in dem sich die Bedingungen der reichen Sporenbildung und der oxydierenden Enzyme treffen. In Bezug auf die Oxydationsenzyme können wir nur soviel feststellen, daß bei der Mutante die Bildung der III. und IV. Substanz ausgefallen ist, d. h. die beiden $\alpha$-Ketol-Seitenkette enthaltenden Substanzen (mit Triphenyltetrazoliumchlorid Reaktion gebende Flecke) sind nicht entstanden. Die Mutante weicht also nur in einer Eigenschaft vom Originalstamm ab : es fehlt ihr das Enzym (-System), das am Kohlenatom 21 eine Oxy-Gruppe bildet.

Die Mutante kann auf Grund ihrer morphologischen Merkmale leicht aufrechterhalten werden.

## Zusammenfassung

Mit Ultraviolettbestrahlung wurde aus dem *Aspergillus niger*-Stamm Nr. 47, der aus Progesteron 11 $\alpha$-Oxyprogesteron, 11-Epicorticosteron und zwei mehr polare Substanzen bildet, eine morphologische Mutante gewonnen, die aus Progesteron nur zwei Oxydationsprodukte lieferte : hauptsächlich 11 $\alpha$-Oxyprogesteron und in Spuren eine mehr polare Substanz.

Die Mutante hat demnach ihr 21-Hydroxygenase-Enzymsystem verloren.

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# FREQUENCY DISTRIBUTION OF THE COMPLEMENT FIXING ANTIBODIES TO ADENOVIRUSES IN SEVERAL GROUPS OF THE HUNGARIAN POPULATION

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(Received April 10, 1959)

Soon after the adenoviruses had been discovered and their pathogenicity demonstrated, investigations were initiated to establish the frequency of adenovirus infections in various groups of the human population [1—11]. For such investigations, owing to its simple technique, the complement fixation (CF) test seems to be most suitable. It is to be noted, however, that the CF antibodies disappear soon after infection has been overcome, thus, previous adenovirus infections can be demonstrated by the test only for short period of time. Up to present, 23 serological types of adenoviruses have been described, each of which shares a common CF antigen. CF antibodies to adenoviruses can thus be detected by using antigens prepared from several strains, or even one single strain, of the adenovirus group.

The occurrence of adenoviruses in Hungary has been demonstrated in three laboratories. We isolated one type 2 and two type 5 strains from surgically removed tonsils [12], two type 3 strains were isolated in Szeged [13] from patients with pharyngoconjunctival fever and a type 4 strain was recovered from an infant with atypical pneumonia at the *Department of Paediatrics No. II of University, Budapest* [14].

So far, no quantitative data on the incidence of adenovirus infections in Hungary have been published. Some data on the frequency of adenovirus CF antibodies in the sera of the Hungarian population are reported in the present report.

## Materials and methods

*CF antigen.* Representative strains of each of the types 1 to 7 adenoviruses were cultured in *Detroit 6* cell cultures as described previously [16—18]. The maintenance fluids were harvested and pooled when the cells had detached from the glass wall. The 7 pools were mixed, then frozen and thawed 5 or 6 times and centrifuged at low speed. The supernatant was stored at —18° C until used as antigen. In some cases the antigen was heated to 56° C for 30 minutes immediately before use in the CF test.

*CF test.* Takátsy's micro technique [15] was employed. Each serum was tested in two or three parallel rows of serial twofold dilutions from 1 : 4 to 1 : 512. The sera were tested also with a control antigen prepared from the maintenance fluids of non-infected *Detroit 6* cultures. For the sake of comparison, a positive standard serum was always titrated simultaneously.

## Results

In four groups of different age distribution and different state of health, altogether 426 persons were examined for CF antibodies to adenoviruses in sera.

Group I. Eighty-five children, mostly below 5 years of age, under orthopaedic treatment following an attack of poliomyelitis.

Group II. Sixty-one, 10 to 20 year-old patients suffering from lung tuberculosis.

Group III. A group of 163 apparently healthy, 18—20 year-old men and women living in different parts of Hungary, applying for admission to the *University Medical School, Budapest.*

Group IV. One hundred and seventeen adults aged 20 years or more, apparently in good health.

The distribution of CF positive sera in the four groups is presented in *Table I.* It can be seen that CF antibody was demonstrated in a number of sera in each group.

**Table I**

*Results of CF tests*

| Group | No. of sera examined | Positive sera | |
|---|---|---|---|
| | | No. | per cent |
| I. | 85 | 41 | 48.2 |
| II. | 61 | 15 | 24.5 |
| III. | 163 | 24 | 14.7 |
| IV. | 117 | 16 | 13.6 |
| Total | 426 | 96 | 22.5 |

**Table II**

*Age distribution of CF positive persons in groups I, II and IV*

| Age years | No. of sera examined | Positive sera | |
|---|---|---|---|
| | | No. | per cent |
| 0— 5 | 64 | 29 | 45.3 |
| 5—10 | 18 | 8 | 44.4 |
| 10—15 | 39 | 13 | 33.3 |
| 15—20 | 25 | 6 | 24.0 |
| 20—30 | 40 | 9 | 22.5 |
| 30—40 | 30 | 4 | 13.3 |
| 40—50 | 13 | 1 | 7.7 |
| over 50 | 34 | 2 | 5.8 |

In *Table II* the persons belonging to the groups I, II and IV have been grouped by age, irrespective of the Roman-numbered group they belong to.

Some inverse relation between age and the frequency of positive sera can be observed.

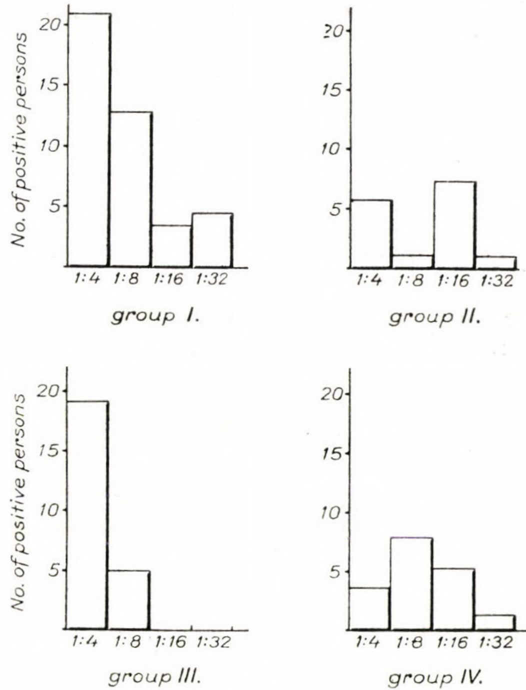


*Fig. 1*

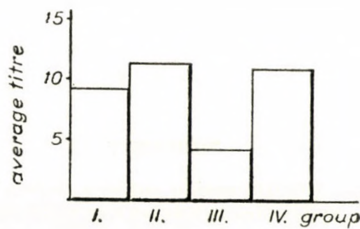


*Fig. 2*

As to the antibody level in the positive sera, the highest titre in each of the groups I, II and IV was equally 1 : 32. In group III it was 1 : 8. The distribution by titre of the positive sera in each of the four groups are presented in *Fig. 1.*

The arithmetic means by group of the reciprocals of titres are illustrated in *Fig. 2.*

## Discussion

The proportion of seropositive persons varied in the four groups examined. It was as high as 48.2 per cent for group I and only 13.6 per cent for group IV. A similar discrepancy was found by ZAIMAN *et al.* [8] who had tested the sera of 54 university students and 65 factory workers. CF antibody to adenoviruses was detected in 76 per cent of the students whereas only 20 per cent of the workers were seropositive. When examining 207 patients showing various symptoms of disease, ANDRIEU *et al.* [10] found 8.7 per cent positive sera. SPENGLER *et al.* [9] reported that among 240 healthy recruits 30 per cent had CF antibodies.

The titres in the positive sera varied between 1 : 4 and 1 : 32. The latter titre is only twofold, *i. e.* one step of dilution, in excess of the highest titre, 1 : 64, reported by the authors cited above.

As to the distribution by age, BALDUCCI *et al.* [11] found CF antibodies to adenoviruses mainly in sera of persons under the age of 30 years. Similarly, of our 77 persons over 30 years only 7, *i. e.* 9.1 per cent, had CF antibodies in contrast to the 25.5 per cent (89/349) positive sera found in the age groups under 30. The inverse relation between age and the proportion of seropositive persons seems to be marked, as shown in *Table II*. However, groups I and II consisted of persons who had lived together for months, and thus cannot be regarded as randomized representatives of the corresponding age groups. In group III, which seemed to be more adequate, fewer seropositive persons (14.7 per cent) were found than expected for 18—20 year-old persons as computed from the figures presented in *Table II*.

In the present work we only intended to obtain some information on the level of CF antibodies to adenoviruses in some groups of the Hungarian population. Further investigations are needed to reveal the actual distribution and aetiological importance of adenoviruses in this country.

## Summary

Serum specimens from 426 Hungarian subjects belonging to four groups have been tested for complement fixing antibodies to adenoviruses. Group I consisted of children under orthopaedic treatment following an attack of poliomyelitis ; group II included patients with pulmonary tuberculosis, between 10 and 20 years of age ; group III and IV consisted of healthy persons aged 18—20 years and more than 20 years, respectively. The proportion of sera with positive reaction was 48.2 per cent in group I, 24.5 per cent in group II, 14.7 per cent in group III and 13.6 per cent in group IV. There was some inverse relation observable between age and this proportion. The highest titre was 1 : 32 in each of the groups I, II and IV, and 1 : 8 in group III.

*Acknowledgement:* We are highly indebted to the leaders of the *Heine-Medin Hospital, Budapest;* of the *State Children Sanatorium, Szabadsághegy, Budapest;* of the *Institute for Health Examination of the University Medical School, Budapest;* and of the *Central Institute of the National Blood Transfusion Service* for supplying the blood specimens. We wish to thank also Miss Eva Egri and Mr. I. Balázs for excellent technical assistance.

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# NEW CONTRIBUTIONS TO PERMANENT HETEROKARYOSIS OF STREPTOMYCES

By

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(Received April 14, 1959)

In a previous paper [1] we reported on the occurrence of spontaneous heterokaryosis in a Streptomyces species. It was stated that this light grey species with straight air mycelia, when inoculated after trituration with glass pearls or exposure to ultraviolet irradiation, yields a dark grey, spiral, sporiferous species that can be isolated. The spiral sporiferous species is identical with *Streptomyces fimbriatus*, while the original species is almost identical with *Streptomyces fasciculus*. It has furthermore been stated that there are significant differences in antibiotic production by *Streptomyces fimbriatus*, the non-sporiferous *Streptomyces fasciculus* resulting after trituration, and the original strain.

According to the pertaining literature, artificially induced heterokaryosis of Streptomyces persists only until the onset of sporogenesis [2—6]. The present report gives an account of new studies concerned with the duration of heterokaryosis.

## Materials and methods

The Streptomyces species used in the present experiments was the same as that employed in the work reported on in our previous paper [1], *i. e.* the genus determined as *Streptomyces fasciculus*, isolated in 1950 from marshy soil collected on the shore of *Lake Balaton*. Of this strain-designated 5a [7] — it was characteristic that in half a year its antibiotic spectrum shrank to the minimum, and that it produced only air mycelia but no spores.

This species was grown on potato-tripton-glucose agar containing 300 g peeled potatoes, 5 g glucose, 20 g agar, in 1000 ml tap water. The potatoes, cut into small slices, were boiled in 1000 ml water for about an hour ; after filtration through fine null and addition of the other constituents, the medium was adjusted to pH 6.8.

The state of heterokaryosis was investigated by two methods. *a)* A thick glass tube was filled with glass pearls and the intervening spaces were filled with tap water ; then the tube was sterilized in an autoclave. Air mycelia were transferred from oblique agar into this tube which was then placed in a shaker and shaken for 30 minutes. Thereafter agar plates were inoculated with an adequate dilution of the triturated air mycelium suspension and incubated for two weeks. *b)* The other method was based on ultraviolet irradiation ; this was made with Hannau-Höhensonne, type 300 S, from a distance of 35 cm for 6, 7, 8, or 9 minutes.

## Results

The original *Streptomyces fasciculus* is presented in *Fig. 1*, showing that the straight air mycelia of light grey colour do not form any spores.

About three to four per cent of the colonies that grew from the incubated mycelium fragments of *Streptomyces fasciculus* triturated with glass pearls,

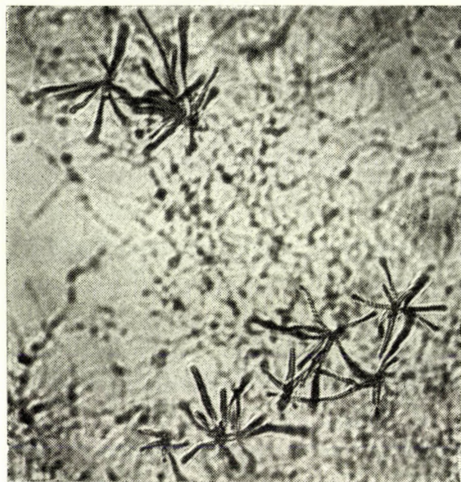

*Fig. 1.* Air mycelia of heterokaryogenic *Streptomyces fasciculus*. Microphotograph of living material. Magnified 280 times

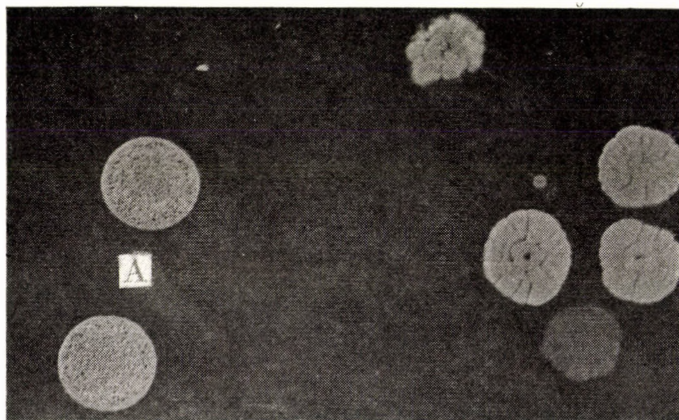


*Fig. 2.* The colonies marked A are sporiferous forms of *Streptomyces fasciculus*, while the other colonies consist of non-sporiferous forms. Natural size

produced and at the same time formed straight spores. Such colonies are presented in *Fig. 2;* the striking difference of the two colonies marked A from the rest is clearly visible. Their surface is homogeneous, of a more intense grey colour and granular form, while the original colonies, some of them of a

lighter grey, display semicircular shapes. A sporiferous hypha producing straight spores — isolated from the colony marked A — is shown in *Fig. 3.*

Another three per cent of the colonies formed by the triturated hyphae bore spiral spores (see *Fig. 4)*. This species identified as *Streptomyces fimbriatus*, is of a more intense grey colour than that bearing straight spores.

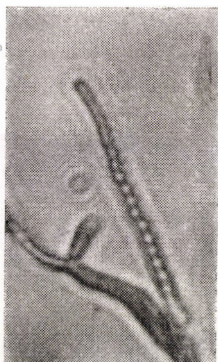

*Fig. 3.* Sporiferous *Streptomyces fasciculus:* straight spore-bearer with spores. Microphotograph of living material. Magnified 1300 times

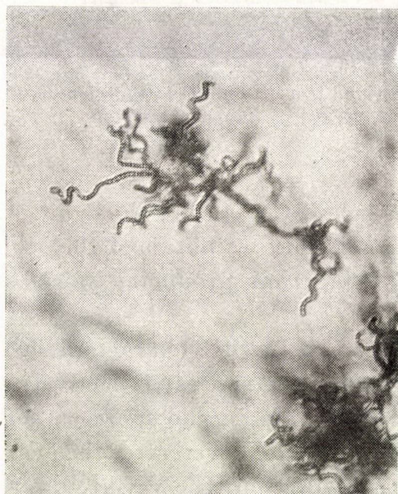

*Fig. 4. Streptomyces fimbriatus* bearing spiral spores. Microphotograph of living material. Magnified 430 times

Finally, approximately 0.5 per cent of the colonies deriving from the triturated hyphae were of a light greenish yellow colour. The colonies showed no peculiar structure, as confirmed by those marked A in *Fig. 5.* They were furthermore characterized by developing only air mycelia and no spores.

In potato agar, aging cultures produced a pink dye diffused in the medium, which lent a pink shade to old cultures. This species has so far defied determination ; it may be identical with *Streptomyces alboflavus.*

The species thus differentiated vary widely as to nutritional conditions.

Investigations have been undertaken to disclose whether some of the isolated genera show homokaryosis. To this end we employed ultraviolet irradiation. It was found that from the original strain the same three species are obtained by ultraviolet irradiation as by trituration. The species resulting from ultraviolet irradiation were exposed to further irradiation : this procedure was repeated with each of the species thus gained, altogether four times. Even after the fourth irradiation, we obtained the other two from the species bearing straight spores, from that producing spiral spores, as well as from the yellow one.

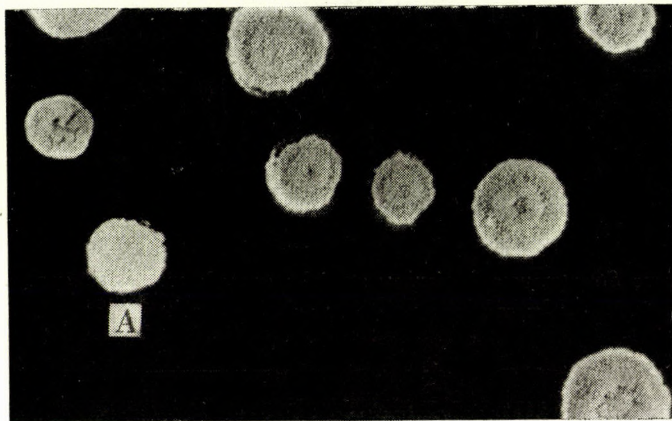


*Fig. 5.* Yellow species derived from *Streptomyces fasciculus*, marked A in the picture. Natural size

These genera possessed a further characteristic feature. In half a year, those bearing spiral and straight spores respectively reverted to the original grey *Streptomyces fasciculus* producing no spores, whereas the species forming yellow air myceliums did not change even after the passage of a year. Following trituration or ultraviolet irradiation, the reverted species again yielded high percentages (20—25%) of spore-bearing species producing spiral or straight spores.

As shown in *Table I*, the antibiotic spectrum of the species obtained by trituration in some cases differs from that of species gained by ultraviolet irradiation. For instance, individuals of *Streptomyces fasciculus* obtained by trituration have a much wider spectrum than those origination after ultraviolet irradiation. The species gained by trituration yields an antibiotic that

**Table I**

*Antibiotic spectrum determined by cross tests*

| Test organisms | Original strain | *Streptomyces fimbriatus* | *Streptomyces fasciculus* | *Streptomyces fimbriatus* | *Streptomyces fasciculus* | Streptomyces spore with yellow air mycelium | |
|---|---|---|---|---|---|---|---|
| | | obtained by trituration | | obtained by ultraviolet irradiation | | obtained by ultraviolet irradiation | obtained by trituration |
| *Staphylococcus aureus* ........ | 0 | 16* | 5.7 | 12 | hardly | 0 | hardly |
| *Bacillus subtilis*... | 0 | 11 | 10 | 16 | 8 | 3 | 3 |
| *Escherichia coli* .. | hardly | 0 | 6.7 | 0 | 0 | 0 | 0 |
| *Sarcina lutea* .... | 12 | 20 | 15 | 22 | 8 | 22 | 20 |
| *Serratia marcescens* | 0 | 0 | 7.8 | 0 | 0 | 0 | 0 |
| *Bacillus radiobacter* | hardly | 5 | 14 | 4.7 | 4 | 0 | 0 |
| *Mycobacterium peregrinum* .... | 6 | 15 | 6 | 7 | 0 | 2 | 2 |
| *Mycobacterium phlei* | 2 | 10 | 3 | 15 | hardly | 0 | 2 |
| *Mycobacterium smegmatis* ..... | 14 | 8 | 3 | 0 | hardly | 0 | 2 |
| *Nocardia rosea*.... | 0 | hardly | 20 | 12 | 5 | 2 | 5 |
| *Streptomyces griseus* | 12 | 10 | 10 | 20 | 2 | 15 | 15 |
| *Penicillium chrysogenum* ......... | 0 | 0 | hardly | 18 | 15 | 5 | 20 |

* Diameter of inhibition zone in mm.

acts on *Escherichia coli,* while that produced by ultraviolet irradiation does not. On the other hand, *Streptomyces fimbriatus* obtained by ultraviolet irradiation is almost similar to that gained by trituration. The yellow species whether obtained by trituration or by ultraviolet irradiation, shows an antibiotic spectrum closely approaching that of the original strain.

*Table I* furthermore reveals that the antibiotic spectrum varies with each species ; moreover, inside these variations there are differences between the species obtained by trituration and those produced by ultraviolet irradiation. Presumably, the numerical proportion of the nuclei of various origin they contain is different in every instance.

## Discussion

The development of various species under the influence of ultraviolet irradiation is not the result of a suppressor effect, as evidenced by the appearance of the same species after trituration with glass pearls.

Heterokaryosis seems to be a permanent phenomenon. This heterokaryogenic condition persists even among the descendants of sporiferous species. Hence the claim put forward in the pertaining literature quoted *(l. c.)* is untenable, because heterokaryosis does not cease with sporulation. This also implies the presence of more than one nucleus in the spore itself, passing on the state of heterokaryosis to descendants. Numerical proportion of the incorporated nuclei of different origin determines the species whose characteristics are to prevail in the progeny. The individual produced will belong to the species represented by the majority of nuclei. This majority is only numerical, since sporiparous species bearing straight or spiral spores have been proved to revert in a few months to a non-sporiferous species corresponding to the original strain. Moreover, by trituration or ultraviolet irradiation the two other species can be obtained from every apparently pure species.

The fact that in time the species reverts to a non-sporiferous form, provides ground for some further considerations. In a state of heterokaryosis it cannot be indifferent which of the conjugate species has received in its cytoplasm the partner's nuclear material at conjugation. This cytoplasmic effect appears to prevail in every case. Nuclei of a species that may have attained majority at the outset will in time be relegated to the background owing to effect of the cytoplasm. The initial state is restored, though not completely, for such reverted strains again yield much higher percentages of the species produced first than does the original strain. The assumption that the cytoplasm may exert such an effect is supported by the experiments of DANIELLI *et al.* with nuclear transfer in Amoeba [8]: they transferred the nucleus of *Amoeba proteus* into *Amoeba discoides* deprived of its own nucleus. The animal survived for six years and multiplied at a normal rate. From our point of view it is essential that the nucleus of *Amoeba proteus* did not transform the cytoplasm of the *Amoeba discoides* which to the end kept most of its morphological character. Heterokaryogenic Streptomyces present a similar phenomenon, with the difference that some kind of nucleus, having reached numerical superiority, temporarily exerts an influence on the phenotype of the species but soon the cytoplasmic effect gains ascendency and gradually suppresses the action of intrusive nuclei, presumably by moderating proliferation of alien ones. Being apparently unable wholly to eliminate conclusively the effect of alien nuclei, the species develops a mixed morphological character.

The species bearing yellow air mycelia possesses the most constant character. A year after isolation from the original strain it was still unchanged although still heterokaryogenic. Owing to the presence of a certain proportion of *Streptomyces fasciculus* and *fimbriatus* nuclei received at conjugation, the colour had shifted towards a light grey ; as to the other morphological features,

it corresponded to *Streptomyces fasciculus*, therefore the original species has also been termed *fasciculus*.

In nature these heterokaryogenic strains appear as intermediary forms. As evidenced by our experiment, their antibiotic spectrum is variable, in some instances even unstable. At the beginning, after isolation from its natural environment, our experimental material had acted on *Escherichia coli*, later it has lost this capacity. The last conjugation may have taken place at a time preceding its isolation from soil, and it might have been at that occasion that the species had acquired its effect on *Escherichia coli*, which effect, however, ceased in the phenotype, due to cytoplasmic action.

The various isolated species showed different antibiotic spectra, but differences were encountered even among individuals of the same species: *e. g.* the antibiotic spectrum of the species bearing loose, straight spores was different from that of the species displaying markedly spiral forms isolated from it. In addition to the changes in morphological character, the responsibility for the differences in antibiotic effect may be ascribed to the nuclear preponderance of one species and to cytoplasmic influences.

KRASSILNIKOV [9] refers to sporogony by notching and by fragmentation. In our experiments the original species forming initially only air mycelia subsequently to produce fragmented mycelia, whereas both *Streptomyces fasciculus*, bearing straight spores, and *Streptomyces fimbriatus*, bearing spiral spores, produce spores by notching. The "spore" formed by KRASSILNIKOV's fragmentation is most probably the result of balanced heterokaryosis.

## Summary

A non-sporiparous, heterokaryogenic species resembling *Streptomyces fasciculus*, which had been in the collection of our department for eight years, has been subjected to trituration with glass pearls or exposed to ultraviolet irradiation. Both methods made it possible to isolate three distinctly differentiated species. Of the three species, two had become sporiferous. One bore straight, the other spiral, spores ; the third differed from the original species only in colour. After having been maintained for a year, the two sporiferous species reverted to a form that was similar to the original species ; upon fresh trituration, this again produced a high percentage of sporiparous species corresponding to the original strain, and a lower percentage of the other two. Thus the various species retained heterokaryosis. Reversion of the two sporiferous species was most probably brought about by cytoplasmic influences.

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# SH. SONNEI CULTURES FERMENTING SALICIN

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(Received April 21, 1959)

**The usual biochemical investigation of intestinal bacteria involves determination of their effect on salicin. Under the influence of enzymes, salicin, containing 2-oxybenzylalcohol-beta-d-glucopyranoside (termed also saligenine-beta-d-glucopyranoside) [1] releases glucose which is broken down by certain intestinal bacteria with formation of acid. Thus, in salicin-peptone water cultures of bacteria fermenting salicin, fermentation of the glucoside is followed by acid reaction. It has been generally accepted that Shigella cultures do not break down salicin [3, 6, 9], consequently the members of the *Enterobacteriaceae* family responsible for the fermentation of this glucoside cannot be regarded as Shigella. The present report shall deal with *Sh. sonnei* cultures that break down salicin.**

## Materials and methods

In the fermentation experiments four kinds of media were used. These have been discussed in detail elsewhere and were

1. Peptone water prepared with Andrade's indicator [9].
2. The same as No 1, only alkalized with N/10 sodium hydroxide to the point of discoloration.
3. Broth prepared with brom-thymol-blue indicator, as recommended by Kauffmann [6].
4. Peptone water with brom cresol-purple indicator.

Salicin was dissolved in such culture media previously sterilized in an autoclave (at 1 per cent concentration in No 1 peptone water, at 0.5 per cent concentration in the other media). Transfer into tubes was followed by one hour's sterilization in Arnold's apparatus. As a rule, a pin-point amount of the one day old agar culture was used as inoculum and incubated at 37° C ; the observation period was thirty days.

*Identification of Shigella cultures* was carried out by the aid of up-to-date morphological, cultural, biochemical, and serological methods on which we have reported elsewhere. Investigation included morphological study of colonies under oblique illumination [14] ; virulence of the strains was checked by conjunctival infection [4, 8, 11—13, 15—17].

*When fermentation had set in*, material from several media of each series was transferred on the surface of agar plates and purity of the agar culture was checked.

*Primary agar slant culture.* After having been isolated, the strains were stored on Dorset media in a refrigerator (at + 4° C). Every time we started with agar slant subcultures derived from this Dorset culture in order that investigations undertaken at various points of time might be carried out on — as far as possible — similar cultures.

## Results

*Sh. sonnei culture fermenting salicin.* The *Sh. sonnei* strain No 88 was isolated in 1958 at the *Bacteriological Department* of the *State Institute of Hygiene* in the course of routine faecal tests ; detailed examinations were initiated a few days following isolation, and the strain was found to break down salicin *(Table I)*. Apart from the breakdown of salicin, strain 88, as shown by our findings, was a (saccharose negative) *Sh. sonnei* culture, yet, according to currently valid views, it ought to have been excluded from the Shigella genus. Since in salicin culture media acid reaction could be demonstrated with varying frequency but at all events unfailingly in every series consisting of an adequate number of culture media *(Tables II* and *III)*, investigations were initiated for the purpose of elucidating the origin of this atypical fermentation.

*Purity of the strain.* Breakdown of salicin may have been due to admixture of contaminating bacteria to the strain. As a matter of fact, our strains were after isolation grown on fresh desoxycholate-citrate (DC) agar and all Dorset cultures were prepared from one and the same DC colony ; however, in other

**Table I**

*Properties of Sh. sonnei strain No 88*
Primary agar slant culture

| Fermentation | | Other tests | |
|---|---|---|---|
| Arabinose ....... | + | Indole | negative |
| Xylose .......... | — | $H_2S$ | negative |
| Rhamnose ...... | + | Gelatin | negative |
| Glucose ......... | + | Ammoniumcitrate | negative |
| Saccharose ....... | — | Voges-Proskauer | negative |
| Maltose ......... | + | Methylred | positive |
| Lactose ......... | +15 | Urea | negative |
| Adonitol ........ | — | Motility | negative |
| Mannitol ........ | + | Conjunctival infection | positive |
| Dulcitol.......... | — | Star-shaped colonies | in great numbers |
| Sorbitol ......... | — | Slide agglutination in serum *S* of *Sh. sonnei* | positive +++ |
| Salicin .......... | +9 | | |
| Inositol | — | Staining | Gram-negative rods |

Key to signs used under the heading fermentation :
\+ acid formation within 24 hours, without the development of gas
+9 acid formation after 9 days
— no acid formation during the 30 day period of observation

instances we had noticed that not even this procedure could ensure the development of absolutely pure cultures in every case. In order to eliminate any possible contamination of *Sh. sonnei* strain No 88, we therefore proceeded as follows.

a) *Serial subcultures on DC agar.* Five subcultures were produced in succession on DC agar, by the use of another isolated colony at every step. Ten specimens each of peptone water Nos 2 and 4 were inoculated with agar slant subcultures grown from the fifth DC culture (immediately and strongly agglutinated by serum *Sh. sonnei S).* Acid reaction appeared in three of the ten tubes containing medium No 2 (after 4, 4 and 7 days, respectively), and in one of the ten tubes containing medium No 4 (after 7 days).

b) *Serial subcultures in dulcitol-peptone water.* Five subcultures were started in succession in dulcitol-peptone water ; after passage through agar plates and agar slant cultures (results of slide agglutination : *Sh. sonnei,* phase II highly positive ; *Sh. sonnei S* and *R* negative), the material derived from the fifth subculture was used for inoculation of 25 tubes containing peptone water No 2. After a latency period of four to fifteen days, acid was formed in seven out of the twenty-five culture media.

c) *Serial conjunctival infection.* Up to the present, keratoconjunctivitis could be elicited in guinea pigs only by *E. coli* 0 : 124 and Shigella cultures ; other intestinal bacteria are destroyed within a few days on the conjunctiva of the guinea pig, while virulent Shigella and *E. coli* 0 : 124 individuals proliferate. The conjunctiva of the guinea pig was used as an elective culture medium when conjunctival infection with *Sh. sonnei* strain 88 culture was carried out five times in succession ; for fresh infection, agar subculture of the only colony grown in ocular secretion was used in each case. The data concerning salicin breakdown by the purified cultures thus obtained have been summarized in *Table II.*

**Table II**

*Capacity of Sh. sonnei strain No 88 to break down salicin in animal passage*

| Conjunctival infection No | In peptone water culture No | | | |
|---|---|---|---|---|
| | 2 | | 4 | |
| | Number of culture media showing | | | |
| | Unchanged reaction | Acid reaction | Unchanged reaction | Acid reaction |
| 2 | 7 | 1 | 5 | 3 |
| 3 | 8 | 2 | 9 | 1 |
| 5 | 9 | 1 | 9 | 1 |

Thus, various methods of purification failed to yield a culture of *Sh. sonnei* strain No 88 free from variants that decompose salicin.

*Purity of substrates.* The possibility had also to be taken into consideration that the salicin preparation employed was not sufficiently and was contaminated with material whose breakdown by *Sh. sonnei* cultures was associated with the formation of acid. The salicin in question was a product of *British Drug Houses, Ltd.*, and its purity was checked by determination of the melting point which was found to be 196° C (KEREKES). Various authors [5, 7] put the melting point of pure salicin between 199 and 201° C, hence the preparation used in our work may have been regarded of satisfactory purity. After crystallization it was tested again. Thirty-five tubes filled with culture medium No 2 prepared with re-crystallized salicin were inoculated with primary agar cultures of *Sh. sonnei* strain No 88 ; acid formation occurred in twenty-four tubes on the sixth to fourteenth day of incubation.

As a control we employed salicin made by the Czechoslovak firm *La Fachema* (our thanks are due to Dr. E. KLECKOVÁ-ALDOVA for the supplies). Twenty-five tubes of No 2 culture medium — prepared with the Czechoslovak product — were inoculated with primary agar culture of *Sh. sonnei* strain No 88 ; acid was formed in every one of the tubes after four to thirty days.

Thus, the splitting of salicin cannot be ascribed to a contamination of the glucoside. Other observations furnished indirect corroboration of this statement. When salicin was dissolved in peptone water at low concentration (0.1 per cent), acid formation nevertheless occurred in part of the tubes inoculated with *Sh. sonnei* strain No. 88, though in this instance the assumed contamination could have been but very slight. Among Shigella cultures, *Sh. sonnei* strains alone proved to be capable of breaking down salicin (see later), which also tends to bar contamination.

*Possible defects in culture media* (the presence in peptone of some compound susceptible to fermentation, infection of tubes that escapes detection by the usual sterility tests [2], any change in salicin at high temperatures) may form sources of error. For inoculation with the strain under review, we therefore used culture media containing no sugar and furthermore carried out the tests whose results are presented in *Table III.* Our findings justify the statement that the observed breakdown of salicin cannot be explained by technical defects ; in subcultures of *Sh. sonnei* strain 88, variants decomposing salicin were demonstrated under widely varying conditions.

*Oxidative or fermentative reaction?* In peptone waters containing carbohydrate, acidification may take place not only in consequence of fermentation but also as a result of oxidative processes. To exclude the latter, salicin-peptone waters from which the absorbed air had been expelled by boiling, were inoculated with primary agar cultures of *Sh. sonnei* strain No 88 and its promptly fermenting variant (see later). After inoculation, half of the tubes were

**Table III**

*The frequency of salicin breakdown by Sh. sonnei strain No 88 in various culture media*
Primary agar slant cultures

| Culture medium | Inoculated culture media, number of tubes showing | | Latency period days |
|---|---|---|---|
| | Unchanged | Acid | |
| | reaction | | |
| No 1 (Andrade's indicator) | 2 | 8 | 4—14 |
| No 2 (Alkaline peptone water) | 20 | 10 | 2—12 |
| No 3 (Kauffmann's broth) | 31 | 9 | 3—12 |
| No 4 (Brom cresol purple indicator) | — | 10 | 4—23 |
| No 1 Seitz filtrate | 2 | 3 | 3—20 |
| No 2 Prior to inoculation stored for alternative 10 day periods in thermostat and at room temperature | 20 | 5 | 4—19 |
| Total | 75 | 45 | |

sealed with sterilized paraffine oil by lay over the culture medium. Acid was still formed, both under aerobic and anaerobic conditions, hence the process may be interpreted only as fermentation.

*Repeated identification of the strain.* Since the above-discussed experimental results provided evidence of strain No 88's unquestionable capacity to ferment salicin, while in the general opinion cultures which break down salicin cannot be regarded as Shigella, we began to wonder whether identification of our strain was not based on some error. All tests were therefore repeated by whose aid our strain might be differentiated from Shigella. On this occasion each test was carried out in five to twenty parallel. The results incontrovertibly confirmed that strain 88 was a culture of *Sh. sonnei.*

*Structure of colonies in cultures of strain No 88.* These investigations led to the elaboration of a new method for the morphological determination of colonies in *Sh. sonnei* cultures [14], which has been employed also for the identification of strain No 88. Each transfer was followed by the development of numerous star-shaped colonies *(Table I)*, with moderately refractive, homogeneous specimens among them. Subcultures derived from the central part of star-shaped colonies yielded a fair number of star-shaped colonies and few moderately refractive, homogeneous ones, whereas growths from the marginal parts of star-like colonies produced exclusively homogeneous, moderately refractive colonies. When serial subcultures were prepared with salicin-peptone water, network colonies alone developed in the fifteenth subculture. Material taken from the centre of star-shaped colonies was agglutinated only by absorbed *S* serum of *Sh. sonnei,* that from the marginal line by phase II, and some-

times by *S*, sera ; moderately refractive homogeneous colonies showed agglutination in the presence of phase II antiserum, while network colonies were agglutinated by phase II, and at times by *R*, antisera. Thus, the morphological features displayed by the structure of strain No 88 colonies were in every respect characteristic of *Sh. sonnei* cultures.

*Studies concerning the antigenic structure of strain No 88.* The antigenic structure of our strain was examined by cross absorption. Rabbits were immunized with vaccine derived from primary agar cultures of *Sh. sonnei* strains Nos 88 and 254 (salicin-negative) killed with formalin. The agglutinating sera thus obtained were absorbed partly by identical material, partly by star-shaped or moderately refractive homogeneous colonies of the other *Sh. sonnei* culture. The capacity of the sera was tested by slide agglutination before and after absorption *(Table IV)*. As revealed by these studies, salicin-positive and salicin-negative cultures have the same antigenic structure.

*Growth of variant fermenting salicin promptly and consistently.* After a latency period of nine days, the indicator showed acid formation in salicin-peptone water inoculated with the primary agar culture of *Sh. sonnei* strain No 88. Material from this culture was transferred with a loop to fresh salicin-peptone water ; discoloration of the culture medium set in after two days. Passage was continued in a similar manner until the fifteenth transfer, but from the third subculture on, acid was found to develop within 24 hours. Serial subcultures thus brought about a remarkable shortening of the latency period preceding breakdown of the glucoside ; after a few passages a variant was produced which fermented salicin in twenty-four hours. This capacity proved to be constant : all of twenty-five simultaneously inoculated tubes of salicin-peptone water culture medium showed acid reaction after incubation for one day. During three months' storage in a refrigerator, the variant kept its capacity for prompt fermentative action on 1 per cent of salicin in both common agar and in Dorset culture media. Concerning other properties, the variant possessed the features summed up in *Table I* as characteristic of primary cultures, with the difference that it split saccharose after thirteen days, did not form any star-shaped colonies, but grew only in moderately refractive homogeneous (seventh subculture) or network (fifteenth subculture) colonies.

No close connexion seems to exist between the enzymes that break down salicin, lactose, and saccharose. Of the eighth salicin-peptone water subculture, passages were made by transfer with a loop to eight tubes of lactose-peptone water and eight tubes of saccharose-peptone water ; the decomposition time of lactose was not shortened but remained eight to fifteen days, and no fermentation of saccharose set in within twenty-one days.

*Breakdown of salicin by various types of Shigella.* The fermentation of salicin *(Table V)* by Shigella cultures freshly isolated (before one to fourteen days) from faeces has also been investigated. Decomposition of salicin was

**Table IV**

*Antigenic structure of salicin-positive and salicin-negative Sonne cultures, tested by cross absorption*

| Serum (1 : 20 dilution) | Results of slide agglutination with | | | |
|---|---|---|---|---|
| | *Sh. sonnei* strain No 88 | | *Sh. sonnei* strain No 254 | |
| | Type of colony | | | |
| | No 1 | No 2b | No 1 | No 2b |
| *Sh. sonnei No 88* | | | | |
| Before absorption | ++++ | +++ | ++++ | +++ |
| After absorption with colony type 88 No 1 | — | ++ | — | ++ |
| 88 No 2b | ++++ | — | ++++ | — |
| 254 No 1 | — | + | — | + |
| 254 No 2b | ++++ | — | ++++ | — |
| *Sh. sonnei No 254* | | | | |
| Before absorption | +++ | ++ | +++ | ++ |
| After absorption with colony type 88 No 1 | — | + | — | + |
| 88 No 2b | +++ | — | +++ | — |
| 254 No 1 | — | + | — | + |
| 254 No 2b | +++ | — | +++ | — |

Remark: Colony type No 1 = star-shaped colony
Colony type No 2b = moderately refractive, homogeneous colony.

**Table V**

*Fermentation of salicin by genus Shigella*

| Type of strain | Investigated strains | |
|---|---|---|
| | Total number | Salicin positive |
| *Sh. dysenteriae* 2 | 5 | — |
| *Sh. flexneri* 1b | 1 | — |
| 2a | 55 | — |
| 2b | 1 | — |
| 3 | 26 | — |
| 4a "A" | 7 | — |
| 4a "B" | 1 | — |
| 6 | 1 | — |
| X | 5 | — |
| Y | 2 | — |
| *Sh. sonnei* | 69 | 8 |
| Total | 173 | 8 |

observed only in *Sh. sonnei* cultures ; however, in these cultures, too, change of colour of the indicator ensued late in every case, after a latency period of four to twenty-six days. Out of the sixty-nine *Sh. sonnei* cultures examined eight strains were found to be salicin-positive, hence late breakdown of salicin appears to be a rather frequent phenomenon in the Sonne group. It deserves mentioning that of the eight salicin-fermenting strains, five proved to be late saccharose-positive, and three were saccharose-negative.

Salicin-negative Sonne strains may come to yield salicin-fermenting variants, provided that a long chain of parallel passages are made by inoculation into salicin-peptone water. When five strains of *Sh. sonnei* — found to be incapable of fermenting salicin in the first test — were each grown in twenty tubes of salicin-peptone water, acid was formed in one culture on the twenty-ninth day.

## Discussion

It is a generally accepted view amounting to a rule that Shigella cultures are incapable of breaking down salicin. In the pertaining literature available we have found only one contrary statement : among the forty-one strains of Sonne bacteria they investigated, ROELCKE *and* EYCKMAYER [10] observed fermentation of salicin in one culture. According to the definition of the *Shigella Commission* (1950), strains belonging to the Shigella group do not break down salicin [6]. As evidenced by our investigations, considerable numbers of Sonne strains, though late and inconsistently, are capable of splitting this glucoside ; a promptly and consistently decomposing variant can be obtained by serial subcultures of such strains. Every other property of the variant capable of breaking down salicin corresponds to the special characteristics of the Sonne group. With due consideration to these circumstances it is hardly tenable that strains decomposing salicin should be excluded from the Shigella genus. However, we do not think that the definition ought to undergo a fundamental modification. The following wording would, we believe, tally with the facts : "In general, Shigella strains do not ferment salicin, but some cultures of *Sh. sonnei* may break down this glucoside, though the process is apt to set in late and irregularly".

As concerns the biochemical qualities of salicin-positive Sonne cultures, they manifest the closest likeness to the *Dispar-Alkalescens* group, type 02, of genus *E. coli.* From the latter it can, however, be easily differentiated by the negative indole reaction ; the regular, prompt breakdown of rhamnose ; by late but unfailingly ensuing fermentation of lactose and the difference in antigenic composition ; finally, by the pathogenicity evidenced in conjunctival infection and on the basis of star-shaped colonies. Intermediate strains are known to be common in the family of *Enterobacteriaceae* [6]. Salicin-

positive cultures of *Sh. sonnei* may in our opinion be regarded as such transitorial strains between Shigella and *E. coli.*

## Summary

(i) Out of sixty-nine *Sh. sonnei* strains identified by the aid of modern morphological, biochemical, and serological methods of procedure, eight cultures were found to ferment salicin late, after a latency period of four to twenty-six days, and inconsistently, in 10 to 100 per cent of peptone water cultures.

(ii) Decomposition of salicin did not occur in cultures of *Sh. dysenteriae* 2 and *Sh. flexneri.*

(iii) A strain causing breakdown of salicin was subjected to detailed study; it has been ascertained that fermentation could not be ascribed to methodical error but was a newly discovered capacity of the strain.

(iv) Our results indicate that it is not correct to exclude salicin-positive strains from the Shigella genus.

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# ÜBER DIE WIRKUNG DER ZÜCHTUNG IN STREPTOMYCES AUREOFACIENS-KULTUREN AUF DIE ANTIBIOTIKUMPRODUKTION EINZELNER STREPTOMYCES-ARTEN

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(Eingegangen am 27. April 1959)

Vor der Entdeckung des transformierenden Effektes der Desoxyribonukleinsäure (DNS) [1] und auch noch danach galt es als einfachste Transformationsmethode, daß man den umzugestaltenden Mikroben mit irgendeinem abgetöteten Mikroorganismus zusammen züchtete (z. B. GRIFFITH [2]) oder den Körper des Donors destruierte und filtriert der Kultur der umzugestaltenden Art zugab [3, 4, 5]. Über die transformierende Wirkung der gemeinsamen Züchtung stehen bisher wenige Angaben zur Verfügung [6].

Am Warschauer Antibiotikum-Symposium haben wir eine Methode der gemeinsamen Züchtung mitgeteilt, die eine Veränderung in der Antibiotikumproduktion einzelner Streptomyces-Arten herbeiführte [7]. Es handelt sich um die Züchtung auf Streptomyces aureofaciens-Kulturen. Wir hatten lediglich hervorgehoben, daß sich in gewissen Fällen das Spektrum der behandelten Streptomyces unter der Wirkung der gemeinsamen Züchtung wesentlich vergrößert. Aus den seither durchgeführten Versuchen ging jedoch hervor, daß die Antibiotikumerzeugung der behandelten Streptomyces nach gemeinsamer Züchtung während einer gewissen Zeit auch abnehmen oder verlorengehen kann. Dieselbe Wirkung ist in gewissen Fällen auch bei der Behandlung mit der aus *Str. aureofaciens* hergestellten DNS wahrzunehmen. Über derartige Versuche wollen wir nachfolgend berichten.

## Material und Methoden

Wie bereits erwähnt, war die transformierende Art *Streptomyces aureofaciens*, von der wir früher festgestellt haben [7], daß ihr vegetatives Myzel bei der Züchtung am Schrägagar in kurzer Zeit (etwa 14 Tagen) zugrunde geht. Auf der zugrunde gehenden Myzelmasse können andere Streptomyces-Arten gut gezüchtet werden. Während der Züchtungsdauer wirkt *Streptomyces aureofaciens* transformierend auf die auf ihr gezüchteten Arten. Bei unseren Versuchen verwendeten wir folgende Arten : *Streptomyces griseus, Streptomyces globisporus, Streptomyces flaveolus* sowie die nicht näher bestimmten, mit xjl, R3, R5, und J bezeichneten Arten, die seit 1951 unserer Artensammlung angehören.

Die Antibiotikumproduktion wurde folgendermaßen ermittelt : Wir legten Metallringe mit gleichem Durchmesser auf eine Glasplatte und gossen in diese unter sterilen Bedingungen eine bestimmte Bouillon-Pepton-Glukoseagarmenge und beimpften den Nährboden mit den Sporen einer der zu untersuchenden Streptomyces-Arten. Von diesen Agarplatten schnitten wir nach 4tägiger Inkubation mit einem zu diesem Zweck konstruierten Apparat [8]

Scheiben heraus, die wir auf ein Testbakterien (*Staphylococcus aureus*, *Bacillus subtilis*, *Escherichia coli*) enthaltende Agarplatte legten. Damit aus der Agarscheibe eine entsprechende Antibiotikummenge vor dem Wachstum der Testbakterien in die Agarplatte diffundieren könne, wurde das Testgefäß 12 Stunden lang bei einer Temperatur von —5° C aufbewahrt. Danach erfolgte die Inkubation bei 28° C, und anschließend maßen wir die Radien der Hemmungszone (*Abb. 1 u. 2*) .

Die Präparation der DNS aus *Streptomyces aureofaciens* nahmen wir nach der Methode von MIRSKY und POLISTER [9] mit einer gewissen Modifikation vor. Das Myzel wurde mit 0,14 *M* NaCl gewaschen, mit Quarzsand zerrieben und zuerst die Ribonukleinsäure mit 0,14 *M* NaCl extrahiert, sodann die DNS bei pH 7 mit 1 *M* NaCl. Anschließend befreiten wir die DNS von ihrem Proteingehalt. 1 *M* NaCl-Lösung wurde mit einem Chloroform-Isoamylalkoholgemisch mehrmals ausgeschüttelt und die Nukleinsäure über die wässrige Phase mit Alkohol gefällt, was wir mehrmals wiederholten. Die auf diese Weise gewonnene Nukleinsäure gab positive Diphenylaminreaktion. Ihr UV-Absorptionsspektrum liegt bei 2600 Å. Auf Grund des Spektrums kann man sie als fast ganz proteinfrei bezeichnen.

## Ergebnisse

Die Streptomyces-Arten, die wir zu transformieren versuchten, hielten wir im allgemeinen solange auf den am Schrägagar befindlichen *Streptomyces aureofaciens,* bis die Sporenbildung begann ; dann wurden sie auf eine 5tägige *Streptomyces aureofaciens*-Schrägagarkultur weitergeimpft. Jede benutzte Art führten wir bis zur 20. Passage, mit Ausnahme von *Str. globisporus,* die wir bis zur 100. Passage weiterimpften.

Wie aus *Tabelle I* hervorgeht, trat in der Antibiotikumerzeugung der Streptomyces-Arten bis zur 20. Passage im allgemeinen Senkung ein. Von den untersuchten Streptomyces-Arten hatten 5 ihre antibiotische Wirkung gegenüber *Staphylococcus aureus* und *Escherichia coli* ganz verloren, während die Antibiotikumproduktion von 2 Arten diesen Bakterien gegenüber wesentlich herabgesetzt war. *Bacillus subtilis* hat demgegenüber insgesamt in 3 Fällen bis zur 20. Passage zum Aufhören der Antibiotikumbildung geführt. Im allgemeinen stellten wir bis zur 5. und 10. Passage Erhöhung, in den weiteren Passagen jedoch Senkung der Antibiotikumproduktion fest. Gegen die transformierende Wirkung von *Streptomyces aureofaciens* wurde der stärkste Widerstand von *Str. globisporus* geleistet. Deshalb führten wir diese Art durch weitere Passagen, wobei wir feststellten, daß der Zustand, der bei den anderen bereits in der 20. Passage beobachtet werden konnte, daß sie nämlich ihre Wirkung auf *Staphylococcus aureus* und *Escherichia coli* verloren, hier bei der 60. Passage eintrat. In *Tabelle I* ist die Antibiotikumerzeugung der 100. Passage angegeben. Es bestand indessen kein wesentlicher Unterschied zwischen den Ergebnissen der 60. und 100. Passage.

Die Verminderung der Antibiotikumproduktion von *Str. globisporus* nach der 30. Passage auf *Str. aureofaciens* ist in *Abb. 1* und *2* deutlich zu sehen. Papierchromatographisch stellten wir 3 Komponenten des von *Str. globisporus* erzeugten Antibiotikums fest. Von der 30. Passage an fällt eine Komponente aus der Produktion ganz aus. Die weiteren Passagen bewirken, daß auch die anderen beiden Komponenten nur in minimaler Menge produziert wurden.

**Tabelle I**

*Veränderung der Antibiotikumproduktion verschiedener Streptomyces-Arten im Verlauf der Passagen auf Str. aureofaciens*

| Auf Streptomyces gezüchtete Arten | Anzahl der Passagen | Größe der ausgeübten Hemmung | | |
|---|---|---|---|---|
| | | *Staphylococcus aureus* | *Bacillus subtilis* | *Escherichia coli* |
| *Streptomyces griseus* | 0 | 4* | 12 | 6 |
| | 5 | 5 | 13 | 4 |
| | 10 | 3 | 11 | 3 |
| | 15 | 3 | 11 | 3 |
| | 20 | 0 | 4 | 0 |
| *Streptomyces xjl* | 0 | 0 | 6 | 0 |
| | 5 | 0 | 5 | 2 |
| | 10 | 0 | 4 | 0 |
| | 15 | 0 | 4 | 0 |
| | 20 | 0 | 2,5 | 0 |
| *Streptomyces R5* | 0 | 4,5 | 5 | 0 |
| | 5 | 5,5 | 3,5 | 0 |
| | 10 | 3,5 | 2,5 | 0 |
| | 15 | 3 | 2,5 | 0 |
| | 20 | 2,5 | 2 | 0 |
| *Streptomyces R3* | 0 | 4 | 0 | 0 |
| | 5 | 8 | 6 | 0 |
| | 10 | 7 | 4 | 0 |
| | 15 | 0 | 0 | 0 |
| | 20 | 0 | 0 | 0 |
| *Streptomyces flaveolus* | 0 | 0 | 0 | 0 |
| | 5 | 4 | 2 | 0 |
| | 10 | 2 | 0 | 0 |
| | 15 | 0 | 0 | 0 |
| | 20 | 0 | 0 | 0 |
| *Streptomyces J* | 0 | 0 | 3 | 0 |
| | 5 | 0 | 3 | 0 |
| | 10 | 12 | 6 | 0 |
| | 15 | 0 | 0 | 0 |
| | 20 | 0 | 0 | 0 |
| *Streptomyces globisporus* | 0 | 12 | 9 | 8 |
| | 5 | 11 | 8 | 4 |
| | 10 | 10 | 6 | 3 |
| | 15 | 11 | 6 | 3 |
| | 20 | 12 | 5,5 | 2,5 |
| | 30 | 5 | 3,5 | 2 |
| | 100 | 0 | 3 | 0 |

* Radius der Hemmungszone in mm, nach der Agarblockmethode gemessen.

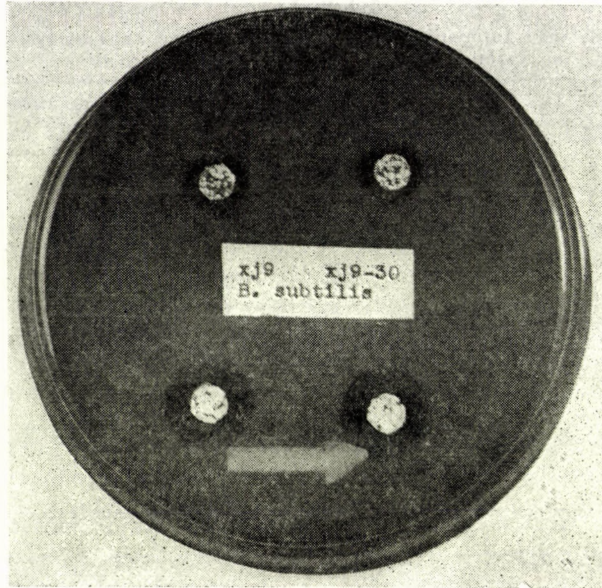


*Abb. 1.* Antibiotikumproduktion von *Streptomyces globisporus* (xjl) gegenüber *B. subtilis*, nach der Agarblockmethode gemessen. Unten, am Pfeil, sieht man die normale, oben die nach der 30. Passage auf *Str. aureofaciens* festgestellte antibiotische Wirkung

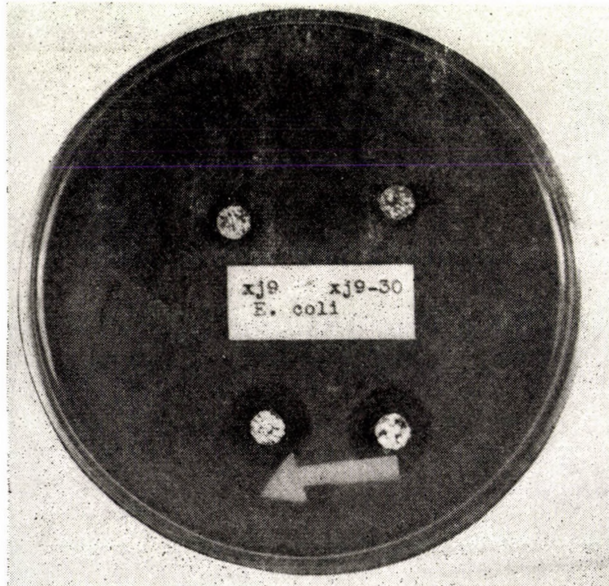


*Abb. 2.* Antibiotikumproduktion von *Streptomyces globisporus* (xjl) gegenüber *E. coli*, nach Agarblockmethode gemessen. Erklärung s. *Abb. 1.*

In *Tabelle II* zeigen wir die veränderte Antibiotikumerzeugung des mit der aus *Streptomyces aureofaciens* gewonnenen DNS weitergezüchteten *Str. globisporus.* Die Behandlung erfolgte auf Schrägagar, von dem wir *Str. globisporus* nach der Sporenbildung auf das neue, DNS-haltige Schrägagar übertrugen. Wie aus *Tabelle II* ersichtlich, geht die Wirkung auf *Staphylococcus aureus* und *Escherichia coli* von der 4. Passage an verloren, während die Wirkung auf *Bacillus subtilis* zunimmt. Dasselbe Resultat erzielten wir, wenn *Str. globisporus* in flüssigem Nährboden unter Zugabe von DNS gezüchtet wurde.

**Tabelle II**

*Veränderung der Antibiotikumproduktion von Str. globisporus im Laufe der in Anwesenheit der aus Str. aureofaciens isolierten Desoxyribonukleinsäure durchgeführten Passagen*

| Anzahl der in Anwesenheit der aus *Str. aureofaciens* gewonnenen DNS durchgeführten Passagen | Größe der Hemmung | | |
|---|---|---|---|
| | *Staphylococcus aureus* | *Bacillus subtilis* | *Escherichia coli* |
| 0 | 7* | 12 | 6 |
| 3 | 5 | 17 | 2 |
| 4 | 0 | 13 | 0 |
| 5 | 0 | 16 | 0 |

* Radius der Hemmungszone in mm, nach der Agarblockmethode gemessen.

## Besprechung

Es wurde festgestellt, daß die Züchtung auf *Streptomyces aureofaciens* im Stadium der ersten Passagen im allgemeinen eine Vergrößerung des antibiotischen Spektrums herbeiführt, das ungefähr von der 15. Passage an wieder kleiner wird, so daß die Antibiotikumproduktion auch ganz aufhören kann. Es gibt auch Arten, die sehr starken Widerstand zeigen und bei denen das Spektrum in dem Maße, das bei den anderen nach der 20. Passage beobachtet werden kann, erst nach zahlreichen Passagen eingeengt wird. Die Behandlung mit der aus *Streptomyces aureofaciens* gewonnenen DNS bewirkt indessen nach ganz wenigen Passagen dasselbe Ergebnis.

Aus unseren Versuchen geht weiterhin hervor, daß die transformierende Wirkung vom Gesichtspunkt der Antibiotikumproduktion nicht in Richtung der Donoreigenschaften in Erscheinung tritt. Die transformierte Art übernimmt nicht das antibiotische Spektrum des Donors. Es handelt sich vermutlich darum, daß die Antibiotikumproduktion einen erblichen Defekt darstellt, der durch gemeinsame Züchtung oder durch Behandlung mit DNS verringert oder in anderen Fällen behoben werden kann. Die Antibiotikum-

erzeugung wird durch den transformierenden Effekt bei einer Art rascher bei der anderen später aufgehoben, was im Zusammenhang mit der Antibiotikumbildung für die stärkere oder schwächere Beschaffenheit der erblich erworbenen Eigenschaft zeugt.

## Zusammenfassung

1. Auf *Streptomyces aureofaciens* wurden 7 Streptomyces-Arten *(Str. griseus, Str. globisporus, Str. flaveolus, Str. xjl, Str. R3, Str. R5* und *Str. J)* gezüchtet und nach Sporenbildung auf die 5tägige vegetative Schrägagar-Myzelkultur von *Str. aureofaciens* überimpft.

2. Nach den ersten Passagen hatte das antibiotische Wirkungsspektrum der Streptomyces zugenommen, das sich jedoch im Laufe der weiteren Passagen verminderte, ja in mehreren Fällen auch ganz verschwand.

3. Dasselbe Ergebnis trat ein, und zwar in wesentlich kürzerer Zeit, wenn die Passagen in einem Nährboden durchgeführt wurden, der aus *Streptomyces aureofaciens* gewonnene Desoxyribonukleinsäure enthielt.

4. Die Antibiotikumproduktion wird im Hinblick darauf, daß sie durch transformierende Wirkung aufgehoben werden kann, als ein erblicher Enzymdefekt angesehen.

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# THE BIOCHEMICAL AND SEROLOGICAL PROPERTIES OF PROTEUS MORGANII

By

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(Received April 28, 1959)

In his original paper, Morgan [1] described the strain that bears his name as follows. "A Gram negative, generally flagellated organism, which produces indole, does not liquefy gelatin and does not form clot in milk. Lactose, maltose, mannitol, sucrose and dulcitol are not fermented by it ; glucose is fermented with slight gas production. Serologically the strains are heterogeneous." This description had well-characterized the strain called later as Morgan's No 1 bacillus. Unfortunately, by describing some strains with different properties, Morgan *and* Ledingham [2] subsequently greatly confused identification of the original strain, which really represented an individual group of bacteria. The numerous investigations into the biochemical and serological properties and the systematic position and pathogenicity of *B. morganii* failed to solve the problem [2—7].

On the basis of their ability to swarm and their antigenic structure, Rauss in 1936 brought evidence that *B. morganii* No 1 strains belonged to the *Proteus* group [8]. These observations were confirmed by Sevin *and* Buttiaux [9], who found that placing these bacteria in the *Proteus* group was reasonable. These opinions were accepted by Bergey, who in the 1939 edition of "*Manual of Determinative Bacteriology*" regarded Morgan's bacillus as a member of the *Proteus* group.

Rauss' above-mentioned investigations did not include the establishment of an antigenic schema. The present examinations have been undertaken as the more extensive knowledge of the taxonomic position of the *Enterobacteriaceae* groups requires the exact determination of the antigenic structure of all groups of bacteria and also the elucidation of the group interrelationships. The investigation was further motivated by Fulton's opinion [10] according to which the *P. morganii* group may be regarded as a separate genus : *Morganella*. In the present study, Kauffmann's recent recommendation, namely that *P. morganii* should be one of the genera of the *Proteus* family, will also be discussed.

## Materials und methods

*Cultures.* From faecal samples taken from infants and children with enteric infections (dyspepsia, dysentery, enteritis) and from the faeces from healthy infants and children, 121 strains were isolated in this Institute. Of these strains, 116 were freshly isolated, while 5 of them were stock cultures. 101 strains of similar origin were obtained from Dr. B. LÁNYI, *State Institute of Hygiene, Budapest.* Thus, altogether 222 strains were examined. *Rauss'* original strains had been lost, so these could not be included.

Desoxycholate citrate agar and Endo agar served for isolating the strains from suspected cases of dysentery and dyspepsia, respectively. The colonies were inoculated onto NÓGRÁDY's polytropic medium [12]. This medium proved to be very useful for primary selection : the powerful decomposition of urea, the lack of $H_2S$ formation and the slight gas production were very characteristic of these bacteria.

*Biochemical reactions* were performed by standard methods [3]. The medium for MØLLER's KCN test was prepared with 0.3 per cent Bacto-peptone as described by EWING [15].

*Serological examinations.* O and H antigens were examined by KAUFFMANN's standard method using tube agglutination and boiled or formalinized suspensions. For preparing the H antigens, flagellar cultures were obtained by passing the strains through U tubes. Thermolabile antigens were examined with alcohol treated suspensions in sera prepared with alcoholized antigens.

## Experimental

### *Biochemical examinations*

The results of the biochemical examinations are summarized in *Table I*. In *Table I* only substances fermented by *P. morganii* are shown. Neither of the strains fermented lactose, mannitol, maltose, xylose, arabinose, rhamnose, dulcitol, salicin, adonitol and inositol.

The fermentation of dextrose, mannose and levulose can be regarded as a constant property of the strains. Galactose was often fermented. Some strains fermented sucrose late, some produced acid from sorbitol, sorbose or raffinose. Only a slight amount of gas was produced.

According to *Table I*, the constant biochemical reactions of *P. morganii* are : decomposition of urea, growth in KCN medium, formation of phenylpyruvic acid from phenylalanine, production of indole and positive methyl red reaction. No gelatinase is produced. Positive Voges-Proskauer reaction or growth in ammonium citrate medium were unfrequently observed, thus these are not typical reactions. Of the strains 30 per cent showed haemolysis on sheep-blood agar plates. According to the biochemical reactions, the strains were divided into 12 biotypes. No relationship was demonstrated between the biotypes and serological groups.

While the results of the fermentation and other biochemical reactions found in the present study perfectly corresponded to those of various authors [9, 13, 16—21], and also to data reported by the *Enterobacteriaceae Subcommittee* and approved at the *VII International Congress of Microbiology*, the findings concerning the $H_2S$ production were discrepant.

According to RAUSS [8] and BLAŠKOVIČ [23], *P. morganii* produces $H_2S$; SEVIN *and* BUTTIAUX [9] state that only part of the strains do so. Accord-

**Table I**

*The biochemical behaviour of P. morganii*
Total number of strains 222

| Fermentation types | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Gas | + | + | + | + | + | + | + | + | + | + | + | + |
| Sucrose | — | — | — | — | — | × | × | — | — | — | — | — |
| Glucose | + | + | + | + | + | + | + | + | + | + | + | + |
| Galactose | + | — | — | — | — | + | + | + | + | + | + | + |
| Levulose | + | + | + | + | + | + | + | + | + | + | + | + |
| Mannose | + | + | + | + | + | + | + | + | + | + | + | + |
| Raffinose | — | — | — | — | — | — | — | + | — | — | — | — |
| Sorbitol | — | — | — | — | — | — | + | — | + | — | — | — |
| Sorbose | — | — | — | — | + | — | — | — | — | — | — | — |
| Indole | + | + | + | + | + | + | + | + | + | + | + | + |
| Methyl red | + | + | + | + | + | + | + | + | + | + | + | + |
| Voges-Proskauer | — | — | — | — | — | — | — | — | — | + | + | — |
| Ammonium citrate (Simmons) | — | — | — | × | — | — | — | — | — | — | × | × |
| Urea | + | + | + | + | + | + | + | + | + | + | + | + |
| Gelatin | — | — | — | — | — | — | — | — | — | — | — | — |
| KCN | + | + | + | + | + | + | + | + | + | + | + | + |
| $H_2S$ | — | — | — | — | — | — | — | — | — | — | — | — |
| Phenylalanine desaminase | + | + | + | + | + | + | + | + | + | + | + | + |
| Number of strains | 185 | 12 | 1 | 1 | 1 | 6 | 2 | 2 | 2 | 4 | 2 | 4 |

Key: + = positive within 1 or 2 days
× = late positive
— = negative

ing to Bergey's 1948 edition, no $H_2S$ is formed; Kauffmann [13, 18] reports *P. morganii* to be a weak $H_2S$ producer. According to Lányi [19], only a small part of the strains produced this substance.

These controversies in the literature have made it necessary to supervise the findings. The examinations being detailed elsewhere [24], only the results will now be summarized briefly. No $H_2S$ was produced by any of the strains in ferrous chloride gelatin or lead acetate agar within 4 weeks of incubation. By using suspended paper strips saturated with lead acetate solution, however, within 24 hours some degree of $H_2S$ production was indicated, even in simple broth. It should be noted that strains known as $H_2S$ negative organisms *(E. coli, Klebsiella)* also showed some degree of $H_2S$ production by this method.

It was shown that *P. morganii* grew well and intensively produced $H_2S$ in a synthetic medium containing buffer solution, glucose, cystine, nicotinamide and calcium pantothenate. By adding agar and ferrous ammonium sulfate to this medium, $H_2S$ production was markedly indicated within 24 hours.

By the addition of 0.1 per cent cystine, a positive $H_2S$ reaction was also obeserved in simple agar containing ferrous ammonium sulfate. From these findings it can be concluded that the peptones generally available contain only a small quantity of substances that can be attacked by the sulfhydrase enzymes of *P. morganii*. In the presence of an adequate compound, such as cystine, the strains produce $H_2S$ abundantly.

### *Determination of antigenic structure*
### *1. O antigens*

*Thermostable antigens. Table II* shows the results of cross-agglutination of boiled suspensions in sera prepared with boiled cultures.

For the sake of simplicity, each O group is represented in *Table II* by only one strain. With the exception of strains 558, 292/2 and 702, the cross-agglutinations were of a low titre. The analysis of the antigenic relationships is summarized as follows.

The cross-absorption tests showing the relationship among strains 558, 292/2 and 702 are demonstrated in *Table III*.

**Table III**

*Antigenic structure of O group 1*

| O antigen suspensions | O sera | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | 558 | | | 292/2 | | | 702 | | |
| | Un-absorbed | Absorbed by | | Un-absorbed | Absorbed by | | Un-absorbed | Absorbed by | |
| | | 292/2 | 702 | | 558 | 702 | | 558 | 292/2 |
| 558 | 5120 | 1280 | 1280 | 640 | — | — | 320 | — | — |
| 292/2 | 2560 | — | — | 5120 | 640 | 640 | 320 | — | — |
| 702 | 320 | — | — | 320 | — | — | 5120 | 2560 | 2560 |
| Factors | a, b | b | b | a, c | c | c | a, d | d | d |

From the results of the cross-absorption tests it is evident that all the three strains contain an antigen that is common to the cultures ("a"), and in addition each possesses a special factor. Regarding the remarkable cross-

**Table VII**

*Cross-agglutinations of H antigens*

| Strains and their antigenic structure | Sera and titres of agglutination | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 558 (1ab :1) | 366 (1ab : 2) | 702 (1ad : 3) | 795 (1a, d : 3,4) | 319/1 (2 : 5) | 741 (3 : 6) | 285 (4 : 7) | 265/a (5 : 8,18) | 550 (6 : 9) | 1457 (11:10, 13) | 614 (12 : 10) | 547 (14 : 12, 13) | 449 (10 : 14) | Pf (21 : 15) | 734 (24 : 16) | 266/56 (26 : 17, 18) | 1594 (29 : 11 19) |
| 558 (1ab : 1) | 6400 | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — |
| 366 (1ab : 2) | — | 6400 | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — |
| 702 (1ad : 3) | — | — | 12800 | 3200 | — | — | — | — | — | — | — | — | — | — | — | — | — |
| 795 (1ad : 3,4) | — | — | 3200 | 12800 | — | — | — | — | — | — | — | — | — | — | — | — | — |
| 319/1 (2 : 5) | — | — | — | — | 3200 | — | — | — | — | — | — | — | — | — | — | — | — |
| 741 (3 : 6) | — | — | — | — | — | 12800 | — | — | — | — | — | — | — | — | — | — | — |
| 285 (4 : 7) | — | — | — | — | — | — | 3200 | — | — | — | — | — | — | — | — | — | — |
| 265/a (5 : 8,18) | — | — | — | — | — | — | — | 12800 | — | — | — | — | — | — | — | — | 200 |
| 550 (6 : 9) | — | — | — | — | — | — | — | — | 12800 | — | — | — | — | — | — | — | — |
| 1457 (11 : 10,13) | — | — | — | — | — | — | — | — | — | 12800 | — | 200 | — | — | — | — | — |
| 614 (12 : 10) | — | — | — | — | — | — | — | — | — | — | 6400 | — | — | — | — | — | — |
| 547 (14 : 12,13) | — | — | — | — | — | — | — | — | — | 200 | — | 12800 | — | — | — | — | — |
| 449 (10 : 14) | — | — | — | — | — | — | — | — | — | — | — | — | 12800 | — | — | — | — |
| Pfeiffer (21 : 25) | — | — | — | — | — | — | — | — | — | — | — | — | — | 12800 | — | — | — |
| 734 (24 : 16) | — | — | — | — | — | — | — | — | — | — | — | — | — | — | 12800 | — | — |
| 266/56 (26 : 17,18) | — | — | — | — | — | — | — | 800 | — | — | — | — | — | — | — | 12800 | — |
| 1594 (29 : 11 : 19) | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | 6400 |

**Table II**

*Cross-agglutinations of O antigens*

| O groups | Strains | Sera and titres of agglutination | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 558 | 292/2 | 702 | 319/1 | 741 | 285 | 265/a | 550 | Czakó | 429 | 352/56 | 264 | 1457 | 614 | 74/56 | 547 | 1644/56 | 369 | 618 | 958 | 658 | 524 | Pfeiffer | 739 | 637 | 734 | 274 | 266/56 | 261/56 | 979 | 1594 |
| | 558 | 5120 | 640 | 320 | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | 160 | — | — | — | — | — | — | — | — | — | — |
| 1 | 292/2 | 2560 | 5120 | 320 | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | 80 | — | — | — | — | — | — | — | — | — | — |
| | 702 | 320 | 320 | 5120 | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | 80 | — | — | — | — | — | — | — | — | — | — |
| 2 | 319/1 | — | — | — | 5120 | 160 | — | 80 | — | — | — | — | — | — | — | — | — | — | — | 80 | — | — | — | — | — | — | — | — | — | 40 | — | — |
| 3 | 741 | — | — | — | 160 | 5120 | — | — | — | — | — | — | — | — | — | — | — | — | — | 80 | — | — | — | — | — | — | — | — | — | — | — | — |
| 4 | 285 | — | — | — | — | — | 2560 | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | 80 | — | — | — | — | — | — | — | — |
| 5 | 265/a | — | — | — | 80 | 80 | — | 1280 | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | 160 | — | — | — |
| 6 | 550 | — | — | — | — | — | — | — | 1280 | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | 80 | — | — | — | — | — |
| 7 | Czakó | — | — | — | — | — | — | — | — | 1280 | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — |
| 8 | 429 | — | — | — | — | — | — | — | — | — | 5120 | 40 | — | — | — | — | 80 | 40 | — | — | — | — | — | — | — | — | 40 | — | — | — | — | — |
| 9 | 352/56 | — | — | — | — | — | — | — | — | — | — | 2560 | — | — | — | — | 80 | — | — | — | — | — | — | 80 | 80 | — | — | — | — | — | — | — |
| 10 | 264 | — | — | — | — | — | — | — | — | — | — | — | 2560 | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | 40 | — | — |
| 11 | 1457 | — | — | — | — | — | — | — | — | — | — | — | — | 2560 | — | — | — | — | — | — | — | — | — | — | — | — | — | 80 | — | — | — | — |
| 12 | 614 | — | — | — | — | — | — | — | — | — | — | — | — | — | 640 | — | — | — | 160 | — | — | — | — | — | — | — | — | — | — | — | — | — |
| 13 | 74/56 | — | — | — | 40 | — | — | — | — | — | — | — | — | — | — | 2560 | — | — | — | — | — | — | — | — | 80 | — | 40 | 40 | — | — | — | — |
| 14 | 547 | — | — | — | — | — | — | — | — | — | 80 | 40 | — | — | — | — | 2560 | 80 | — | — | — | — | — | — | — | — | — | — | — | — | — | — |
| 15 | 1644/56 | — | — | — | — | — | — | — | — | — | 40 | — | — | — | — | — | 40 | 5120 | — | — | — | — | 80 | — | — | — | — | — | — | — | — | — |
| 16 | 369 | — | — | — | — | — | — | — | — | — | — | — | — | — | 160 | — | — | — | 5120 | — | — | — | 40 | — | — | — | — | — | — | — | — | — |
| 17 | 618 | — | — | — | 160 | 160 | — | 80 | — | — | — | — | — | — | — | — | — | — | — | 640 | — | — | — | — | — | — | — | — | — | — | — | — |
| 18 | 958 | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | 2560 | — | — | — | — | — | — | — | — | — | 160 | — |
| 19 | 658 | 160 | 80 | 80 | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | 1280 | — | — | — | — | — | — | — | — | — | — |
| 20 | 524 | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | 40 | — | — | — | — | 2560 | — | — | — | — | — | — | — | — | — |
| 21 | Pfeiffer | — | — | — | — | — | — | — | — | — | — | 80 | — | — | — | — | — | — | — | — | — | — | — | 2560 | — | — | — | — | — | — | — | — |
| 22 | 739 | — | — | — | — | — | — | — | — | — | — | 40 | — | — | — | 40 | — | — | — | — | — | — | — | — | 5120 | — | 40 | — | — | — | — | — |
| 23 | 637 | — | — | — | — | — | — | — | — | — | — | — | 40 | — | — | — | 40 | — | — | — | — | — | — | — | — | 2560 | — | — | — | — | — | — |
| 24 | 734 | — | — | — | — | — | — | — | — | — | — | — | — | — | — | 40 | — | — | — | — | — | 80 | — | — | 80 | — | 10240 | — | — | — | — | — |
| 25 | 274 | — | — | — | — | — | — | — | — | — | — | — | — | 80 | — | — | — | — | — | — | — | — | — | — | — | — | — | 5120 | — | — | — | — |
| 26 | 266/56 | — | — | — | — | — | — | 80 | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | 80 | — | — | 5120 | — | — | — |
| 27 | 261/56 | — | — | — | — | — | — | — | — | — | — | — | 80 | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | 5120 | — | — |
| 28 | 979 | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | 80 | — | — | — | — | — | — | — | — | — | 2560 | — |
| 29 | 1594 | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | 2560 |

reaction given by the common antigen, the strains were considered as representatives of 3 subgroups of O-antigenic group 1.

Despite of their apparently small importance, most of the other, low-titre relationships were also studied. These relationships were found to be of the ab—ac variety, *i. e.* in addition to the factor arbitrarily designated as "a", which accounted for the cross-reaction, both of the examined strains possessed a special factor of their own. The common factor was, however, of no practical significance, which was clearly shown by the low cross-agglutination titres and also by the fact that weak or no cross-agglutination was observed with slide agglutination at dilutions 1 : 40. One example of these antigenic relationships is given in *Table IV*.

**Table IV**

*Antigenic relationships among some P. morganii strains*

| O antigen suspensions | O sera | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | 319/1 | | | | 741 | | | |
| | Un-absorbed | Absorbed by | | | Un-absorbed | Absorbed by | | |
| | | 618 | 265/a | 741 | | 319 | 618 | 265/a |
| 319/1 | 5120 | 5120 | 5120 | 5120 | 160 | — | — | — |
| 618 | 160 | — | — | — | 160 | — | — | — |
| 265/a | 80 | — | — | — | 80 | — | — | — |
| 741 | 160 | — | — | — | 5120 | 5120 | 5120 | 5120 |

According to Table IV, a minor common factor is responsible for the cross-agglutination of the 3 strains examined. The examination of other strains giving cross-reaction yielded a similar result.

By O antigen analysis, the strains were divided into 29 O groups. O group 1, to which the largest number of strains belonged, could be subdivided into 3 subgroups. The distribution of strains among the O groups will be shown in the antigenic schema *(Table XI)*.

*Thermolabile antigens.* Rauss [25] demonstrated the complete antigenic identity of *E. coli* O 112ac, B11 and a *P. morganii* strain. The latter contained a B-like antigen, serologically identical with B11 ; in an antiserum prepared with the corresponding living strain, however, its living suspension gave a higher titre than its boiled one. This finding suggested that the thermolabile antigen occurring in this strain, although serologically identical with antigen B11, differed from it functionally, as instead of exerting an inhibitory action, it promoted the agglutination of the living culture. Indeed, living *P. morganii* strains frequently gave a higher titre than their boiled suspensions. In antisera prepared with alcohol treated suspensions some reactions were

observed between strains not related by either O or H antigens. These agglutinations were, however, always unilateral. Some examples are shown in *Table V.*

**Table V**

*Unilateral agglutination of P. morganii strains in sera prepared with alcoholized antigens*

| Sera against alcoholized antigens | | Living cultures | | Titre of agglutination |
|---|---|---|---|---|
| Strain | O group | Strain | O group | |
| 319/1 | II | 658 | XIX | 1280 |
| 658 | XIX | 739 | XXII | 640 |
| 741 | III | 658 | XIX | 320 |
| 739 | XXII | 352 | IX | 320 |

Absorption by the homologous boiled suspension caused perfect exhaustion of the sera. Living, alcoholized or formalinized suspensions of the unilaterally related strains removed the heterologous agglutinins but left unaffected the homologous ones. The heterologous agglutinins were totally absorbed also by boiled suspensions *(Table VI).*

**Table VI**

*Absorption of agglutinins from serum "739" prepared with alcoholized antigen*

| Strains | Titre of agglutination | | | |
|---|---|---|---|---|
| | Unabsorbed serum *739* | Serum *739* absorbed by strain | | |
| | | *739* 100° C | *352* living | *352* 100° C |
| 739 living | 5120 | — | 5120 | 5120 |
| 739 100° C | 1280 | — | 1280 | 1280 |
| 352 living | 320 | — | — | — |
| 352 100° C | — | — | — | — |

The absorption tests indicated that the thermolability of the substance responsible for the increased agglutinability has the same peculiarities as B antigen. No explanation has been obtained from the tests as to the mechanism of the increased agglutinability of living cultures.

### *2. H antigens*

The cross-agglutination of formalinized broth cultures in H antisera is shown in *Table VII.* The sera were prepared with broth cultures that had previously been passed through semisolid agar and finally killed with for-

malin. In order to eliminate completely the sources of error, O agglutinins were removed from each of the H sera by the boiled suspension of the homologous strain.

For the sake of simplicity, *Table VII* shows only sera corresponding to the various H types, the homologous titres and one prototype of the strains giving agglutination in more than one of the sera. Te occurrence of H antigens in the various O groups will be shown in the diagnostic antigenic schema *(Table XI)*.

According to *Table VII*, the occurrence of cross-agglutinations among the H types was unfrequent. The relationship between the cross-reacting antigens was studied in absorption tests. It should be noted that only the examination of prototypes is presented here ; in reality all strains were tested against all sera and those giving cross-agglutination were all submitted to cross-absorption. The antigenic structure of prototypes containing complex flagellar antigens is indicated in *Tables VIII, IX* and *X*.

**Table VIII**

*H antigenic relationships between strains 702 and 795*

| Strains | Serum 702 | | Serum 795 | |
|---|---|---|---|---|
| | Unabsorbed | Absorbed by strain 795 | Unabsorbed | Absorbed by strain 702 |
| 702 | 12800 | — | 3200 | — |
| 795 | 3200 | — | 12800 | 3200 |
| Antigenic structure | 702 = lad : 3 | | 795 = lad : 3,4 | |

The cross-agglutination between these two strains was caused by a common flagellar antigen, which was well-developed in strain 702, and which might therefore be regarded as the characteristic type antigen of strain 702. In strain 795, however, the same factor was of secondary importance, the determinant being antigen 4, which completely lacked in strain 702.

**Table IX**

*H antigenic relationships between strains 265/a and 266*

| Strains | Serum 265 | | Serum 266 | |
|---|---|---|---|---|
| | Unabsorbed | Absorbed by strain 266 | Unabsorbed | Absorbed by strain 145 |
| 265/a | 12800 | 12800 | 200 | — |
| 266 | 800 | — | 12800 | 3200 |
| Antigenic structure | 265/a = 5 : 8,18 | | 266 = 26 : 17,18 | |

In the example shown in *Table IX*, both strains had different character istic type antigens in addition to a common partial antigen of minor importance.

Like in the above example, in the one shown in *Table X*, the antigenic relationship was due to a minor common factor.

No phase variation of the H antigens could be demonstrated.

**Table X**

*H antigenic relationships between strains 1457 and 547*

| Strains | Serum 1457 | | Serum 547 | |
|---|---|---|---|---|
| | Unabsorbed | Absorbed by strain 547 | Unabsorbed | Absorbed by strain 1457 |
| 1457 | 12800 | 12800 | 200 | — |
| 547 | 200 | — | 12800 | 12800 |
| Antigenic structure | 1457 = 11 : 10,13 | | 547 = 14 : 12,13 | |

*Antigenic schema of P. morganii*

Based on the investigations detailed in this study, a diagnostic antigenic schema was established *(Table XI)*.

By well-defined thermostable antigens the 222 strains were divided into 29 O groups. O group 1 was subdivided into 3 subgroups, since the members of this group in addition to a common partial antigen characteristic of the group, contained 3 different factors specific for the subgroups ("b", "c" and "d"). The frequency distribution of the strains was as follows. Commonest were the strains belonging to O group 1 (54 per cent). O groups 2 and 3 contained 7 and 3 per cent of the strains, respectively. The percentage of strains in other O groups was even less. Relationships of the ab—ac variety were observed between some groups.

By the H antigens the groups were divided into serotypes. In 222 strains 19 different H antigens were demonstrated, of which 5 had a complex antigenic structure. Since several of the H antigens were found to figure in more than one O group, the total number of serotypes was 57. Flagellar antigens 1 to 8 were the antigens most frequently encountered. As it was impossible to recover motile forms from 6 of the strains, the type determination for those could not be performed.

The presence of thermolabile antigen has reliably been demonstrated in one serotype only (29 : 19), therefore the systematic significance of these antigens remains unknown.

The interrelationships between *P. morganii* and other *Enterobacteriaceae* groups will be discussed in another paper.

**Table XI**

*Diagnostic antigenic schema*

| Groups | Antigens | | Number of strains within | |
|---|---|---|---|---|
| | "O" | "H" | types | groups |
| I | lab | 1 | 36 | 60 |
| | lab | 2 | 4 | |
| | lab | 3 | 2 | |
| | lab | 3,4 | 1 | |
| | lab | 5 | 5 | |
| | lab | 6 | 7 | |
| | lab | 7 | 3 | |
| | lab | 8,18 | 1 | |
| | lab | 9 | 1 | |
| | lac | 1 | 2 | 12 (119) |
| | lac | 2 | 1 | |
| | lac | 3 | 2 | |
| | lac | 3,4 | 4 | |
| | lac | 5 | 1 | |
| | lac | H— | 2 | |
| | lad | 1 | 1 | 47 |
| | lad | 2 | 1 | |
| | lad | 3 | 27 | |
| | lad | 3,4 | 4 | |
| | lad | 5 | 6 | |
| | lad | 6 | 6 | |
| | lad | 7 | 1 | |
| | lad | 10,13 | 1 | |
| II | 2 | 2 | 1 | 16 |
| | 2 | 3 | 2 | |
| | 2 | 5 | 11 | |
| | 2 | 6 | 2 | |
| III | 3 | 5 | 2 | 7 |
| | 3 | 6 | 5 | |
| IV | 4 | 2 | 1 | 6 |
| | 4 | 3 | 1 | |
| | 4 | 7 | 4 | |
| V | 5 | 8,18 | 4 | 6 |
| | 5 | 10,13 | 2 | |

*Diagnostic antigenic schema (continued)*

| Groups | Antigens | | Number of strains within | |
|---|---|---|---|---|
| | "O" | "H" | types | groups |
| VI | 6<br>6 | 9<br>— | 4<br>1 | 5 |
| VII | 7<br>7<br>7 | 5<br>12,13<br>— | 2<br>2<br>1 | 5 |
| VIII | 8 | 1 | 5 | 5 |
| IX | 9 | 16 | 4 | 4 |
| X | 10 | 14 | 3 | 3 |
| XI | 11 | 10,13 | 3 | 3 |
| XII | 12 | 11 | 3 | 3 |
| XIII | 13<br>13 | 6<br>10,13 | 1<br>2 | 3 |
| XIV | 14<br>14 | 12,13<br>— | 2<br>1 | 3 |
| XV | 15 | 9 | 2 | 2 |
| XVI | 16 | 11 | 2 | 2 |
| XVII | 17 | 8,18 | 1 | 1 |
| XVIII | 18 | — | 1 | 1 |
| XIX | 19 | 5 | 1 | 1 |
| XX | 20 | 10,13 | 1 | 1 |
| XXI | 21 | 15 | 1 | 1 |
| XXII | 22 | 1 | 1 | 1 |
| XXIII | 23 | 10,13 | 1 | 1 |
| XXIV | 24 | 16 | 1 | 1 |
| XXV | 25 | 12,13 | 1 | 1 |
| XXVI | 26 | 17,18 | 1 | 1 |
| XXVII | 27 | 10,13 | 1 | 1 |
| XXVIII | 28 | — | 1 | 1 |
| XXIX | 29(B)* | 19 | 1 | 1 |

* B-like thermolabile antigen

### *Practical diagnosis of P. morganii*

Serological diagnosis can mostly be performed by the slide agglutination technique, as the strains do not contain antigens inhibiting the agglutination of living bacteria.

In determining the O antigens, the cross-reactions must be taken into consideration. Most of these, however, are not apparent in slide agglutination carried out with O sera diluted 1 : 40. Cross-reactions giving a titre of 1 : 160 or more can usually be observed by slide agglutination. In these cases the serum in question should be absorbed by the strain showing heterologous agglutination in the highest dilution. Experience has shown that absorption by one strain simultaneously removes occasional heterologous agglutinins for other strains. Subdivision of O group 1, however, can exclusively be performed by absorbed sera containing factors b, c and d *(Table III)*.

For type determination the culture should at first be passed in U tubes or inoculated into tubes containing semisolid agar. For tube agglutination the strain is transferred into broth ; the culture is finally killed with formalin. For slide agglutination the strains are inoculated on the middle of a 1 per cent agar plate and incubated at 30° C [8]. The fine layer of swarming is used for slide agglutination in sera diluted 1 : 50 to 1 : 100. Alternatively, the strain may be inoculated into the condensation water of a 1 per cent agar slant and the growth spreading over the surface of the medium may be agglutinated.

Typing was made more convenient by employing 7 different O and 3 different H polyvalent sera. The compositions of O sera were as follows. 1 = 1ab, 1ac, 1ad ; 2 = 2—5 ; 3 = 6—9 ; 4 = 10—14 ; 5 = 15—19 ; 6 = 20—24 ; 7 = 25—29. Each of the pools contained the corresponding O sera in equal proportions. The order of sera was based upon the prevalence of the O groups. These sera were used at a dilution of 1 : 20. The O group of a strain showing agglutination in one of the pooled O sera was determined by slide agglutination in the separate O sera. Of the strains, 70 per cent gave agglutination in O polyvalent sera 1 to 3.

The composition of H polyvalent sera was as follows: 1 = 1—6 ; 2 = 7, 9, 11, 14, 16, 19 ; 3 = 8, 18 + 10, 13 + 12, 13 + 17, 18. Type determination was also performed in the separate sera. Complex antigens could be determined only by agglutination in absorbed sera. *(Tables VIII, IX, X.)* Of the serotypes 65 per cent gave agglutination in H polyvalent serum 1.

### *The systematic position of P. morganii*

Opinions found in earlier literature are confusing. Bahr *and* Thomsen [3] and Jordan *et al.* [4] classified this organism as belonging to the *Escherichia* group. The views of Besson *and* de Lavergne [5] and of Magheru [6],

who placed it in the *Salmonella* group, were adopted by BERGEY's, 1930 edition. Various descriptions state the inconstant behaviour of the strains. The obvious explanation of such findings is that various bacteria have been believed to be Morgan's bacillus. MORGAN *and* LEDINGHAM [2] distinguished 8 types, which were hardly connected by more than that they were all Gram-negative and non-lactose fermenting organisms. THJØTTA [7] suggested that Morgan's bacillus was a member of a heterogenous group of bacteria and therefore proposed the name "metacolon". It has been made clear later that Morgan's bacillus No 1, or *P. morganii* as referred to to-day represented the only individual group, while *B. morganii* No 2—8 were probably members of other *Enterobacteriaceae* groups.

RAUSS [8] showed that *B. morganii* No 1 comprised a serologically heterogeneous, but biochemically well-defined group. Taking into consideration its swarming property and its serological behaviour, he regarded it as a characteristic fermentation group of Proteus bacteria.

These results were confirmed by SEVIN *and* BUTTIAUX [9], in whose opinion the organism undoubtedly belongs to the *Proteus* group.

In *Bergey*'s, 1939 edition it was included into the *Proteus* group and COWAN [20] also regarded it as a member of that group.

In KAUFFMANN's *Enterobacteriaceae* [13] a generic rank has been given to *Proteus*, and *P. morganii* is one of the 4 species considered as belonging to this genus.

FULTON [10] regards *P. morganii* as a separate genus and proposed the name *Morganella*. Later KAUFFMANN [18] has also recommended that, on the basis of its biochemical behaviour, *P. morganii* should be excluded from the genus *Proteus* and a new genus, *Morganella* should be created. In EWING's [21] opinion, *P. morganii* has to be excluded from the genus *Proteus* and the new genus *Morganella*, in addition to *M. morganii*, should include *M. rettgeri* and *M. inconstans (Providencia)*.

The more exact identification by recently employed biochemical reactions of organisms belonging to the family *Enterobacteriaceae* gave rise to some diversities of opinion as to the systematic classification. Until a final agreement can be reached, in Rome (1953) the *Enterobacteriaceae Subcommittee* came to a decision that temporarily in the family *Enterobacteriaceae* instead of "genus" and "species" the more general terms "group" and "type" should be used [26]. This simplification undoubtedly solved some of the present difficulties, but made the dividing line between genus and species indistinct and caused the confusion of the two units.

In its 1958 report, the *Enterobacteriaceae Subcommittee* [22] regards the taxonomic position of *P. morganii* doubtful and recommends the revision of the problem in the light of recent investigations.

In his recent work, KAUFFMANN [11] stresses the importance of a more exact classification of *Enterobacteriaceae.* According to the principles of biological classification, the term tribe means the totality of related genera, while the related species constitute the genera. Each group of bacteria having individual characteristics within the genus, should be regarded as a separate species.

Considering the results of recent investigations, the relationship between *P. morganii* and the *Proteus* group can be summarized as follows.

According to the present classification, the *Proteus* group contains 4 subgroups : *P. vulgaris, mirabilis, morganii* and *rettgeri.* These subgroups are related by their common biochemical character, namely no fermentation of lactose, growth in KCN medium, formation of urease and glutamic acid decarboxylase and production of phenylpyruvic acid from phenylalanine [13, 18, 20, 22],

*P. morganii* corresponds to these requirements, thus it may be included in the *Proteus* group and its taxonomic position may be regarded as definite. On the basis of its biochemical behaviour (production of indole, no liquefaction of gelatin, formation of $H_2S$ in amounts detectable only by special methods and characteristic fermentation reactions) and its antigenic properties, *P. morganii* constitutes a well-defined subgroup within the *Proteus* group.

Considering the definitions mentioned above, the *Proteus* group (Proteae) should be regarded as a tribe of the family *Enterobacteriaceae,* since it is made up of individually distinguishable large units corresponding to the listed general properties. These units or subgroups, as referred to at present, are, in reality, genera. Accordingly, *P. morganii* belongs to a separate genus as it sharply differs from other genera *(P. Hauseri, P. Rettgeri)* of the tribe Proteae. In accordance with KAUFFMANN's view, we consider *P. morganii* as a representative of one of the genera of the tribe Proteae. This conception requires the naming of the new genus. On the basis of priority, the name *Morganella* is justified. Thus, in our opinion *P. morganii* should be called *Morganella morganii,* as the only known species of the new genus.

## Discussion

The biochemical behaviour of *P. morganii* strains examined in the present study corresponded to the results of our earlier observations [8] and of those of other workers [9, 13, 16—21]. The results recorded for the production of $H_2S$ were, however, discrepant. *P. morganii* has been found to produce $H_2S$ in amounts easily detectable by the usual reagents, provided that the culture medium contained cystine [24]. In media commonly employed for this reaction $H_2S$ production cannot be determined by the standard methods. As shown by the investigations, however, in adequate medium any organ-

ism belonging to *Enterobacteriaceae* can produce $H_2S$ [24]. In agreement with EWING [2], we think that $H_2S$ production should be examined in media regarded at present as standard for this biochemical reaction. The degree of sensitivity provided by the standard tests yield a basic method of differentiation ; its change would only cause confusion. Accordingly, *P. morganii* has to be recognized as an $H_2S$-negative organism.

Our earlier results have been confirmed by the present serological examinations [8]. Previously, 48 strains had been divided into 17 groups on the basis of the O antigens. In the first and largest O group 3 subgroups had been distinguished. One third of the strains belonged into O group 1, while other O groups were represented only by 1 or 2 strains.

These previous results have been supported by the present investigation, performed on a broader scale. One large and 28 small groups have been distinguished. The largest of them, O group 1, contained 54 per cent of the strains, and as in the previous studies, it could be subdivided into 3 subgroups. Considerably less strains belonged to each of the other O groups ; O group 2 and 3 contained 7 and 3 per cent of the strains, respectively. Even less strains belonged to each of the remaining O groups. By comparing the present data with those of the other authors, the following conclusion can be made. The earliest of the data are those of LEWIS [27], who could determine 73 per cent of 242 strains by means of 16 sera. SOMPOLINSKY [28] divided 78 per cent of 110 strains into 5 groups by 5 sera. Of the strains 65 per cent belonged to the first groups ; in the present investigations 54 per cent of the strains belonged to O group 1. Accordingly, the distribution observed cannot be attributed to a mere chance.

As to H antigens, no data are available with the exception of those of our earlier examinations, where within a relatively small number of strains 7 various H antigens were distinguished. The antigenic analysis of 222 strains performed in the present study yielded 19 H antigens, 5 of which showed a complex structure. Many of the H antigens were found in more than one O group, thus the total number of serotypes was 57.

The unquestionable presence of a thermolabile antigen was shown in only one strain ; this factor was serologically identical with *E. coli* B antigen 11. Practically thermolabile factors could be found in every strain, which instead of inhibiting O agglutination, promoted it. As no pure antisera reacting exclusively with these antigens could be prepared, studying the nature or function of the thermolabile factors was not possible.

On the basis of the present studies, the antigenic schema of *P. morganii* has been established. The schema includes 29 O groups with 3 subgroups and 57 serotypes. O groups 1 to 5 contained 70 per cent of the isolated strains. The reality of this finding is clearly shown by comparing it with data of other authors, and also by the fact that *P. morganii* strains recently isolated

could be included in O groups 1 to 5 (and in serotypes within these groups) in a similar distribution. The serogroups and serotypes listed in the antigenic schema are far from containing every O and H antigen occurring in the genus *P. morganii*. After these investigations had been brought to an end, several strains were isolated containing either O or H and sometimes both O and H antigens different from those included in the antigenic schema. However, the antigenic structure of frequently encountered serotypes probably figures in the antigenic schema.

In the present report the antigenic interrelationships between *P. morganii* and other *Enterobacteriaceae* genera have not been dealt with. This will be discussed in a subsequent work.

The revision of the taxonomic position of *P. morganii* has also been suggested. The creation of the tribe Proteae has been recommended, which would among others include the *P. morganii* group under the name *Morganella* as one of its genera. Within the genus Morganella, for the time being *M. morganii* would be the only species.

## Summary

(i) The biochemical behaviour and antigenic structure of 222 *P. morganii* strains have been studied.

(ii) *P. morganii* is biochemically well-defined and distinguishable from other members of the *Proteus* group.

(iii) The 222 strains were divided into 29 groups including 3 subgroups and by 19 H antigens into 57 serotypes.

(iv) The existence of a thermolabile antigen was undoubtedly shown in only one of the serotypes.

(v) The antigenic schema of *P. morganii* has been established.

(vi) Problems of the taxonomic position of *P. morganii* have been discussed.

*Acknowledgement.* The authors wish to thank Dr. B. LÁNYI, *State Institute of Hygiene, Budapest*, for providing *P. morganii* strains of his collection.

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

## I. PHAGE-TYPING OF POLYRESISTANT STAPHYLOCOCCUS AUREUS STRAINS

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As a result of the wide application of antibiotic treatment, the incidence of resistant *Staphylococcus aureus* strains has greatly increased all over the world. With the introduction of antibiotic therapy the development of resistance to penicillin had been first observed ; various examinations performed later have shown that, in addition to penicillin, the same strains may acquire resistance to other antibiotics. As the occurrence of "polyresistant" strains, resistant against two or more antibiotics, becomes more and more frequent, there is no doubt that the broadening of the resistance spectrum of *Staphylococcus aureus* is still in progress. In the light of these findings it is no wonder that increased attention is paid to the various properties of the "potentially pathogenic" *Staphylococcus aureus* strains, both from theoretical and practical points of view. Several authors have studied the phage typing of staphylococci with different patterns of antibiotic sensitivity [2, 4, 5, 6, 7, 10, 11]. From the results of these investigations the following conclusions can be drawn.

(i) *Staphylococcus aureus* strains sensitive to antibiotics are approximately evenly distributed in the various phage groups.

(ii) Although most of the penicillin-resistant strains fall into phage group I, they are encountered in all the other phage groups.

(iii) Staphylococci resistant to any of the broad spectrum antibiotics or to 2, 3 or 4 antibiotics fall most frequently into phage group III. Within group III these strains correspond chiefly to phage pattern 47/53/54/77.

(iv) Development of resistance to antibiotics may cause an alteration in the phage type of the strain.

(v) *Staphylococcus aureus* strains belonging to phage group III produce resistant mutants more easily than members of other groups.

The present investigations were performed to study the changes in the characteristic properties and phage-sensitivity of *Staphylococcus aureus* strains which had acquired resistance to four or five different antibiotics.

## Materials and methods

The pathogenic *Staphylococcus aureus* strains used in this study were isolated from adult patients and from patients and staff at the infants' department of a hospital. The pathogenicity of the 355 *Staphylococcus aureus* strains isolated was tested by examining the liquefaction of gelatin, formation of pigment, fermentation of mannitol and production of haemolysin and coagulase.

Sensitivity to antibiotics was tested by the paper disc method using 6 different antibiotics, namely penicillin, streptomycin, chloromycetin, terramycin, aureomycin and erythromycin. Agar plates were seeded with 18 hour broth cultures diluted 1 : 100 with saline. The inoculated media were dried for 20 minutes and then the paper discs were placed on the plates. Readings were made after 24 hours at 37° C. Determination of sensitivity was in some cases checked by the tube dilution method.

Phage typing was carried out by the method of WILLIAMS and RIPPON [13] using the following phages

Group I : 29, 52, 52/a, 79, 80
Group II. 3/a, 3/b, 3/c, 55, 71
Group III : 6, 7, 42/E, 47, 53, 54, 70, 73, 75, 77
Group IV : 42/D.

## Results

Of the 355 *Staphylococcus aureus* strains 240 were isolated from adult patients. The remaining 115 strains were obtained from the infants' department. In both groups most of the strains were isolated from skin or throat conditions, some from other infections. The antibiotic sensitivity of the strains is presented in *Table I*.

**Table I**

*Sensitivity of Staphylococcus aureus strains to various antibiotics*
Total number of strains, 355

| | Sensitive | | Resistant | |
|---|---|---|---|---|
| | No | % | No | % |
| Penicillin | 40 | 12.0 | 315 | 88.0 |
| Streptomycin | 266 | 75.0 | 89 | 25.0 |
| Chloromycetin | 237 | 67.0 | 118 | 33.0 |
| Terramycin | 275 | 77.6 | 80 | 22.4 |
| Aureomycin | 275 | 77.6 | 80 | 22.4 |
| Erythromycin | 345 | 97.2 | 10 | 2.8 |

According to *Table I*, of the 355 strains examined, to penicillin 315 (88 per cent), to chloromycetin 118 (33 per cent), to streptomycin 89 (25 per cent) to terramycin 80 (22.4 per cent) and to aureomycin 80 (22.4 per cent), were resistant. The number of erythromycin-resistant strains was only 10 (2.8 per cent).

Of the strains, 34 (9.6 per cent) were sensitive to all antibiotics tested. A resistance to one of the antibiotics was shown by 182 cultures (51.2 per cent), these were chiefly penicillin-resistant. Fifty strains (14.1 per cent) were resistant to two antibiotics. Against 3 or 4 and 5 or 6 antibiotics 24 (6.7 per cent), and

65 (18.4 per cent), of the cultures were resistant, respectively. Thus the number of "polyresistant" strains, *i. e.* of those with a very broad spectrum of resistance, was 65. This number was considerable by itself, since it meant that the antibiotics commonly used were ineffective against nearly 1/5 of the isolated strains.

In *Table II* the phage group distribution of *Staphylococcus aureus* strains is shown.

**Table II**

*Phage group distribution of Staphylococcus aureus strains*

| | No. | % |
|---|---|---|
| Phage group I | 126 | 35.3 |
| Phage group II | 34 | 9.5 |
| Phage group III | 39 | 11.0 |
| Phage group IV | 2 | 0.56 |
| Cross-reacting strains | 47 | 13.2 |
| Non-typable strains | 107 | 30.4 |

According to *Table II*, 35.3 per cent of the strains belonged to phage group I, 9.5 per cent to phage group II, 11.0 per cent to phage group III and 0.6 per cent to phage group IV. Cross-reactions were given by 13.2 per cent of the strains. The high number of non-typable strains was striking; out of 355 strains 107 belonged to these, suggesting the necessity of more detailed examinations as to the connection between antibiotic sensitivity and phage groups.

In *Table III* the antibiotic sensitivity of strains belonging to various phage groups is presented.

**Table III**

*Relation between the antibiotic sensitivity and phage groups of Staphylococcus aureus strains*

| Antibiotic sensitivity pattern | No of strains | Phage groups | | | | Cross-reacting strains | Non-typable strains | |
|---|---|---|---|---|---|---|---|---|
| | | I | II | III | IV | | No | % |
| Sensitive to all antibiotics | 34 | 15 | 8 | 3 | 1 | 3 | 4 | 11.8 |
| Resistant to 1 antibiotic | 182 | 89 | 18 | 15 | 1 | 31 | 28 | 15.4 |
| Resistant to 2 antibiotics | 50 | 18 | 7 | 10 | — | 9 | 6 | 12.0 |
| Resistant to 3 or 4 antibiotics | 24 | 4 | 1 | 5 | — | 2 | 12 | 50.0 |
| Resistant to 5 or 6 antibiotics | 65 | — | — | 6 | — | 2 | 57 | 88.0 |
| Totals | 355 | 126 | 34 | 39 | 2 | 47 | 107 | |

Although *Staphylococcus aureus* strains sensitive to all the tested antibiotics or resistant to one or two antibiotics occurred in every phage group, they fell mainly into phage group I (44 per cent). Of the strains resistant only to penicillin and of those resistant to two of the antibiotics, 48 and 36 per cent respectively, belonged to this phage group. Of the strains belonging to the above sensitivity groups, 15.4 per cent gave cross-reactions and 3 strains (14.2 per cent) were non-typable.

Of the 24 strains resistant to 3 or 4 antibiotics, 12 could not be included in any of the phage groups. When phage typing the "really polyresistant" strains, which were resistant to 5 or 6 antibiotics, a significant increase in the occurrence of non-typable strains was observed. Out of the 65 polyresistant strains 2 gave cross-reactions and only 6 could be typed as belonging to phage group III (9 per cent). Neither of the strains belonged to phage groups I, II or IV. Of these strains 57 (88 per cent) could not be typed with the phages used. Thus among strains with multiple resistance the frequency of non-typable cultures was strikingly higher than among other strains.

The changes affecting the typability of strains with a broad spectrum of resistance are clearly shown in the extreme right column of *Table III*. While the percentage frequency for non-typable strains among the sensitive cultures and among those resistant to one or two antibiotics was only about 12—15 per cent, among the strains resistant to 3 or 4 antibiotics it was 50 per cent and, finally, among strains resistant to 5 or 6 antibiotics it was as high as 88 per cent. All the typable polyresistant strains fell into phage group III.

Of the strains examined 39 belonged to phage group III. Taking into consideration that, according to the literature, the majority of cultures resistant to antibiotics are members of phage group III, our strains falling into this group were more extensively studied. Phage typing of these strains was repeatedly performed not only with the R. T. D., but also with concentrated phage suspensions. Thus, according to the presence or absence of cross-reactions with concentrated phage suspensions, the strains could be divided into subroups. Part of the strains might be included in the corresponding phage group simply by examination with the concentrated phages. Under the latter circumstances 21 strains gave no cross-reaction, therefore they might directly be included in phage group III. The remaining 18 phage group III strains could exactly be typed only by using the R. T. D. Almost none of the polyresistant strains belonging to phage group III gave cross-reactions in the concentrated phage suspensions ; accordingly, they represented a more stable kind of subgroup within phage group III. Although cultures sensitive to all antibiotics or resistant to one or two antibiotics were encountered in phage group III, these belonged mainly to the less stable kind of phage subgroup.

By discussing separately the 115 strains isolated in the department for infants, the above observations become more demonstrative. The 115 cultures

were possibly nosocomial ones, as they had been isolated from patients treated for a longer period or from the staff. Out of 115 strains, 42 (36.5 per cent) were polyresistant. The antibiotic sensitivity and phage groups of these strains are shown in *Table IV*.

**Table IV**

*Antibiotic sensitivity and phage groups of Staphylococcus aureus strains isolated in an infants, department*

| Antibiotic sensitivity pattern | No of strains | Phage groups | | | | Cross-reacting strains | Non-typable strains |
|---|---|---|---|---|---|---|---|
| | | I | II | III | IV | | |
| Sensitive to all antibiotics ..... | 4 | 2 | 1 | 1 | — | — | — |
| Resistant to 1 or 2 antibiotics . | 64 | 33 | 7 | 5 | — | 12 | 7 |
| Resistant to 3 or 4 antibiotics . | 5 | — | — | — | — | 1 | 4 |
| Resistant to 5 or 6 antibiotics . | 42 | — | — | 1 | — | 2 | 39 |

Of the strains shown in Table IV, 68 were sensitive to all the antibiotics or resistant only to 1 or 2 of them. Of these strains, 51.5 per cent fell into phage group I. Seven strains (10 per cent) were non-typable. Staphylococci resistant to 5 or 6 antibiotics occurred very frequently (42 out of 115 strains or 36.5 per cent). Of the latter 42 strains 39 were non-typable. One out of the 3 typable strains was lysed by phages 47, 53, 54 and 77, thus it belonged to phage group III ; the remaining 2 cultures gave cross-reactions. The results appear to show that strains with a very broad resistance spectrum are mostly nosocomial ones and, parallel with the development of multiple resistance, the possibility of typing them is reduced or stopped.

## Discussion

If there is any relation between the antibiotic resistance and phage typability of organisms, the factors responsible for this seem to be connected with the surface properties of the cells. In the action of both antibiotics and phages the first step is their contact or linkage to the surface of the cell. These actions may be due to an electrostatic effect and, in addition, they may be influenced by other properties of the surface. The composition of substances constituting the cell surface and their stereospecificity undoubtedly play a very important part in both processes.

The present results differ from those of other authors in two respects, namely, on the one hand in the high number of strains resistant to 5 or 6 different antibiotics and, on the other, in the fact that nearly one third of the strains could not be typed with the phages used. Of the *Staphylococcus aureus* strains of KNIGHT *and* HOLZER [5] 5 per cent was non-typable. BARBER *et*

*al.*[3] found even less such strains. It should, however, be noted that the strains of these authors had a narrower spectrum of resistance. According to Barber *et al.* [2, 3], the sensitive strains are fairly evenly distributed in all the phage groups. In the present investigations 44 per cent of the sensitive strains fell into phage group I. A similar distribution was found for *Staphylococcus aureus* strains resistant to 1 or 2 antibiotics. Parallel with the broadening of the resistance spectrum, however, a fundamental change in the frequency of distribution was observed, as the number of strains falling into phage groups I, II and III became considerably less frequent while the number of non-typable strains gradually increased. Whereas only 12 to 15 per cent of the strains sensitive to all, or resistant to 1 or 2, of the antibiotics were non-typable, among strains resistant to 3 or 4 antibiotics this percentage was 50 ; among the polyresistant strains the occurrence of non-typable cultures reached 80 per cent. According to the observations of other authors and results of the present studies, the typable strains showing multiple resistance belonged to phage group III. The occurrence of such strains, however, was unfrequent.

As regards the phage types in relation to antibiotic sensitivity, it should be noted that of the *Staphylococcus aureus* strains isolated by Knight [5] from 1932 to 1938, only 20 per cent belonged to phage group III ; of the strains isolated by the same author in 1953 and 1954, 60 per cent belonged to this phage group. A large number of the latter strains showed multiple resistance. According to Gould [4], the resistant mutants of a strain originally belonging to phage group I were members of phage group III. The examinations of Váczi [8] and Wallmark [12] showed that development of resistance to antibiotics causes a partial or total reduction in the frequency of typable strains. According to the present results, of the strains examined only 11.0 per cent belonged to phage group III. Many of the remaining strains were non-typable, probably in account of their broad spectrum of resistance.

The predominance of non-typable strains may be explained by a difference existing between the composition of surface in sensitive and polyresistant cells. In consequence of this, the primary contact with the reactive groups of the surface may be inhibited in resistant cells. A difference between the composition of the surface of sensitive and polyresistant cells of certain *E. coli* strains was indicated by Váczi *and* Incze [9b] ; the experiments of Váczi, Hadházy *and* Katona [9] showed that polyresistant strains were markedly less phagocyted than sensitive ones. Further investigations concerning this problem are in progress.

## Summary

The antibiotic sensitivity and phage type of 355 pathogenic *Staphylococcus aureus* strains have been compared.

(i) Of the strains, 34 were sensitive to all antibiotics, 182 were resistant to one, 50 to two, 24 to three or four, and 65 to five or six, antibiotics.

(ii) Of the strains, 35.3 per cent belonged to phage group I, 9.5 per cent to phage group II, 11 per cent to phage group III, 0.6 per cent to phage group IV. 13.2 per cent of them gave cross-reactions and 30.4 per cent was non-typable.

(iii) Parallel with the broadening of the resistance spectrum the frequency of non-typable strains was considerably increased. Of the polyresistant *Staphylococcus aureus* strains, 88 per cent was non-typable.

(iv) For the inhibition of phage adsorption and of penetration of antibiotics, the possible responsibility of an alteration in the composition of the surface of polyresistant strains has been suggested.

*Acknowledgement.* The authors are indebted to Dr. M. Eörsi, *State Institute of Hygiene, Budapest*, for providing the type phages.

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# VIROLOGICAL INVESTIGATIONS DURING THE 1957 EPIDEMIC OF POLIOMYELITIS IN HUNGARY

By

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(Received May 16, 1959)

In the summer of 1957, Hungary experienced a severe poliomyelitis epidemic as it has been described by Rudnai [1]. Molnár [2] reported that mainly type 1 poliovirus strains were isolated from paralytic cases, while cases of abacterial meningitis, being particularly frequent during the epidemic in some areas of the country, equally yielded poliovirus type 1 and other enterovirus strains.

We also isolated some strains of poliovirus and of other enteroviruses from patients with paralytic poliomyelitis and carried out neutralization tests during the epidemic. The results are presented in this paper.

## Materials and methods

*Faecal specimens* from patients with paralytic poliomyelitis were obtained from the *László Central Hospital of Infectious Diseases, Budapest*, and from the *County Hospital of, Nyiregyháza.* Collecting specimens from the latter hospital seemed to be of special interest since the *Nyiregyháza* area is neighbouring the *East-Szabolcs* area which was affected by an unusually severe poliomyelitis epidemic in 1956 [1].

*Tissue cultures.* Human embryonic fibroblast cultures were used thoroughly. Plasma clot explants, as described previously [3], were employed at the beginning. Subsequently, mainly for the neutralization tests, a less time consuming technique, the trypsinized cell method, with cells growing on the glass wall was introduced.

*Standard antisera.* Type specific monkey sera to types 1, 2 und 3 poliovirus were used. The sera were prepared and kindly supplied by the *Virus Laboratory* of the *Schweizerisches Serum- und Impfinstitut, Bern, Switzerland.*

*Rabbit antisera* were prepared by the intravenous inoculation of adult rabbits. Each rabbit received four injections of 1 ml spaced 3, 3 and 14 days apart. Four weeks after the last injection the rabbits were bled out.

*Neutralization tests.* Sera were inactivated at 56° C for 30 minutes before tested. The working dilution of the standard sera was 1 : 5, that of rabbit antisera and of patients' sera was 1 : 20. Each dilution was mixed with an equal volume of virus suspension made up to contain 100 CPD50 per tissue culture tube. The serum-virus mixture was incubated at 37° C for one hour.

*Suckling mice.* Two-day-old mice were inoculated with 0.1 ml of the viral preparation to be tested, 6 to 10 mice with each, intraperitoneally.

## Experimental

From patients with paralytic poliomyelitis 55 faecal specimens were examined and thereof 21 cytopathogenic strains were isolated. The 37.5 per cent yield agrees with the results obtained by other authors [4]. Thirteen of

the isolates were neutralized by type 1, one strain by type 2 poliomyelitis antiserum.

Seven isolates were not neutralized even with a mixture of the standard antisera to the three types of poliomyelitis virus. The same strains failed to cause any symptom when inoculated into suckling mice. Thus, the seven isolates were neither poliovirus nor Coxsackie virus strains. We therefore supposed them to be members of the ECHO group of viruses. To obtain more information, rabbits were immunized with the isolates and the antisera were tested against the isolates themselves, as well as against the reference strains of the types 1 to 9, 11 to 15, 17 and 19 of ECHO virus. (The reference strains were obtained from the *State Institute of Hygiene, Budapest.)* Reference strains of the types 16 and 18 were not available. The type 10 strain used in our experiments could not be cultivated in human embryonic fibroblasts. The results of the neutralization tests are summarized in *Table I.*

A definite cross neutralization was demonstrated between the strains L. J., K. I. and the reference strain ECHO 11. The S. M. strain exhibited only partial crossing with the same reference strain. Although antiserum to S. M. strain neutralized the reference strain well, the strain itself was poorly neutralized by the antiserum prepared against the reference strain. Such a partial crossing is characteristic of some strains, called ECHO-prime strains [6], belonging to types 5, 7, 9, 10, 11 and 13. A definite cross neutralization was similarly demonstrated between the strain V. R. and the reference strain ECHO 7. Cross neutralization between the strains B. J. and O. E. was also demonstrated but the corresponding antisera did not neutralize any of the reference strains tested. None of the strains, except the homologous one were neutralized by the B. I. antiserum.

The results indicate that strains L. J., K. I. and S. M. were ECHO 11 strains, strain V. R. belonged to ECHO type 7 whereas the remainder belonged to 2 distinct, yet undetermined types.

All the seven strains exerted a marked cytopathogenic effect in human fibroblasts and so did the ECHO reference strains except type 10. In monkey kidney the B. J. and O. E. strains exerted a weak effect while the remainder were strongly cytopathogenic.

The high incidence of strains unidentifiable with standard poliomyelitis antisera has raised the question whether the paralytic cases yielding the viruses had all been caused by poliovirus, or had in some cases the isolates been paralytogenic. To elucidate this question, 214 children under orthopaedic treatment in the *State Heine-Medin Hospital, Budapest,* following an attack of poliomyelitis, were examined for neutralizing antibodies to the 3 types of poliovirus. The results are presented in detail in *Table II.*

As shown in *Table II,* all the 214 sera contained neutralizing antibodies to at least one type of the virus. Fifty sera neutralized two types, 11 sera

**Table I**

*Identification of the isolates unidentifiable with type specific poliomyelitis antisera—Neutralization tests*

| Rabbit antisera | Isolates | | | | | | | Reference strains | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | Poliovirus | | | ECHO virus | | | | | | | | | | | | | | | |
| | LJ | KI | SM | BJ | OE | BI | VR | 1 | 2 | 3 | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 11 | 12 | 13 | 14 | 15 | 17 | 19 |
| LJ | + | + | + | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | + | — | — | — | — | — | — |
| KI | + | + | + | — | — | — | — | — | — | — | | | | | | | | | | + | | | | | | |
| SM | + | + | + | — | — | — | — | — | — | — | | | | | | | | | | + | | | | | | |
| BJ | — | — | — | + | + | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — |
| OE | — | — | — | + | + | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — |
| BI | — | — | — | — | — | + | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — |
| VR | — | — | — | — | — | — | + | — | — | — | — | — | — | — | — | — | + | — | — | — | — | — | — | — | — | — |
| ECHO 11 | + | + | + | — | — | — | — | | | | — | — | — | — | — | — | — | — | — | + | — | — | — | — | — | — |
| ECHO 7 | | | | — | — | — | + | | | | — | — | — | — | — | — | + | — | — | — | — | — | — | — | — | — |

**Table II**

*Neutralizing antibodies to the 3 types of poliovirus in the sera of 214 poliomyelitis convalescents*

| Age group | <6 months | | | 6—11 months | | | 1—2 years | | | 3—5 years | | | 6—9 years | | | 10—14 years | | | 15 years and over | | | Total | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Persons having antibodies to one type** | | | | | | | | | | | | | | | | | | | | | | | | |
| Antibody type | 1 | 2 | 3 | 1 | 2 | 3 | 1 | 2 | 3 | 1 | 2 | 3 | 1 | 2 | 3 | 1 | 2 | 3 | 1 | 2 | 3 | 1 | 2 | 3 |
| No of sera | 4 | — | — | 13 | — | 1 | 96 | 7 | — | 25 | — | — | 3 | — | 2 | 2 | — | — | — | — | — | 143 | 7 | 3 |
| Total | 4 | | | 14 | | | 103 | | | 25 | | | 5 | | | 2 | | | 0 | | | 153 | | |
| **Persons having antibodies to two types** | | | | | | | | | | | | | | | | | | | | | | | | |
| Antibody type | 1+2 | 1+3 | 2+3 | 1+2 | 1+3 | 2+3 | 1+2 | 1+3 | 2+3 | 1+2 | 1+3 | 2+3 | 1+2 | 1+3 | 2+3 | 1+2 | 1+3 | 2+3 | 1+2 | 1+3 | 2+3 | 1+2 | 1+3 | 2+3 |
| No of sera | — | — | — | 2 | 1 | — | 19 | 4 | 1 | 3 | 9 | — | 1 | 4 | — | — | 4 | — | 1 | 1 | — | 26 | 23 | 1 |
| Total | 0 | | | 3 | | | 24 | | | 12 | | | 5 | | | 4 | | | 2 | | | 50 | | |
| **Persons having antibodies to three types** | | | | | | | | | | | | | | | | | | | | | | | | |
| No. of sera | — | | | — | | | 1 | | | 1 | | | 4 | | | 2 | | | 3 | | | 11 | | |
| Grand total | 4 | | | 17 | | | 128 | | | 38 | | | 14 | | | 8 | | | 5 | | | 214 | | |

three types. Two hundred and three sera (95 per cent) had type 1 antibodies whereas type 2 and type 3 antibodies were demonstrated in as few as 45 (21 per cent) and 38 sera (18 per cent), respectively. Such a distribution of antibodies agrees well with the summarized distribution by type of the 94 poliovirus strains which had been isolated in 3 Hungarian laboratories [2, 5] during the 1957 epidemic. Eighty-four strains (89 per cent) belonged to type 1, 7 to type 2 and 3 to type 3.

## Discussion

Our experimental results confirm the conclusion drawn by other Hungarian authors [2, 5] suggesting that the 1957 poliomyelitis epidemic in Hungary, like the most extensive epidemics elsewhere *(e.g. Denmark* 1952, *Stockholm* 1953) was caused by the type 1 poliovirus. In Hungary, as it has been reported by Rudnai [1], the number of cases notified in 1957 (2334) was more than twice the highest yearly incidence reported before. In 1954 and 1955, however, when only moderate epidemics were observed, type 2, resp. type 3 poliovirus strains were predominant. The 1956 epidemic characterized by an unusually high morbidity at the north-eastern corner of the country was due to the type 1 virus. Spread of this epidemic from the north-eastern focus could be followed early in 1957.

Another question of interest has been, whether the isolated strains other than poliovirus might have been responsible for typical paralytic poliomyelitis cases. Soviet authors [7] have recently described that Coxsackie A7 strains recovered from paralytic poliomyelitis cases caused typical paralytic poliomyelitis in experimental monkeys. The pathogenicity of some types of the ECHO viruses also seems now more important than thought before.

Our studies have not yielded further evidence of the paralytogenic ability of enterovirus strains other than poliovirus. Antibodies to at least one type of poliovirus were demonstrated in the sera of all the convalescents who were examined. These had been serious cases of poliomyelitis with orthopaedic consequences. Unfortunately, paired sera from the patients involved in the isolation experiments were not available but from patients B. I. and L. J. Antibodies to type 1 poliovirus and to the homologous strain were demonstrated in both specimens.

## Summary

Twenty-one cytopathogenic virus strains have been isolated from the faeces of paralytic poliomyelitis cases in 1957 during a severe epidemic. Of the strains, 13 have been identified as type 1, one as type 2 poliovirus, three strains proved to be type 11, one strain type 7 ECHO virus. Two of the three strains belonging to ECHO virus type 11 seemed to be identical with the ECHO 11 reference strain, while the third behaved like ECHO-prime strains.

Two hundred and fourteen convalescent sera from patients under orthopaedic treatment were tested for neutralizing antibodies to the 3 types of poliovirus. All the sera contained antibodies to at least one type, 95 per cent of the sera to the type 1, of poliovirus.

*Acknowledgements.* We are indebted to Dr. U. Krech *(Schweizerisches Serum- und Impfinstitut)* for type specific sera. We also wish to thank Dr. L. Lukács *State Heine-Medin Hospital)* for serum specimens and the *Virus Deparment of the State Institute of Hygiene, Budapest* for supplying ECHO reference strains.

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A kiadásért felel az Akadémiai Kiadó igazgatója Műszaki felelős: Farkas Sándor
A kézirat nyomdába érkezett: 1959. VIII. 4. — Terjedelem: 8 (A/5) ív, 16 ábra, 2 melléklet

1959/49735 — Akadémiai Nyomda, Budapest — Felelős vezető: Bernát György

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# BEITRÄGE ZUR BIOSYNTHESE VON CYANOCOBALAMIN IN PROPIONIBACTERIUM SHERMANII-KULTUREN

Von

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(Eingegangen am 21. März 1959)

Die zur Familie *Propionibacterium* zählenden Bakterien produzieren bekanntlich in großer Menge zur Vitamin $B_{12}$-Gruppe gehörende Verbindungen. Von *Propionibacterium shermanii* wurde nachgewiesen [1, 2], daß es auf üblichen Nährböden beinahe reinen Faktor-B (Aethiocobalamin) erzeugt. Gibt man indessen 1—2 mg/l 5,6-Dimethylbenzimidazol als Präkursor zur Kultur, so entsteht reines Cyanocobalamin. Der in den Kulturen von *P. shermanii* entstehende Faktor-B wandelt sich auch dann zu Vitamin $B_{12}$ um, wenn man den Präkursor einige Tage nach Beimpfung der Kultur zugibt [3]. In der 4tägigen Kultur geht diese Umgestaltung in Anwesenheit von 1—2 $\mu$g/ml Präkursor in 24—36 Stunden fast vollkommen vor sich.

Es wurde weiterhin nachgewiesen, daß sich *P. shermanii* auch in Schüttelkulturen gut entwickelt, aber in diesem Fall sehr wenig Substanz mit Vitamin $B_{12}$-Aktivität entsteht. Die optimale Vitamin $B_{12}$-Produktion erzielt man in stehenden Kulturen, in denen man laut Literaturangaben [1, 3] nach der *E. coli*-Plattenmethode 2—4 $\mu$g/ml Aktivität fand. Die Ursache der Differenz zwischen der Vitamin $B_{12}$-Produktion von Schüttel- und Stehkulturen wurde bisher nicht näher untersucht.

In den Bereich des Cyanocobalamin-Stoffwechsels der Bakterien rechnet auch die bekannte Erscheinung, daß zahlreiche Mikroorganismen das im Nährboden gelöste Vitamin $B_{12}$ oder einen Teil davon binden [4—9]. Ebenso bekannt ist auch die Tatsache, daß gewisse Mikroorganismen dem Nährboden zugegebenes Vitamin $B_{12}$ spalten. Dies wurde z. B. im Zusammenhang mit *Streptomyces griseus* festgestellt [10].

In vorliegender Arbeit wollen wir über Untersuchungen berichten, in deren Verlauf wir Einblick in die Einzelheiten der Biosynthese und Bindung von Cobalaminen in *P. shermanii*-Kulturen zu gewinnen suchten.

Einerseits untersuchten wir die Bindung des dem Nährboden zugegebenen Vitamins $B_{12}$ und im Zusammenhang damit die Frage, ob *P. shermanii* ein Enzymsystem enthält, mit dem es Vitamin $B_{12}$ abzubauen imstande ist, anderseits suchten wir die Eigenheiten der Vitamin $B_{12}$-Produktion durch Schütteln ventilierter und statischer Kulturen festzustellen. Endlich beschäf-

tigten wir uns mit der Frage, wie sich mit Formalin abgetötete Kulturen in bezug auf den Vitamin $B_{12}$-Stoffwechsel verhalten.

## Methodik

*Stamm.* Bei den beschriebenen Versuchen benutzten wir einen *P. shermanii*-Stamm, der uns von der Milchversuchsanstalt in Magyaróvár zur Verfügung gestellt worden war.

*Nährboden.* In sämtlichen Versuchen verwendeten wir folgenden Nährboden: Maisextrakt (auf Trockensubstanz gerechnet) 2%, Glukose 2%, präzipitierter $CaCO_3$ 0,8%, NaCl 0,5%, $Co(NO_3)_2 \cdot 6\,H_2O$ 5 mg/l, 5,6-Dimethylbenzimidazol 1 mg/l. — Das pH wurde mit NaOH auf 7 eingestellt und die Lösung bei 120° C während 20 Minuten sterilisiert [11].

*Züchtungsmethode in Stehkulturen.* In Erlenmeyer-Kolben zu 500 ml wurden 400 ml des vorstehend beschriebenen Nährbodens sterilisiert und die Kolben mit 20—25 ml einer auf demselben Nährboden gezüchteten 48stündigen Bakteriensuspension beimpft. Die Züchtung erfolgte bei 28° C, die Kolben wurden täglich einmal gründlich geschüttelt.

*Züchtungsmethode in ventilierten Kulturen.* In diesem Fall verwendeten wir 100 ml Nährboden in Erlenmeyer-Kolben von 500 ml. Der Nährboden wurde mit 5 ml 48stündigem, in einer Stehkultur gewonnenem Inokulum beimpft. Die Kolben wurden bei 28° C in einer rotierenden Schüttelmaschine mit 200—220 Drehungen/min und einer Bewegungsamplitude von etwa 5 cm geschüttelt.

*Bestimmung der Vitamin $B_{12}$-Aktivität.* Diese erfolgte mit der *E. coli*-Mutante auf großen Platten nach der Agarplattenmethode von TSCHAJKOWSKAJA und DRUZHINJINA [12]. Die untersuchten Materialien wurden 30 Minuten bei 100° C erschlossen.

*Kristallines Vitamin $B_{12}$.* Wir benutzten kristallines Vitamin $B_{12}$ (Chinoin), das laut Papierchromatographie im Gemisch sek. Butanol-Wasser und photometrischer Untersuchung der separierten Flecke weniger als 2% Faktor enthielt.

*Radioaktives kristallines Vitamin $B_{12}$.* Für unsere Versuche stellten wir selber markiertes Cyanocobalamin auf folgende Weise her: Im Glaskolben zu 50 Liter wurden unter Anwendung des oben beschriebenen Stammes und Nährbodens 50 l *P. shermanii*-Kultur zubereitet, mit dem Unterschied, daß wir dem Nährboden statt des üblichen Kobaltnitrates 3,5 mg/l $Co^{60}(NO_3)_2 \cdot 6\,H_2O$ beimengten; das $Co^{60}$-Salz wies nach Lösung im Nährboden 1 $\mu$C/l Aktivität auf. Aus der 6tägigen Kultur wurde das kristalline Vitamin $B_{12}$ präpariert, das bei der Elektrophorese im sauren Medium bzw. bei der mit wassergesättigtem sek. Butanol durchgeführten aufsteigenden Chromatographie einen Reinheitsgrad (auf Trockensubstanz bezogen) von 97—98% zeigte.

*Bestimmung der Radioaktivität.* Die Bestimmung der Radioaktivität erfolgte in der Flüssigkeitsphase, in 50 ml-Kammern, in denen die Flüssigkeit die GM-Röhre in einem zylindrischen Gefäß konzentrisch umgab. Im allgemeinen gaben wir 25 ml des zu untersuchenden Materials mit 25 ml Wasser verdünnt in die Zählkammer und stellten die Zahl der Anschläge dreimal 2 Minuten lang fest. Obgleich das Instrument kalibriert war, gaben wir die Resultate, nachdem wir nicht absolute, sondern nur relative Werte benötigten, der Einfachheit halber in cpm (Anzahl der Anschläge) je 25 ml/min an.

Wenn nicht anders angegeben, wurden stets zwei oder mehr Parallelversuche durchgeführt.

## Versuchsergebnisse

1. *Vitamin $B_{12}$-Bindungsfähigkeit der Propionibacterium shermanii-Kulturen.*

Den 48stündigen *P. shermanii*-Kulturen wurden verschiedene Mengen kristallines Vitamin $B_{12}$ zugegeben und die Kulturen 2 Stunden später kristallklar zentrifugiert (30 Minuten, 4500 Dreh./min). Anschließend ermittelten wir den Vitamin $B_{12}$-Gehalt der Supernatans. In dem aus der 48stündigen Kultur entnommenen Kontrollzentrifugat fanden wir Vitamin $B_{12}$-Aktivität nur in geringen Spuren. Die Ergebnisse sind in *Tabelle I* zusammengefaßt.

**Tabelle I**

*Bindung von Vitamin $B_{12}$ während 2 Stunden in 48stündigen P. shermanii-Kulturen*

| Zugegebenes Vitamin $B_{12}$ µg/ml | Im Supernatans der zentrifugierten Kultur gefundene $B_{12}$-Aktivität µg/ml | Gebundenes Vitamin $B_{12}$ µg/ml |
|---|---|---|
| 1,25 | Spuren | 1,25 |
| 3,15 | 1,53 | 1,62 |
| 6,30 | 4,53 | 1,77 |
| 0,00 | Spuren | 0,00 |

Im weiteren untersuchten wir, wie sich das Schicksal des zugegebenen Vitamins $B_{12}$ nach Ablauf bestimmter Zeitspannen gestaltet. Zu diesem Zweck gaben wir mit $Co^{60}$ markiertes Cyanocobalamin in verschiedenen Mengen zu 72stündigen *P. shermanii*-Kulturen und bestimmten im Zentrifugat (in der reinen Supernatans) der zu verschiedenen Zeitpunkten entnommenen Proben den Vitamin $B_{12}$-Gehalt und die Radioaktivität. Hierbei gingen wir von der Voraussetzung aus, daß wenn das zugeführte Vitamin $B_{12}$ abgebaut wird, die daraus freigesetzten Zersetzungsprodukte und mit diesen das radioaktive $Co^{60}$ wieder in Lösung übergehen und dann die im Supernatans gemessene Radioaktivität verhältnismäßig höher sein wird als seine Vitamin $B_{12}$-Aktivität. Falls jedoch das Verhältnis der Vitaminaktivität zur Radioaktivität dasselbe ist wie bei Versuchsbeginn, so kann das als Zeichen dafür genommen werden, daß das zugeführte und von den Bakterien assimilierte Cyanocobalamin nicht gespalten wurde. Die Resultate veranschaulicht *Tabelle II.*

Wie aus *Tabelle I* ersichtlich, binden die 48stündigen Kulturen von *P. shermanii* in 2 Stunden 1,6—1,7 µg/ml Vitamin $B_{12}$. Geben wir zum Nährboden weniger Vitamin als diese Menge, so sättigen sich die Bakterienzellen nicht mit Vitamin $B_{12}$ ; wenn wir mehr zuführen, so bleibt der Überschuß in Lösung.

Wie *Tabelle II* entnommen werden kann, nehmen die der Kultur zugegebene Vitamin $B_{12}$-Menge und die radioaktive Wirkung im Zentrifugat (d. h. im Supernatans) der zu verschiedenen Zeitpunkten entnommenen Proben in Abhängigkeit von der Zeit nach und nach ab. Aus der parallelen Senkung der beiden Werte darf geschlossen werden, daß es sich nicht um die Spaltung von Vitamin $B_{12}$, sondern um seine Bindung an die Bakteriensubstanz handelt.

In *Tabelle II* ist ferner zu sehen, daß die Relation zwischen Vitamin $B_{12}$-Aktivität und Radioaktivität gewisse Schwankungen zeigt. Diese beruhen wahrscheinlich auf experimentellen Fehlern und sind schon deshalb nicht von wesentlicher Bedeutung, weil beide Werte am Ende der drei Versuche beinahe auf Null sanken.

**Tabelle II**

*Im Supernatans der zentrifugierten Kultur zu verschiedenen Zeitpunkten ermittelte Vitamin $B_{12}$-Aktivität und Radioaktivität nach Zugabe von mit $Co^{60}$ markiertem Cyanocobalamin in der 72. Stunde*

| Zugegebene | | Zeitp. der Messung (Stunden) | Im Supernatans ermittelte | | |
|---|---|---|---|---|---|
| $B_{12}$-Akt. $\mu g/ml$ | Radioakt. Anschl./min | | $B_{12}$-Akt. $\mu g/ml$ | Radioakt. Anschl./min | Radioakt./$B_{12}$-Aktivität |
| 1,2 | 148 | 120 | Spuren | 0 | — |
| | | 168 | Spuren | 0 | — |
| 3,0 | 376 | 120 | 1,6 | 174 | 108,7 |
| | | 168 | 1,4 | 145 | 103,6 |
| | | 240 | 1,1 | 125 | 113,6 |
| | | 504 | 0,7 | 84 | 120,0 |
| 6,0 | 750 | 120 | 3,6 | 370 | 102,8 |
| | | 168 | 1,2 | 145 | 120,8 |
| | | 312 | 0,27 | 30 | 111,1 |

2. *Wirkung von kristallinem Cyanocobalamin auf die Biosynthese des Vitamins $B_{12}$.*

Zu 72stündigen Stehkulturen von *P. shermanii* gaben wir kristallines Vitamin $B_{12}$ und stellten zu verschiedenen Zeitpunkten die Vitamin $B_{12}$-Aktivität der vollständigen Kultur fest. Die Kulturen wurden, wie im methodischen Teil mitgeteilt, durch Kochen erschlossen. *Tabelle III* enthält die Resultate.

**Tabelle III**

*Vitamin $B_{12}$-Aktivität der P. shermanii-Kulturen nach Zugabe von kristallinem Cyanocobalamin in der 72. Stunde nach der Beimpfung, zu verschiedenen Zeitpunkten gemessen*

| | Alter der Kultur (Stunden) | Biologische Aktivität ($\mu g/ml$) | | | | | |
|---|---|---|---|---|---|---|---|
| | | ins-gesamt* | produ-ziert** | ins-gesamt | produ-ziert | ins-gesamt | produ-ziert |
| Zugegebene Vitamin $B_{12}$-Menge | 72 | 1,2 | — | 3,0 | — | 6,0 | — |
| Festgestellte Vitamin $B_{12}$-Aktivität zur angegebenen Zeit | 96 | 3,0 | 1,8 | — | — | — | — |
| | 120 | 4,36 | 3,16 | 5,30 | 2,30 | 9,40 | 3,40 |
| | 168 | 4,42 | 3,12 | 5,70 | 2,70 | 10,10 | 4,10 |
| | 312 | — | — | 6,90 | 3,90 | 10,50 | 4,50 |
| | 504 | — | — | — | — | 11,40 | 5,40 |
| | 600 | — | — | 7,20 | 4,20 | — | — |

* insgesamt = die in der Kultur gefundene Gesamtaktivität
** produziert = die in der Kultur erzeugte Aktivität,
d. h. Gesamtaktivität — zugegebenes Vitamin $B_{12}$

Wird die in den Nährboden eingemessene Vitamin $B_{12}$-Menge von der ermittelten Vitamin $B_{12}$-Aktivität abgezogen, so macht die erzeugte Cyanocobalamin-Aktivität in der 168. Stunde 3,2 ; 2,7 bzw. 4,10 $\mu$g/ml aus und nimmt bei den weiteren Züchtungen noch etwas zu. Es scheint demnach, daß die Erzeugung der Vitamin $B_{12}$-Aktivität durch das der jungen Kultur zugegebene Vitamin $B_{12}$ erhöht wird. *P. shermanii* verhält sich in dieser Hinsicht anders als einzelne untersuchte *Streptomyces griseus*-Stämme [10], die in Schüttelkulturen durchschnittlich 0,4 $\mu$g/ml Vitamin $B_{12}$-Aktivität erzeugten. Wenn wir dem Nährboden weniger Cyanocobalamin beimengten, so wurde diese Menge von den Mikroorganismen gebunden und nur soviel Vitamin $B_{12}$ synthetisiert, daß die Gesamtmenge 0,4 $\mu$g/ml betrug. Wurde mehr als 0,4 $\mu$g/ml Vitamin $B_{12}$ zum Nährboden gegeben, so produzierte der Mikroorganismus überhaupt kein Vitamin $B_{12}$, sondern inaktivierte zum Teil den über 0,4 $\mu$g vorhandenen Überschuß. Bei den *P. shermanii*-Kulturen wurde die Vitamin $B_{12}$-Biosynthese der Kultur von dem in den Nährboden eingeführten Vitamin $B_{12}$ nicht herabgesetzt. Weiterhin scheint es, daß *P. shermanii* kein Vitamin $B_{12}$ spaltendes Enzym erzeugt.

3. *Wirkung von Formalin auf den Cyanocobalamin-Stoffwechsel der Propionibacterium shermanii-Kulturen.*

*P. shermanii*-Kulturen behandelten wir mit 0,1 bzw. 0,2% (40%igem) Formalin. Von der 0,2% Formalinlösung wird die Kultur innerhalb einer Stunde, von der 0,1%igen binnen 24 Stunden abgetötet.

Fernerhin stellten wir fest, daß die Anwesenheit von 0,1—0,2% Formalin auf das Ergebnis der nach der *E. coli*-Plattenmethode vorgenommenen Bestimmung keinen Einfluß ausübt, wenn die Bestimmung in 20facher oder größerer Verdünnung durchgeführt wird. Diese Feststellung gilt sowohl für reine Cyanocobalaminlösungen als auch für *P. shermanii*-Kulturen, ob diese nun 5,6-Dimethylbenzimidazol als Präkursor enthalten oder nicht. Von Formalin wurde die Aktivität der erwähnten Lösungen bzw. Kulturen selbst in 6 Tagen nicht beeinflußt, wenn das Untersuchungsmaterial inzwischen bei Zimmertemperatur aufbewahrt worden war.

**Tabelle IV**

*Wirkung von 0,1 bzw. 0,2%, 40% Formalin auf die Biosynthese von Vitamin $B_{12}$ in P. shermanii-Kulturen*

| Behandlung der Kultur | Zeitpunkt der Formalinzugabe Stunde | Vitamin $B_{12}$-Aktivität in $\mu$g/ml in der Stunde | | |
|---|---|---|---|---|
| | | 72. | 96. | 120. |
| 0,1% Formalin | 72 | 6,16 | 5,88 | 6,02 |
| 0,2% Formalin | 72 | 5,54 | 5,28 | 5,16 |
| Kontrolle, ohne Formalin | — | 6,00 | 8,68 | 8,66 |

Zugleich beobachteten wir, daß die Biosynthese von Vitamin $B_{12}$ nach bugabe von 0,1% Formalin zum Stillstand kommt. Die diesbezüglichen Anga-Zen veranschaulicht *Tabelle IV.*

Nunmehr gaben wir 0,28 $\mu$g/ml kristallines Vitamin $B_{12}$ zu formalinbehandelten 120stündigen *P. shermanii*-Kulturen. Nach 2stündigem Stehen bei 25° C wurden die Kulturen scharf abzentrifugiert und die Vitamin $B_{12}$-Aktivität der reinen Supernatans untersucht. Die Resultate zeigt *Tabelle V,* aus der hervorgeht, daß die mit Formalin abgetöteten *P. shermanii*-Zellen — wenn auch in verringertem Maße — die Fähigkeit behalten, Vitamin $B_{12}$ aus dem Nährboden zu binden.

**Tabelle V**

*Die Vitamin $B_{12}$-Bindungsfähigkeit der P. shermanii-Kultur binnen 2 Stunden, nach Vorbehandlung mit Formalin bzw. Aufkochen*

| Abtötung der Kultur | | Zugabe v. Vit. $B_{12}$ | | $B_{12}$-Aktivität im Supernatans $\mu$g/ml | $B_{12}$-Gesamtaktivität der Kultur $\mu$g/ml |
|---|---|---|---|---|---|
| Art | Zeitpunkt | Menge $\mu$g/ml | Zeitpunkt (Stunde) | | |
| Kontrolle (lebende Kultur) ............ | — | 0,28 | 120 | Spuren | 1,98 |
| 0,1% Formalin ..... | 72 | 0,28 | 120 | 0,03 | 2,12 |
| 0,2% Formalin ..... | 72 | 0,28 | 120 | 0,04 | 1,82 |
| Aufkochen .......... | 72 | 0,28 | 120 | 1,93 | 2,02 |

Endlich führten wir folgenden Versuch durch: zu Präkursor nicht enthaltende *P. shermanii*-Kulturen gaben wir in der 120. Stunde nach der Beimpfung 1 $\mu$g/ml 5,6-Dimethylbenzimidazol und 0,1 bzw. 0,2%,40%ige Formalinlösung. Die Vitamin $B_{12}$-Aktivität bestimmten wir in der 120. und 144. Stunde nach der *E. coli*-Plattenmethode. Die Ergebnisse sind in *Tabelle VI* wiedergegeben.

**Tabelle VI**

*Wirkung der Abtötung mit Formalin auf die Umgestaltung des Faktors-B zu Vitamin $B_{12}$ in P. shermanii-Kulturen*

| Behandlung der Kultur | Zeitpunkt der Präkursorzugabe Stunde | Vitamin $B_{12}$-Aktivität $\mu$g/ml | | Senkung % |
|---|---|---|---|---|
| | | 120. Stunde | 144. Stunde | |
| 0,1% Formalin in d. 120. Stunde .. | 120 | 7,48 | 5,67 | 24,4 |
| 0,2% Formalin in d. 120. Stunde .. | 120 | 6,85 | 5,02 | 26,7 |
| Kontrolle (ohne Behandlung) ..... | 120 | 7,38 | 5,70 | 22,8 |

Bei der Bestimmung nach der *E. coli*-Plattenmethode ergibt der Faktor-B bekanntlich einen etwa dreifach größeren Wert als die gleiche Cyanocobalamin-

menge. Die Senkung der biologischen Aktivität ist daher, nachdem die Möglichkeit der Zersetzung des Vitamins auf Grund der früheren Versuche ausgeschlossen werden kann, mit großer Wahrscheinlichkeit (wenn auch konkrete Beweise nicht vorliegen) darauf zurückzuführen, daß sich der Faktor-B unter der Wirkung des Präkursors zu Vitamin-$B_{12}$ umgestaltet. Diese Umwandlung geht in den mit Formalin behandelten und in den unbehandelten Kulturen gleicherweise vor sich, da die biologische Aktivitätssenkung annähernd gleiche Werte zeigt. Die Kultur wird demnach von Formalin abgetötet, aber es scheint, daß das Enzymsystem, welches den Faktor-B zu Vitamin $B_{12}$ umbildet, seine Aktivität behält.

4. *Wirkung der Ventilation auf den Cyanocobalamin-Stoffwechsel der Propionibacterium shermanii-Kulturen.*

Die Vitamin $B_{12}$-Aktivität der Schüttelkulturen betrug nach 120stündiger Züchtung durchschnittlich 0,15 $\mu$g/ml. Die Inokulummenge machte 5% aus, und mit ihr waren der Kultur ungefähr 0,10—0,12 $\mu$g/ml Vitamin $B_{12}$-Aktivität zugeführt worden. In der Schüttelkultur besitzt demnach *P. shermanii* praktisch nicht die Fähigkeit, Cyanocobalamin bzw. Faktor-B zu erzeugen.

Hiernach untersuchten wir, ob die Schädigung, die in der Schüttelkultur (offenbar durch Sauerstoff) auf das cyanocobalaminsynthetisierende System von *P. shermanii* einwirkt, aufrechterhalten bleibt, wenn die Kultur wieder in Ruhezustand kommt.

Um diese Frage zu klären, nahmen wir zwei Versuche vor. Im ersten stellten wir einen Kolben nach 72stündigem Schütteln bei 28° C in den Thermostaten und ließen die Kultur im Ruhezustand weiter wachsen. Die Vitamin $B_{12}$-Aktivität der auf diese Weise 96 Stunden weitergezüchteten Kultur betrug 0,28 $\mu$g/ml, d. h. kaum mehr als die der nur geschüttelten Kulturen.

Im anderen Versuch wurde das Inokulum in der Schüttel- bzw. Stehkultur bereitet und der Kultur in 5%iger Menge beigegeben und dann beide unter statischen Bedingungen 168 Stunden in den Thermostaten gestellt. Beide Inokula ergaben nahezu übereinstimmende Werte : aus der Stehkultur 4,5 $\mu$g/ml, aus der Schüttelkultur 4,88 $\mu$g/ml. Daraus geht hervor, daß sich das aus der Schüttelkultur von *P. shermanii* stammende Inokulum in der Stehkultur in bezug auf die Vitamin $B_{12}$-Produktion ebenso verhält wie der aus der Stehkultur stammende Impfstoff. Durch die Ventilation wird das Inokulum also nicht geschädigt.

Die in *Tabelle VII* zusammengefaßten Versuchsergebnisse geben über die Einzelheiten Auskunft.

Im weiteren ergab sich die Möglichkeit, daß die Biosynthese von Vitamin $B_{12}$ eventuell auch in Schüttelkulturen ungestört vor sich geht, jedoch zugleich ein Teil des erzeugten Vitamins $B_{12}$ abgebaut wird. Zur Klarstellung der Frage führten wir zwei Versuche durch.

**Tabelle VII**

*Biosynthese der Vitamin $B_{12}$-Aktivität in P. shermanii-Schüttel- bzw. Stehkulturen sowie in den mit diesen beiden auf verschiedene Weise hergestellten Inokula beimpften Stehkulturen*

| Art des Inokulums | Züchtungsart | Zeit Stunde | Vitamin $B_{12}$-Aktivität μg/ml | Bemerkung |
|---|---|---|---|---|
| Stehkultur | Schüttelkultur | 120 | 0,15 | |
| Stehkultur | Schüttelkultur | 72 | | |
| | weiterhin in Stehkultur gehalten | 96 | 0,28 | d. h. in d. 168. Stunde nach d. Beimpfung gemessen |
| Stehkultur | Stehkultur | 120 | 3,17 | Stehkontrolle |
| Stehkultur | Stehkultur | 168 | 4,50 | |
| Schüttelkultur | Stehkultur | 168 | 4,88 | |

Der einwöchigen Stehkultur im Kolben entnahmen wir 100 ml, die in der Schüttelmaschine weitere 8 Tage geschüttelt wurden. Inzwischen bestimmten wir die Vitamin $B_{12}$-Aktivität, konnten aber feststellen, daß diese während des Schüttelns unverändert geblieben war.

Diesen Versuch wiederholten wir mit der Modifikation, daß wir zur einwöchigen Stehkultur kristallines Cyanocobalamin gaben und sie hiernach schüttelnd weiter wachsen ließen. Im Laufe des Schüttelns trat keine Senkung der Vitamin $B_{12}$-Aktivität ein. Der niedrige Vitamin $B_{12}$-Gehalt der Schüttelkultur kann daher nicht darauf zurückgeführt werden, daß die entstehenden Vitamin $B_{12}$-aktiven Substanzen unter der Wirkung der Ventillation sogleich abgebaut werden. Diesen Versuch zeigt *Tabelle VIII.*

**Tabelle VIII**

*Wirkung der Ventilation auf die Vitamin $B_{12}$-Aktivität der P. shermanii-Kulturen*

| Art und Alter der untersuchten Kultur Stunden | Dauer des Schüttelns Tage | Vitamin $B_{12}$-Aktivität μg/ml | |
|---|---|---|---|
| | | vor dem Schütteln | nach dem Schütteln |
| Stehkultur, 168 | 3 | 4,88 | 4,62 |
| | 8 | | 4,90 |
| Stehkultur, 168 mit krist. Vitamin $B_{12}$ ergänzt | 8 | 7,00 | 7,10 |

Sodann untersuchten wir die Umwandlung des Faktors-B zu Vitamin $B_{12}$ in Schüttelkulturen. Zu diesem Zweck gaben wir 1 $\mu$g/ml Präkursor zu 96stündigen *P. shermanii*-Stehkulturen, die auf einem 5,6-Dimethylbenzimidazol nicht enthaltenden Nährboden gezüchtet worden waren, und ließen die Kolben 24 Stunden schütteln. Die Vitamin $B_{12}$-Aktivität bestimmten wir in der 96. und 120. Stunde und fanden eine sehr wesentliche Senkung, die auf eine Umwandlung des Faktors-B deutet. Die in der 96. Stunde festgestellte Vitamin $B_{12}$-Aktivität betrug 9,53 $\mu$g/ml, die in der 120. Stunde, d. h. unter 24stündiger gleichzeitiger Einwirkung des Präkursors und der Ventilation ermittelte 4,87 $\mu$g/ml, was einer 49%igen Senkung entspricht.

## Besprechung

Die Untersuchung der Cyanocobalamin-Bindungsfähigkeit der *P. shermanii*-Kulturen ergab, daß eine 48stündige Stehkultur in 2 Stunden 1,6—1,7 $\mu$g/ml Vitamin $B_{12}$ bindet. Wird die Vitamin-Bindung nach längerer Zeit untersucht, so tritt ein noch wesentlich höheres Ergebnis zutage, was einerseits darauf beruhen dürfte, daß ältere Kulturen mehr Bakterienmasse (die sog. Biomasse) enthalten, anderseits darauf, daß der Bindungsprozeß mehr Zeit in Anspruch nimmt und nach 2 Stunden nicht abgeschlossen ist, sondern möglicherweise tagelang währt.*

Vohra Pran und Mitarbeiter [13] haben nachgewiesen, daß Formalin mit Cyanocobalamin eine Komplexverbindung bildet, die aber weder über ein charakteristisches Spektrum verfügt noch chromatographisch von Vitamin $B_{12}$ getrennt werden kann und im wesentlichen dieselbe biologische Aktivität wie Vitamin $B_{12}$ besitzt. Die Verbindung konnte erst nachgewiesen werden, als man Cyanocobalamin mit radioaktivem Formalin behandelte und bei der Chromatographie die Radioaktivität mit dem dem Vitamin entsprechenden roten Fleck zusammen wanderte. Anläßlich der Durchführung unserer Versuche waren uns diese Resultate noch nicht bekannt. Der Mitteilung von Vohra Pran ist nicht zu entnehmen, ob Formalin auch mit dem an die Zellsubstanz gebundenen Vitamin $B_{12}$ einen Verbindungskomplex bildet. Tatsache ist jedoch, daß die Bakterien das Vitamin aus der Formalin und Cyanocobalamin enthaltenden Lösung binden.

Im Rahmen unserer Versuche haben wir drei leicht verfolgbare Prozesse untersucht und gefunden, daß die Zunahme der Vitamin $B_{12}$-Aktivität (ob es sich nun um Cyanocobalamin- oder um Faktor-B-Produktion handelt)

* Es sei bemerkt, daß ältere — 12-14tägige — Kulturen nur noch einen ganz unbedeutenden Teil der zugegebenen Vitamin $B_{12}$-Menge binden, also nicht behauptet werden kann, das Ausmaß der Bindung sei nur von der Menge der Biomasse abhängig. Eher dürfte es vom physiologischen Zustand der Bakterien abhängen.

durch die Anwesenheit von 0,1—0,2% Formalin gehemmt wird, während diese Menge die Umwandlung des Faktors-B zu Vitamin $B_{12}$ und die Bindung des Vitamins aus der Lösung durch die Bakterien nicht beeinflußt.

Dieses Ergebnis steht im Einklang mit der Tatsache, daß Formalin die Funktion einzelner Fermente selektiv lähmt, dagegen andere Fermentsysteme nicht schädigt. So hat man z. B. nachgewiesen [14], daß die Lactat-Dehydrogenase und DPNH-Diaphorase bei der Fixierung von Geweben mit Formol, d. h. bei hoher Formalinkonzentration, ihre Aktivität bewahren, während die beta-Oxy-buttersäure-dehydrase zum Teil, die Succin-dehydrase hingegen völlig inaktiviert wird.

Bei Schüttelkulturen stellten wir fest, daß die Biosynthese von Vitamin $B_{12}$ fast auf Null sinkt, während die Umgestaltung des Faktors-B zu Vitamin $B_{12}$ ebenso vor sich geht wie in normalen oder formalinbehandelten Stehkulturen. Weiterhin fanden wir, daß die Vitamin $B_{12}$ synthetisierende Fähigkeit stark ventilierter Bakterien nicht endgültig verlorengeht und im Stoffwechsel dieser Zellen keine tiefgreifenden Veränderungen eintreten, weil die aus Schüttelkulturen beimpften Stehkulturen eine beträchtliche Menge Vitamin $B_{12}$-Aktivität produzieren, nicht weniger als die aus Stehkulturen beimpften Kulturen. Die Cyanocobalaminbindung der Schüttelkulturen vermochten wir infolge methodischer Schwierigkeiten nicht zuverlässig festzustellen.

## Zusammenfassung

Die Untersuchung des Cyanocobalamin-Stoffwechsels von *Propionibacterium shermanii*-Kulturen ergab folgende Resultate:

1. 48stündige Kulturen binden in 2 Stunden 1,6—1,7 $\mu$g/ml Cyanocobalamin. Nach Verlauf einer längeren Zeit kommt es noch zur Steigerung der Bindungsfähigkeit.

2. Das in überflüssiger Menge anwesende Vitamin $B_{12}$ wird von den Kulturen nicht abgebaut, und das zugegebene Vitamin $B_{12}$ ist auf die Produktion der Vitamin $B_{12}$-Aktivität ohne wesentlichen Einfluß.

3. Von 0,1—0,2% Formalin wird die Kultur abgetötet. Eine derartige Kultur verliert ihre Fähigkeit zur Synthese von Vitamin $B_{12}$, bewahrt aber die Fähigkeit, das der Lösung zugegebene Cyanocobalamin zu binden und den Faktor-B (in Anwesenheit eines Präkursors) zu Vitamin $B_{12}$ umzubilden.

4. Stark ventilierte (geschüttelte) Kulturen verlieren die Fähigkeit, Vitamin $B_{12}$ zu synthetisieren, bewahren dagegen die Fähigkeit, den Faktor-B zu Vitamin $B_{12}$ umzugestalten. Auch im Falle starker Ventilation ist die Kultur nicht imstande, Cyanocobalamin abzubauen.

5. Die aus der Schüttelkultur beimpfte Stehkultur erzeugt ebensoviel Vitamin $B_{12}$-Aktivität und verhält sich im allgemeinen auf dieselbe Weise wie die aus der Stehkultur beimpfte Stehkultur.

*

Den Herren Á. Jász und T. Lengyel *(Laboratorium für Kernchemie des Chemischen Forschungsinstitutes der Ungarischen Akademie der Wissenschaften)*, deren freundliche Hilfe und Fachunterstützung die Arbeit mit dem radioaktiven Co-Isotopen ermöglichte, sei auch an dieser Stelle gedankt.

Ebenso danken wir Frau G. Mertha *(Institut für Pharmakoindustrielle Forschung)* für die Präparation des radioaktiven Vitamins $B_{12}$ und Á. Balázs *(Chinoin)* für die chromatographischen Untersuchungen bzw. für die photometrische Messung der von den Chromatogrammen abgelösten Substanzen.

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# STUDIES ON A CL. PERFRINGENS PHAGE

By

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(Received May 22, 1959)

COWLES [1] in 1934, was the first to isolate an anaerobic phage, a phage lysing some strains of *Cl. tetani*. Subsequently, phages lysing "gas gangrene bacilli" were isolated by ZAEVA *et al.* [2] who suggested the therapeutic use of their phages in war-injuries. KRUEGER *et al.* [3] recovered a *Cl. perfringens* phage from sewage in 1947. In 1955, GUELIN [4] reported on the isolation, propagation and multiplication of a phage of the same species.

The objects of the investigations to be described were, (i) to establish appropriate techniques for our present and subsequent studies on anaerobic phages ; (ii) to obtain data on the heat stability and immunogenicity of GUELIN's *Cl. perfringens* phage. A one-step growth curve of the phage will also be presented.

## Materials and methods

*Bacterium and phage.* The *Cl. perfringens* strain and its phage used in this work were kindly supplied by Mme GUELIN *(Institut Pasteur, Paris)*. The bacterium was maintained in HOLMAN's medium at room temperature fresh cultures being made at 3 or 4 week's intervals.

To obtain bacteria for all the experiments described below, 24-hour HOLMAN cultures were subcultured into HARTLEY's broth, and the subcultures were incubated at 37° C for 4 hours.

*Propagation of phage.* Boiled and immediately cooled HARTLEY broth was inoculated with 1/20 volume four-hour HARTLEY broth culture of bacterium, and phage was added to make $10^4$—$10^5$ infecting particles/ml. Ninety minutes' incubation at 37° C led to clouding visible by the naked eye. By the end of the 3rd hour, however, the culture had cleared up completely or almost completely. The lysed culture was then filtered through a Seitz EK pad and titrated as described below. The filtered phage preparation was stored in sealed ampoules at +4° C. Preparation signed 808/3a, which was exclusively used in our experiments, had lost one logarithmic unit by the end of the 2nd month of storage, whereas by the methods used no further change in titre was detectable within the subsequent year.

*Phage titrations*, GRATIA's agar layer technique was adapted to anaerobic conditions.

WILSON—BLAIR's agar, as modified by THOMPSON [5], was used, with the only difference that the concentration of ferrous sulphate was reduced. The ferrous sulphate stock solution contained 0.8 g $FeSO_4$ in 10 ml distilled water. The medium consisted of 10 ml 20 per cent $Na_2SO_3$ (freshly dissolved), 5 ml 20 per cent glucose, and 1 ml ferro-sulphate stock solution diluted 1 to 10 and made up to 100 ml with 2 per cent agar broth.

The medium was poured into Petri dishes 9 cm in diameter, 20 ml into each, and allowed to harden. A second layer was then poured over the surface of the basal layer. The surface layer consisted of 0.5 ml test bacterium, and volumes varying from 0.1 to 1.0 ml of the phage

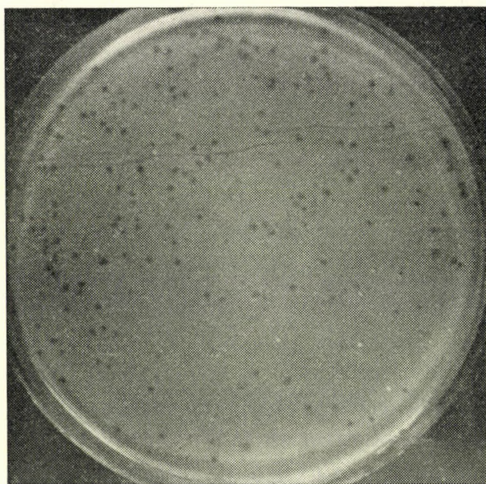

*Fig. 1.* Plaques by *Cl. perfringens* phage 808/3a in the modified Wilson—Blair' medium

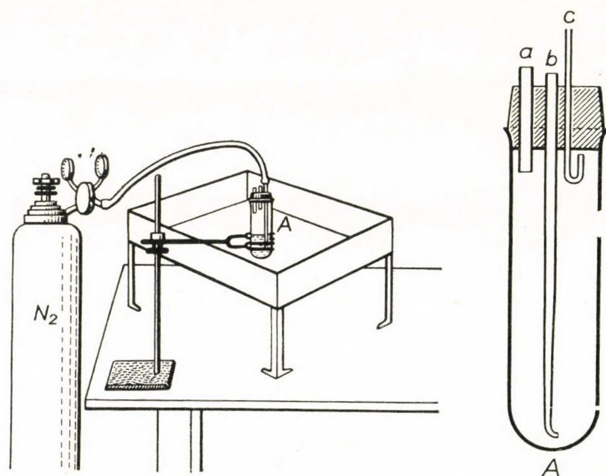


*Fig. 2.* Apparatus for establishing one-step growth curve of phage 808/3a

*Explanations:*
*a* = tube for taking samples.
*b* = tube for introducing nitrogen gas.
*c* = ventil tube.

dilution to be tested, made up to 10 ml with a mixture composed of equal volumes of the above medium and nutrient broth. After being allowed to dry at room temperature for at least an hour, the Petri dishes were covered and incubated at 37° C overnight. The following morning plaques were counted on a white background. The plaques were well-visible, surrounded by the bacterial culture assuming a grey colour from the metallic iron *(Fig. 1)*.

*Heat sensitivity* of the phage was assayed at 60° C in a water bath. Samples were taken every 30 minutes.

*Antiphage.* Rabbits were immunized intravenously with a phage preparation containing $10^8$ plaque forming units (PFU)/ml. Each of the rabbits was given $3 \times 1$ ml at one-week intervals. The third injection was followed by 6 more injections, 2 ml each, given every other day. Blood was taken and antibody assayed 7 days after the last injection. The neutralization test was performed as described by ADAMS [6].

*One step growth curve* of the phage was determined essentially according to ADAMS' method [6], except that we established anaerobic conditions for the bacterium—phage system by constructing a simple apparatus illustrated in *Fig. 2.*

Before each experiment Hartley broth was measured into the cotton-plugged tube *A* (growth tube), for the last-step dilution of the bacterium—phage mixture. The medium was then boiled and immediately cooled. The cotton plug was changed for a bored rubber stopper holding tubes *a*, *b* and *c*. The growth tube was connected through tube *b* with $N_2$ bottle. The fluid was stirred by introducing nitrogen gas, 220 to 250 bubbles/min for 10 minutes.

A typical one-step curve experiment was performed in 4 steps, (i) mixing phage and bacterium; (ii) adsorption in a 37° C water bath; (iii) dilution of the bacterium—phage mixture (for the last step of dilution the mixture was introduced into tube *A* through tube *a)* ; (iv) taking samples at intervals.

Before each experiment the phage preparation was titrated as described above and the bacterium count was determined in a haemocytometer. To estimate the extent of adsorption, ADAMS' method was adopted. The bacterium—phage mixture was diluted and centrifuged, and the PFU count in the supernatant was determined.

## Experimental

*Heat stability. Table I* shows a typical experiment, demonstrating the rate of inactivation at 60° C of phage 808/3a.

**Table I**

*Inactivation of the Cl. perfringens phage 808/3a at 60° C*

| Minutes at 60° C | $10^6 \times$ PFU/ml |
|---|---|
| 0 | 20 |
| 30 | 3.2 |
| 60 | 1.6 |
| 90 | 0.58 |
| 120 | 0.31 |
| 150 | 0.24 |

It is clearly seen that the activity of the phage preparation was rapidly reduced in the first 30 minutes. Thereafter inactivation was slower. At 150 min 1.2 per cent of the initial phage activity was demonstrable.

*Neutralization test.* A typical quantitative neutralization test with an antiphage to phage 808/3a is shown in *Table II.*

**Table II**

*Neutralizing capacity of antiphage N° 449**

| Serum dilution | PFU in 4 parallel plates | Average PFU |
|---|---|---|
| 1 : 10 | 0, 0, 0, 0, | 0 |
| 1 : 50 | 0, 0, 0, 0, | 0 |
| 1 : 100 | 6, 3, 3, 8, | 5 |
| No serum | 96, 111, 98, 83 | 97 |

* For a description of the experiment, see in the text.

The phage preparation used contained 1.9 $\times$ $10^7$ PFU/ml. Of this preparation 0.1 ml was mixed with 0.9 ml diluted serum or Hartley broth and incubated in a 37° C water bath for 10 minutes. The mixtures were then diluted and 0.1 ml of the 1 : 2000 dilution was poured onto each plate.

It is apparent that 95 per cent of the phage activity was neutralized by the 1 : 100 dilution of serum. The velocity constant ($C$) was computed as suggested by Adams [6]

$$C = 2.3 \frac{1000}{10} \log \frac{97}{5} = 294$$

*Multiplication of phage 808/3a.* A one-step curve illustrating the first cycle of multiplication is shown in *Fig. 3.*

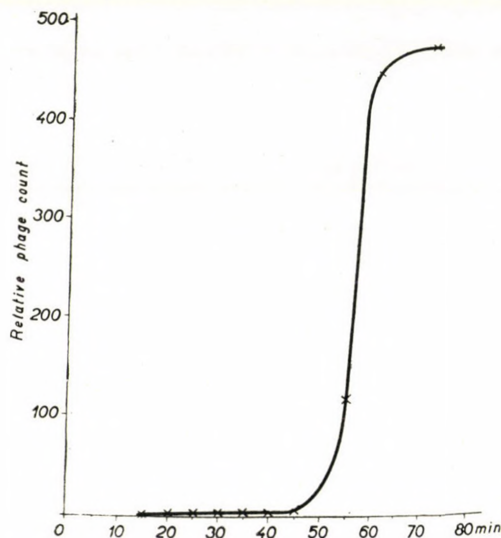


*Fig. 3.* One-step growth curve of phage 808/3a

*Table III* shows the data in detail.

**Table III**

*Numerical data of the growth curve of phage 808/3a*

| Time of incubation min. | Dilution | PFU/ml in 3 parallel plates | | | $10^7 \times$ average PFU/ml adsorption mixture | Relative* phage count |
|---|---|---|---|---|---|---|
| 15 | $10^0$ | 296 | 240 | 216 | 5 | 1.0 |
| 20 | $10^0$ | 386 | 404 | 464 | 8.3 | 1.9 |
| 25 | $10^0$ | 484 | 574 | 600 | 11 | 2.6 |
| 30 | $10^{-1}$ | 86 | 90 | 99 | 18 | 4.5 |
| 35 | $10^{-1}$ | 83 | 78 | 77 | 16 | 4.0 |
| 40 | $10^{-1}$ | 90 | 87 | 88 | 18 | 4.5 |
| 45 | $10^{-1}$ | 73 | 75 | 70 | 15 | 3.7 |
| 55 | $10^{-2}$ | 228 | 174 | 262 | 440 | 119 |
| 65 | $10^{-3}$ | 74 | 72 | 102 | 1650 | 440 |
| 75 | $10^{-3}$ | 82 | 85 | 94 | 1740 | 462 |
| | Burst size, | 452 | | | | |

* Relative phage count $= \frac{C-B}{A-B}$

$A$ = PFU added/ml; $B$ = PFU unadsorbed/ml;
$C$ = PFU demonstrated at the given time.

The adsorption mixture contained $5 \times 10^7$ PFU of phage and $2 \times 10^8$ bacterial cells/ml. Thus, multiplicity of infection was 0.25. The mixture was incubated in a 37° C water bath for 3 minutes* then, diluted 1 in 200 000 chilled Hartley broth in 5 steps. The last step of dilution was performed in tube *A* of the apparatus shown in *Fig. 2*, as described above. Samples were taken at intervals, as given in *Table III*. The samples, either undiluted or diluted, were immediately poured over the surface of plates, as described under *Materials and methods*.

*Table III* and *Fig. 3* show that, if the moderate increase of the relative phage count between 15 and 30 minutes is disregarded, latency lasts about 45 minutes and is followed by a steep rise lasting 20 minutes. The usual stationary phase begins thereafter. Burst size calculated from the average relative phage count of the stationary phase is 452.

* Unadsorbed phage was also determined. Of the adsorption mixture, 0.1 ml was added to 9.9 ml chilled Hartley broth in a centrifuge tube, with 3000 r.p.m. for 10 minutes and in the supernatant the PFU count was determined. In the experiment presented in *Fig. 3*, 75 per cent of phage were adsorbed. The relative phage count (r) was calculated by the formula given in the footnote to *Table III*.

## Discussion

The use of solid media in titrating aerobic phages is certainly preferable to the use of fluid media because of the numerous sources of error in the latter's relations. This view has not been accepted for anaerobic phages. GOLD *and* WATSON [7] emphasize that titration on solid media of anaerobic phages is troublesome. Instead they use a fluid medium containing molasses, some salts and vitamin C. Introducing our simple techniques described in the present paper has made the solid-medium principle preferable in titrating an anaerobic phage.

Our first aim was to find a medium in which *Cl. perfringens* grows without any anaerobic apparatus. Such a medium is WILSON—BLAIR's as modified by THOMPSON. *Cl. perfringens,* however, blackens the medium so diffusely that plaque counting is difficult. Iron concentration was, therefore, reduced to 1 : 10 in the basal layer and to 1 : 20 in the surface layer. In such a milieu the transparent plaques were well-visible as surrounded by the grayish culture of bacterium. According to GRATIA, the phage—bacterium mixture should be poured over the surface of the basal layer in 2.5 ml soft agar. Since this layer is too thin to cultivate *Cl. perfringens,* we made up the mixture with soft agar to 10 ml. Using such a thick layer was expected to give rise to new sources of error inherent in the plaques covering one another and in the lack of plaque formation in the superficial layer where *Cl. perfringens* grows barely. To test the reproducibility of the results, we titrated a phage preparation in 20 plates and calculated PEARSON's coefficient of variation for 3 plates. The result was $\pm$ 12.8 per cent. According to HORVÁTH *and* ALFÖLDI [8], coefficients of variation for aerobic phage titrations are $\pm$ 3.5 to $\pm$ 8.2 per cent for the method of HORVÁTH *and* ALFÖLDI, $\pm$ 13 per cent for that of HERSHEY, and $\pm$ 6.9 per cent for that of JONES *and* KRUEGER. Taking into account that possible modifications aiming at a further reduction of the sources of error would need more equipment, we must be satisfied by the accuracy of our method.

Our experiments give no explanation why the phage count was slightly but definitely increasing during the latency. Further investigations are in progress.

## Summary

1. A method for the titration on solid medium of a phage of *Cl. perfringens* has been presented.

2. A simple apparatus for investigating the multiplication of the phage has been constructed.

3. In heat stability and immunogenic capacity the phage does not significantly differ from the aerobic phages so far tested.

4. The one-step growth curve of the phage has been determined. In single-infected cells latency lasts about 45 minutes, the rise period about 20 minutes. Burst size was 452.

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# THE EFFECT OF REMOVAL OF AVAILABLE WATER ON CELL FORM AND KINETICS OF GROWTH OF A STRAIN OF SACCH. CEREVISIAE VAR. ELLIPSOIDEUS

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(Received May 28, 1959)

One of the earliest methods of preserving perishable foods is the removal of water by drying, evaporation, addition of sugar, salt, *etc.* The basis of all these operations is the fact that, for the maintenance of their life processes, the deteriorating agents, *i.e.* the microorganisms, need water. Growth of the microbial cell depends upon the water content of the medium. The decisive point, however, is not the percentual water content but its availability, the "state" of water in the medium, *i.e.* its "hydrature" [14]. This may be characterised directly by the relative vapour pressure or the relative humidity of the material and indirectly by the osmotic pressure or the freezing point depression of the aqueous phase of the foodstuff. Thus, foods may be preserved by lowering their hydrature below the minimum hydrature supporting growth of the microorganisms causing deterioration.

By this lowering of hydrature the physical and chemical characteristics of the surroundings of the cell are altered, and this induces changes in the cell itself as far as water content, cell volume, cell structure, *etc.* are concerned. In concentrated solutions, *i.e.* in those of low hydrature, the cell shrinks, it undergoes plasmolysis or, if the cell wall is not rigid, cytorrhysis [15] ; in media of low concentration swelling, plasmoptysis, occurs. In the course of these processes changes in the "bound" water content of the cell (bound in various ways, *e.g.* as hydrate water, or by van der Waals, or Donnan forces, or bound mechanically, *etc.*) may occur [2] and these changes may harm certain important polymers (proteins, polysaccharides, nucleic acids, *etc.*) and especially the enzyme systems of the cell [3, 4, 5].

It was decided to study some of these changes with special regard to cell form and growth kinetics, in a yeast strain kept in salt and glycerol solutions of varying concentrations.

## Materials and methods

The microorganism studied was a single-cell culture of the wine yeast *Saccharomyces cerevisiae* var. *ellipsoideus*, Tokaj 22, grown in Petri dishes on the following medium : 0.4 per cent meat extract, 20 per cent sweet whey, 10 per cent (1 + 10) yeast extract, 1 per cent glucose, 0.2 per cent peptone, and 2 per cent agar (the pH was adjusted to 7.2).

To study the *changes in form*, cell suspensions were prepared by careful scraping of the yeast growth off the surface of the Petri dish cultures and suspending portions of it in NaCl and glycerol solutions of various strengths. The solutions were made up of analytically pure substances and distilled water. One loopful of each suspension was placed on each of two slides. The preparations were immediately covered with cover-slips and each sealed with melted petrolatum around the edges of the cover-glass.

After standing at 8° C for 20 hours, microphotogrammes were made from these preparations at a magnification of about 300 diameters. Of each concentration level 6 photogrammes and their paper prints (linear magnification : 2.5) were made at 3 points along an imaginary diagonal line over the cover-slip : in the middle and at two corners of each of the 2 preparations.

Evaluation of the pictures was effected by placing a piece of film containing the positive picture of a millimeter paper reduced to half its original size (a network with a ruling of 0.5 mm was obtained in this way) over the cells and recording the lengths of their long ($A$) and short ($B$) axes. At each concentration level, 120 cells (20 cells from each photogramme) were measured at random. From the data the volume ($V$) of each cell was calculated assuming that the cells were ellipsoidal and their invisible axis (that perpendicular to the plane of the picture) was the same length as the measured shorter axis [1, 11].

Thus :

$$V = \frac{\pi}{6} \cdot A \cdot B^2$$

Salt concentrations applied were as follows : 0 (distilled water), 0.1, 0.2, 0.4, 0.8, 1.6, 3.2, 6.4, 9.6, 12.8, 16.0, 19.2, 22.4 and 25.6 per cent (w/w) NaCl.

The concentrations of the glycerol solutions were so chosen as to be isoosmotic with the above salt solutions, *i.e.:* 0, 0.35, 0.7, 1.4, 2.4, 4.5, 8.7, 16.6, 23.9, 30.8, 37.5, 44.2 and 51 per cent (w/w) of glycerol. These values were derived by interpolation along a curve constructed from the data on isoosmotic solutions of SCATCHARD *et al.* [9]. The concentration of glycerol corresponding to 25.6 per cent NaCl could not be computed.

*Changes in the kinetics of growth* were studied with the same yeast strain by turbidimetric measurements, carried out with a modified Pulfrich Stufenphotometer [12], in NaCl and glycerol solutions of various strengths.

The medium contained 0.18 per cent peptone, 0.46 per cent sucrose and 0.23 per cent $KH_2PO_4$ in a (1 + 100) yeast decoction. The pH was adjusted to 4.5 by HCl. The concentration of NaCl was varied from 2.50 to 10.75 per cent, using a concentration ratio of 1.2 (2.50, 3.00, 3.60, 4.32, 5.16, 6.22, 7.46, 8.96, 10.75 per cent NaCl). To the solutions, distributed and sterilised in test tubes, $6.6 \times 10^5$ cells of *Sacch. cerevisiae* var. *ellipsoideus*, Tokaj 22 were added per ml and turbidity (growth) was measured as a function of time using an S 66 red Pulfrich filtre and a water bath of 30 ± 0.25° C applied for incubation. — The concentration of glycerol was adjusted to 0, 2.5, 4.9, 9.6, 18 and 32 per cent (w/w) of $CH_2OH \cdot CHOH \cdot CH_2OH$. According to SCATCHARD *et al.* [9] these concentrations are isoosmotic with 0, 0.85, 1.75, 3.55, 7.00 and 13.35 per cent of NaCl, respectively. Inoculation was made with $1.2 \times 10^6$ cells per ml of the inoculated solution. Incubation and measurements were effected as mentioned previously.

Evaluation of the kinetic data obtained in 5 parallel measurements was carried out according to the method described elsewhere [13].

## Results

*Changes in cell volume.* — The first observation made was the fact that very great differences in size exist between cells derived from the same single-cell culture. Thus, *e.g.* a range of variation from 80 to 3500 "cubic units" was observed quite frequently. (Since one "unit" equalled 0.5 mm on the photographic print, and this corresponded to 0.67 $\mu$, the above range means from 24 to 1050 $\mu^3$. In order to simplify calculations, data will always be recorded in "units" ($u$) and "cubic units" ($u^3$), respectively.) Since we were primarily concerned with finding out whether the increase in osmotic pressure caused a statistically significant change in cell volume, the wide range of variation

made it necessary to study the frequency distribution ($f$ = number of cells out of 120) of cell volumes ($V$) (*Fig. 1*).

It may be seen from *Fig. 1* that the frequency distribution curves show great variation in form. Nevertheless, the real normal form of distribution can in no case be detected. Nearly all curves are asymmetrical showing strong,

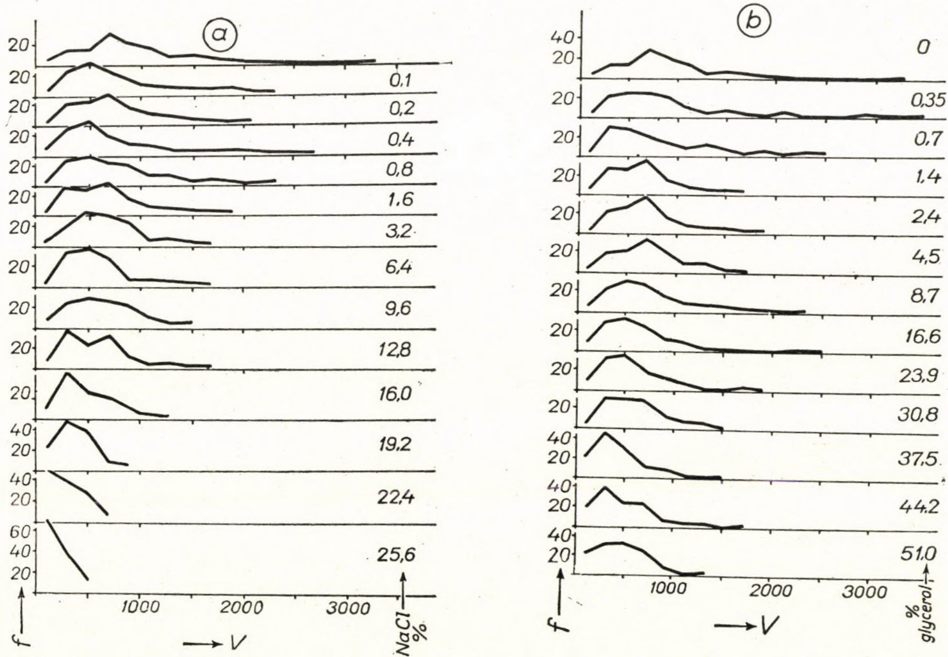


*Fig. 1.* Frequency distribution of the volumes of yeast cells in various NaCl and glycerol solutions

positive skewness [6] owing to the presence of a number of cells much larger than the average.

It was therefore decided to study the nature of the frequency distribution of cell volumes. It was found that most of the curves follow the laws of the log-normal distribution. This was made probable by probit transformation of the data, and was confirmed by fitting the latter to the normal curve. As usual, fitting was based on calculation of the mean ($M$) and standard deviation ($s$) of the data. From these the theoretical distribution was determined. Goodness of fit was evaluated by comparing the theoretical and actual values using the $\chi^2$ test.

It has been found that while the probit lines of most of the original distributions showed pronounced curvature, the probit lines obtained from the logarithmically transformed data were nearly straight, indicating a log-normal distribution.

Similarly, while fitting the original data to the normal curve resulted in $\chi^2$ values as large as to warrant the rejection of the assumption of normal distribution, most of the logarithmically transformed data gave excellent fits.

**Table I**

*Distribution of the volumes of yeast cells suspended in NaCl and glycerol solutions. Comparison with the normal and the log-normal distributions, respectively*

| Suspending medium | Solution isoosmotic with % NaCl | V | | | log V | | |
|---|---|---|---|---|---|---|---|
| | | $\chi^2$ | degrees of freedom | $P$ | $\chi^2$ | degrees of freedom | $P$ |
| Water | 0 | 13.09* | 5 | < 0.025 | 4,17* | 1 | < 0.05 |
| Sodium chloride | 0.1 | 37.23*** | 6 | <0.0005 | 0.79 | 1 | >0.30 |
| | 0.2 | 17.02** | 4 | <0.005 | 4.17 | 3 | >0.20 |
| | 0.4 | 53.0*** | 6 | <0.0005 | 2.90 | 2 | >0.20 |
| | 0.8 | 25.29*** | 5 | <0.0005 | 0.90 | 1 | >0.30 |
| | 1.6 | 20.47*** | 4 | <0.0005 | 0.75 | 2 | >0.60 |
| | 3.2 | 12.86* | 4 | <0.025 | 15.32*** | 2 | <0.0005 |
| | 6.4 | 29.00*** | 3 | <0.0005 | 1.83 | 2 | >0.40 |
| | 9.6 | 6.84 | 4 | >0.10 | 2.36 | 2 | >0.30 |
| | 12.8 | 21.48*** | 3 | <0.0005 | 0.02 | 1 | >0.80 |
| | 16.0 | 16.82*** | 2 | <0.0005 | 0.19 | 1 | >0.60 |
| | 19.2 | 0.35 | 1 | >0.50 | 3.72 | 1 | >0.05 |
| | 22.4 | 20.02*** | 1 | <0.0005 | 3.64 | 1 | >0.05 |
| | 25.6 | 15.36*** | 2 | <0.0005 | 0.40 | 1 | >0.50 |
| Glycerol | 0.1 | 36.45*** | 7 | <0.0005 | 0.15 | 1 | 0.70 |
| | 0.2 | 47.13*** | 7 | <0.0005 | 1.02 | 1 | >0.30 |
| | 0.4 | 14.42** | 4 | <0.01 | 1.58 | 2 | >0.40 |
| | 0.8 | 19.29*** | 4 | <0.001 | 0.13 | 1 | >0.70 |
| | 1.6 | 9.35 | 4 | >0.05 | 5.32* | 1 | <0.025 |
| | 3.2 | 19.86*** | 4 | <0.001 | 3.15 | 2 | >0.20 |
| | 6.4 | 23.18*** | 5 | <0.0005 | 1.19 | 1 | >0.20 |
| | 9.6 | 19.44*** | 4 | <0.001 | 1.07 | 1 | 0.30 |
| | 12.8 | 14.30** | 3 | <0.005 | 0.89 | 2 | >0.60 |
| | 16.0 | 19.47*** | 3 | <0.0005 | 3.41 | 1 | >0.05 |
| | 19.2 | 15.87** | 3 | <0.005 | 1.61 | 1 | >0.20 |
| | 22.4 | 0.84 | 2 | >0.60 | 10.70** | 1 | <0.005 |

*Notation:* $P$ = probability of compliance with the normal distribution
* = significant deviation from the normal distribution
** = highly significant deviation from the normal distribution
*** = very highly significant deviation from the normal distribution

The situation is illustrated by *Table I* and *Fig. 2*. The latter shows the fitting of the data obtained in the 0.4 per cent NaCl solution. (The dotted lines in *Fig. 2* represent normal distributions. $f$ = number of cells out of 120.)

As may be seen from *Table I*, the frequency distribution of cell volumes is, in most of the cases (22 in 26), of the log-normal type.

This phenomenon which, according to our knowledge, has not yet been reported in the literature, seems to be of some interest in the taxonomy of yeasts. Recent results of Kocková—Kratochvilová *and* Necásek [7] cast doubts on some methods of morphological identification of yeasts. According to Lodder *and* Kreger-Van Rij [8] there exist yeast species that possess the same biochemical, physiological characteristics and can be distinguished

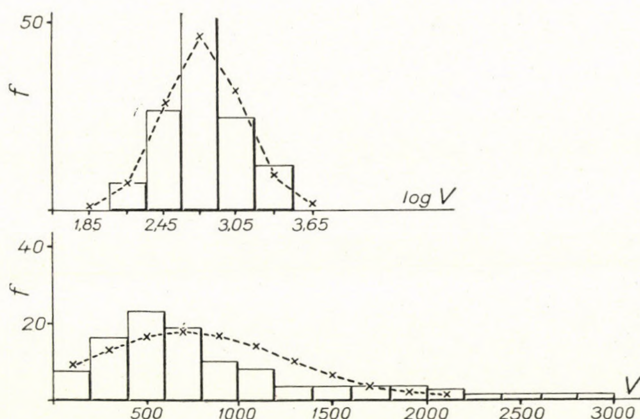


*Fig. 2.* Comparison of the distribution of the volumes of yeast cells, suspended in 0.4 per cent NaCl, to the normal and the log-normal distributions, respectively

only by differences in the size of their cells. This possibility was questioned by the former authors [7]. It is believed that the above-described nature of the frequency distribution of cell volumes may help to shed fresh light on the problem by making possible the statistical analysis and evaluation of differences in volume of cells of physiologically similar yeast species allegedly differing in size. The usual method of identification based on the range of variation, as measured on only 20 cells, seems to lead to illusory conclusions.

In the evaluation of the effect of osmotic pressure on cell volume, data were, therefore, transformed logarithmically. After calculating the means ($\overline{\log V}$) and standard deviations significance of the differences in the mean volumes of cells suspended in isoosmotic glycerol and salt solutions was determined by the usual $t$ test. The results are given in *Table II* and illustrated by *Fig. 3*.

**Table II**

*Comparison of the effect of NaCl and glycerol on the mean volume of yeast cells suspended in solutions of various strengths*

| NaCl % (w/w) | Glycerol % (w/w) | Means of log V | Standard deviation of log V $s$ | $t$ | $P$ |
|---|---|---|---|---|---|
| 0 | 0 | 2.84302 | — | — | — |
| 0.1 | — | 2.79023 | 0.2748 | | |
| — | 0.35 | 2.82284 | 0.2640 | 0.94 | 0.35 |
| 0.2 | — | 2.80036 | 0.2276 | | |
| — | 0.7 | 2.79274 | 0.2703 | 0.24 | 0.81 |
| 0.4 | — | 2.76765 | 0.2917 | | |
| — | 1.4 | 2.73755 | 0.2402 | 0.87 | 0.39 |
| 0.8 | — | 2.81030 | 0.2480 | | |
| — | 2.4 | 2.78521 | 0.2059 | 0.98 | 0.33 |
| 1.6 | — | 2.76263 | 0.2342 | | |
| — | 4.5 | 2.78270 | 0.2420 | 0.65 | 0.52 |
| 3.2 | — | 2.78019 | 0.2276 | | |
| — | 8.7 | 2.76765 | 0.2465 | 0.41 | 0.68 |
| 6.4 | — | 2.69741 | 0.2104 | | |
| — | 16.6 | 2.71497 | 0.2546 | 0.58 | 0.54 |
| 9.6 | — | 2.75009 | 0.2474 | | |
| — | 23.9 | 2.69239 | 0.2667 | 1.74 | 0.08 |
| 12.8 | — | 2.68487 | 0.2489 | | |
| — | 30.8 | 2.70995 | 0.2218 | 0.83 | 0.40 |
| 16.0 | — | 2.61466 | 0.2492 | | |
| — | 37.5 | 2.56195 | 0.2348 | 1.67 | 0.10 |
| 19.2 | — | 2.52435 | 0.2408 | | |
| — | 44.2 | 2.59709 | 0.2709 | 2.20 | 0.03* |
| 22.4 | — | 2.37634 | 0.2814 | | |
| — | 51.0 | 2.58703 | 0.2676 | 5.95 | $<$0.001 *** |
| 25.6 | — | 2.24593 | — | — | — |

* significant deviation
*** very highly significant deviation

As may be seen, generally speaking, the standard errors do not show great variation. The $t$ test reveals that the geometric means of the volumes of cells suspended in isoosmotic salt and glycerol solutions do not differ significantly from one another. Only in the case of the last 2 compared concentrations do significant differences exist between the action of the two plasmolytic agents. Nevertheless, it must be stated here that in these NaCl suspensions the photogrammes were not very clear-cut and so the data are more of relative rather than of absolute value.

The above data may, therefore, be interpreted in such a way that — at least from the point of view of volume changes — the above two plasmolytic

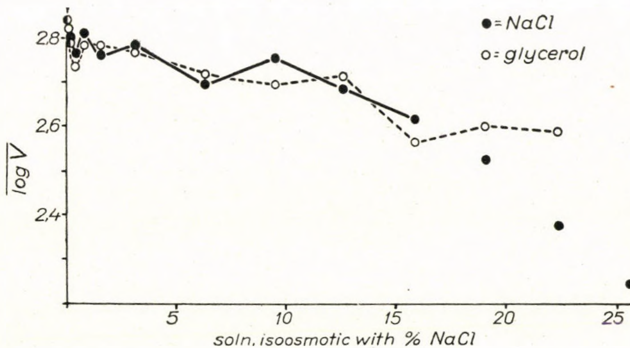


*Fig. 3.* The effect of increasing the concentration of NaCl and glycerol on the volume of yeast cells. *Note:* The values at 19.2, 22.4 and 25.6 per cent NaCl (full circles) were measured on unclear photogrammes (see *Table IV)*

agents, while widely differing in chemical nature, exert the same action in isoosmotic concentrations. This may be regarded as an indication that the degree of cytorrhysis is independent of the material characteristics of the plasmolytic agent and depends only upon the value of osmotic pressure.

*Changes in the ratio of cell diameters.* — In order to characterize shrinkage of cells more precisely, changes in the ratio of cell diameters have also been studied. It could be expected that, in the course of cytorrhysis, shrinkage does not occur uniformly in all directions. The cell may retain its original dimensions more tenaciously in the direction of one or the other of the axes. Thus, it may occur that in solutions of higher osmotic pressure the ratio of diameters ($A/B$) increases (elongation) or decreases.

Knowing the longer and shorter diameters of yeast cells suspended in the various salt and glycerol solutions, the frequency distribution of their ratio ($A/B$) has been established. Generally speaking, these distributions also show deviations from the normal. Compliance with the Gaussian distribution becomes better if the distribution of the logarithms of the ratios is considered. This is clearly demonstrated in *Table III*.

**Table III**

*Distribution of the diameter ratios of yeast cells suspended in NaCl and glycerol solutions. Comparison with the normal and the log-normal distributions, respectively*

| Suspending medium | Solution isoosmotic with % NaCl | $\frac{A}{B}$ | | | $\log \frac{A}{B}$ | | |
|---|---|---|---|---|---|---|---|
| | | $\chi^2$ | degrees of freedom | $P$ | $\chi^2$ | degrees of freedom | $P$ |
| Water | 0 | 11.33* | 3 | < 0.025 | 5.40 | 2 | > 0.05 |
| Sodium chloride | 0.1 | 3.45 | 3 | > 0.30 | 0.92 | 2 | > 0.60 |
| | 0.2 | 17.90** | 4 | < 0.005 | 5.55 | 2 | > 0.05 |
| | 0.4 | 6.25 | 3 | 0.10 | 9.45** | 2 | < 0.01 |
| | 0.8 | 9.49* | 3 | < 0.025 | 1.32 | 2 | > 0.50 |
| | 1.6 | 7.29 | 3 | > 0.05 | 0.61 | 2 | > 0.70 |
| | 3.2 | 3.21 | 3 | > 0.30 | 1.20 | 2 | > 0.50 |
| | 6.4 | 13.25* | 4 | < 0.025 | 1.75 | 2 | > 0.40 |
| | 9.6 | 4.72 | 3 | > 0.10 | 1.53 | 2 | > 0.40 |
| | 12.8 | 8.78* | 3 | < 0.05 | 2.11 | 2 | > 0.30 |
| | 16.0 | 1.82 | 3 | > 0.60 | 7.52* | 2 | < 0.025 |
| | 19.2 | 22.92*** | 6 | < 0.001 | 15.34** | 4 | < 0.005 |
| | 22.4 | 9.54 | 5 | > 0.05 | 1.81 | 3 | > 0.60 |
| | 25.6 | 15.32* | 6 | < 0.025 | 7.62 | 5 | > 0.10 |
| Glycerol | 0.1 | 4.10 | 3 | > 0.20 | 2.03 | 2 | > 0.30 |
| | 0.2 | 3.55 | 3 | > 0.30 | 0.94 | 2 | > 0.60 |
| | 0.4 | 13.70** | 3 | < 0.005 | 10.03** | 2 | < 0.01 |
| | 0.8 | 8.40 | 4 | > 0.05 | 5.59 | 2 | > 0.05 |
| | 1.6 | 4.58 | 3 | > 0.20 | 1.02 | 2 | 0.60 |
| | 3.2 | 15.88** | 4 | < 0.005 | 7.21* | 2 | < 0.05 |
| | 6.5 | 1.01 | 3 | 0.80 | 1.33 | 2 | > 0.50 |
| | 9.6 | 0.48 | 3 | > 0.90 | 1.63 | 2 | > 0.40 |
| | 12.8 | 3.92 | 3 | > 0.20 | 0.90 | 2 | > 0.60 |
| | 16.0 | 8.97 | 4 | > 0.05 | 4.76 | 3 | > 0.10 |
| | 19.2 | 6.59 | 4 | > 0.10 | 1.92 | 2 | > 0.30 |
| | 22.4 | 8.84* | 3 | < 0.05 | 3.57 | 2 | > 0.10 |

*Notation:* $P$ = probability of compliance with the normal distribution
* = significant deviation from the normal distribution
** = highly significant deviation from the normal distribution
*** = very highly significant deviation from the normal distribution

*Fig. 4* shows the frequency distribution curves ($f$ = number of cells out of 120) of the original and logarithmically transformed $A/B$ data in various NaCl solutions, together with their probit curves (in order to avoid confusion, probit data have not been connected).

Nevertheless, it may be observed that even the distribution of original $A/B$ values is not as skew as that of the $V$ data. The arithmetic means of the $A/B$ series are quite close to the geometric means. This is shown in *Table IV*.

Thus, for the study of the effect of osmotic pressure on the ratio of cell diameters a comparison of arithmetic means seems to be sufficient, and logarithmic transformation of the data not necessary.

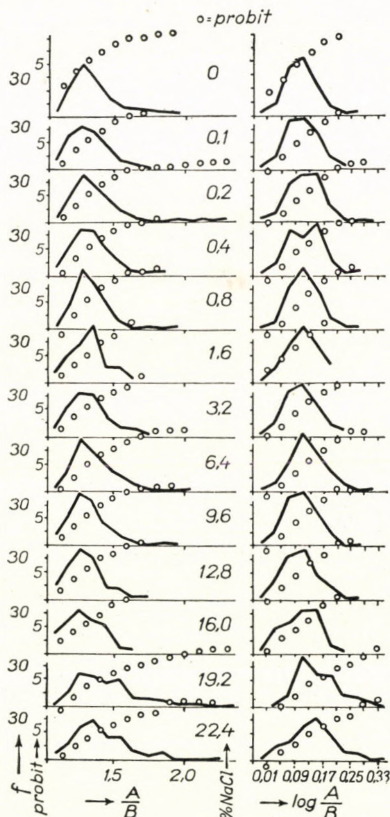


*Fig. 4.* Frequency distribution of the diameter ratios of yeast cells in NaCl solutions

Comparison of the means of $A/B$ values in various NaCl and glycerol solutions showed the ratio of diameters to be essentially independent of osmotic pressure. Considerable differences could only be observed in the last three NaCl concentrations. This, however, may be explained by the unclear photographs obtained in these solutions, a fact mentioned previously.

It may be stated, therefore, that the shrinkage of yeast cells in solutions of higher osmotic pressure occurred proportionately in all directions.

*Changes in the kinetics of growth.* — From the growth curves obtained in solutions containing various concentrations of NaCl and glycerol, the effect

**Table IV**

*The effect of NaCl and glycerol on the mean diameter ratio of yeast cells suspended in solutions of various strengths*

| Solutions corresponding to per cent NaCl (w/w) | Means of diameter ratios $\overline{(A/B)}$ | | | |
|---|---|---|---|---|
| | arithmetic | | geometric | |
| | NaCl | Glycerol | NaCl | Glycerol |
| 0 | 1.291 | | 1.283 | |
| 0.1 | 1.283 | 1.313 | 1.276 | 1.311 |
| 0.2 | 1.323 | 1.311 | 1.312 | 1.311 |
| 0.4 | 1.315 | 1.288 | 1.307 | 1.285 |
| 0.8 | 1.301 | 1.321 | 1.295 | 1.307 |
| 1.6 | 1.288 | 1.283 | 1.282 | 1.280 |
| 3.2 | 1.289 | 1.317 | 1.280 | 1.312 |
| 6.4 | 1.334 | 1.305 | 1.326 | 1.298 |
| 9.6 | 1.292 | 1.299 | 1.285 | 1.293 |
| 12.8 | 1.277 | 1.283 | 1.269 | 1.278 |
| 16.0 | 1.274 | 1.322 | 1.267 | 1.314 |
| 19.2* | 1.441 | 1.317 | 1.425 | 1.313 |
| 22.4* | 1.383 | 1.299 | 1.369 | 1.280 |
| 25.6* | 1.377 | — | 1.359 | — |

* unclear photogrammes.

on the lag phase (*L:* time, in hours, necessary to reach a cell count of $10^6$ per ml), on the growth rate constant ($k$) and on total growth ($G$) has been calculated. The results of two series of experiments (one with NaCl and one with glycerol) performed at two different dates, in quintuplet, are summarized in *Table V*.

It is evident that lengthening of the lag occurs at osmotic pressures corresponding to NaCl concentrations greater than about 5 per cent. It must be noted here that the differing (though moving in parallel) $L$ values in the two experimental series may be explained by the higher value of the inoculum suspended in the glycerol solutions.

The growth rate constant begins to decrease at about 5 per cent NaCl, while it shows a continuous decrease from the smallest concentration onwards in glycerol solutions.

Total growth decreased smoothly both in NaCl and in glycerol solutions.

## Discussion

*Comparison of changes in cell form and in the kinetics of growth.* — The results of all the above experiments are summarized in *Fig. 5*.

**Table V**

*The effect of NaCl and glycerol on the lag, rate of growth and total growth of yeast cells*

| Suspending medium | Solutions corresponding in per cent (w/w) NaCl | L (hours) | | k (hour$^{-1}$) | | G ($10^6$/ml) | |
|---|---|---|---|---|---|---|---|
| | | $M_L$ | $s_L$ | $M_k$ | $s_k$ | $M_G$ | $s_G$ |
| Sodium chloride | 0 | 6.0 | 0.82 | 0.42 | 0.051 | 24.5 | 0.52 |
| | 2.50 | 6.0 | 0.83 | 0.42 | 0.050 | 20.7 | 0.60 |
| | 3.00 | 6.0 | 0.85 | 0.42 | 0.054 | 20.4 | 0.30 |
| | 3.60 | 6.0 | 0.80 | 0.42 | 0.050 | 19.8 | 0.40 |
| | 4.32 | 6.0 | 0.22 | 0.37 | 0.009 | 17.6 | 0.25 |
| | 5.18 | 7.5 | 0.22 | 0.35 | 0.008 | 13.6 | 0.31 |
| | 6.22 | 9.5 | 1.19 | 0.35 | 0.023 | 12.1 | 0.49 |
| | 7.46 | 12.5 | 0.48 | 0.22 | 0.008 | 9.1 | 0.44 |
| | 8.96 | 19.5 | 0.50 | 0.13 | 0.026 | 6.5 | 0.22 |
| | 10.75 | 38.0 | 2.36 | 0.06 | 0.027 | 4.8 | 0.09 |
| Glycerol | 0 | 2.0 | 0.27 | 0.51 | 0.021 | 34.0 | 0.27 |
| | 0.85 | 2.0 | 0.22 | 0.43 | 0.025 | 29.0 | 0.66 |
| | 1.75 | 2.0 | 0.22 | 0.42 | 0.014 | 28.0 | 1.89 |
| | 3.55 | 3.0 | 0.27 | 0.37 | 0.006 | 25.0 | 0.65 |
| | 7.00 | 5.5 | 0.42 | 0.24 | 0.006 | 18.0 | 0.56 |
| | 13.35 | 145.0 | 11.0 | 0.03 | 0.005 | 3.0 | 0.30 |

*Note :* $M$ = mean value
$s$ = standard error

It can be seen that the geometric mean of cell volumes decreases quite abruptly up to concentrations corresponding to about 1 per cent NaCl. At higher concentrations a slower decrease ensues. In contradiction to this, $k$ and $G$ values showed a continuous decrease from the smallest concentration onwards ; $k$ values of the NaCl series behaved in an exceptional manner.

Comparison of the curves makes it probable that the stronger shrinkage of cells suspended in solutions of lower concentration has no decisive effect on life processes of cells (at least not in relation to growth). At higher concentrations, however, in spite of the slowing down of shrinkage, the retardation of physiological processes continues with unaltered intensity. Thus, increasing the removal of water beyond a certain critical level causes a relatively stronger inhibition of growth. In other words the relatively strong "initial" changes in degree of swelling experienced at lower osmotic pressures have no impressive effect on the building-up and budding processes of cells. Beyond a certain amount of de-swelling, however, further removal of water causes a relatively heavy decrease in the intensity of life processes. It seems probable that the

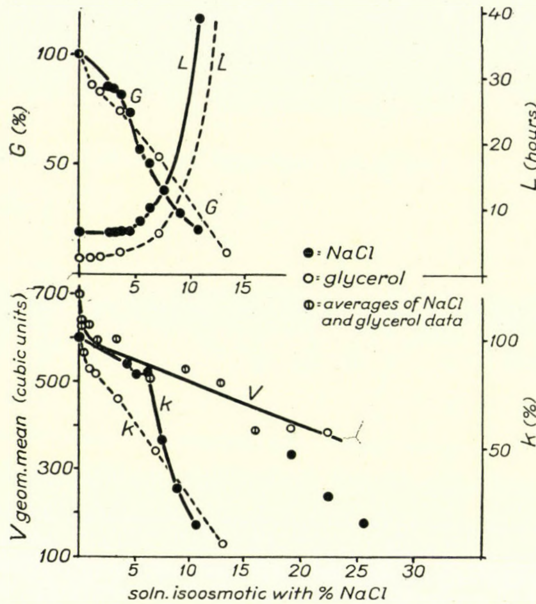


*Fig. 5.* Comparison of the shrinkage and the growth of yeast cells suspended in NaCl and glycerol solutions. *Note:* Owing to technical difficulties, the last value of *L* in the glycerol series (145 hrs. at 13.4 per cent ; *Table V)* is not shown

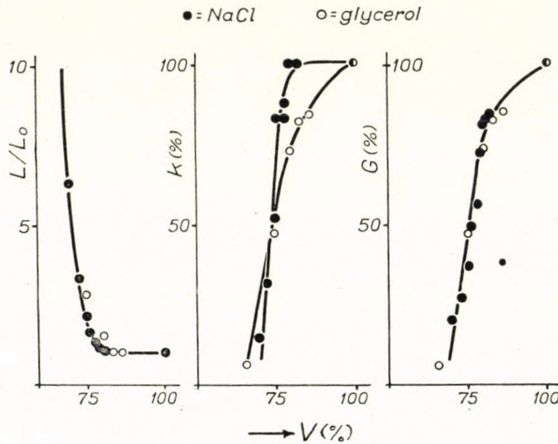


*Fig. 6.* Changes in lag, growth rate and total growth accompanying changes in volume of yeast cells in NaCl and glycerol solutions

above-mentioned critical shrinkage is accompanied by a stronger change, a decrease in the "state of water", in the "hydrature" [14] of the cell, and that this latter change is the direct cause of the retardation of growth.

This point is well illustrated by the correlation between shrinkage and growth lag. It may be seen in *Fig. 5* that while beyond an osmotic pressure

corresponding to about 2 per cent NaCl, the decrease in cell volume becomes a rather even, slow process, the length of the lag period undergoes an abrupt change at about 5 per cent NaCl.

All this is made more clear by *Fig. 6* where changes in $L$, $k$ and $G$ have been related to the geometric mean of cell volumes ($V$) interpolated from the line in *Fig. 5*. $V$, $k$ and $G$ are expressed as percentages of their respective values in the control solution (not containing NaCl or glycerol).

It is apparent that lag and total growth, as functions of cell volume, undergo identical changes in NaCl and in glycerol solutions : the values obtained in glycerol and in NaCl are situated along the same lines. Only the growth rate constant showed some deviation in the two solutions. It is evident from all three correlations that beyond a decrease in volume of about 15 per cent a considerable and fairly abrupt change in growth sets in. This seems to be the critical decrease in volume beyond which a more marked decrease in the hydrature of cells occurs.

The limit for growth of the yeast strain investigated was around 13 per cent NaCl. This corresponds to an osmotic pressure of 125 atm or 91 per cent, relative humidity at 18° C.

Computation, according to the method described elsewhere [13] of the concentrations necessary to attain 50 per cent inhibition of growth rate, resulted in the following values : 7.7 per cent of NaCl and a glycerol concentration isoosmotic with 6.7 per cent NaCl. These data correspond to osmotic pressures of 66 and 56 atm, respectively, and to equilibrium relative humidites of 95 and 95.6 per cent, respectively. This seems to be a further confirmation of the fact that NaCl and glycerol, while being chemically quite different compounds, exert their action on the growth of yeast in an identical manner, *i. e.* by lowering the hydrature of the medium. Thus, the specific chemical action of NaCl, often mentioned in the literature, could not be demonstrated here. On the contrary, it may be observed in *Figs. 5* and *6* that, in lower concentrations, NaCl exerted a somewhat weaker inhibition than did glycerol.

It is interesting to note that evaluation of the data of SCHACHINGER *and* HEISS [10] gives a similar value for a 50 per cent inhibition of fermentation of *Sacch. ellipsoideus* in the case of sucrose, glucose, fructose or invert sugar. This concentration corresponds to an equilibrium relative humidity of 95 per cent. Thus, comparison of the data of SCHACHINGER and HEISS with our own results seems to strengthen the view that growth *and* fermentation of wine yeasts are mainly functions of the hydrature of the medium, independently of the chemical nature of the plasmolytic substance.

## Summary

It has been shown that the frequency distribution of cell volumes is log-normal in character. This finding provides a tool for a more critical survey of the taxonomy of yeasts based on cell size and for the statistical evaluation of the cytorrhytic effect of various osmotic agents.

Comparison of the data on the kinetics of growth and on cell form and size showed that the two plasmolytic agents, NaCl and glycerol, so widely differing in chemical nature and material characteristics, exert a similar action on the cell : both act as substances simply enhancing osmotic pressure and not possessing specific effects (*cp.* salt effect). Increasing the concentration brings about a relatively strong initial change in the degree of swelling of the cytoplasm without apparent harm to its building-up and budding processes. Beyond a certain degree of de-swelling, further removal of water caused a severe reduction in the intensity of life processes. This critical decrease in cell volume may be accompanied by a stronger decrease in the hydrature of the cell and this latter may be the direct cause of the slowing-down of growth.


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

## II. PHAGOCYTOSIS OF ANTIBIOTIC SENSITIVE AND RESISTANT STAPHYLOCOCCUS AUREUS STRAINS

By

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(Received May 28, 1959)

In recent years, parallel with the increasing frequency of infections with *Staphylococcus aureus,* the diseases due to this organism have become more severe and respond less and less to antibiotic treatment. The present study has been undertaken to examine the relation of antibiotic sensitivity to phagocytosis of staphylococci. Considering the close connection between the virulence and phagocytosis of an organism, this aspect seemed to be suitable for determining whether the broadening of the resistance spectrum exerted any influence on the resistance of staphylococci against the non-specific immune response of the host. The phagocytic activity of rat leukocytes has been examined *in vitro* on antibiotic sensitive and polyresistant (*i. e.* resistant to several antibiotics) *Staphylococcus aureus* strains and a comparison has been made between the phagocytosis of resistant strains isolated from infections and the phagocytosis of a strain made resistant to antibiotics *in vitro.*

### Materials and methods

Pathogenic *Staphylococcus aureus* strains were isolated from various infections. All strains produced golden yellow pigment, haemolysin, gelatinase and coagulase and all of them fermented mannitol.

*Sensitivity to antibiotics* was tested by the paper disc method, as described in the first part of the present series of reports [14]. The strains employed were either sensitive to all of the tested antibiotics, or resistant to five or six of them (polyresistant strains). As a control, *Staphylococcus aureus* Duncan was used throughout the experiments.

*Phagocytosis* was examined by the method of Wright [16] and Ludány *and* Vajda [7]. Surviving leukocytes were obtained from the abdominal cavity of rats and suspended in Ringer's solution. To 0.6 ml leukocyte suspension 0.2 ml rat serum and 0.2 ml suspension of the organism under investigation were added. The final concentration of leucocytes in this system was 7000/$\mu$l. The suspensions of the *Staphylococcus aureus* strains tested were previously diluted to give equal bacterial counts ; within one experiment the addition of bacterial suspensions to the phagocytic systems was performed practically at the same time. Incubation lasted for 15 minutes at 37° C. The suspensions were then centrifuged at a low speed and the deposit was stained by Gram's method. The phagocytic index was calculated by counting the number of phagocytized cocci in 500 leucocytes. The limits of error of the method were $\pm$ 10 per cent.

*Treatment of staphylococci with various substances.* Sensitive and polyresistant *Staphylococcus aureus* strains were suspended in buffer solution and diluted to give equal bacterial counts. The suspensions were treated at pH 8.0 with 1 per cent trypsin for 90 minutes and with $1 \times 10^{-4}$ M $KJO_4$ for 15 minutes at pH 6.0 ; with 0.1 per cent sodium duodecyl benzenesulphonate for 15 minutes, with 0.1 per cent cetyl pyridinium bromide (Sterogenol) for 15 minutes

and with 0.01 per cent pancreatic lipase for 60 minutes. The last mentioned material was prepared as described by BOISSONAS [17]. The mixtures were incubated at 25° C and during incubation they were gently shaken. The suspensions were then centrifuged, washed twice in saline and finally resuspended in a buffer solution of pH 7.2. After checking the bacterial count, the organisms were tested for phagocytosis and the result was compared with that given by the untreated cells.

## Results

In *Table I* the phagocytosis of a sensitive and two polyresistant staphylococci isolated from patients and that of *Staphylococcus aureus* strain Duncan were compared. The results are expressed in phagocytic index.

**Table I**

*Phygocytic index of sensitive and polyresistant Staphylococcus aureus strains*

| | Strains | | | |
|---|---|---|---|---|
| | Duncan | 88 (sensitive) | 68 (polyresistant) | 1153 (polyresistant) |
| Experiment 1 | 0.45 | 0.27 | 0.24 | 0.18 |
| Experiment 2 | 0.87 | 0.47 | 0.30 | 0.22 |
| Experiment 3 | 1.12 | 0.65 | 0.22 | 0.42 |
| In relation to strain Duncan | 1 | 0.56 | 0.35 | 0.33 |

Calculated from the data of the three separate experiments, *Table I* presents the average of the phagocytic indices of the sensitive and the two polyresistant strains as related to the mean phagocytic index of strain Duncan. The results show that the polyresistant strains were phagocyted at a lower rate than the sensitive ones. Considering the limits of error and the differences in the individual strains, the experiments were repeated on a larger number of sensitive and polyresistant strains, as follows. The 18-hour broth cultures of 9 sensitive and 9 polyresistant strains were diluted with saline. After the determination of the number of bacteria in a Helber-Glynn chamber, the suspensions were diluted to give equal bacterial counts. Then by pipetting equal samples from each of the suspensions, the 9 sensitive cultures were mixed. Likewise, a similar mixture was prepared from the suspensions of the 9 polyresistant strains. The bacterial counts were again determined and provided that the results were within the limits of error, the suspensions were used for the experiments.

*Table II* shows the results of these experiments. Each of the four series represents the mean phagocytic index obtained in 4 separate examinations ; altogether 16 separate experiments were carried out both with the sensitive and the polyresistant suspensions. The phagocytic indices of the two kinds of

**Table II**

*Average of phagocytic indices of sensitive and polyresistant strains*

| | Series of experiments* | | | |
|---|---|---|---|---|
| | 1 | 2 | 3 | 4 |
| Sensitive strains .................. | 1.15 | 0.81 | 0.62 | 0.83 |
| Polyresistant strains ............. | 0.78 | 0.50 | 0.42 | 0.51 |
| Difference in per cent ............ | 32 | 37 | 32 | 38 |
| | Average : 34 per cent | | | |

* In each series the mean values obtained in 4 separate experiments are shown.

the suspensions were simultaneously determined in each series of experiments. It is seen that in every series the phagocytic index of polyresistant bacteria was lower than that of the sensitive ones ; the differences ranged from 32 to 38 per cent (34 per cent on the average). Accordingly, the polyresistant strains were phagocytized at a lower rate than the sensitive ones.

*Table III* compares the phagocytosis of a sensitive *Staphylococcus aureus* strain and that of its variant which had been made resistant to 200 $\mu$g/ml chloramphenicol by culturing the original strain in gradually increasing concentrations of the antibiotic. The results clearly show that the resistant variant was phagocyted at a higher rate than the sensitive strain (by 71 per cent on the average). Accordingly, in contrast with the organisms acquiring

**Table III**

*Phagocytosis of a sensitive Staphylococcus aureus strain and of its variant made resistant in vitro to 200 $\mu$g/ml chloramphenicol*

| | Sensitive | Resistant | Difference in per cent |
|---|---|---|---|
| Experiment 1 ................ | 0.69 | 1.10 | 57 |
| Experiment 2 ................ | 0.69 | 1.20 | 71 |
| Experiment 3 ................ | 0 66 | 1.18 | 78 |
| Experiment 4 ................ | 0.71 | 1.29 | 81 |

resistance *in vivo,* the variant made resistant to antibiotics *in vitro* was phagocyted more easily than the sensitive strains.

For studying the part played by the cell surface in phagocytosis, before performing the experiments the sensitive and resistant bacteria were treated with substances acting at different sites.

*Table IV* indicates the phagocytic indices of organisms which prior to the experiments had been exposed to 1 per cent trypsin for 90 minutes and

were then washed. This treatment did not considerably influence the phagocytosis of sensitive and polyresistant staphylococci. The original difference between the sensitive and polyresistant strains was, however, left unchanged.

**Table IV**

*Phagocytosis of Staphylococcus aureus strains treated with trypsin*

| | Phagocytic index | | | |
|---|---|---|---|---|
| | Sensitive strains | | Polyresistant strains | |
| | Treated with trypsin | Untreated | Treated with trypsin | Untreated |
| Experiment 1 | 0.90 | 0.89 | 0.62 | 0.65 |
| Experiment 2 | 1.00 | 1.05 | 0.79 | 0.84 |
| Experiment 3 | 1.54 | 1.24 | 0.69 | 0.75 |
| Experiment 4 | 1.17 | 1.04 | 0.54 | 0.63 |
| Experiment 5 | 1.01 | 1.02 | 0.64 | 0.63 |
| Mean values | 1.12 | 1.05 | 0.65 | 0.70 |
| Difference in per cent | +7 | | —5 | |

*Table V* shows the results of experiments carried out with organisms treated with $1 \times 10^{-4}$ M $KJO_4$ for 15 minutes. The rate of phagocytosis of the treated organisms was considerably higher than that of the untreated cells.

**Table V**

*Phagocytosis of Staphylococcus aureus strains treated with $KJO_4$*

| | Phagocytic index | | | |
|---|---|---|---|---|
| | Sensitive strains | | Polyresistant strains | |
| | Treated with $KJO_4$ | Untreated | Treated with $KJO_4$ | Untreated |
| Experiment 1 | 1.43 | 1.02 | 0.84 | 0.49 |
| Experiment 2 | 1.95 | 1.26 | 0.77 | 0.47 |
| Experiment 3 | 1.66 | 0.82 | 0.78 | 0.51 |
| Experiment 4 | 1.00 | 0.49 | 0.47 | 0.39 |
| Mean values | 1.51 | 0.89 | 0.71 | 0.46 |
| Difference in per cent | +69 | | +53 | |

The phagocytic index of the sensitive and resistant bacteria was 69, resp. 53 per cent higher than that obtained for the corresponding untreated organisms. This result points to the presence of surface components (polysaccharide

materials) attacked by $KJO_4$. As regards these components, there was, however, no essential difference between sensitive and resistant suspensions.

It is known that the electrophoretic mobility, *i. e.* the charge, of cells with surface lipids increases on exposure to anionic surface active agents [4]. In subsequent experiments the staphylococci were therefore treated with sodium duodecyl benzenesulphonate. The results are shown in *Table VI.*

**Table VI**

*Phagocytosis of Staphylococcus aureus strains treated with sodium duodecyl benzenesulphonate*

| | Phagocytic index | | | |
|---|---|---|---|---|
| | Sensitive strains | | Polyresistant strains | |
| | Treated | Untreated | Treated | Untreated |
| Experiment 1 | 2.29 | 2.29 | 2.73 | 1.43 |
| Experiment 2 | 2.25 | 1.91 | 2.56 | 1.06 |
| Experiment 3 | 2.27 | 2.88 | 3.18 | 1.63 |
| Experiment 4 | 2.73 | 3.27 | 2.21 | 1.73 |
| Mean values | 2.38 | 2.58 | 2.67 | 1.46 |
| Difference in per cent | —7 | | +82 | |

From *Table VI* it is evident that while the anionic surface active agent did not significantly affect the phagocytosis of sensitive staphylococci, it increased the rate of phagocytosis of polyresistant strains by nearly 100 per cent.

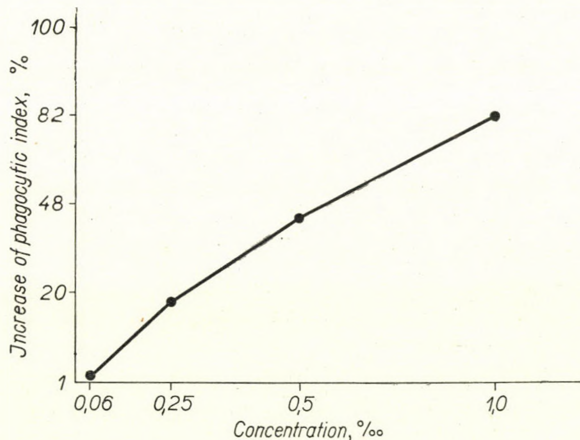


*Fig. 1.* The phagocytosis of polyresistant *Staphylococcus aureus* strains treated with various concentrations of sodium duodecyl benzenesulphonate

As shown by *Fig. 1,* the rate of phagocytosis of polyresistant strains increased in proportion with the concentration of the anionic surface active agent.

*Fig. 1* shows that bacteria treated with 0.006 per cent sodium duodecyl benzenesulphonate were phagocyted at the same rate as the controls. The rate of phagocytosis increased at a concentration of 0.025 per cent by 20 per cent, at a concentration of 0.05 per cent by 48 per cent, and at a concentration of 0.1 per cent by as much as 82 per cent. These experiments show that the surface of polyresistant cells contains a lipid material which, probably in linkage with the polysaccharides, renders the surface properties of polyresistant strains considerably different from those of sensitive cultures.

The treatment of the resistant cells with the cationic surface active substance, Sterogenol, did not significantly alter their phagocytosis. Neither was phagocytosis of sensitive strains influenced by Sterogenol. The *conditio sine qua non* for successful treatment of bacteria with these agents was the application of concentrations not interfering with the agglutination (or clumping) of the bacterial cells.

In subsequent experiments the cells were treated with pancreatic lipase in a buffer solution of pH 8.0, at room temperature. The phagocytic indices of the sensitive and resistant bacteria showed no significant differences as compared to those of the untreated ones. It was, however, striking that if the suspensions were treated with the anionic surface active substance after they had been exposed to lipase, the phagocytosis-promoting effect of the former agent was significantly decreased or completely blocked. This change did not occur on applying a lipase previously exposed to 60° C heat for 1/2 hour ; in this case the increase in phagocytosis of polyresistant strains was similar to that shown by strains unexposed to lipase *(Table VII)*.

**Table VII**

*Phagocytosis of lipase-treated Staphylococcus aureus strains after exposure to sodium duodecyl benzenesulphonate*

| Agent | Phagocytic index | |
|---|---|---|
| | Sensitive strains | Resistant strains |
| Lipase | Unchanged | Unchanged |
| Lipase + anionic agent | Unchanged | Unchanged |
| Inactivated lipase + anionic agent | Unchanged | Highly increased |

## Discussion

The finding that polyresistant *Staphylococcus aureus* strains are phagocyted at a lower rate than the sensitive ones may be explained by an alteration in the composition and physicochemical properties of the surface of the former bacteria. Similarly, it was shown by VÁCZI *et al.* [14] that the broadening of

the resistance spectrum of *Staphylococcus aureus* was rendering most of the strains non-typable by phages. This observation indicates a difference between the surface of sensitive and polyresistant strains. The cell surface is well-known to play a fundamental role in the properties and biological functions of micro-organisms as also in phagocytosis. According to FINDLAY *and* BRAUN [5], ALEXIEFF [1], BROWN *and* BROOM [2], MUDD *et al.* [10] and others, the degree of phagocytosis depends on the surface charge of the object to be ingested. MELCZER *and* KISS [8] explained the mechanism of reticuloendothelial storage by the electric potencial difference between the phagocyte and the corpuscle to be phagocyted.

To quote some pertaining data from the literature, according to SUSSMAN *and* LOWRY [18], the charged surface results in the formation of an ionic double layer around the cell. This circumstance may play an important part not only in phage adsorption but also in phagocytosis. A linkage of the bacteria to the cell surface by electrostatic forces may be regarded as a primary step in the latter process. DYAR *and* ORDAL [4] showed that on the surface of Staphylococci the proportion of a lipid material to an other, ionised surface factor was responsible for the quality and quantity of electric charge of the cell surface. They could prove by electrokinetic assays that Staphylococci producing under certain cultural conditions an increased quantity of lipid materials contained amphoteric substances, the charge of which — in contrast to cells poor in lipids — was reversed only at pH 3.0. The examinations of LERCHE [6] on the electrophoretic mobility of *Staphylococcus aureus* revealed that the minute quantities of lipid in the surface of Staphylococci were strongly negative.

The difference in the composition of cell surface undoubtedly affects the charge of the cell. The present observations, by establishing differences in the phagocytosis of sensitive and polyresistant strains, revealed a change in the surface composition and consequently in the surface charge of the latter ones. This change is clearly shown by the highly increased rate of phagocytosis of polyresistant strains treated with the anionic surface active agent. The surface lipids have an increased negative charge in solutions containing anionic surface active agents. The higher rate of phagocytosis shown by the polyresistant strains treated with sodium duodecyl benzenesulphonate may be explained by the solubility of the hydrocarbon chain in the surface lipids. This way the charge of the cells is affected by the polar groups of the agent and consequently their opsonization and phagocytosis will be promoted. The effect of the anionic surface active agent on the polyresistant strains shows that the surface of these organisms, in contrast to the sensitive ones, is abundant in lipids. VÁCZI *et al.* [13, 15] showed the importance of surface lipids, chiefly of the cephalin-type phosphatides, in the regulation of the permeability of the cell and in the development of resistance to antibiotics. The antiphagocytic activity of lipid

materials was emphasized by MIKATA *et al.* [9], who could prove the considerable protection afforded by the cephalin-type phosphatides : the intravenous inoculation of rabbits with *Streptococcus viridans* and these substances gave rise in most cases to experimental endocarditis, indicating that the cephalin-type phosphatides exerted a protective action on the injected bacteria, which otherwise would have easily been phagocyted. The close connection between the biochemical changes brought about in the cell by penicillin and the structure of the cell surface has been demonstrated in recent years by SALTON *and* SHAFA [12] and PARK *and* JOHNSON [11]. The connection between the antibiotic resistance and phagocytosis of Staphylococci may accordingly be explained by a change brought about by the antibiotics in the composition of the muco-complex material of the cell wall.

In the present investigations the difference between cells which had acquired antibiotic resistance *in vivo* and *in vitro* has been clearly shown by the fact that the two kinds of cells behaved quite opposedly as regards phagocytosis. Some data as to the important role of surface lipids in the biological properties and functions of the bacterial cell have also been revealed.

## Summary

The phagocytosis by rat leukocytes of antibiotic sensitive and polyresistant (resistant to several antibiotics) *Staphylococcus aureus* strains has been compared.

1. The rate of phagocytosis of polyresistant strains was 34 per cent lower than that of sensitive strains.

2. The rate of phagocytosis of strains which had been made resistant *in vitro* was 71 per cent higher than that of sensitive strains.

3. Treatment of staphylococci with trypsin did not considerably alter their phagocytosis.

4. Exposure of both sensitive and resistant staphylococci to potassium periodate considerably increased their phagocytosis (by 69 and 53 per cent).

5. The rate of phagocytosis of resistant *Staphylococcus aureus* strains showed a considerable (82 per cent) increase if the organisms had previously been treated with an anionic surface active agent (sodium duodecyl benzenesulphonate). The same treatment did not affect the phagocytosis of sensitive cultures.

6. The phagocytosis-promoting effect of sodium duodecyl benzenesulphonate on resistant organism was proportional to the concentration of the agent.

7. No change was observed in the phagocytosis of either sensitive or resistant strains after they had been treated with a cationic surface active agent.

8. The anionic surface active agent failed to promote the phagocytosis of resistant cells previously treated with pancreatic lipase. No such effect was exerted by inactivated lipase.

9. The examinations have furnished new proof of the role of various constituents of the cell surface, chiefly of lipids, in the biological properties and functions of the cell (phagocytosis, antibiotic resistance).

*Acknowledgement:* The authors are indebted to Dr. I. SZEGŐ, *Laboratory for Dairy Research, Budapest*, for a supply of sodium duodecyl benzenesulphonate.

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# RELATIONS BETWEEN IMMUNE SUBSTANCES AND PROTEIN FRACTIONS

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(Received June 10, 1959)

In the study of protein fractions and antibodies, preparative electrophoresis is assuming an increasing importance. Various authors have sought different lines of approach in their endeavours to elucidate the question and have suited their methods to materials of varying activity.

Tiselius *and* Kabat [1] drew their conclusions from electrophoretic analyses performed prior to and following specific absorption of serum. By this method they localized egg-white precipitins to gamma-globulin. Peter *et al.* [2] studied rabbit immune serum in vertical cellulose columns with Bockmüller's electrophoretic apparatus. Stelos [3] analysed haemolysins by the starchblock method. Scheiffarth *et al.* [4] reported on the simple procedure of agglutination electrophoresis performed on the pherograms. Gedin *and* Porath [5] separated antibodies by two-dimension paper electrophoresis with continuous migration. For his antigen and antibody investigations, Backhausz [18] elaborated a new method of agar electrophoresis which is based on the principle of immuno-electrophoresis but leads to more accurate findings.

Preparative electrophoresis has brought a remarkable advance, particularly in research work concerned with the correlations of immune substances and protein fractions, because not only isolation of purified immune substances but also elimination of inert components became feasible. Thus it becomes possible to determine the grade of purity of a protein. The immuno-electrophoretic studies of Grabar [6] and Schultze [7] have greatly promoted our knowledge as regards determination of purity in proteins and the question of fractions that are found pure when judged by antigenity. Besides the serologically pure fractions, information concerning the correlation between active substances and protein fractions is of great importance.

For the study of immune substances from these aspects we have developed a new method [8], by combining paper electrophoresis with agar diffusion. Bactericidal substances [9], antistreptolysins [10], rabbit and dog haemolysins [11] have been studied by this method.

The immune substances under investigation were localized chiefly to gamma-globulins, in a lesser degree between gamma- and beta-globulins. In the literature numerous data refer to the antibody-supporting protein fractions ranging between beta- and gamma-globulins ; these researches resulted in the differentiation of gamma-1 or beta-2 and T-fractions, and in recognition of their significance as regards immune substances [12—15].

In some experiments distribution to several fractions has been observed, which permits the inference that the distribution of immune substances does not always reflect the true connection. In the case of haemolysins, the work of PETER [2] and STELOS [3] — to be discussed in detail later — refers to this question, but in conjunction with other immune substances, too, one finds such data, whose enumeration has to be omitted. Presumably, the cause of such distributions resides in the methods employed.

These considerations have induced us to repeat our own experiments in order to identify the origin of spread of the antibody to several fractions. If we suppose that in the investigated cases the immune substances, on grounds of specificity, were bound to gamma-globulins alone, their exact relationship can be approached only by studying the factors of the methods. Among these, the amount of the applied serum appeared important. By means of our method we therefore investigated the distribution obtained at electrophoresis of varying quantities of serum and repeated elution of the same strip of paper.

## Materials and methods

Electrophoresis was performed with the method of GRASSMANN *and* HANNIG [16] and the pherograms were evaluated with Eppendorff's photometer. Localization of immune substances was carried out by cutting up of the pherograms and subsequent diffusion in agar containing the antigen.

As serum immune rabbit serum lysing sheep erythrocytes and high titre (over 1 : 720) human antistreptolysin serum were used. Haemolysis observed in the agar, respectively its inhibition in the case of antistreptolysin, were recorded and the effect was computed from the area and the degree of reaction. The details of the method have been described in an earlier report [8].

To study the correlation between the amount of serum and the distribution of haemolysins, 40, 60, and 80 cu. mm amounts of the serum to be investigated were dropped on paper strips. After halving, 20, 30, and 40 cu. mm amounts of the serum, resp. its separate parts were examined as to haemolysis. With antistreptolysins, 20, 40, or 60 cu. mm quantities were used, of which the half, 10, 20, and 30 cu. mm, served for the determination of immune substances. The haemolysins were eluted with complement diluted in MAYER's buffer [17] at a ratio of 1 : 5, the antistreptolysins with streptolysin-0 reagent. In the Figures the effect is demonstrated by columns drawn at the appropriate place of the fractions. The presented Figures illustrate single experiments selected from among several assays producing identical results. The measure of migration displayed slight differences in the single pherograms ; this might have been due to variations in the migration rate and to the superimposed position of one paper strip over the other. Hence maximum albumin migration seen in the Figures was from 9,5 to 14 cm.

## Results

As shown in *Fig. 1,* the distribution of haemolysin was influenced by the amount of the applied serum. In the case of 40 cu.mm serum, the immune

substances were localized to the gamma-globulin area. Their course ran parallel with that of gamma-globulin. A minute part of the antibodies had reached the area between the beta- and gamma-globulins ; the fractions gamma-1 and beta-2-globulin could not be regarded as typical immune substance supporting fractions *(Fig. 1a)*. When the quantity of the serum was increased, the effect of the immune substance extended to beta-globulin. Upon electrophoresis of 60 cu.mm serum, haemolysis appeared in part of the beta-globulins *(Fig. 1b)*. On a further increase in the amount, lysis was encountered even within the alfa-globulin area *(Fig. 1c)*. In the two latter cases, single fractions

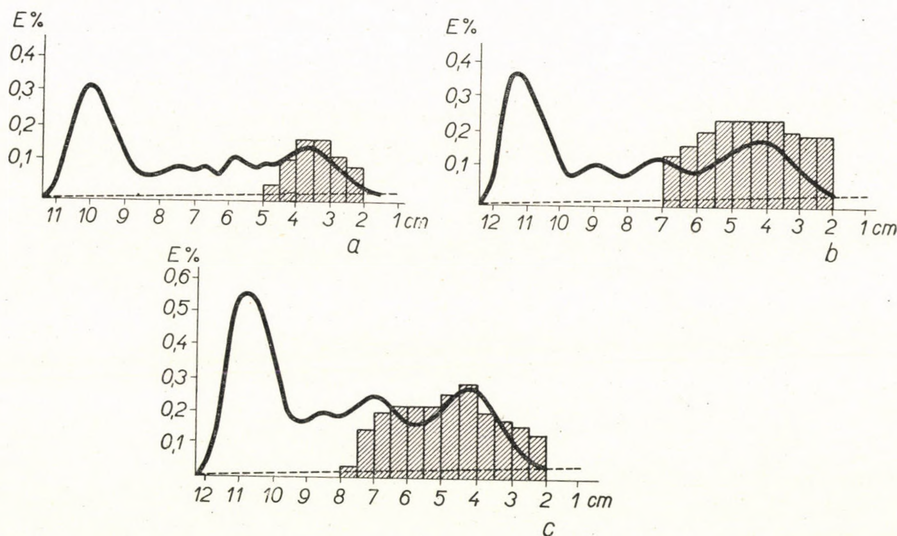


*Fig. 1*

did not clearly separate. With amounts of 80 cu.mm there was extensive haemolysis, but the fractions did not sharply separate.

According to the above, the spread of haemolysins could thus be extended to the faster migrating beta- and alfa-globulins by increasing the quantity of serum.

Next, it appeared necessary to undertake similar experiments with antistreptolysins. In these, the effect was expressed as complete or partial inhibition. In *Fig. 2*, partial inhibition is indicated by blank columns, complete inhibition by shaded columns. Whereas at a low protein concentration, with 20 cu.mm, antistreptolytic activity was localized to gamma-globulin *(Fig. 2a)*, in the case of 40 cu.mm it spread to the start of beta-globulin. On a further increase in the quantity, to 60 cu.mm, it even attained the maximum of beta-globulin *(Fig. 2c)*. These investigations clearly demonstrated the spread of antistreptolysins and haemolysin, as well as the extension of immune substances to the faster components on increasing the amount of serum.

Subsequently, such experiments were performed with haemolysins that the first elution was followed by a second one, by placing the strips on a second blood agar. On the hypothesis that the measure of dissolution depends on the antibody content, we intended to determine in this way the antibody maximum and to examine at the same time the feasibility of elution, as well as to determine the ratio of the antibodies left behind after one elution. For adequate

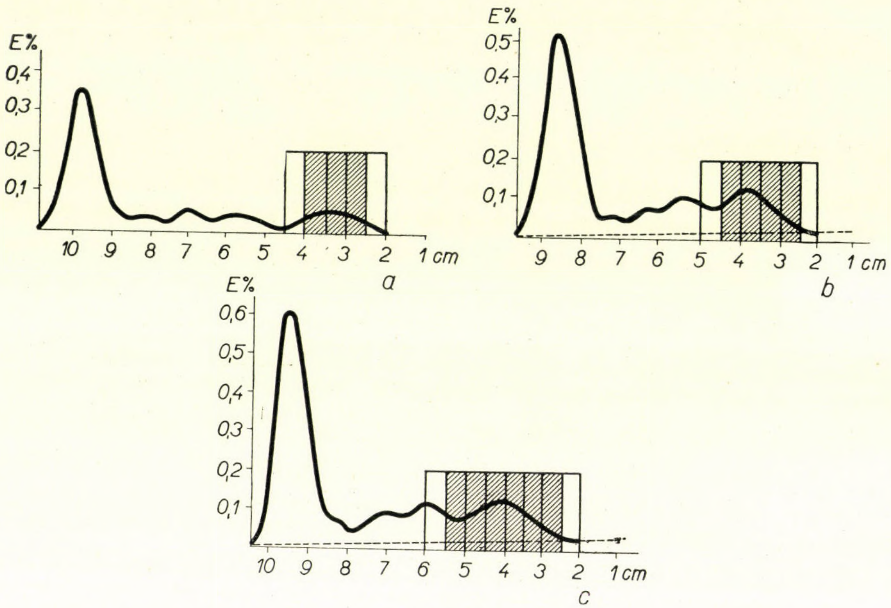


*Fig. 2*

evaluation, the size and degree of haemolytic areas seen in the agar were recorded, in order to gain correct data on antibody distribution.

These experiments are presented in *Fig. 3*. Low-titre haemolytic serum was run for one curve, high-titre haemolytic serum for the other. As revealed by *Fig. 3*, with the low-titre serum (1 : 1000) haemolysis was the most marked in the gamma-globulin area, as regards both the degree of lysis and its intensity *(Fig. 3a)*. The effect is reflected in the size and lining of the columns ; the black ones express complete lysis, the shaded ones medium, and the blank ones, slight lysis. In part „*a*" of *Fig. 3*, the maximum effect corresponds to the gamma-globulin peak, and weaker lysis appears in the area between the gamma and beta fractions (lower row of columns). However, when a strip was subjected to a second elution, the effect was not so pronounced as with the first elution, but its peak still corresponded to the maximum of gamma-globulin and followed this curve almost completely (upper row of columns).

Part "*b*" of *Fig. 3* presents the results of experiments performed in a similar manner with high-titre (1 : 10 000) rabbit serum. In extent and intensity alike, the degree of haemolysis showed two maxima, one of which corresponded to gamma-globulin, while the other fell between the gamma- and beta-globulins, to the faster migrating gamma-globulin parts. Lysis was marked not only in the gamma-1 fraction, but also in beta-globulin (lower row of columns). After repeated elution of the paper strips (upper row of columns), the maximum was seen in gamma-globulin, appearing parallel to its curve.

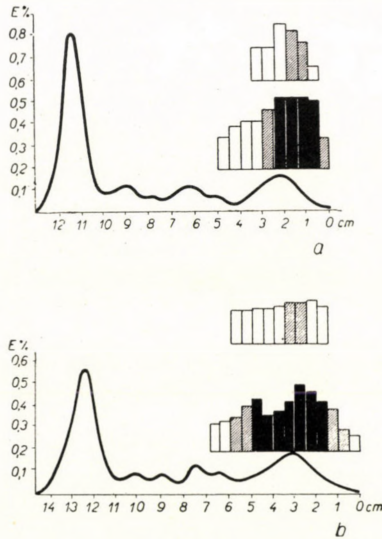


*Fig. 3*

The two series of experiments permit the inference that in pherograms the greatest amounts of immune substances occur in the gamma-globulin area. In case of repeated elution, the effect is localized to this fraction alone. From a comparison of the degree of lysis and the quantity of immune substances, the conclusion may be drawn that the said area would seem to be the site of their maximum concentration, and that with the use of appropriate amounts of serum they are localized exactly to the gamma-globulin fraction.

## Discussion

In the linkage of immune substances to protein fractions, varying connections have been found. Concerning haemolysins, the studies of PETER *et al.* [2] and STELOS [3] fully agree with our own investigations [11] in that gamma-globulin is the chief carrying protein of these antibodies. There is, however, disagreement as regards the role of faster migrating gamma-globulins. In

addition to the results obtained by PETER and ourselves [12], STELOS' [3] findings indicate that haemolysins may occur even in alfa-2-globulins. Such a distribution has been reported to occur also with other immune substances. For instance, BACKHAUSZ [18] in the course of investigations concerned with the separation of antitoxic gamma-globulin, succeeded in demonstrating antitoxic activity even in the areas of albumin migration and observed a permanently increasing antibody effect with a peak coinciding with the maximum of gamma-globulins.

In our earlier studies dealing with antistreptolysins [10] we also observed immune bodies to be present in the slow migrating beta-globulin, beside the faster gamma-globulin. The above experiments, however, have induced us to adopt the view that the effect of faster migrating components may also be attributed to gamma-globulin. It is particularly striking that upon decreasing the amount of serum, immune activity coincides with the peak of gamma-globulin, while an increase of the applied amount of serum produces a gradual spread of the immune substance to components with faster mobility. This has been observed to occur with both haemolysins and antistreptolysins.

The effect of the quantity of serum on the distribution of immune substances has been observed by TISELIUS *and* KABAT [1] who found the antibodies contained in dilute porcine immune serum exclusively in gamma-globulin. On the strength of our own experiments we must share the opinion of SEHON, HARTER *and* ROSE [19] who claim that, as a fractionation method, free electrophoresis can be used only in the presence of very small quantities of globulins. In paper electrophoresis these factors may play an important role, as evidenced by the above experiments ; in accordance with the quoted statements and our own experiments, here, too, only small amounts of proteins are amenable to the study of relations. The wider distribution observed with the use of larger amounts of sera have to be interpreted on this basis. STELOS [3] doubtlessly found the haemolysin distribution spreading to the alfa-globulins on electrophoresis of large quantities of serum in starch block. In the same sense should be interpreted the findings of BACKHAUSZ [18], who made agar electrophoresis with large amounts of serum and noted a wide-spread of immune substances.

However, if in the experiments of STELOS [3] the Forssmann lysins had been absorbed, the remaining isolysins were always found in close connection with gamma-globulin. Considering the small amount of isolysins, it is clear that for the observed localization the low concentration was responsible. This again confirms the view that experiments for determining localization should be carried out with small quantities.

The wide-spread observed by PETER [2] and later by STELOS *and* TALMAGE [20] in their haemolysin experiments may be explained on the same basis, and the same applies to our own earlier experiments and the above-mentioned observations made with increased quantities of serum.

It should be remembered that by certain methods gamma-globulins can be separated into further fractions, as shown by MICHELAZZI [21]. At varying pH values and ionic strength this author was able to fractionate haemolytic rabbit gamma-globulin into three or four subfractions, and the specific antibodies were found in the faster migrating part. This, however, applies to the fractions of a previously precipitated protein which had undergone some change as compared to the native fractions. Therefore, this observation cannot be discussed in the same light as the former one, because in some of their properties such fractions may differ from the native serum, as has been demonstrated by HEIDELBERGER, KRUEGER *and* DEUTSCH [22], KUHNS [23], and SCHULTZE *et al.* [14].

Summing up, the experiments have supported our hypothesis that in studies on the localization of immune substances correct results can be ensured only by working with small amounts of serum and at low protein concentrations. This was shown also by our experiments in which only 5 cu.mm of serum was run on the paper strip. Activity could then be observed only in a small, narrow part of the fraction corresponding exactly to the gamma-globulin peak ; owing to the low protein concentration, however, densitometric reading was not feasible [24].

## Summary

As regards the relations between immune substances and protein fractions, there is a certain disagreement between the data of various authors. The cause of these deviations has been sought for in the amount of serum used. The present investigations have revealed that, with both haemolysins and antistreptolysins, the distribution of immune substances is influenced by the quantity of serum examined, in dependence on the protein content. In the presence of large quantities of serum, immune substance activity spreads to the faster migrating components. The immune substances which after application of small quantities had reached only the gamma-globulin, gradually spread to beta- and alfa-globulins if the quantity of serum had been increased. The same phenomenon was elicited by subjecting an eluted paper strip to a second elution ; since lysis depends on protein concentration, localization was more accurate after the second elution. In these experiments the originally extensive spread of immune substance was narrowed to gamma-globulin, and maximum activity coincide with the gamma-globulin's peak. On running minute amounts of serum, immune bodies were detected only at the maximum of gamma-globulin.

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# COMBINED FERMENTATION

## I. TRICHOTHECIUM–PENICILLIUM FERMENTATION*

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(Received June 13, 1959)

The yield of antibiotics produced by microorganisms can generally be increased in two different ways, on the one hand, by employing mutagenic agents which bring about mutants producing a constantly increased amount of antibiotic and, on the other, by introducing favourable changes in the composition and the pH of the medium, or altering the technology of the fermentation. In the present experiments an increased production of trichothecin [1], an antibiotic formed by *Trichothecium roseum* was obtained by a method differing from the above ones. Our work was based on the following observation. The Trichothecium strain was streaked on agar plates containing trichothecin. Discrete colonies were transferred to agar slants and subsequently into shake flasks, in which finally the quantity of the produced trichothecin was assayed. In one series of shake cultures nine strains produced 0—210 μg/ml trichothecin, while one culture (strain Tr III. 189/c 27) yielded 704 μg/ml. At first it was thought that an extremely good antibiotic producer variant was isolated on the trichothecin containing plates. On the next day, however, it was found that this culture had been contaminated with a Penicillium strain. From this culture both the inoculated Trichothecium and the contaminant Penicillium strains were isolated. The latter was designated as $P_2$. As earlier observations suggested the possibility of the association of the contaminant with the highly increased trichothecin yield, the investigations to be described have been performed.

### Materials and methods

*Tr. roseum* strain Tr III. 189/c was obtained from the series of strains used by VÖRÖS *and* SZABÓ [2]. The Penicillium strain isolated from the contaminated culture belonged to the Asymmetrica—velutina group (deposited in the stock culture collection of the *State Institute of Hygiene, Budapest,* as *Penicillium sp.* "a—13"), termed in this laboratory $P_2$.

*Medium for the inoculum and fermentation cultures.* Throughout the experiments medium "Tr VIII" was used. It contained 0.2 per cent ammonium tartrate, 1.0 per cent sucrose, 0.025 per cent baker's yeast extract solids, 0.1 per cent corn steep solids and 5.0 per cent malt extract,

* Presented partly at the Meeting of the *Association of Hungarian Microbiologists,* November 10, 1958, Budapest.

at pH 5.2. Yeast extract was prepared by suspending 1.0 kg baker's yeast in 2.0 l tap water, autoclaving the suspension at 120° C for 30 minutes and leaving it to stand for at least two days. The clear supernatant containing 3.3—4.2 per cent solids and 2.1—2.2 mg/ml total nitrogen was used.

The medium was dispensed in 100 ml portions in 500 ml Erlenmeyer flasks. Fermentation was performed at 26° C on a modified flour mill plansifter used as a rotary shaker making a circle 30 mm in diameter and operating at 320 cycles per minute.

*Inoculation of the shake cultures* was performed with a vegetative culture of Trichothecium. This one was started from well-sporing agar slant cultures : to each of a number of such cultures 5 ml saline and glass beads were added and the tubes were shaken. In preparing the inoculum culture a certain amount (usually 1.0 ml) of the spore suspension was added to a flask containing 100 ml of medium "Tr VIII". In some experiments the number of spores was counted in a Bürker chamber and the shake flasks were inoculated with a known number of spores. The spore suspension of the Penicillium strain was similarly prepared, except that in preparing it, the spores from each agar slant culture were suspended in 8.0 ml of 1 : 8000 diluted sodium lauryl sulphonate. These suspensions of Trichothecium and Penicillium strains contained $1.0—2.8 \times 10^6$ and $200—800 \times 10^6$ spores per ml, respectively.

In the inoculum culture (first generation) the elementary colonies of Trichothecium easily formed a deposit ; their average diameter was 0.4 mm. This culture was mostly used in 2.0 ml amounts for seeding the shake cultures (second generation). The latter cultures were agitated for 24 hours and then superinoculated with a 24 hour vegetative Penicillium culture. When Penicillium spores were used as a superinoculum, the shake flasks were seeded simultaneously with the vegetative inoculum of Trichothecium (first generation) and with the spore suspension of Penicillium. Samples were taken as a routine at the 64th, 88th, 112th, 136th and 160th hour of cultivation. The samples were examined for pH and trichothecin production. Every experiment was performed in three parallel flasks. The peak yield of antibiotic produced in each flask throughout the incubation was noted, and the values obtained in three flasks were averaged (average of peak yields).

*Examination of the pH of the cultures* was carried out with Merck's "Universal" indicator paper. The pH of certain cultures was checked with an electric pH meter.

*Assay of trichothecin production* was performed by VÖRÖS' method [3] at the *Research Institute for Plant Protection, Budapest.*

*Fermentation experiments* were made in 5 l JOHAN—LOVREKOVICH glass fermentors. The fermentors were inoculated with shake cultures (first generation), which had been prepared as described above. The fermentors had paddle-type stirrers operating at 500 r. p. m. The cultures were aerated with 1 l air per 1 l of medium per min and the evaporated water was replaced from time to time. From the cultures two samples were taken daily.

*Paperchromatographic identification of trichothecin* was performed by DR. A. SZENTIRMAI, *Institute for Pharmacoindustrial Research, Budapest.* In the experiments, Schleicher and Schüll's 2043b paper was used. In part of the examinations the paper was impregnated with crude rubber dissolved in benzene and as a solvent absolute methanol was applied. Alternatively, for other chromatograms the paper was impregnated with silicic acid and petroleum ether served as a solvent. For identification the biological method using *Saccharomyces carlsbergensis* was applied, as recommended by DR. J. VÖRÖS.

## Experimental

(i) The Trichothecium strain which produced a high quantity (704 μg/ml) of antibiotic when contaminated with Penicillium, was examined first in pure culture. It was shown that the strain *per se* yielded only 155 μg/ml of trichothecin.

(ii) The range of scattering of the trichothecin produced was studied by collecting the averages of peak yield obtained in shake flasks within six months by the strain used in our experimental work. These data divided into four groups according to the amount of trichothecin produced are shown in *Table I.*

**Table I**

*Average of peak values of trichothecin obtained in triplicate shake flasks*

| μg/ml trichothecin produced (groups according to order of magnitude) | | | |
|---|---|---|---|
| 0—100 | 100—200 | 200—300 | 300—400 |
| 36* | 160 | 203 | 303 |
| 93 | 181 | 205 | 303 |
| | 185 | 207 | 310 |
| | | 210 | 316 |
| | | 223 | 323 |
| | | 228 | 400 |
| | | 250 | |
| | | 251 | |
| | | 271 | |
| | | 290 | |
| | | 296 | |

* Inoculated from a poorly sporing culture.

Accordingly, in half of the shake flasks (triplicate) the trichothecin yield was between 200 and 300 μg/ml. Only 6 of the cultures yielded more than 300 μg/ml (5 produced 303—323 μg/ml and one as much as 400 μg/ml).

The highest concentrations of the trichothecin produced in 5 l fermentors were 95, 130, 150, 210, 250, 260, 280 and 308 μg/ml.

As is usual in shake cultures and small fermentors, the results of experiments carried out at different times varied considerably. The antibiotic yields in cultures seeded with the same inoculum at the same time into the same batch of medium were, however, fairly close to each other. *E. g.* in two parallel experiments the highest yields in triplicate shake flasks averaged 296 and 250, while in fermentors 260 and 250 μg/ml, respectively.

(iii) As it was clear that *per se* our Trichothecium strain was unable to produce higher amounts of trichothecin in the medium used, in subsequent experiments a reproduction of the results obtained in the culture contaminated with Penicillium was attempted. Therefore, an inoculum (first generation) was prepared from the contents of the flask which, as mentioned in the introductory part of this report, had been contaminated with a Penicillium strain. Seeding three shake flasks with this contaminated inoculum, the trichothecin produced amounted to 405, 575 and 750 μg/ml. In parallel experiments with the same, but pure, culture of Trichothecium the peak values were 155, 155 and 155 μg/ml. Thus the observation could be reproduced that a Trichothecium culture when contaminated with Penicillium yielded a much higher

amount of trichothecin than the pure culture. The fermentation performed with the simultaneous cultivation of two different microorganisms was called combined fermentation.

In subsequent experiments the circumstances influencing the stimulation of antibiotic production in combined fermentations were investigated.

(iv) The quantitative relation between the two strains was examined as follows. Shake flasks (second generation) were seeded with equal amounts of a 24 hour vegetative Trichothecium inoculum. After shaking for 24 hours these cultures were superinoculated with a 24 hour vegetative Penicillium culture. The Penicillium culture was applied in various amounts ranging from 1 to 10 or sometimes from 20 to 30 per cent of the Trichothecium culture (second generation). Using the same medium, shake flasks with pure cultures of Trichothecium and Penicillium, respectively, were prepared as controls. When the Penicillium inoculum was increased within certain limits, the amount of antibiotic produced was 2 or 3, occasionally 5 times more than the yields in pure cultures. On inoculating still higher quantities of Penicillium, trichothecin was no more produced. In these cases the production of trichothecin often did not cease in all but only in one or two of the three parallel flasks.

The amount of Penicillium inoculum causing trichothecin production to cease in at least one out of three parallel cultures was called the critical quantity (or critical point). In this particular culture usually a small amount of antibiotic was detectable, which did not exceed the corresponding effect of 0.1—0.5 I. U./ml. penicillin. The critical quantity may vary with different strains, or even with the same strain in different runs *(Table II)*.

**Table II**

*The effect of various quantities of Penicillium inoculum on the trichothecin production by Trichothecium in shake cultures*

| No. of experiment | Penicillium inoculum as percentage of second generation Trichothecium | | | | | |
|---|---|---|---|---|---|---|
| | 0 | 1 | 2 | 10 | 20 | 30 |
| | Maximum production of trichothecin, $\mu$g/ml | | | | | |
| XXVII | 190 | 850 | . | 850 | 760 | 0 |
| | 190 | 850 | . | 1140 | 0 | 0 |
| | 235 | 475 | . | 0 | 0 | 700 |
| XXXIII | 300 | 210 | . | 0 | . | . |
| | 270 | 300 | . | 950 | . | . |
| | 300 | 300 | . | 1140 | . | . |
| XXXII | 87 | . | 115 | 0 | . | . |
| | 96 | . | 0 | 0 | . | . |
| | 96 | . | 550 | 0 | . | . |

(v) For obtaining the highest trichothecin production, the most suitable time for Penicillium superinoculation had to be determined. A 24-hour vegetative Penicillium shake culture was used for superinoculation. *Table III* presents the trichothecin production by Trichothecium cultures superinoculated with Penicillium at various times of incubation.

**Table III**

*Superinoculation of Trichothecium shake cultures at various times of incubation with various amounts of Penicillium inoculum*

| No. of experiment | Age of Trichothecium culture at time of superinoculation, hours | Percentage of Penicillium inoculum | | | | |
|---|---|---|---|---|---|---|
| | | 0 | 1 | 2 | 5 | 10 |
| | | Peak yield of trichothecin, $\mu g/ml$ | | | | |
| XXVII | 24 | 190 | 475 | 760 | 700 | 850 |
| | | 190 | 850 | 0 | 0 | 1140 |
| | | 235 | 850 | 0 | 0 | 0 |
| | 44 | 190 | 260 | 405 | 570 | 290 |
| | | 190 | 290 | 435 | 570 | 320 |
| | | 235 | 300 | 470 | 630 | 475 |
| XXIX | 22 | 150 | 270 | 350 | . | 400 |
| | | 165 | 310 | 380 | . | 700 |
| | | 165 | 630 | 450 | . | 750 |
| | 74 | 150 | 160 | . | . | 150 |
| | | 165 | 160 | . | . | 150 |
| | | 165 | 165 | . | . | 160 |

According to the experiments shown in *Table III*, the Penicillium inoculum employed at the 74th hour of cultivation of Trichothecium did not increase the trichothecin yield. Production increased when the Penicillium was inoculated after 44 hours of incubation. The highest rise, however, was observed when the vegetative Penicillium inoculum was employed at 22—24 hours ; in these cases sometimes even small inocula acted as critical quantities.

(vi) Considering the marked scattering in the results, it was attempted to make the experiments more reproducible by determining the Trichothecium—Penicillium ratio by means of spore counts instead of the relative volumes of vegetative mycelium suspensions. Two inoculum shake flasks (first generation) were seeded to contain at the start 25 000, resp. 2500 Trichothecium spores per ml. After 24 hours of incubation, shake flasks containing 100 ml medium were seeded (second generation) with 2 ml quantities of these inoculum cultures (*i. e.* with cultures started from 50 000, resp. 5000 spores). Thus the 1.0 ml culture of the second generation was grown from 500, resp. 50 spores per ml.

These shake cultures (second generation) were inoculated simultaneously with the Trichothecium inoculum with varied numbers of Penicillium spores. *Table IV* shows the trichothecin production by such cultures. Trichothecium

**Table IV**

*Trichothecin production by combined Trichothecium-Penicillium shake cultures started from various numbers of spores*

| Trichothecium 2nd generation. No. of spores per ml at start | Penicillium, No. of spores per ml superinoculated | Ratio of Trichothecium and Penicillium spores | Trichothecin $\mu g/ml$ (average of 3 flasks) |
|---|---|---|---|
| 500 | 0 | . | 296 |
| 500 | 14 000 | 1 : 28 | 300 |
| 500 | 140 000 | 1 : 280 | 503 |
| 50 | 0 | . | 250 |
| 50 | 1 400 | 1 : 28 | 340 |
| 50 | 14 000 | 1 : 280 | 463 |
| 50 | 140 000 | 1 : 2800 | (0, 0, 600) |

*per se* produced 296, resp. 250 $\mu g/ml$ trichothecin. To raise the production to 503 $\mu g/ml$, the Trichothecium culture started from 500 spores had to be superinoculated with 140 000 Penicillium spores. Alternatively, the culture started from 50 Trichothecium spores produced a similar quantity of antibiotic (463 $\mu g/ml$), after it had been inoculated with 14 000 Penicillium spores. Maximum antibiotic production was thus obtained only when the number of the two kinds of spores corresponded to a certain proportion. The inoculation with 14 000 Penicillium spores of Trichothecium cultures started from 500 spores was perfectly ineffective. Likewise, the production by cultures started from 50 Trichothecium spores was hardly influenced by the inoculation of 1400 Penicillium spores. Despite the use of triplicate cultures, deviations ranging from 10 to 20 per cent may, of course, occur in such biological experiments, particularly with the small volumes used in shake cultures.

(vii) As shown by *Table V*, the results obtained in 5 l fermentors were more close to each other. It is seen that the fermentation of pure Trichothecium cultures started from 500, resp., 50 spores per ml yielded 260, resp., 250 $\mu g/ml$ trichothecin. To raise production as high as 420 $\mu g/ml$, the Trichothecium cultures started from 500 spores per ml needed to be superinoculated with 140 000 Penicillium spores per ml. Proportionally, each ml of the culture started from 50 spores needed the addition of 14 000 Penicillium spores to produce the antibiotic in the same amount. When a culture started from 50 Trichothecium spores was superinoculated with Penicillium to give a ratio of spores of 1 : 2800, production was still more (520 $\mu g/ml$).

**Table V**

*Trichothecin production by combined Trichothecium-Penicillium fermentor cultures started from various numbers of spores*

| Trichothecium 2nd generation, No. of spores per ml at start | Penicillium No. of spores per ml superinoculated | Ratio of Trichothecium and Penicillium spores | Trichothecin $\mu$g/ml |
|---|---|---|---|
| 500 | 0 | . | 260 |
| 500 | 14 000 | 1 : 28 | 300 |
| 500 | 140 000 | 1 : 280 | 420 |
| 50 | 0 | . | 250 |
| 50 | 14 000 | 1 : 280 | 420 |
| 50 | 140 000 | 1 : 2800 | 520 |

(viii) *Table VI* presents the course of a characteristic combined fermentation, showing the variations in trichothecin production and pH of the culture. All shake cultures (second generation) were started with an inoculum originating from 50 Trichothecium spores per ml. From *Table VI* it is clear that the critical point fell between the ratios 1 : 60 and 1 : 100. As compared with flasks 2 and 3, in flasks 4 the antibiotic production started irregularly and the pH level showed a different course. No trichothecin was produced in flasks inoculated with ratios of Trichothecium-Penicillium spores 1 : 100 or 1 : 1000. Variation of the pH in the latter flasks resembled more and more that obtained in the pure Penicillium culture. In these flasks a small amount of antibiotic acting like penicillin could be detected (0.1—0.5 I. U./ml).

(ix) *Microscopic examination.* In pure shake flask cultures Trichothecium hyphae formed characteristic bundles resembling tufts of hair. Characteristic, comparatively early, spore formation was shown by some of the hyphae. In combined fermentations yielding high levels of antibiotic the filaments became thicker and shorter and often showed beaded forms. Oidium-like forms were also frequent. Sporulation was weak and prolonged. In part of the flasks containing more than 500 $\mu$g/ml trichothecin, groups of crystals were seen. The crystals were soluble in chloroform, in ordinary and polarized light they showed under the microscope the usual pattern of trichothecin crystals. The filaments of Penicillium were not easy to distinguish from those of Trichothecium. The former ones were mostly shorter than those belonging to Trichothecium, had more branches and formed a network. In the medium used, Penicillium cells occurred occasionally as swollen or cyst-like structures. Penicillium spores were seldom encountered.

(x) The Trichothecium strain used in the combined fermentation experiments produced usually 150—250 $\mu$g/ml trichothecin in pure culture. From unknown reasons the sporulation of this strain on solid media was sometimes

**Table VI**

*Combined Trichothecium — Penicillium fermentation using as a superinoculum increasing numbers of Penicillium spores*

| Designation of flask | Ratio of Trich. and Penic. spores 2nd gen. | Age of culture, hrs. Trichothecin µg/ml 64 | 88 | 112 | 136 | 160 | Average of peak yields µg/ml | Age of culture, hrs. pH 0 | 64 | 88 | 112 | 136 | 160 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1a | Tricho- | 110 | 175 | 210 | 210 | 210 | | 5.1 | 6.0 | 6.2 | 5.8 | 6.0 | 6.8 |
| b | thecium | 110 | 190 | 210 | 230 | 200 | 223 | 5.1 | 6.0 | 6.2 | 5.7 | 6.0 | 6.8 |
| c | control* | 190 | 230 | 230 | 230 | 220 | | 5.1 | 6.0 | 6.2 | 5.7 | 6.0 | 6.8 |
| 2a | | 720 | 980 | 1110 | 840 | 1350 | | 5.1 | 5.8 | 5.8 | 5.5 | 5.8 | 6.8 |
| b | 1 : 10 | 720 | 1200 | 840 | 1050 | 630 | 1176 | 5.1 | 5.8 | 5.8 | 5.5 | 5.8 | 6.8 |
| c | | 720 | 980 | 930 | 930 | 600 | | 5.1 | 5.8 | 5.8 | 5.5 | 5.8 | 6.8 |
| 3a | | 490 | 1090 | 1190 | 1005 | 1260 | | 5.1 | 5.6 | 6.0 | 5.4 | 5.8 | 6.5 |
| b | 1 : 30 | 720 | 1440 | 1005 | 1230 | 1230 | 1330 | 5.1 | 5.6 | 6.0 | 5.5 | 5.8 | 6.5 |
| c | | 335 | 1200 | 1110 | 930 | 1290 | | 5.1 | 5.6 | 6.0 | 5.5 | 5.8 | 6.5 |
| 4a | | 11 | 21 | 110 | 460 | 400 | | 5.1 | 5.0 | 4.5 | 5.4 | 4.0 | 6.5 |
| b | 1 : 60 | 175 | 950 | 980 | 1110 | 1110 | 783 | 5.1 | 5.0 | 4.5 | 5.4 | 4.0 | 6.5 |
| c | | 14 | 28 | 280 | 560 | 780 | | 5.1 | 5.0 | 4.5 | 5.5 | 4.0 | 6.5 |
| 5a | | 0 | 0 | 0 | 0 | 0 | | 5.1 | 4.5 | 3.0 | 4.0 | 3.5 | 4.0 |
| b | 1 : 100 | 0 | 0 | 0 | 0 | 0 | 0 | 5.1 | 4.5 | 3.0 | 4.0 | 3.5 | 4.0 |
| c | | 0 | 0 | 0 | 0 | 0 | | 5.1 | 4.5 | 3.0 | 4.0 | 3.5 | 4.0 |
| 7a | | 0 | 0 | 0 | 0 | 0 | | 5.1 | 3.0 | 3.0 | 3.0 | 3.0 | 3.0 |
| b | 1 : 1000 | 0 | 0 | 0 | 0 | 0 | 0 | 5.1 | 3.0 | 3.0 | 3.0 | 3.0 | 3.0 |
| c | | 0 | 0 | 0 | 0 | 0 | | 5.1 | 3.0 | 3.0 | 3.0 | 3.0 | 3.0 |
| 8a | Penic. control** | 0 | 0 | 0 | 0 | 0 | | 5.1 | 3.0 | 3.0 | 3.0 | 3.0 | 3.0 |

* Pure culture of Trichothecium
** Pure culture of Penicillium

poor. When suspending this type of agar slant culture in the usual 5.0 ml saline, the suspension contained only a small number of spores. (The spores were not counted in these experiments.) Using the suspension of the poorly and the well-sporing cultures of the same strain as inocula for combined fermentation, the results shown in *Table VII* were obtained. It is seen that, as compared to its pure culture, in combined fermentation the weakly sporing strain exhibited a relatively high antibiotic production.

(xi) For superinoculation, Penicillium strain $P_2$ was mostly used. In some experiments other strains were applied, namely the good penicillin producer strain Wis Q 176, and the white strain "N. H. Novihybrid" [4]. The latter strain was obtained from the *Institute for Pharmacoindustrial Research, Budapest*. Similarly to strain $P_2$, in combined fermentation both of these strains increased the trichothecin production.

**Table VII**

*Trichothecin production in combined fermentation by a poorly and a well-sporing culture of the same Trichothecium strain*

| Sporulation of Trichothecium | Penicillium inoculum as percentage of Trichothecium second generation | | |
|---|---|---|---|
| | 0 | 1 | 10 |
| | Peak yield of trichothecin, μg/ml | | |
| Poor | 35 | 190 | 950 |
| | 40 | 300 | 1130 |
| | 35 | 300 | 750 |
| Good | 300 | 1040 | 0 |
| | 350 | 1260 | 150 |
| | 300 | 1040 | 870 |

Killed and washed Penicillium mycelium was ineffective in combined fermentation, as well as the filtrate of Penicillium cultures. No extract with an effect promoting trichothecin production has so far been prepared from Penicillium cultures. No increase was obtained in the trichothecin level after the addition to the culture at the 18th and 65th hours of incubation of 1.5—80 I. U./ml of potassium salt of penicillin G.

(xii) The mutual effect of the Trichothecium and Penicillium strains used was investigated on malt extract agar plates as follows. Across the surface of separate plates a diagonal line was streaked with the culture under investigation. After incubation for 48 and 72 hours the culture of the other organism was streaked across the plate vertical to the line of growth of the originally inoculated strain. When inoculating the Penicillium strain onto the plate already streaked with Trichothecium, the former grew freely at the very edge of the latter, *i. e.* the growth of Penicillium was not inhibited by Trichothecium. In contrast to this, when inoculating Trichothecium against Penicillium, the former grew for two days only outside a zone 22—25 mm from the edge of the Penicillium culture. After two days, however, a scanty growth occurred within the zone. Consequently, the Penicillium culture inhibited the growth of Trichothecium, but did not destroy it, *i. e.* it exerted an antibiotic but no mycocidal effect.

## Discussion

In manufacturing antibiotics, great effort is made to keep the operation "sterile", which means that the fermentor should not contain organisms other than the antibiotic producer. In penicillin production the fermentation of Penicillium strains is regarded as particularly sensitive to certain contaminations. It should be noted, however, that in one case the authors were able to observe

a contamination which allowed a penicillin production considered at that time a peak yield. Trichothecium fermentations proved to be less sensitive, moreover the artificial infection of such fermentations with Penicillium strains resulted in an antibiotic production 2—4 times higher than that obtained with pure cultures. Antibiotic production under these circumstances has been termed combined fermentation.

The question arose whether during combined fermentation perhaps an antibiotic biologically more active than trichothecin was produced. This possibility was excluded, as the crystal type of the antibiotic isolated from combined fermentations was identical with that of the trichothecin obtained from pure cultures. Furthermore, the two substances had the same melting point (118° C). Paper chromatography carried out at the *Institute for Pharmacoindustrial Research* also revealed the identity of the two substances. Thus, in combined fermentation the trichothecin yield was undoubtedly increased.

According to CRAVERI [5], his Streptomyces strain in a medium containing peptone, corn steep liquor and corn meal produced 400 units/ml of antibiotic. The yields of antibiotic were considerably increased when the 24 hour culture of the Streptomyces was inoculated with a bacterium sensitive to the antibiotic in question. The best result was achieved with *Corynebacterium equi,* which caused a production of 1800—2000 units/ml. After four days this bacterium disappeared from the culture. If Streptomyces and Corynebacterium were inoculated at the same time, only 600 units/ml of antibiotic were produced. By adding a lysate instead of living bacteria, the rise in production was less.

These observations differ from those of our own. For stimulating production, CRAVERI used strains sensitive to the antibiotic. In the present experiments the higher yields of trichothecin were obtained with a Penicillium strain insensitive to trichothecin. On the other hand, the Penicillium strain was active against Trichothecium. CRAVERI was unable to isolate the inoculated bacteria on the fourth day. In the present experiments the Penicillium strain could be isolated throughout the combined fermentation. CRAVERI did not examine the effect of various quantities of the inoculum ; according to the present findings, this should be regarded as of basic importance.

As to the mechanism of the stimulatory effect of Penicillium on trichothecin production by Trichothecium, either some kind of "growth factor" must be formed by the Penicillium or its action must be due to metabolic products promoting the growth of Trichothecium. According to this hypothesis, in the presence of Penicillium the mycelium of Trichothecium would grow more abundantly and consequently produce more antibiotic. Experiments, however, have shown that this is not the case. *E. g.* in one experiment in the control shake culture the peak level of trichothecin was 220 $\mu$g/ml and the dry weight of the mycelium was 2.45 per cent. In the culture superinoculated with Penicillium at a ratio of 1 : 30 the maximum production was 980 $\mu$g/ml, with

2.03 per cent dry weight of the mycelium. Accordingly, the higher trichothecin production was not connected with a higher weight of the mycelium.

Solving this problem is far from being easy, since not even the high antibiotic production achieved in fermenting pure cultures can be satisfactorily interpreted. In fermentors — perhaps due to some pathological metabolism — antibiotics are often produced at much higher concentration than in nature. The high antibiotic yields in combined fermentation may likewise be connected with changes in metabolism. This seems to be supported by the finding that in combined fermentation the morphology of Trichothecium filaments is distinctly aberrant from the picture shown under normal conditions, *i. e.* in flasks containing a pure culture. The morphological changes affecting sensitive bacteria exposed to subantibiotic concentrations of antibiotics are well-known [6]. The morphological change of the filaments in our combined fermentations may similarly be due to a subinhibitory effect, since, as the critical point is reached, the antibiotic activity of Penicillium inhibits the growth of Trichothecium and also the production of trichothecin. As the effect cannot be brought about with penicillin, the possible role of another kind of Penicillium-produced antibiotic may be supposed; it is known that some Penicillium strains produce two or three different antibiotics.

The alteration in the antibiotic producing ability of Trichothecium in combination with Penicillium is not constant. Penicillium, therefore, is not a mutagenic agent under these circumstances and, accordingly, it does not give rise to new Trichothecium variants. When isolating the Trichothecium strain from a combined fermentation where a higher trichothecin production has been reached, and subsequently culturing it without Penicillium, only the lower production characteristic of pure cultures will be obtained.

Considering all the above facts, on the basis of the present investigations the high antibiotic yields in combined Trichothecium—Penicillium fermentations cannot be attributed to a "growth factor". From the aberrant morphology of Trichothecium growing in such fermentations the role of a pathological metabolism may perhaps be concluded. The stimulation of trichothecin production, however, might also be due to an "activity factor" or some "precursor" formed by Penicillium. The final elucidation of the problem requires further investigations.

## Summary

Fermentation of *Trichothecium roseum* resulted in a greatly increased trichothecin production if the culture had been superinoculated with a *Penicillium sp.* This procedure has been termed combined fermentation. High antibiotic yields were obtained only when the superinoculation with Penicillium had been performed after a certain period of incubation and the Trichothecium and Penicillium cultures had been inoculated in a certain proportion. On agar plates Penicillium inhibited the growth of Trichothecium, while Trichothecium was not inhibitory to Penicillium. In combined fermentation with Penicillium, Trichothecium suffered characteristic microscopic morphological changes.

*Acknowledgements:* The authors gratefully acknowledge the aid in the present study of Drs. I. HORVÁTH and A. SZENTIRMAI from the *Institute for Pharmacoindustrial Research, Budapest* and of Dr. J. VÖRÖS and Mrs. H. TURÁNYI, from the *Research Institute for Plant Protection, Budapest.*

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# MEGACINS

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(Received June 21, 1959)

In some previous studies it has been demonstrated that in certain strains of *B. megaterium* ultraviolet irradiation induces lysis connected with the release of a bacteriocin-like antibacterial substance, megacin [1, 2]. The phenomenon had been first observed in one of our collection strains designed as *B. megaterium* "216". Up to now, mostly the megacin produced by this single strain has been examined. Megacin of strain 216 was found to be heat labile and very sensitive to the action of crystalline pepsin and chymotrypsin, suggesting the substance to be of protein character. This supposition was further supported by the demonstration that megacin has an antigenic activity as well. The homologous antibody precipitates megacin and neutralizes its bactericidal effect [3].

The antibacterial spectrum of the substance was found to be very narrow [4]. The observations on its mechanism of action presently available suggest that megacin exerts its bactericidal effect by increasing the permeability of the cytoplasmic membrane of *B. megaterium*, resulting in the effusion of cytoplasm [5].

As to the megacins produced by other strains of *B. megaterium*, our knowledge is rather scarce. Only the antibacterial spectrum of the megacin from strains 216 and 119 has been examined [4]. Both these megacins exhibited essentially similar characteristics.

Some more recent studies [6] on a number of *B. megaterium* strains isolated from different soil samples revealed a 50 per cent incidence of megacinogeny. In these experiments the following two criteria were taken as signs for the presence of megacin production.

(i) Lysates of the strain should exhibit a bactericidal effect on a mutant of *B. megaterium* used as standard indicator strain.

(ii) The production of antibacterial principle should be enhanced by ultraviolet irradiation.

Naturally, these signs do not prove anything else but the presence of megacinogeny, while they are entirely insufficient to decide whether the mega-

cins actually produced are all identical or more or less different according to the actual strain of *B. megaterium* examined.

Our earlier studies already suggested that the megacin produced by *B. megaterium* 216 must be a particular one, as no similarly effective bactericidal action was exhibited by the lysates of any of the 17 strains markedly inducible by ultraviolet irradiation out of 200 random strains of *B. megaterium* [6]. Lysates of strain 216 were usually effective in dilutions 1 to 40 000, while those of the other strains never had activity titres higher than 1 to 100.

The present study was made in order to detect whether the differences observed indicated only the different activity of megacins produced by different strains of *B. megaterium*, or there are certain further intrinsic differences to be supposed.

## Materials and methods

As to the methodical and technical details and also to the characteristics of the bacteria used — if not described below — our earlier reports should be consulted.

*Bacterium strains. B. megaterium* strain 216, and strains Mut-C used as standard indicator, furthermore the phage resistant strains designed as 202*m*, 207*m* and 208*m* have already been described [7, 8] as also the standard laboratory strains KM, W899, W-0—7—11 and 337a [8]. Strains of *B. megaterium* designed by figures higher than 500 were isolated in connection with another experiment [6].

The chromococci No 9, No 1 and No 111 (the second and the third isolated recently) were identified as *M. aurantiacus* according to BERGEY's manual (6th edition). Strains of *B. anthracis* and *B. cereus* were characterized earlier [9]. The other authentic strains used were those maintained in our laboratory.

*Media.* Horse meat extract peptone medium was prepared according to the conventional rules and contained 1 per cent final concentration of peptone. The YDC medium (yeast extract enzymic digest of casein medium) and its method of preparation were described in an earlier publication [2]. The synthetic medium for megacin production was prepared as described by ALFÖLDI [10].

*Megacin preparations.* 200 ml of synthetic medium in a 1 liter Erlenmeyer flask was inoculated by a 16 hours old YDC agar slant culture of the appropriate megacinogenic strain. The inoculum was prepared so as to contain 2 to $3 \times 10^6$ cells per ml. Cultures were incubated at 37° C under constant shaking until their optical density had reached the value of 0.5. This figure corresponded to $10^8$ cell count per ml of fluid. After the appropriate cell concentration had been reached, the culture was dispensed into enamelled dishes 50 ml each, and irradiated under vigorous shaking by a low tension mercury lamp (Hanau Germicid, 20 W). The intensity of radiation was 1200 erg/mm$^2$. Calibration of the lamp was performed as described by LATARJET *et al.* [11]. After irradiation the samples were pooled and reincubated under constant shaking for 4 hours. Under these experimental conditions no remarkable lysis occurred in the cultures, while the megacin yield was much higher than in similarly treated cultures of low optical density [12].

The megacin content of the supernatant of the centrifuged cultures was 10 to 500 units per ml, when titrated on Mut-C indicator strain. *B. megaterium* strain 216 was an exception, usually producing 40 000 units of megacin per ml under the same experimental conditions.

Crude supernatants were only exceptionally used in the present experiments. Partially purified megacin preparations were obtained from strains 216, 527, 638, 675 and 689 of *B. megaterium*. Purification and concentration was performed by adsorption to, and elution from, anion cellulose, as described earlier [3]. One single lot of concentrate was prepared from each type of megacin, and used in all the present experiments. The lot number of each preparation corresponds to the protocol number of the appropriate strain of *B. megaterium.*

*Examination of megacin sensitivity of bacteria.* From young agar slant cultures of the bacteria to be examined a suspension of 0.1 optical density was prepared each and 1 ml of the suspension was added to an equal amount of melted broth agar and the mixture was layered

onto the surface of an appropriate agar plate. As to the further details of the method and the determination of megacin units, we refer to an earlier report from this laboratory [5].

The highest dilution of the preparation causing complete inhibition of bacteria inoculated into the soft agar layer was taken as the titration end point. Inhibition was indicated by the presence of a bacterium-free area around the spot where the megacin-containing fluid had been dropped.

*Preparation of protoplasts.* In contrast to our earlier work, instead of strain KM, the Mut-C strain of *B. megaterium* was used for protoplast preparation. Appropriate cells of Mut-C strain were obtained aerated shaken broth cultures incubated at 37° C. Two hundred ml of a culture of 0.6 optical density (0.7 mg/ml dry bacterium cells) was centrifuged and the sediment washed with buffered saline. Resuspension was made in 13 ml of PM (protoplasting medium) [5], containing 0.3 ml sucrose. Protoplasts were obtained by adding 150 $\mu$g per ml of crystalline lysozyme to the final suspension prepared as described above.

*Examination of protoplast disintegration.* To a series of test tubes containing 0.9 ml of the above protoplast preparation each, and incubated at 37° C, 0.1 ml of a twofold dilution series of megacin was added. After 15 minutes of incubation, 0.2 ml of a 4 per cent formaldehyde solution was added to each tube as a fixative. Samples were then taken and examined under the phase contrast microscope and the proportion of protoplasts transformed to ghost-like structures was estimated. The highest dilution of megacin causing disintegration of most of the protoplasts within 15 minutes was taken as the titration end point.

As the method exhibited many sources of error, it could only be regarded as semi-quantitative. Nevertheless, values obtained in different experiments were always found to be within the same range of magnitude, so the accuracy of the method had met the requirements in respect of the problem actually studied.

*Immune sera.* As to the preparation and standardization of anti-megacin immune, sera one of our earlier publications should be consulted [3].

## Experimental

*Antibacterial effect of different megacin concentrates, as measured on different strains of B. megaterium.* In some previous experiments the sensitivity to megacin 216 of the different strains of *B. megaterium* was found to exhibit remarkable differences. The 6 individual megacin concentrates obtained from 6 different strains were also examined as to their effectiveness, using 5 different strains of *B. megaterium* as indicator. The experiment was repeated 3 times at different occasions ; the geometrical means of the titre values obtained are presented in *Table I.*

*Table I* shows that the particularly high activity of megacin concentrate 216 could clearly be demonstrated for all but the KM indicator strains tested ; the effect on KM strain was peculiarly moderate. The activity titres of the other megacin preparations were rather weak when tested by Mut-C indicator strain. Nevertheless, the same preparations were sufficiently effective when certain other strains (578, 535) of *B. megaterium* were used as indicator.

Megacin activity studies were further extended to 11 additional strains of *B. megaterium*. Part of them, including the phage resistant mutants Mut-C, 202*m*, and 208*m*, was lacking both lysogenic and megacinogenic character, while the others exhibited either megacinogeny or lysogeny. The results are given in *Table II.*

As *Table II* reveals, all the strains examined were sensitive to the different megacin preparations, though to a varying degree. No relation whatever could

**Table I**

*Antibacterial titres of megacin concentrates for different indicator strains of B. megaterium*

| Megacin concentrate | Megacin titres for the indicators* | | | | |
|---|---|---|---|---|---|
| | MUT-C | KM | 202*m* | 578[1] | 538[2] |
| 216 | 160 | 1.0 | 100 | 80 | 100 |
| 527 | 0.16 | 6.4 | 0.2 | 6.4 | 6.4 |
| 575 | 0.2 | 0.01 | 0.2 | 1 | 0.6 |
| 638 | 1.2 | 0.2 | 1.6 | 5 | 5 |
| 675 | 2.5 | 0.8 | 5 | 19 | 12 |
| 689 | 3.2 | 0.2 | 1.6 | 9.5 | 6.4 |

* Figures represent $1/10^3$ of the values actually obtained.
[1] Non-lysogenic, megacinogenic.
[2] Lysogenic and megacinogenic.

**Table II**

*Antibacterial titres of megacin concentrates for different strains of B. megaterium*

| Strains and characteristics | Megacin concentrates* | | | | | |
|---|---|---|---|---|---|---|
| | 216 | 527 | 575 | 638 | 675 | 689 |
| MUT-C (1) | 160 | 0.16 | 0.2 | 3.2 | 3.2 | 6.4 |
| 207*m* (1) | 160 | 0.2 | 0.01 | 3.2 | 3.2 | 3.2 |
| 208*m* (1) | 40 | 1.6 | 0.01 | 6.4 | 6.4 | 6.4 |
| 522 (2) | 40 | 3.2 | 0.2 | 3.2 | 3.2 | 3.2 |
| 337 (2) | 4 | 0.8 | 0.01 | 0.8 | 0.8 | 1.6 |
| 583 (L) | 4 | 6.4 | 0.2 | 3.2 | 3.2 | 3.2 |
| 551 (L) | 80 | 1.6 | 0.1 | 0.8 | 1.6 | 1.6 |
| W 0-7-11 (L) | 4 | 1.6 | 0.2 | 3.2 | 3.2 | 3.2 |
| W 899 (L) | 4 | 0.4 | 0.1 | 1.6 | 0.8 | 0.8 |
| 296 (M) | 160 | 6.4 | 0.1 | 0.8 | 0.8 | 0.8 |
| 525 (M) | 40 | 6.4 | 0.2 | 3.2 | 3.2 | 3.2 |

* Figures represent $1/10^3$ of the values actually obtained

*Notes ;* (1) Phage-resistant, neither lysogenic, nor megacinogenic
(2) Phage-sensitive, neither lysogenic, nor megacinogenic
(L) Lysogenic
(M) Megacinogenic

be detected between the megacin sensitivity and the other properties, *i. e.* megacinogeny, lysogeny and phage resistance of the strains examined. Some data suggested the lysogenic strains to be somewhat less sensitive to the action of the different megacins, but the number of examinations did not permit to draw definite conclusions. In contrast to this observation stands the fact that the non-lysogenic strain 337 exhibited essentially similar megacin sensitivity as its lysogenic variant W 0—7—11. The remarkable sensitivity to megacin 216 of the phage resistant mutants was already observed in some of our previous experiments.

*Sensitivity of protoplasts from B. megaterium to megacin preparations of different origin.* It has been observed earlier that megacin concentrate 216 affected the cytoplasmic membrane of *B. megaterium,* causing the cytoplasm to escape from the cell. Protoplasts of *B. megaterium* become ghost-like structures in the presence of megacin. We consider this protoplast-destructive effect of megacin to be its specific way of action [5]. It seemed therefore important to examine whether the megacin preparations recently investigated had any similar effect and whether some relation could be detected between the protoplast-destructive and the antibacterial activity of the individual preparations. As there were wide variations in the antibacterial titres of each individual megacin, depending on the sensitivity of the indicator strain actually used, a comparison of the protoplast-destructive and of the antibacterial effect seemed to be reliable only if values had been referred to the highest antibacterial titres observed. *Table III* presents the maximum antibacterial titres of the individual megacins as compared to their protoplast-destructive activity titres.

**Table III**

*Protoplast-destructive effect of different megacin concentrates*

| Megacin | Maximum antibacterial titre (A) | Protoplast-destructive titre (B) | A/B |
|---|---|---|---|
| 216 | 160 000 | 10 000* | 16* |
| 527 | 6 400 | 1 000 | 6.4 |
| 575 | 1 000 | 100 | 10 |
| 638 | 6 400 | 4 000 | 1.6 |
| 675 | 19 000 | 4 000 | 4.8 |
| 689 | 10 000 | 8 000 | 1.3 |

* No higher dilutions tested.

As the figures in *Table III* show, all megacin concentrates from the 6 different *B. megaterium* strains exhibited a high protoplast-destructive activity. Depending on the actual preparation, 1.3 to 16 units of megacin/ml were found to transform most of the protoplasts to ghost-like structures within 15 minutes. In spite of the semi-quantitative character of the protoplast destruction method, it yielded results sufficiently correct to prove that the antibacterial activity of all the megacins tested was brought about by the same mechanism, *i. e.* the destruction of the osmotic barrier of the cell membrane.

*Antibacterial spectra of the megacin preparations.* The criterion of an antibacterial effect was the presence of a growth inhibition zone around the drop of megacin preparation carried onto the surface of an appropriately inoculated soft agar layer. When the concentrated preparation was ineffective, the test strain was regarded as megacin-resistant. All the 6 different megacin concentrates were ineffective for the following Gram positive organisms (figures in brackets represent the number of strains tested) : *Micrococcus pyogenes*, var. *aureus* (3), *Micrococcus pyogenes* var. *albus* (1), *Micrococcus pyogenes*, var. *citreus* (1), *Micrococcus tetragenus* (1), *Micrococcus lysodeikticus* (1), *Streptococcus faecalis* (2), *Corynebacterium xerosis* (1), *Corynebacterium ljubinski* (1), *B. cereus* (8), *B. subtilis* (7). Ten further strains of Gram positive micrococci isolated from air and forming pink, glossy and slimy colonies on agar plate were also found to be resistant to the action of all the 6 megacin preparations tested, while each of them was sensitive to lysozyme.

Megacine preparations were all ineffective for the following Gram negative organisms tested : *Shigellae* (4), *Salmonellae* (7), *Proteus vulgaris* (1), *Proteus mirabilis* (1), *Proteus morganii* (1), *Proteus rettgerii* (1), *Pseudomonas aeruginosa* (3), *Faecalis alcaligenes* (1), *Serratia marcescens* (1), *Escherichia coli* (6), *Aerobacter aerogenes* (1).

Some strains tentatively identified as *Micrococcus aurantiacus* exhibited a certain degree of sensitivity to all megacin preparation. The antibacterial titres for these strains varied between $10^{-2}$ to $10^{-4}$, depending on the kind of megacin used. Megacins 675 and 689 were more effective (titre, $10^{-4}$) than megacin 216 (titre, $10^{-3}$), a remarkable observation, since the opposite was found when *B. megaterium* was used as an indicator.

The megacin sensitivity of *B. anthracis* merits to be discussed separately. In some earlier studies out of the 43 strains of *B. anthracis* 8 had been found to be moderately sensitive to rough lysates of *B. megaterium* 216. The specificity of this effect appeared questionable [5] and therefore the experiment was repeated with some strains, using six different megacin concentrates. The results are presented in *Table IV*.

Only some of the 19 strains of *B. anthracis* tested were sensitive to megacins 216, 527 and 575, while almost all of them exhibited a moderate or a weak sensitivity to the other three megacin preparations.

**Table IV**

*Megacin sensitivity of different B. anthracis strains*

| Sign of the strain | Characteristics of the strain | Megacin sensitivity (3) | | | | | |
|---|---|---|---|---|---|---|---|
| | | 216 | 527 | 575 | 638 | 675 | 689 |
| A 66 | Acapsulogen (1) | 0 (4) | 0 | 1 | 100 | 100 | 80 |
| A 68 | Acapsulogen | 0 | 0 | 0 | 0 | 0 | 0 |
| A 69 | Capsulogen | 0 | 0 | 1 | 160 | 160 | 160 |
| A 72 | Capsulogen | 0 | 0 | 1 | 10 | 160 | 160 |
| A 74 a | Capsulogen | 0 | 0 | 1 | 100 | 160 | 100 |
| A 74 b | Acapsulogen | 1 (5) | 1 | 1 | 10 | 100 | 10 |
| A 76 | Capsulogen | 0 | 0 | 1 | 100 | 320 | 100 |
| A 77 | Capsulogen | 0 | 0 | 0 | 10 | 10 | 10 |
| A 91 | Capsulogen | 0 | 0 | 0 | 1 | 10 | 10 |
| A 98 T | Capsulogen | 0 | 0 | 10 | 10 | 10 | 10 |
| A 100 | Capsulogen, full virulent | 0 | 0 | 0 | 10 | 10 | 10 |
| A 101 | Capsulogen vaccine II. (2) | 0 | 0 | 0 | 10 | 10 | 10 |
| A 102 | Capsulogen vaccine II. | 0 | 0 | 0 | 1 | 10 | 10 |
| Davis | Capsulogen asporogen | 0 | 0 | 0 | 10 | 10 | 10 |
| Vollum S | Capsulogen full virulent | 0 | 0 | 0 | 1 | 0 | 0 |
| Vollum R | Acapsulogen, non virulent | 0 | 0 | 0 | 0 | 0 | 0 |
| NPA, S | Capsulogen, full virulent | 0 | 0 | 1 | 10 | 10 | 10 |
| NPA, R | Acapsulogen, non virulent | 0 | 0 | 0 | 1 | 10 | 1 |
| XWS | Capsulogen, moderately virulent | 0 | 1 | 1 | 10 | 10 | 10 |

*Remarks ;* (1) Capsule production was tested on serum-agar in 20% $CO_2$ atmosphere.
(2) "Deuxième vaccine" according to Pasteur.
(3) Highest dilution causing inhibition of growth.
(4) Ineffective even undiluted.
(5) Effective only undiluted.
Titration performed in a tenfold series. Concentrates effective in dilutions higher then 1 to 10 were titrated in a twofold series.

*The serological specificity of different megacins.* Rabbits, when immunized with megacin concentrates from strain 216 of *B. megaterium,* produced specific antibody giving precipitation and complement fixation reaction with the homologous megacin preparations. Moreover, these sera had the ability to neutralize the antibacterial action of the homologous megacin [3]. Some very active sera were effective even in several thousands-fold dilutions, when tested against a hundred units of megacin.

In the present study, a medium-effective anti-megacin serum prepared with megacin 216 was examined under standardized conditions [3]. After standing for 24 hours at 4° C, the mixture of 100 units of megacin 216 and of an 1 to 500 dilution of the homologous serum was found to lack an antibacte-

rial effect when tested appropriately on an indicator plate. No effect was observed when the test was made with a 1 to 20 dilution of an immune serum obtained after immunization with washed suspensions of *B. megaterium* 216.

Megacin neutralization tests were then carried out with different dilutions (from 1 to 2 to 1 to 2000) of anti-megacin 216 serum and 10 to 100 units of 16 different megacin preparations. In no case was neutralization of the antibacterial effect observed.

To examine the possible serological relations between the different *B. megaterium* strains used in this experiment, agglutination tests were performed with an immune serum obtained by immunization by means of washed suspensions of *B. megaterium* 216, using the 16 different strains as antigens. The wide variety in the surface antigenic structure of *B. megaterium* strains was made responsible for the fact that no agglutination was observed in any of these tests.

Preparation of anti-megacin serum was also attempted using megacin preparations other than the 216. In this experiment 5 ml single doses of the megacin concentrates 575 and 689 were injected intravenously at 15 occasions on every second day to one rabbit each. In spite of the intensive immunization, neither of the animals developed antibodies neutralizing the homologous megacin preparations.

## Discussion

All megacin preparations obtained from any strain were uniformy found to exert an antibacterial effect on all the *B. megaterium* and *Micrococcus aurantiacus* strains examined. Nevertheless, depending on the actual strain yielding the megacin, the intensity of the antibacterial effect exhibited a wide variety. The mode of action of megacin was found to be its destructive action on the cytoplasmic membrane of protoplasts from *B. megaterium*, resulting in the formation of ghost-like structures. Thus, every megacin preparation appeared to have the ability to destroy the osmotic barrier of the protoplasts. When compared to the corresponding antibacterial titre, the protoplast-destructive activity of all the megacin preparations examined was found to be within the same order of magnitude. This observation suggests the grade of sensitivity of the single *B. megaterium* strains to depend on the possibility of megacin exerting a destructive activity on the cytoplasmic membrane through the cell wall. The cell walls of the different strains apparently permit penetration of the colloidal megacin through the cell membrane to a different extent.

The antibacterial spectrum of the different megacins is essentially similar and very narrow. Besides *B. megaterium* and the above-mentioned Micrococcus species, only certain strains of *B. anthracis* were found to be sensitive and even those only moderately. As remarkable differences were found between the

efficiency of the different megacins on *B. anthracis,* it may be supposed that the mode of action of megacins on *B. anthracis* may be similar to that exerted on *B. megaterium.* Could this be proved by further experiments, the permeability of the cell wall of *B. anthracis* will turn out to be responsible for the differences observed in bacterial sensitivity.

As to the immunobiological characteristics of the different megacin preparations, striking differences were demonstrated. The megacin obtained from *B. megaterium* 216 had peculiarly good antigenic properties [3] — as demonstrated also earlier — and gave rise to a highly specific antibody, as revealed by the present study. This antibody was found to be ineffective in neutralizing the antibacterial action of other megacins, thus suggesting sharp differences in the antigenic structure of megacin 216 and of the others. Our attempts to produce antibodies by immunizing with 2 other megacins have failed. These results, however, are not regarded as definitive, considering that the concentrates of lower activity might be weaker antigens.

In connection with the specific immunological characteristics of megacin 216, it seems to be worth mentioning that the strain yielding this megacin has a most particular antigenic structure. In some earlier studies of this laboratory [8] it was found to be unrelated to 23 strains of *B. megaterium* tested. The present examination of 16 additional strains yielded similar results.

The iso-antagonistic antibacterial substances produced by different strains of *B. megaterium* appear to have a similar if not identical way of action. Thus their designation with the same name seems to be justified. There are, however, remarkable immunobiological differences between them. As to the spectrum of their antibacterial action, certain minor differences were encountered. These apparently depend on the different ability of the single megacins to penetrate the cell wall. Strain 216 of *B. megaterium* has been unique in that it produces the antibacterial substance in much greater amounts than any other strain and in that the megacin produced by it is of antigenic character.

Strain 216 has now been studied for several years and one of our collection strains, *B. megaterium* 119, had in one instance been found to display characteristics quite similar to the former. Unfortunately, recent samples taken from the frozen spores of strain 119 exhibited characteristics different from those observed earlier [13] ; they were less inducible by ultraviolet irradiation and also their lysates were of very low titre. There is, however, no doubt about the identity of the strain, since it gives a positive agglutination reaction with the specific immune serum prepared during our earlier studies.

## Summary

The iso-antagonistic antibacterial principles from different strains of *B. megaterium* have similar or identical ways of action. Their denomination with the common name "megacin" seems thus to be justified. A further proof of the above statement is the great similarity of their antibacterial spectra. Megacins exert their action on the cytoplasmic membrane of the sensitive bacteria. The cytoplasmic membrane is destroyed by megacin which thus abolishes the osmotic barrier of the bacteria. In spite of these biological similarities, certain serological differences could be demonstrated in the antigenicity and immune specificity of the different megacins.


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# LYSOGENY AND BACTERIOCINOGENY IN B. MEGATERIUM

By

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(Received June 25, 1959)

Megacinogeny, a lethal biosynthesis other than lysogeny has recently been described for strains of *B. megaterium* [1, 2, 3]. Differentiation between lysogenic and megacinogenic strains has become a fairly simple task since IVÁNOVICS [5] had succeeded in isolating indicator strains entirely resistant to megaterium phage, while very sensitive to the action of megacin.

In some preliminary studies [4] on 16 *B. megaterium* strains from our collection, megacinogeny was found to be rather frequent, while only one strain exhibited a lysogenic property. Further examinations carried out on 200 strains of *B. megaterium* originating from different soil and faeces samples revealed a 46 per cent incidence of megacinogeny [9, 10], while out of the 16 most readily inducible megacinogenic strains selected from this group only two were found to be lysogenic [3]. Thus the occurrence of the megacinogenic property appeared to be remarkably more frequent than the lysogenic one.

The present study was made in order to determine more exactly the incidence of megacinogeny and lysogeny both separately and simultaneously in 100 different *B. megaterium* strains isolated from random faeces and soil samples.

## Materials and methods

*Strains.* Strains of *B. megaterium* recently isolated and tested for megacinogeny in this laboratory [3] were used in the present study. The 100 strains to be examined were taken from our collection according to their increasing record numbers without any particular selection.

For the demonstration of lysogeny, the following standard, phage sensitive *B. megaterium* strains were used : KM, Sensitive, Mutilate and 337 b. Later some of the collection strains also proved appropriate for being used as indicators. These were strains 501, 510, 511, 533, 583, 546, 522, 536, 557, 559, 561, 564, 573, 585, 589 and 595.

To demonstrate megacinogeny, the MUT-C strain, the phage-resistant variant of *B. megaterium* isolated [5] from the standard laboratory strain *B. megaterium* "mutilate" was used.

*Media.* YP (yeast-peptone) agar medium, commonly used in this laboratory for similar experiments [2], served for maintenance and every other purpose in the present study.

*Preparation of indicator plates.* From a 20 hours old culture of the indicator strain on YP agar slant a suspension of $1.2 \times 10^7$ cells/ml was prepared by saline. To 1 ml of this suspension an equal amount of melted YP agar was added and the mixture was layered onto the surface of a YP agar plate.

*Ultraviolet irradiation.* A low tension type "Hanau" mercury vapour lamp was used. The energy emitted was at 50 cm distance 24 ergsec$^{-1}$/mm$^2$. Wave length was 2537 Å. For calibration of the UV lamp, the method of LATARJET *et al.* [6] was used.

As to the other methodical details we refer to some earlier reports from this laboratory [2, 3, 8].

## Results

Strains were selected by the replica plating method of LEDERBERG *and* LEDERBERG [7]. Plating was made on YP agar plates in a standard pattern using 30 different strains. After 18 hours of incubation, separate replicas were taken from each plate onto the surface of phage sensitive and phage resistant indicator plates. Each replica was made in duplicate. One of these was left untreated, the other replica was irradiated with UV light before culturing. Reincubation was performed at 37° C for 24 hours.

Evaluation of the results was made according to the following principles. (i) If none of the indicator plates displayed an inhibition zone round the replicated colony, the strain was regarded as lacking both lysogenic and megacinogenic properties. (ii) If inhibition was observed on the phage sensitive indicator plate, the strain was considered lysogenic. (iii) If inhibition was present not only on the phage resistant, but also on one or more of the phage sensitive indicator plates, considering that phage-sensitive indicator strains are at the same time sensitive also the megacin, this group was regarded both megacinogenic and lysogenic, or only megacinogenic.

Remarkable differences were observed in the phage sensitivity of the numerous phage-sensitive indicator strains tested simultaneously. Thus, no proper demonstration of lysogeny was possible by checking with a single indicator strain only. *Table I* presents some data supporting our above observations.

**Table I**

*Examination of some B. megaterium strains by the replica plating method, using different indicator strains*

| Indicator strains | Strains examined | | | | |
|---|---|---|---|---|---|
| | 512 | 501 | 514 | 560 | 588 |
| KM | — | + | + | — | + |
| 337 b | — | — | + | — | + |
| Sensitive | — | + | + | + | — |
| Mutilate | — | + | + | + | — |
| *Mut-C | — | — | + | + | — |

* phage-resistant mutant

As mentioned above, the observation of inhibition zones on both the phage-sensitive and the phage-resistant indicator cultures was usually a sign of the simultaneous presence of megacinogeny and lysogeny. Were the lysogenic plaques exhibiting a typical appearance on the phage-sensitive indicator plates, there was no difficulty in their identification ; intensely lysogenic and at the same time moderately megacinogenic strains could be recognized by direct inspection.

On the other hand, the mere observation of inhibition zones on the appropriate replica plates did not suffice for the demonstration of lysogeny in strains displaying at the same time an intensive megacinogeny. From such strains fluid cultures were prepared and phage demonstration was carried out by dropping their centrifugates onto the surface of phage-sensitive indicator plates.

Out of the 100 strains examined, 53 were found to have affected both the phage-sensitive and the phage-resistant indicator plates. Lysogenic property could be demonstrated directly by replica plating in 25 out of the 53 strains, while a fluid culture was needed in 28 cases.

Summing up, the following pattern of distribution was found for the two characteristics examined :

| | |
|---|---|
| Lysogenic only | 15 |
| Both lysogenic and megacinogenic | 19 |
| Megacinogenic only | 34 |
| Neither lysogenic nor megacinogenic | 32 |
| Total | 100 |

Thus the sum of lysogenic strains was 34, and that of megacinogenic ones 53 of a total of 100 strains of *B. megaterium*. The simultaneous presence of both of the lethal biosyntheses was observed in a total of 19 strains. The incidence of megacinogeny among lysogenic strains was 56 per cent, while that among non-lysogenic ones 52 per cent. Megacinogenic strains exhibited lysogeny in 36 per cent and non-megacinogenic ones in 32 per cent.

These figures, however, do not permit to conclude to some correlation or regularity in the frequent simultaneous occurrence of lysogeny and megacinogeny in ***B. megaterium*** strains.

We refrain from presenting here all the details obtained while examining the above 100 strains with the 16 indicator strains (6 only lysogenic and 10 neither lysogenic nor megacinogenic) originating from the very 100 strains under study. We only mention that on rough checking by the replica plating method no signs of cross-protection were revealed between the two lethal biosyntheses.

It is remarkable that all the lysogenic strains were detected by the KM, 337 b, Sensitive and Mutilate indicator strains and no further ones were found when testing was repeated by the 16 additional indicator strains.

## Summary

The incidence of lysogeny and megacinogeny has been examined in hundred recently isolated *B. megaterium* strains. Lysogeny occurred in 34 per cent and megacinogeny in 53. The simultaneous incidence of megacinogeny and lysogeny was 19 per cent. No genetic correlation between the two lethal biosyntheses could be demonstrated and their occurrence was found to be essentially independent. No signs of a cross-protection were revealed.

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# THE DESIGN AND ANALYSIS OF AN IMMUNOLOGICAL ASSAY*

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(Received August 17, 1959)

## 1. The problem

In many fields of applied biological science, the strengths of materials that are to be recommended for practical use because of the effects they produce on living matter (*e. g.* insecticides for the control of plant and animal pests, and drugs in therapeutic medicine) can be assessed satisfactorily only by means of standardized test procedures using living matter. Moreover, frequently attempts to obtain an absolute measure of potency prove useless because environmental factors and other chance influences cause substantial fluctuations in effects from one occasion to another ; instead, potency may be assessed relative to a standardized material of the same kind, in the expectation that this relative potency will show greater stability. Many techniques of this kind are in regular use, and are known as *biological assays* ; an extensive literature of statistical methods for the evaluation of the experimental results exists, and with some aspects of this I am concerned to-day.

One important class of biological assays arises in immunology in connexion with the comparison and standardization of vaccines. Test animals may be given different doses of vaccine, and subsequently "challenged" with a standard inoculum of disease ; for each vaccine dose, the animals can be divided into those adequately protected and those succumbing to disease, and the relation between dose and proportionate effect provides the basis for potency estimation. The use of "proportion of animals affected" as the measure of response to the applied dose introduces a number of complications into the statistical theory that are absent when the response is a continuous variate (as in the assay of oestrogens by reference to weights of uteri in treated animals).

I propose to take a typical immunological assay and describe various alternative schemes of calculation for deriving an estimate of relative potency and its precision. These methods differ in difficulty of theory, amount of arithmetical labour, and validity for their purpose ; unfortunately the first two

* Read at the *State Institute of Hygiene*, Budapest, September 11, 1959.

properties tend to be inversely related to the third! Discussion of statistical analysis will lead to some consideration of how such an assay ought to be planned for optimal results relative to the total effort expended upon it. The data are from a comparison of two forms of typhoid vaccine reported by Ikić [13] ; they are presented in *Table I.* Three doses of each vaccine (measured as number of organisms given at each of two inoculations) were used on batches of nearly 100 mice, and subsequently all mice were exposed to a fixed challenge dose of a strain of typhoid. The table records the number of deaths and the proportionate death rate in each group.

The main problem for discussion is that of how to estimate the potency of the phenolized vaccine (P) relative to the alcoholized (A). The *relative potency*, $\varrho$, is defined to be a multiplicative factor such that doses of the two vaccines will be on average equally effective if the number of organisms in the alcoholized dose is $\varrho$ times that in the phenolized ; if $\varrho$ exceeds 1, the phenolized is the more potent, and if $\varrho$ is less than 1, the alcoholized is the more potent.

**Table I**

*Data from an assay of typhoid vaccines*

| Vaccine dose* | Alcoholized vaccine (A) | | | Phenolized vaccine (P) | | |
|---|---|---|---|---|---|---|
| | No. of mice | Deaths | Death rate | No. of mice | Deaths | Death rate |
| 250M | 94 | 21 | 0.223 | 96 | 5 | 0.052 |
| 50M | 98 | 38 | 0.388 | 96 | 20 | 0.208 |
| 10M | 96 | 64 | 0.667 | 97 | 56 | 0.577 |

* M is used to symbolize a factor of $10^6$.

## 2. Inspection of data

Evidently any estimate from these data must give P a relative potency greater than 1. A dose 50M of P has a slightly lower death rate than 250M of A, suggesting that $\varrho$, the ratio of equally effective doses, is about 5. On the other hand, the death rate for 10M of P lies between those for 50M and 10M of A, suggesting that P lies between 5 and 1. Taken together, a value of between 3 and 4 for $\varrho$ appears to be indicated.

Some may prefer to look at this graphically. The death rates could be graphed against a scale of dose or, more usefully since a ratio of doses is to be investigated, against the logarithm of dose ; in the latter graph, the curves relating P and A death rates to log dose ought to be of the same shape but a fixed horizontal distance, log $\varrho$, apart. Freehand sketches of the curves could be used instead of the argument of the previous paragraph in order to

give a rough estimate of log $\varrho$, though few would have much confidence in such curves sketched from three points each.

These methods are strongly subjective, and suffer from the further grave disadvantage that they provide no information on the precision of the potency estimate, in terms of a standard error or in any other way. They cannot be recommended for any final analysis of an assay. None the less, it is often useful to employ one of them as soon as data are obtained, both in order to have a provisional value available until the definitive analysis is complete and as a rapid check on the reasonableness of the results.

### 3. Simple interpolation

Hereafter, all calculations will be made with reference to a logarithmic dose scale. Because successive doses bore the ratio 5 to one another, it is convenient to use logarithms to base 5 and to take 50M as the zero of the scale. The three values of $x$, the log doses for the test groups, are then 1, 0, —1, and any value of $x$ will correspond to the true dose

$$5^{x} \times 50\text{M}$$

or

$$10^{0.699x} \times 50\text{M} \tag{3.1}$$

One very simple procedure for analysis is to interpolate linearly between the death rates so as to estimate log doses corresponding to equal rates. For example, for 50% mortality we may estimate :

$$\text{A:} \quad x_{50} = -\frac{0.500-0.388}{0.667-0.388} = -0.402$$

$$\text{P:} \quad x_{50} = -\frac{0.500-0.208}{0.577-0.208} = -0.791$$

and therefore an estimate, R, of $\varrho$ is obtainable as

$$\log_5 \text{R} = 0.791-0.402 = 0.389,$$

whence

$$\text{R} = 10^{0.699 \times 0.389} = 1.87. \tag{3.2}$$

An obvious objection to this method is that it utilizes only part of the data, four doses (however many the total number used). If a different death rate had been chosen as the point of comparison, chance irregularities of the

records might cause the estimate to be very different. For example, if from *Table I* similar calculations are made for 25% mortality, the result is

$$R = 4.61. \qquad (3.3)$$

Although an approximate standard error for one of these estimates could perhaps be found, the method of estimation is so unsatisfactory that this is scarcely worth obtaining.

## 4. The Dragstedt-Behrens method

Doubtless concerned by the failure of simple interpolation to utilize all the data, DRAGSTEDT *and* LANG [7] commented that an animal affected by any particular dose would presumably also have been affected by any greater dose, and that one unaffected by a particular dose presumably would have been unaffected by any lesser dose. They therefore proposed to cumulate numbers of affected and unaffected animals. For example, for the alcoholized vaccine in *Table I*, their argument is that mice protected by a dose of 50M would also have been protected had they received 250M, and that mice protected by 10M would have been protected by 50M or 250M; similarly, mice that died after 50M would have died after 10M, and so on.

Application of this argument to the data yields *Table II*, in which S is the total number of mice protected by a dose or by any lesser dose, T is the total dead at a dose or any higher dose. The method associates the proportion T/(S + T) with each dose, and uses linear interpolation in this series for estimating the value of $x$ at which it would be 0.5 exactly as in Section 3. Hence

$$\text{A}: \quad x_{50} = -\frac{0.500 - 0.391}{0.794 - 0.391} = -0.270$$

$$\text{P}: \quad x_{50} = -\frac{0.500 - 0.176}{0.664 - 0.176} = -0.664$$

The estimate of relative potency is obtained from

$$\log_5 R = 0.394 \qquad (4.1)$$

and is

$$R = 1.89. \qquad (4.2)$$

Essentially the same method was independently proposed by BEHRENS [1].

Although the ratios T/(S + T) have a superficial resemblance to estimates of death rates at the various doses, comparison with *Table I* indicates a difference characteristic of the method : the ratios in *Table II* show a steeper

trend in relation to $x$ than do the death rates as directly computed, because of a tendency for each ratio to deviate from 0.5 more markedly than the corresponding death rate. It can be proved that the process will always overestimate the steepness of the dose-response relation, and therefore cannot properly be used to estimate, say, the dose for 25% mortality. If the true relation is symmetrical about the 50% point, and if the doses used are also symmetrically situated with respect to this point (on the logarithmic scale), the process will still estimate that 50% point, though the user must not imagine that he is estimating on the basis of the apparent numbers of animals shown in *Table II*.

**Table II**

*Cumulative totals of protected and dead mice required for* DRAGSTEDT—BEHRENS *and* REED—MUENCH *methods*

| log dose x | Vaccine A | | | Vaccine P | | |
|---|---|---|---|---|---|---|
| | S | T | T/(S+T) | S | T | T/(S+T) |
| 1 | 165 | 21 | 0.113 | 208 | 5 | 0.023 |
| 0 | 92 | 59 | 0.391 | 117 | 25 | 0.176 |
| —1 | 32 | 123 | 0.794 | 41 | 81 | 0.664 |

The condition of symmetric placing of log doses cannot be guaranteed, and the method cannot seriously be recommended. No satisfactory measure of precision is available, and the only reason for including the method is that it is regrettably popular with some people. It can be used as a rapid method of obtaining a provisional value, but has no particular merits that are not shared either by the SPEARMAN—KÄRBER method (Section 6) or by graphical methods (Section 8).

## 5. The Reed-Muench method

A slight variant on the DRAGSTEDT—BEHRENS method was proposed, apparently quite independently, by REED *and* MUENCH [16]. This consists in estimating the 50% point for each vaccine by simultaneous estimation in the S and T columns of *Table II* so as to find a value of $x$ for which they could be expected to be equal. Thus for A

$$92 + x\,(92-32) = 59 + x\,(59-123)$$

or

$$\text{A:} \quad x_{50} = -\frac{92-59}{123+92-59-32} = -0.266$$

$$\text{P:} \quad x = -\frac{117-25}{81+117-25-41} = -0.667$$

whence

$$R = 5^{0.401} = 1.91 \tag{5.1}$$

If the number of subjects is the same for every dose, REED—MUENCH and DRAGSTEDT—BEHRENS must give very similar results. Probably REED *and* MUENCH did not intend their method to be used unless the number of subjects per dose is constant, but in *Table I* the differences are too small to matter. So similar are the methods that many users write of having employed REED—MUENCH when in fact their method was DRAGSTEDT—BEHRENS. Both are open to the same objections and both appear to have little to be said for them as practical methods to-day.

## 6. The Spearman-Kärber method

One other even more obvious method of estimating the 50% point from records of proportions responding at different doses has often been used. Biologists frequently credit it to KÄRBER [14], but the earliest formal description of it known to me is that by SPEARMAN [17]; it has probably been independently suggested by many authors. Suppose that groups of subjects are tested at a series of doses whose logarithms are (in descending order) $x_1$, $x_2$, ... $x_k$, and that the proportions showing the characteristic response, death or whatever it may be, are $p_1, p_2, \dots p_k$. Then the decrement in death rate as the dose of vaccine is increased from $x_i$ to $x_{i-1}$ is estimated as $p_i - p_{i-1}$. If the doses are sufficiently close together, this difference in death rate could be associated with an average log dose $\frac{1}{2}(x_{i-1} + x_i)$. The mean log dose at which death occurs can then be estimated by further averaging, subject to one important condition: the $x_i$ must span the whole range of doses from those at which death never occurs to those at which death always occurs, for only then can the estimation be based upon the whole distribution of individual susceptibility. The estimate is

$$x_{50} = \Sigma \tfrac{1}{2}(p_i - p_{i-1})(x_{i-1} + x_i). \tag{6.1}$$

The condition strictly requires something more than merely that $p_1 = 0$, $p_k = 1$, for that could happen by chance even when $x_1$, $x_k$ are not sufficiently extreme to guarantee that death will *never* and will *always* occur respectively. If the doses are in a geometric progression, the $x_i$ will be equally spaced, say

$$x_i - x_{i-1} = d$$

for all $i$. Equation (6.1) can then be rearranged in the simpler form

$$x_{50} = x_k - \tfrac{1}{2} d + d \sum_{i=1}^{k} p_i. \tag{6.2}$$

This estimation does not require that $n_i$, the number of subjects tested at $x_i$, be constant. If successive doses are close together, there may be pairs of doses for which $p_{i-1}$ exceeds $p_i$, even though their expectations may be in the reverse order, but equation (6.1) or (6.2) can still be used. Moreover, since each $p_i$ arises from binomial sampling on $n_i$ individuals, its variance can be estimated from the experimental data as

$$V(x_{50}) = d^2 \sum \frac{p_i(1-p_i)}{n_i - 1} \qquad (6.3)$$

if (6.2) applies, with a fairly obvious modification if (6.1) must be used.

In practice, the condition on the range of doses tested may be so far from being fulfilled that even the extreme values of $p_i$ are not 0 and 1. In such circumstances, many experimenters have analysed their results with the assumption, explicit or implicit, that the next higher dose or the next lower dose would have satisfied the condition had they been tested. At best this is of doubtful validity and at worst it encourages an entirely uncritical attitude towards the numerical records of an experiment. Possibly the assumption is reasonable when an extended series of doses has been tested and the extremes have just failed to reach 0 and 1. For the data in *Table I*, to assume that doses of 1,250M would necessarily give complete protection and that doses of 2M would fail to protect any mice is clearly unsafe, yet without it SPEARMAN—KÄRBER estimation must be discarded as unusable. Here purely as illustration of the arithmetic, equations (6.2) and (6.3) will be applied with this assumption. Then, since $x_k = -1$, and $d = 1$

$$\text{A}: \ x_{50} = -1.5 + 0.223 + 0.388 + 0.667 = -0.222,$$

$$\text{P}: \ x_{50} = -1.5 + 0.052 + 0.208 + 0.577 = -0.663.$$

Hence

$$\log_5 R = -0.441.$$

Addition of the two expressions for variance gives

$$V(\log_5 R) = \frac{0.223 \times 0.777}{93} + \frac{0.338 \times 0.612}{97} + \ldots + \frac{0.577 \times 0.423}{96}$$

$$= 0.01144$$

$$= (0.1070)^2$$

The sampling distribution of the estimate will not be Normal, but as an approximation limits of error with 95% probability may be taken as $1.96 \times 0.1070$ on either side of $\log R$, that is to say at 0.231, 0.651.

Hence

$$R = 2.03, \tag{6.4}$$

with lower and upper 95% limits at

$$\left.\begin{aligned} R_L &= 1.45, \\ R_U &= 2.85. \end{aligned}\right\} \tag{6.5}$$

Further comment on this method is made in Section 12.

## 7. Transformations

Although all of the methods so far discussed are superficially sensible, none makes any pretence of employing a mathematical model that represents the manner in which the data arise. Two methods of approach to this can be tried, one starting from consideration of individual variation in susceptibility to the vaccine and the other from consideration of the probability that a particular dose will provide protection. Different though these are, they lead to closely related computational techniques.

The one method employs the concept of the *threshold dose* for each individual, a dose such that any greater amount would protect, whereas it or any lesser amount would fail to protect. Survival or death of a subject under test will depend upon whether or not the dose of vaccine exceeds the threshold for that subject. On the logarithmic scale, the frequency distribution of threshold doses may be written

$$f(x)\,dx. \tag{7.1}$$

In accordance with general biometric practice, it is natural to seek to approximate to this by the Normal distribution

$$\frac{1}{\sigma\sqrt{2\pi}}\,e^{-\frac{(x-\mu)^2}{2\sigma^2}}\,dx \tag{7.2}$$

despite the difficulty of accumulating evidence sufficient to support or to controvert this approximation.

The probability that a subject chosen at random from the population will fail to be protected by a dose whose logarithm is $X$, and will therefore die after challenge is

$$P(X) = \int_X^\infty \frac{1}{\sigma\sqrt{2\pi}}\,e^{-\frac{(x-\mu)^2}{2\sigma^2}}\,dx. \tag{7.3}$$

If $Y$ is defined by

$$P(X) = \int_{Y}^{\infty} \frac{1}{\sqrt{2\pi}} e^{-\frac{1}{2}u^2} du, \tag{7.4}$$

$Y$, which may be termed the ***normal equivalent deviate*** (NED) of $P$, then satisfies

$$Y = (X - \mu)/\sigma. \tag{7.5}$$

Now $Y$ can easily be tabulated as a function of $P$, and equation (7.5) shows it to be linearly related to $X$. Therefore, if the NED values corresponding to the experimental $p_i$ are tabulated against $x_i$, equation (7.5) may be expected to arise as in some sense a linear regression of NED on $x$; the parameter $\mu$ is both the mean of the distribution of threshold values of $x$ and the 50% response point, because of the symmetry of (7.2), and can be estimated as the point on the line for which $Y = 0$.

This transformation has been used very extensively. The idea has been developed independently by several writers notably GADDUM *and* BLISS of whom FECHNER [8] appears to have been the first. I have elsewhere [10] summarized the history. In recent years, the practice has been to increase the NED by 5 so as to have a quantity, known as the *probit* of $P$, that is rarely negative; the sole reason for this is to reduce the risk of arithmetical error in the calculations for estimating the parameters, and with modern computational techniques the advantage perhaps returns to the NED itself, which will therefore be used here.

The alternative method of approach is to specify the model directly in terms of the probability that an individual will fail to be protected when the log dose is $X$, a decreasing function of $X$. In some circumstances, there may be little case for invoking the concept of a distribution of threshold values, and a reasonable supposition may be that this probability conforms to an autocatalytic or logistic function, say

$$P(X) = \frac{1}{1 + e^{-(\alpha - \beta X)}}. \tag{7.6}$$

If $Y$ is defined by

$$P(X) = \frac{1}{1 + e^{-Y}} \tag{7.7}$$

or

$$Y = \log_e [P/(1-P)] \tag{7.8}$$

then

$$Y = \alpha - \beta X. \tag{7.9}$$

BERKSON [2] has termed $Y$ the *logit* of $P$; this transformation also has been suggested by others. Except for extremely large or small $P$, the logit transform of $P$ behaves very similarly to the NED, being approximately twice the NED. For this reason, $\frac{1}{2}Y$ is sometime used instead of $Y$, or even $(\frac{1}{2}Y + 5)$ as an analogue of the probit. Here the logit as defined by (7.7) or (7.8) will be used. By virtue of (7.9), some form of linear regression of logit on $x$ can again be used in order to estimate the parameters, and the 50% point can be estimated by finding where this line has $Y = 0$.

Despite their dissimilar origins, the calculations appropriate to the estimation of parameters on the normal and on the logistic hypothesis are very similar. Indeed, the logit transformation can alternatively be regarded as deriving from an assumption that the distribution (7.1) is

$$\frac{\beta e^{-(\alpha-\beta x)}\, dx}{[1 + e^{-(\alpha-\beta x)}]^2}, \tag{7.10}$$

a frequency distribution that is somewhat similar to the Normal in general appearance. These two are perhaps the most interesting members of a large family of distributions, one other member being mentioned in Section 11. I have discussed theory relating to this family more fully elsewhere [11].

## 8. Graphical estimation

The transformations introduced in Section 7 provide a quick and easy method of graphical estimation of $\varrho$. With the NED transformation, the NED value for each dose can be plotted against $x$. If the comparison between the two vaccines can be expressed simply by a relative potency, so that doses whose average effects (as measured by the proportion of subjects protected) are in a constant ratio whatever the proportionate effect chosen as a basis for comparison, then the horizontal distance between the regression lines represented by equation (7.5) must be constant; in other words, the two regression lines must be parallel. Hence a graphical estimate of $\varrho$ can be rapidly formed by drawing two parallel lines on the diagram that by eye inspection appear to fit satisfactorily.

*Fig. 1* is such a diagram based upon the data of *Table I;* the NED's from which it is drawn may be found in *Table III.* A little experience in these analyses is needed in order to develop skill, since greater weight should be attached to NED's near to zero than to those that are very different from zero, as will become apparent in Section 9. Moreover, if for any dose $p = 0$ or $p = 1$ the corresponding NED is infinite, a complication that is taken account of in the computations of Section 9; in graphical estimation, all that can be done is to make some allowance for a point far from the fitted line with little weight.

In *Fig. 1*, the horizontal distance between the lines is 0.650. Hence the estimate of $\varrho$ is

$$R = 5^{0.650}$$
$$= 2.85 . \quad (8.1)$$

Despite the subjective nature of this analysis, experience with one set of data in which deviations from the lines were much more marked than here [9] suggests that even relatively inexperienced workers will often achieve by this

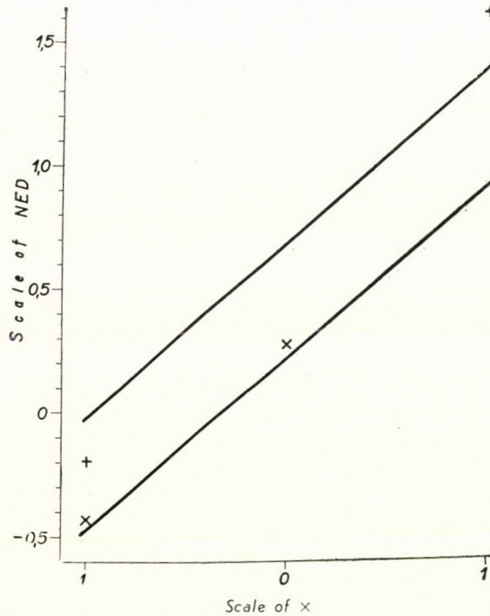


*Fig. 1.* Graphical representation of relation between NED and logarithm of dose for Table I, with parallel regression lines fitted by eye.

× Alcoholized vaccine (A)
+ Phenolized vaccine (P)

graphical method results very close to those from extensive computations ; errors in placement and slope of the lines often compensate, so as to leave the horizontal distance almost unaltered from the best possible estimate. Nevertheless, few will choose to depend solely on this method for routine work because it may go seriously wrong with exceptional data.

Although no assessment of the precision of this estimate can be read directly, various authors have proposed simple arithmetic procedures by which a standard error or limits of error at an assigned probability can be obtained at least approximately. I have elsewhere given a number of references ; possibly

the most useful is the very ingenious scheme proposed by LITCHFIELD *and* WILCOXON [15], though some may think the elaborate system of nomograms so complicated that they would as soon undertake the calculations of Section 9. The data at present being used as illustration fall outside the range of these nomograms because of the large number of subjects per dose, and therefore the method cannot satisfactorily be tried on them.

The same type of graphical estimation could be used in association with any other transformation that enables a linear regression to be used as a representation of the data. The diagram similar to *Fig. 1* for logits is not shown here ; it gave an estimate

$$\begin{aligned} R &= 5^{0.668} \\ &= 2.93. \end{aligned} \qquad (8.2)$$

In fact, this diagram and *Fig. 1* are so similar that obviously the difference between (8.1) and (8.2) can be attributed to the chances of drawing lines on eye judgment. Undoubtedly methods of assessing precision analogous to those of LITCHFIELD *and* WILCOXON and others could be constructed for logits, but as far as I know this has not been done.

## 9. NED estimation

The generally accepted procedure for numerical analysis of data with the aid of the NED transformation is to estimate the parameters according to the principle of maximum likelihood. Equation (7.5) may be rewritten as

$$Y = \alpha + \beta x \qquad (9.1)$$

by redefinition of parameters, and the object is to obtain two such equations for the two vaccines subject to the constraint that they represent parallel lines :

$$\left.\begin{aligned} Y_A &= a_A + bx \\ Y_P &= a_P + bx \end{aligned}\right\} \qquad (9.2)$$

These correspond to *Fig. 1*, but with numerical fitting instead of visual judgment. The relative potency is then obtained from the horizontal distance between the lines, as in Section 8 ; for the present example, this gives

$$\log_5 R = (a_P - a_A)/b. \qquad (9.3)$$

The estimates $a_A$, $a_P$, $b$ are determined in such a way that the joint likelihood of all the observations, the product of the binomial probability functions for all doses, when the probability of death at a particular dose is equation (7.3) or

$$P(X) = \int_{\alpha+\beta X}^{\infty} \frac{1}{\sqrt{2\pi}} e^{-\frac{1}{2}u^2} du, \tag{9.4}$$

is maximized.

The mathematical theory cannot be discussed here ; it has been described for probits in many places [10—11], and the main difference here is that tabulated probits must be reduced by 5.0. Adequate tables of various functions required can be found in various places, in the books already mentioned and in FISHER and YATES [12]. Much of the experimentation in which these methods are used is such that the probability of the characteristic response increases with increasing dose, so that $P$ is usually defined as the complement of the expression on the right hand side of equation (7.4). Here an increase in dose increases protection and therefore decreases the probability of death ; the positive association of NED with dose is maintained by equation (7.4), but a little care is needed in interchanging the roles of $P$ and $(1-P)$ in standard tables. An alternative outlook is to regard protection, not death, as the response, to take $P$ as the probability of protection, and then to use the standard tables. In what follows, only a condensed account of the computations will be given, it being assumed that tables of the functions required are available.

*Table III* contains the greater part of the calculations for potency estimation from *Table 1*. In it, $n$, $r$, and $p$ represent the number of subjects per dose, the number dead, and the proportion dead respectively. Next is shown the NED of $p$ according to equation (7.4) ; the symbol $Y$ is in practice reserved for the next column. If any $p$ were 0 or 1, no entry would be made in the NED column. These last are the values plotted in *Fig. 1*, and the graphical fitting of regression lines in *Fig. 1* is an important first step in the iterative process that leads to maximum likelihood estimation. From *Fig. 1* are read values of $Y$ on the lines, for each $x$ used experimentally, 1 decimal digit being almost always adequate. If $Z$ is defined by

$$Z = \frac{1}{\sqrt{2\pi}} e^{-\frac{1}{2}Y^2} \tag{9.5}$$

and $P$ is related to $Y$ by equation (7.4), the *weighting coefficient*, $w$, is

$$w = \frac{Z^2}{P(1-P)}, \tag{9.6}$$

and the column *nw* can be completed. Also, the *working equivalent deviate*, *y*, is

$$y = Y + \frac{P}{Z} - p \cdot \left(\frac{1}{Z}\right); \qquad (9.7)$$

this will usually agree well with the figure in the NED column, and large discrepancies should be checked carefully. The functions $w$, $Y + \frac{P}{Z}, \frac{1}{Z}$ are in standard tables. Note that (9.7) gives a finite value to $y$ even when $p = 0$ or $p = 1$, and it therefore provides the way of dealing with these extremes.

**Table III**

*NED calculations (maximum likelihood)*

| $x$ | $n$ | $r$ | $p$ | NED | Y | $nw$ | $y$ | $nwx$ | $nwy$ |
|---|---|---|---|---|---|---|---|---|---|
| *Vaccine A* | | | | | | | | | |
| 1 | 94 | 21 | .223 | 0.76 | 0.9 | 44.3 | 0.75 | 44.3 | 33.225 |
| 0 | 98 | 38 | .388 | 0.28 | 0.2 | 61.5 | 0.28 | 0.0 | 17.220 |
| —1 | 96 | 64 | .667 | —0.43 | —0.5 | 55.8 | —0.43 | —55.8 | —23.994 |
| | | | | | | 161.6 | | —11.5 | 26.451 |
| *Vaccine B* | | | | | | | | | |
| 1 | 96 | 5 | .052 | 1.63 | 1.4 | 29.0 | 1.59 | 29.0 | 46.110 |
| 0 | 96 | 20 | .208 | 0.81 | 0.7 | 51.0 | 0.81 | 0.0 | 41.310 |
| —1 | 97 | 56 | .577 | —0.19 | 0.0 | 61.8 | —0.19 | —61.8 | —11.742 |
| | | | | | | 141.8 | | —32.8 | 75.678 |

| | $1/\Sigma nw$ | $\bar{x}$ | $\bar{y}$ |
|---|---|---|---|
| A : | 0.006188 | —0.071 | 0.164 |
| P : | 0.007052 | —0.231 | 0.534 |

| | $(xx)$ | $(xy)$ | $(yy)$ |
|---|---|---|---|
| A : | 100.1 | 57.219 | 40.06 |
| | —0.8 | 1.882 | — 4.33 |
| | 99.3 | 59.101 | 35.73 |
| P : | 90.8 | 57.852 | 109.01 |
| | —7.6 | 17.505 | —40.39 |
| | 83.2 | 75.357 | 68.62 |

$$b = (59.101 + 75.357)/(99.3 + 83.2)$$
$$= 134.458/182.5$$
$$\doteq 0.7368$$
$$Y_A = \bar{y}_A + b\,(x - \bar{x}_A) = 0.216 + 0.737x$$
$$Y_P = \bar{y}_P + b\,(x - \bar{x}_P) = 0.704 + 0.737x$$

The next step is to calculate linear regressions of $y$ on $x$, with each $y$ carrying weight $nw$ and with the regressions constrained to be parallel. The arithmetic shown as part of *Table III* is of standard form, and need not be described in detail. For example, for the sum of products for vaccine A :

$$\begin{aligned} \Sigma nw(x-\bar{x})(y-\bar{y}) &= \Sigma nwxy - (\Sigma nwx)(\Sigma nwy)/\Sigma nw \\ &= 57.219 - (-11.5)(26.451)/161.6 \\ &= 59.101. \end{aligned}$$

Having obtained regression equations of the form of (9.2), the next step is to compare them with the values of $Y$, the expected NED, used in the cycle of computation. If there is any appreciable difference, a second cycle must be computed in the same way but using a new column of $Y$ taken from the equations just calculated. Iteration continues until agreement is satisfactory. Discrepancies up to 0.1 in corresponding values of $Y$ at the beginning and end of a cycle can usually be neglected as unimportant. Here, to 1 decimal, there are no changes in $Y$ and further iteration is unnecessary unless the accuracy obtainable by working to an extra decimal is required.

Next tests of the adequacy of the model are required. The total sum of squares of deviations for $y$, $(35.73 + 68.62)$, can be partitioned according to standard analysis of $\chi^2$ procedure. The fitting of two separate regression coefficients would account for a component

$$\frac{(59.101)^2}{99.3} + \frac{(75.357)^2}{83.2} = 103.43$$

with 2 degrees of freedom, whereas a common regression coefficient accounts for

$$\frac{(134.458)^2}{182.5} = 99.06$$

with 1 d. f. *Table IV* shows the $\chi^2$ with 2 d. f. for residual variation about regression lines to be negligibly small ; that for deviations from parallelism is just significant at the conventional 0.05 probability, suggesting that the same value of $\beta$ may not apply to the two vaccines. If this be true, no representation of relative potency by a single numerical value is possible, and the whole basis of the assay breaks down. However, here one may suspect that the large $\chi^2$ is really due to chance, in compensation for the very low $\chi^2$ for residual variation ; the total, 5.29 with 3 d. f., is nowhere near to statistical significance.

**Table IV**

*Analysis of $\chi^2$ for NED maximum likelihood calculations*

| | d. f. | | Component of $\chi^2$ | |
|---|---|---|---|---|
| Average regression | 1 | | 99.06 | |
| Deviations from parallelism | 1 | | 4.37 | |
| Regressions for A and P | | 2 | | 103.43 |
| Residual | | 2 | | 0.92 |
| Total | | 4 | | 104.35 |

If this fear of the invalidity of the assay is set aside, the potency of $P$ relative to $A$ can be obtained from equation (9.3):

$$\log_5 R = \bar{x}_A - \bar{x}_P - \frac{\bar{y}_A - \bar{y}_P}{b} \tag{9.8}$$

$$= 0.160 + \frac{0.370}{0.7368}$$

$$= 0.662,$$

or

$$R = 2.90. \tag{9.9}$$

Now $\bar{y}_A$, $\bar{y}_P$, $b$ are maximum likelihood estimators of a type whose distribution may be expected to be approximately Normal even in samples of moderate size, because of the close analogy with ordinary means and regression coefficients. Moreover, because the weights used in the calculations are "true weights", reciprocals of variances, it follows that

$$V(\bar{y}_A - \bar{y}_P) = (\Sigma_A nw)^{-1} + (\Sigma_B nw)^{-1}, \tag{9.10}$$

$$V(b) = \Sigma_{xx}^{-1}, \tag{9.11}$$

where $\Sigma_{xx}$ represents the quantity $\Sigma nw\,(x-\bar{x})^2$ summed over $A$ and $P$, the denominator in the calculation of $b$. The covariance of $(\bar{y}_A - \bar{y}_P)$ and $b$ is zero. Although an asymptotic variance for the expression in (9.8) can now be found by a well-known (and too often used) formula for the variance of a ratio, this can be seriously misleading if $b$ is small relative to its standard error. A better way of representing the precision of $R$ is to select a suitable level of probability,

say 0.95, and to calculate lower and upper limits of error at this probability by FIELLER's Theorem. The formula for the limits is

$$\bar{x}_A - \bar{x}_P - \left[\frac{\bar{y}_A - \bar{y}_P}{b} \pm \frac{t}{b}\left\{(1-g)\, V(\bar{y}_A - \bar{y}_P) + \left(\frac{\bar{y}_A - \bar{y}_P}{b}\right)^2 V(b)\right\}^{\frac{1}{2}}\right] \div (1-g), \tag{9.12}$$

where $t$ is the normal deviate corresponding *bilaterally* to the selected probability and

$$g = \frac{t^2\, V(b)}{b^2}. \tag{9.13}$$

So for the example, at probability 0.95, $t = 1.960$, and

$$g = \frac{(1.960)^2}{(0.7368)^2 \times 182.5} = 0.0388.$$

Then the limits are

$$0.160 + \left[0.502 \pm \frac{1.960}{0.7368}\left\{0.9612 \times 0.01324 + \frac{(0.502)^2}{182.5}\right\}^{\frac{1}{2}}\right] \div 0.9612$$

$$= 0.160 + (0.502 \pm 0.316) \div 0.9612$$

$$= 0.354,\ 1.011.$$

Hence, by conversion to the scale of doses in the usual way, the limits are

$$\left.\begin{aligned} R_L &= 1.77 \\ R_U &= 5.09 \end{aligned}\right\}. \tag{9.14}$$

Note that in this method the 50% response points for the two vaccines did not need to be computed. They can be estimated very easily from the two regression lines, but the relative potency is independent of any particular level of response.

As a check on the statement that this one cycle of the calculations was sufficient, a second cycle was computed as described above and taking $Y$ to 2 decimals. The new regressions lines are

$$Y_A = 0.2184 + 0.7396x,$$

$$Y_P = 0.7042 + 0.7396x,$$

almost identical with the old. The $\chi^2$ tests are little changed and the new estimate is

$$R = 2.88 \tag{9.15}$$

with

$$\left.\begin{aligned} R_L &= 1.75 \\ R_U &= 5.05 \end{aligned}\right\}. \tag{9.16}$$

## 10. Logit analysis

An exactly parallel form of analysis can be followed when the logit transformation is regarded as appropriate. The only differences are that equation (9.4) is replaced by equation (7.6), and that the weighting coefficient and working equivalent deviate are respectively

$$w = P\,(1-P), \tag{10.1}$$

$$y = Y + \frac{1}{1-P} - \frac{P}{P\,(1-P)}. \tag{10.2}$$

I shall not show details of the calculations. The regression lines after the first iterative cycle are

$$\left.\begin{aligned} Y_A &= 0.396 + 1.236\,x \\ Y_P &= 1.259 + 1.236\,x \end{aligned}\right\} \tag{10.3}$$

and are in close agreement with the provisional values of $Y$ used in the analogue of *Table III*. *Table V* corresponds to *Table IV*. I must confess that the size of the $\chi^2$ for deviations from parallelism again raises serious doubts about the validity of this particular assay ; it suggests that either some unsuspected disturbance to test conditions occurred or that vaccines $A$ and $P$ are not simply comparable by means of a relative potency. However, as the data are being used primarily for illustrating computations, these anxieties will be ignored.

**Table V**

*Analysis of $\chi^2$ for logit maximum likelihood calculations*

| | d. f. | | Component of $\chi^2$ | |
|---|---|---|---|---|
| Average regression | 1 | | 89.21 | |
| Deviations from parallelism | 1 | | 5.34 | |
| Regressions for A and P | | 2 | | 94.55 |
| Residual | | 2 | | 0.60 |
| Total | | 4 | | 95.15 |

From (10.3), the same process as in Section 9 leads to

$$R = 2.81. \tag{10.4}$$

Equation (9.12) applies without alteration, on the understanding that $w$ everywhere is now defined by (10.1), and the same type of calculation gives

$$\left.\begin{aligned} R_L &= 1.70 \\ R_U &= 4.91 \end{aligned}\right\}. \tag{10.5}$$

In various papers, and definitively in 1953, BERKSON [3] has advocated an alternative method of estimating the parameters for the logistic model. He bases this upon the minimization of a $\chi^2$ for the residual variation after a regression equation of logit on $x$ has been fitted. His $\chi^2$ is not defined in exactly the usual manner, since it uses observed frequencies at one point where the accepted practice in contingency tables is to use expectations, but the formulae are asymptotically identical as the numbers of subjects per dose increase, and indeed the method is asymptotically equivalent to maximum likelihood both being fully efficient in the limit. The method has some obvious merits in ease of computation and in the fact that one cycle of calculation always suffices without iteration.

Computationally, the method involves finding weighted linear regressions of the observed logit of $p$ on $x$, instead of the working value given by (10.2), with the weighting coefficient also taken from $p$ instead of from the provisional $P$. In fact, if we now write

$$w = p\,(1-p) \tag{10.6}$$

$$y = \log\,[p/(1-p)] \tag{10.7}$$

instead of (10.1), (10.2), the computations may be put in the form of *Table VI*. In his paper, BERKSON has tabulated $w$ and $wy$; therefore $y$ need not be explicitly shown in *Table VI*, as $nw$ and $nwx$ can be entered immediately. Apart from the slight differences already mentioned, the calculations are so similar to those of *Table III* as to require no comment. *Table VII* has been constructed in the same way as *Tables IV* and *V*. BERKSON, probably rightly, recommends the more laborious procedure of finding $\chi^2$ as

$$\chi^2 = \sum \frac{n\,(p-P)^2}{P\,(1-P)}\,, \tag{10.8}$$

where $P$ is now taken to correspond to the fitted regression lines at the foot of *Table VI* in order to give the residual with 3 degrees of freedom after estima-

tion of parallel regressions. If a test of parallelism were wanted, this would need to be repeated on lines fitted independently to $A$ and $P$, to give a $\chi^2$ with 2 d. f., and then the difference taken as $\chi^2$ with 1 d. f. With values of $n$ as large as here, the two methods will be nearly equivalent.

**Table VI**

*Logit calculations (minimum $\chi^2$)*

| $x$ | $n$ | $r$ | $p$ | $nw$ | $nwx$ | $nwy$ |
|---|---|---|---|---|---|---|
| *Vaccine A* | | | | | | |
| 1 | 94 | 21 | .223 | 16.290 | 16.290 | 20.332 |
| 0 | 98 | 38 | .388 | 23.275 | 0.000 | 10.604 |
| —1 | 96 | 64 | .667 | 21.322 | —21.322 | —14.813 |
| | | | | 60.887 | —5.032 | 16.123 |
| *Vaccine B* | | | | | | |
| 1 | 96 | 5 | .052 | 4.733 | 4.733 | 13.738 |
| 0 | 96 | 20 | .208 | 15.811 | 0.000 | 21.149 |
| —1 | 97 | 56 | .577 | 23.678 | —23.678 | —7.353 |
| | | | | 44.222 | —18.945 | 27.534 |

| | $1/\Sigma nw$ | $\bar{x}$ | $\bar{y}$ |
|---|---|---|---|
| A : | 0.016424 | —0.0826 | 0.2648 |
| P : | 0.022613 | —0.4284 | 0.6226 |

| | $(xx)$ | $(xy)$ | $(yy)$ |
|---|---|---|---|
| A : | 37.612 | 35.145 | 40.505 |
| | —0.416 | 1.332 | —4.269 |
| | 37.196 | 36.477 | 36.236 |
| B : | 28.411 | 21.091 | 70.437 |
| | —8.116 | 11.796 | —17.144 |
| | 20.295 | 32.887 | 53.293 |

$$b = 69.364/57.491 = 1.2065$$

$$Y_A = 0.364 + 1.206x$$

$$Y_P = 1.139 + 1.206x$$

When the sums of squares and products and the regression lines have been found, the estimation of relative potency proceeds as for maximum likelihood. Details need not be shown as the formulae are unaltered; BERKSON,

**Table VII**

*Analysis of $\chi^2$ for logit minimum $\chi^2$ calculations*

| | d. f. | | Component of $\chi^2$ | |
|---|---|---|---|---|
| Average regression | 1 | | 83.69 | |
| Deviations from parallelism | 1 | | 5.37 | |
| Regressions for A and P | | 2 | | 89.06 |
| Residual | | 2 | | 0.47 |
| Total | | 4 | | 89.53 |

indeed, uses an asymptotic standard error for the logarithm of the relative potency, but occasions when this way of expressing the precision of a ratio is misleading are so frequent that it seems better to adopt the practice of always using FIELLER's Theorem. The results are

$$R = 2.81 \tag{10.9}$$

and

$$\left. \begin{aligned} R_L &= 1.68 \\ R_U &= 4.94 \end{aligned} \right\} . \tag{10.10}$$

This minimum $\chi^2$ method encounters difficulties if any $p$ is 0 or 1. Full treatment of the situation, which of course often occurs if the $n$ are much smaller than in *Table I*, would re-introduce the iteration that BERKSON has otherwise eliminated. He has proposed various simple expedients as reasonable compromises, but his discussion of this problem appears incomplete and is perhaps the weakest part of his paper.

## 11. Angle analysis

One other transformation deserves comment; it is based upon a transformation that is often used outside the field of bioassay for observations expressed as percentages. It consists in taking

$$P = \cos^2 (\alpha + \beta x), \tag{11.1}$$

and defining an angle transform of $P$ by

$$P = \cos^2 Y, \tag{11.2}$$

so that again the linear relation

$$Y = \alpha + \beta x \tag{11.3}$$

appears. One convenient feature is that the weighting coefficient for maximum likelihood estimation is constant :

$$w = 4. \tag{11.4}$$

The working angle, however, takes no particularly simple form and still requires to be computed from special tables as

$$y = Y + \tfrac{1}{2} \cot Y - \operatorname{cosec} 2Y. \tag{11.5}$$

Here $Y$, $w$, and $y$ are of course expressed in radian measure. The tables usually employed are in degrees, for which

$$w = \frac{4\pi^2}{(180)^2} = 0.0012185. \tag{11.6}$$

In practice, time is saved by using $n$ alone as the weight throughout the main processes of calculation and introducing $w$ as multiplier or divisor in appropriate positions at the end ; the estimate of $\varrho$ is unaffected by this simpler weighting, but its precision must take account of $w$. In many assays for which $n$ is also constant at all doses, unit weight can be used in calculation and the factor $nw$ introduced at the end.

The data in *Table I*, when analysed by the angle method, give *Table VIII* corresponding to *Tables IV* and *V*. Here there is no sign of invalidity, either from non-parallelism of the regression lines (*i.e.* inequality of $\beta$ for the two

**Table VIII**

*Analysis of $\chi^2$ for angle maximum likelihood calculations*

| | d. f. | | Component of $\chi^2$ | |
|---|---|---|---|---|
| Average regression | 1 | | 114.70 | |
| Deviations from parallelism | 1 | | 2.92 | |
| Regressions for A and P | | 2 | | 117.62 |
| Residual | | 2 | | 1.97 |
| Total | | 4 | | 119.59 |

preparations) or from heterogeneous residual deviations. The final findings are

$$R = 2.94 \tag{11.7}$$

and

$$\left.\begin{aligned} R_L &= 1.80 \\ R_U &= 5.20 \end{aligned}\right\}. \tag{11.8}$$

## 12. Comparison of methods

Discussion of the relative merits of the various methods of estimating relative potency must not lean too heavily upon the evidence of one numerical example, which may be far from typical. The data in *Table I* are "good" in the sense that they conform well to smooth dose-response relations (linear after any of the transformations used in Sections 8—12), and a method that behaves anomalously with this example must be viewed with grave suspicion. On the other hand, the number of different doses tested was small, which may prevent certain methods showing to best advantage. For ease of discussion, the various estimates from *Table I* have been collected into *Table IX*.

**Table IX**

*Summary of estimates of relative potency for vaccines A and P*

| Method | Estimate | Lower limit | Upper limit |
|---|---|---|---|
| Inspection | 3—4 | — | — |
| Interpolation | 1.87 | — | — |
| DRAGSTEDT—BEHRENS | 1.89 | — | — |
| REED—MUENCH | 1.91 | — | — |
| SPEARMAN—KÄRBER | 2.03 | 1.45* | 2.85* |
| Graphical NED | 2.85 | — | — |
| Graphical logit | 2.93 | — | — |
| NED (Max. Lik.) | 2.90 | 1.77 | 5.09 |
| Logit (Max. Lik.) | 2.81 | 1.70 | 4.91 |
| Logit (Min. $\chi^2$) | 2.81 | 1.68 | 4.94 |
| Angle (Max. Lik.) | 2.94 | 1.80 | 5.20 |

* Of questionable validity: see text.

Although a rough estimate by inspection of the data should always be formed, for it is at least a safeguard against gross errors of calculation, no one would claim any finality for it. Objections to the methods of simple inter-

polation, DRAGSTEDT—BEHRENS, and REED—MUENCH have already been mentioned in Sections 3—5 ; these seem to me overwhelming, since the only compensating merit for the methods is their numerical simplicity, and this is shared by others (SPEARMAN—KÄRBER and graphical) that are in general more trustworthy. I think the time has come when these methods should be completely abandoned. There can be little doubt that the best estimate of relative potency from *Table I* is of the order of 2.8—2.9 yet these three methods indicated about 1.9. DRAGSTEDT—BEHRENS and REED—MUENCH might be expected to perform somewhat better if more doses at closer intervals were tested, but failure to space the doses symmetrically about the 50% point can lead to discrepancies such as are found here, and both the limitation to equal spacing and the lack of any measure of precision are additional disadvantages.

The SPEARMAN—KÄRBER method is perhaps the best of the objective yet rapid methods, provided that a wide range of doses has been covered and that the interval between successive doses is not great. If the doses corresponding to threshold protection for individual subjects are truly lognormal in distribution, and if the levels of dose used in an assay (with equal numbers of subjects at all doses) satisfy this condition, the SPEARMAN—KÄRBER method can be of high efficiency ; use of the NED transformation or some equivalent procedure for obtaining the maximum likelihood estimator may reduce the variance only by about 2%. If the threshold doses in reality derive from the logistic probability model, under the same condition the SPEARMAN—KÄRBER method is equivalent to maximum likelihood [6]. As already indicated, the assay in *Table I* is a somewhat extreme case for the use of SPEARMAN—KÄRBER; the assumption that the next doses in each series would give $p = 0$, $p = 1$ cannot find much support in the data, and I should be reluctant to use the method except for a rough preliminary figure. *Table IX* shows the potency estimate to be low, and the limits of error are really wholly untrustworthy because they rest on a false assumption.

Although in certain circumstances SPEARMAN—KÄRBER can be of high efficiency, the question arises whether or not these circumstances should be permitted to arise! If little is known in advance about the substances under assay, the investigator may feel obliged to use many closely-spaced doses, but he must recognize that those at the extremes near to $P = 0$ or $P = 1$ in reality contribute little information. If existing information enables him to make a reasonable guess at the 50% dose and the slope of the dose-response relation for each preparation, he will be wise to concentrate his effort at fewer doses and to avoid the extremes. An assay with 3 doses of each preparation corresponding to $P = 0.15—0.25$, $0.45—0.55$, $0.75—0.85$ may have twice the precision of one with the same total number of subjects distributed regularly over a much wider range of doses ; in other words, half as many subjects suffice to give an equally trustworthy estimate of relative potency. Another good design

is that with four doses of each preparation, chosen to have approximately $P = 0.2, 0.4, 0.6, 0.8$. Except for the rare situation in which the investigator prefers to use twice as many subjects rather than to embark on calculations such as those of *Tables III* or *VI*, he should not willingly design his assays so as to be suitable for SPEARMAN—KÄRBER. I have discussed this more fully elsewhere [11, Chapters 19 and 20].

I have made no previous mention of moving average methods of estimation [18, 19, 20]. These are closely allied both to SPEARMAN—KÄRBER and to simple interpolation, and share many of the advantages and faults of these. Despite THOMPSON's strong advocacy, I have difficulty in seeing that their slightly more laborious calculations yield estimates that are appreciable improvements on SPEARMAN–KÄRBER. They are of restricted applicability, as they require equally spaced doses, at least four for each preparation, and their efficiency depends to a major extent upon the span of the moving average in relation to the variability of the material, a factor that cannot often be predicted.

Any comprehensive discussion of the merits of the methods of analysis of quantal assay data must depend, implicitly or explicitly, upon the underlying mathematical model that truly represents the probability of response. Fortunately, the methods so far considered in this Section appear to be not particularly sensitive to the exact model, and what has been said of them is probably applicable for many alternative models within a wide range of those that are reasonably sensible. The first consideration in choosing between the methods of Section 7—11 ought of course to be the "correct" model, but unfortunately this is never known with certainty. Whatever theoretical argument may be raised in support of a Normal distribution of thresholds (leading to the use of the NED), a logistic probability of response (leading to the use of the logit), or some alternative, only exceedingly extensive records from an experiment could discriminate between them in the sense of showing one to be in accordance with the data and the others significantly rejected ; if such records were available, they would probably demonstrate *all* simple models to be false and show that a more complicated equation with additional parameters is needed! In practice, it seldom matters which model is adopted as a basis for the analysis. Clearly in *Table IX* the estimates and limits are for all practical purposes the same whether NED, logit, or angle is used.

A simple graphical method based upon one of these three transformations is comparable with SPEARMAN—KÄRBER for speed and is perhaps the easiest way of rapidly obtaining a value for $R$ close to the result of the eventual calculations, but it is open to objection on account of its subjectivity. Methods of combining a graphical approach with some simple calculations, in order to give a measure of precision, can be valuable in routine work, but their use should always be controlled by someone familiar with the principles and

practice of statistical analysis in bioassay, as a safeguard against gross errors or the uncritical use of graphical estimation when special circumstances make it inappropriate. The LITCHFIELD—WILCOXON method is an excellent example. Users should remember that the approximate standard errors obtained assume that the graphical fitting of regression lines has followed optimal procedures ; they make no allowance for the human errors and biases inherent in graphical analysis.

Both the NED and the logit systems have theoretical bases, but I scarcely think anyone would suggest that the angle transformation corresponds to any exact truth about the nature of the response mechanism. Because of the constancy of $w$, angle analysis can represent considerable economy of time when $n$ is constant at all doses, and its adoption may be defended as unlikely to give conclusions materially different from NED or logit. The finite range of the transformation ($0 \leq Y \leq 90$) gives some trouble when very extreme doses occur. I would not myself recommend the angle method, unless perhaps for series of assays in which $n$ was almost always constant and $p$ almost always lay between 0.1 and 0.9. I cannot think that it really matters whether the NED or the logit is preferred, unless there are circumstances making one of the two models seem *a priori* especially likely to approximate to reality.

Although BERKSON's minimum $\chi^2$ method of estimation has been illustrated only for logits, for which it is especially convenient, it can easily be adapted to the other transformations [4]. It is asymptotically the same as maximum likelihood, in the sense that the two must become more closely alike as all values of $n$ are increased. It has the advantage of requiring only one cycle of calculation, whereas in theory maximum likelihood calculations should be indefinitely iterated. On the other hand, the procedure for dealing with zero or total response ($p = 0$ or 1) is unsatisfactory, and the estimation maybe a little strange in behaviour for extreme rates of response other than 0 or 1. The advantage of not needing to iterate is easily exaggerated: good data, analysed by someone with experience, will seldom require more than one cycle of maximum likelihood calculation. The theory of statistical inference has advanced tremendously in the past 30 years, but there still remains great difficulty in discussing fully the performance of alternative principles of estimation in fairly small samples. The large sample merits of maximum likelihood are well-known, but it would be rash to assert that such estimates are always the best in relatively small series of observations dependent upon a complicated model. BERKSON, indeed, has produced extensive numerical evidence that his minimum $\chi^2$ method (with NED's or logits) is at least as good and perhaps slightly better. My own inclination and recommendation, with either transformation, is still to rely upon the well-tried maximum likelihood techniques, but I am well aware that a satisfactory theoretical study of the small sample precision and bias of the various estimators is greatly needed.

For the experienced computor, there is much to be said for organizing maximum likelihood iterative calculations so that each cycle gives a correction to be added to what has gone before, instead of being a repetition of the whole regression calculation. BERKSON [5] and others have illustrated this.

Not least of the advantages of all these later methods, relative to SPEARMAN—KÄRBER and others, is that they all provide tests of significance of deviations from the underlying assumptions of the assay, which appear as linearity and parallelism of the regression equations of the transformed proportionate responses upon the logarithms of the doses.

## 13. Summary

The main statistical characteristics of an immunological assay for comparing the potencies of two vaccines, by reference to the proportions of test animals protected from a standard challenge dose of infective organisms, have been briefly described. Many different computational procedures for statistical analysis and the estimation of relative potency have been suggested, and the chief of these are illustrated here on a particular example. Most of these methods are not now to be recommended, and some (such as the popular REED—MUENCH) ought to be abandoned.

For rapid approximate evaluation of potency, the SPEARMAN—KÄRBER method and various graphical techniques seem the most satisfactory. The former is especially good if the assay was conducted by test of equal numbers of subjects at many doses, these doses being equally spaced logarithmically with a narrow interval and, most important of all, spread over a wide range from certainty of response to certainty of no response. These conditions, however, are far from ideal in the planning of an assay if previous information enables effort to be restricted to a few doses closer to the level of 50% response, and SPEARMAN—KÄRBER is neither efficient nor entirely trustworthy as a method of analysis for a well-planned assay.

For general use, maximum likelihood estimation, using either the normal equivalent deviate or the logit transformation, appears to be the best choice. In practice, there is seldom any strong reason for preferring one to the other. A minimum $\chi^2$ method can be used as an alternative to maximum likelihood, but is perhaps a little less satisfactory as a standard routine.

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# PRACTICAL EXPERIENCES IN BIOLOGICAL TITRATION*

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(Received September 3, 1959)

## 1. Introduction

In the present report we wish to present a review of our work concerning biological assays, particularly of the results which are thought to have some general interest. Most of these results have been published. In the present review, however, the questions will be discussed in some more statistical detail.

## 2. Testing of disinfectants

*2.1.* The problem presented by the titration of disinfectants in a new form was rendered acute by the standardization of quaternary ammonium compounds. Manufacture of these new, highly efficient, disinfectants has been taken up by the Hungarian pharmaceutical industry and the compounds have been given trials in Hungary.

In connection with disinfectants, the necessity of biological standardization in addition to chemical control has long been taken into consideration. Chemical analysis of the active substance content does not suffice in itself there being numerous factors — such as solubility, surface tension, lowering of potency in proteinic media, and other less sharply definable properties — that may decisively influence the disinfectant's efficiency. The biological assay of disinfectants is justified further by the need to check their effects under the conditions they are put to use.

In the international literature on the biological assay of disinfectants, determination of the phenol coefficient is a generally accepted method. *Salmonella typhi* and *Staphylococcus aureus* serve as test organisms. Dilution series are prepared from both the disinfectant to be tested and the phenol used as standard ; in both series the concentration is established which does not kill the test organisms at room temperature, *i.e.* 20° C, in five minutes, but

* Read at the *Biometrical Symposium*, *Budapest*, September 7—9, 1959.

does in ten minutes. To compute the phenol coefficient, the efficient concentration of the preparation under investigation is divided by the corresponding phenol concentration.

For the titration of disinfectants the phenol coefficient has been employed also in Hungary. However, certain results drew attention to the inadequacy of this procedure and to the necessity of considering some important factors, such as the influence of temperature and changes in the concentration of the disinfectant. In some cases the failure of disinfection was ascribable to one of these two factors.

The determination of the phenol coefficient, though very much like the current methods of biological standardization does not meet a fundamental requirement of standardization, namely, that the preparations should not differ but in concentration. This is the principal reason why the phenol coefficient can provide but scanty information about the value of a disinfectant.

Accordingly, the simple comparative method of biological standardization cannot be applied in testing new disinfectants. This, of course, does not yet imply that the use of the phenol coefficient should be abandoned, since this value may still furnish some general information concerning the comparative efficiency of various disinfectants.

*2.2.* In the case of a new disinfectant, in addition to determining the phenol coefficient, the influence of temperature and of changes in concentration should be investigated. It was necessary to revert to the findings of CHICK, and CHICK and WATSON in order to elucidate these questions.

CHICK, investigating the effect of 6 per cent phenol on *Salmonella typhi*, found the following relation between temperature and the process of disinfection,

$$\frac{k'}{k} = \Theta^{(T'-T)}$$

where $k$ and $k'$ are the constants of the velocity of reaction at temperatures $T$ and $T'$, respectively, and $\Theta$ is the temperature constant characteristic of the disinfectant and the test organism. Such experiments must, of course, be performed with samples of equal concentration. The velocity constant is

$$k = \frac{1}{t} \log \frac{B}{b},$$

where $t$ stands for time of exposure,

$B$ for the number of living organisms at the beginning of exposure,

$b$ for the number of organisms at the end of exposure.

If $t$ is the time of efficient exposure, *i.e.* the shortest period needed to the sterilization of the preparation, the value of $b$ may be regarded as 1. In this case

$$k = \frac{1}{t} \log B .$$

If in parallel experiments carried out at different temperatures the initial number of germs agree, and efficient $t$ exposure time is a well-defined value, then

$$\frac{k'}{k} = \frac{t}{t'}$$

This consideration has made it possible to omit the time-consuming and hardly reproducible germ counting. Instead, the exposure time was made to vary with rather short intervals in the ordinate of time, and ten parallel examinations were performed for each exposure time at the same temperature.

The equation

$$\frac{t}{t'} = \Theta^{\ (T'-T)}$$

yields rectilinear regression on arith-log grid. Slope of the straight line is $-\lg \Theta$. Regression is computed in the usual manner. Standard deviation of exposure time was found to be independent of temperature, thus test of linearity can be performed by analysis of variance. Variance of the slope is also determined as usual.

In this Institute, DR. SZITA studied the effect of disinfectants, using *Salmonella typhi* as the test organism. His experimental findings agreed with CHICK's above relation in every case [1]. Value $\Theta$ is an important characteristic of disinfectants; the more its $\Theta$ value approximates unity, the more applicable the disinfectant. A high $\Theta$ value means that the disinfectant, effective at a given temperature, may fail to be effective at a slightly lower one.

*2.3.* The relation of the concentration and efficiency of a disinfectant have been elucidated by WATSON, on the basis of CHICK's original experiments carried out also with phenol. According to the CHICK—WATSON relation, the time of efficient exposure is in inverse ratio to the $n$th power of the disinfectant's concentration. The values of $n$ is characteristic of the disinfectant. Hence the relation is $t \cdot C^n = a$, where the time of efficient exposure is $t$, and concentration is $C$. It gives a log-log linear regression. In this Institute, DR. SZITA investigated the disinfectants used in Hungary [2] in this regard. *Salmonella typhi* was employed as test organism. To determine the effecient exposure time, ten parallel tests were made. The results were evaluated as described above. Linearity could be verified in every case, *i.e.* CHICK and WATSON's relation

has proved to be valid. The exponent $n$, the slope of the regression line, is an important characteristic of the disinfectant; the nearer approximates $n$ the unity, the more applicable, the disinfectant. A disinfectant with a high $n$ value may fail to exert any effect if, for some reason, a slightly lower concentration is applied.

*2.4.* The effect of disinfectants is usually tested on vegetative forms of microorganisms, and disinfectants are generally used to destroy the vegetative forms, not the spores, of bacteria. However, from the point of view of disinfection, this question is of considerable importance; a series of investigations into the sporicidal action of various disinfectants has therefore been carried out at this Institute. On the basis of these tests, from among the disinfectants used in Hungary only formalin and Neomagnol have proved to be sporicidal. Since, however, the sporicidal action of Neomagnol was found to depend on the pH, until elucidation of this aspect the tests had to be restricted to formalin. An excellent spore forming strain of *Cl. sordellii* was used as test organism. The investigations were intended to estimate the disinfectant's sporicidal characteristics regarding temperature and concentration [3, 4].

As expected, higher concentration of the disinfectant and longer times of exposure were required for killing spores than for killing vegetative forms. Statistical analysis — suitable for the described procedure — revealed that the relation between temperature and efficiency of a desinfectant on vegetative forms was valid also for spores, with the difference that the slope was somewhat steeper. Investigations into the relation of concentration and efficiency, however, failed to verify the sought log-log linear relation. The painstaining experiment, when repeated by DR. SZITA, led to the same result. Analysis of variance indicated a significant departure from linearity in every case. On the other hand, the regression of logconcentration on the exposure time gave a good fit for rectilinearity. Thus our findings failed to confirm the validity of the CHICK—WATSON relation in the case of spores.

There is no doubt that relations like those existing between temperature and efficiency on the one hand, and between concentration and efficiency, on the other, prevail only within certain limits. The validity of the first relation cannot extend beyond certain limits because over a certain temperature heat itself will kill microorganisms, while below a certain concentration no disinfecing action will take place. However, the discrepancies in the sporicidal effect of formalin are more general suggesting that the situation must be governed by other factors. In fact, in the case of spores the relation between formalin concentration and the efficient time of exposure might be expressed, instead of the CHICK—WATSON formula, by a simple log-linear regression complying with WEBER—FECHNER's law. Since further studies concerning this question have not yet been undertaken, our results cannot to be considered final.

By means of the procedures discussed we were able to establish for the biological testing of disinfectants a new system which in assays may furnish a good basis by the use of simple statistical methods. Elaboration of some aspects concerning the system of tests is in progress.

### 3. Animal experiments for the titration of biologicals

*3.1.* Animal experiments usually provide excellent information concerning the efficiency of a serum or vaccine. In the work of this Institute in standardizing serobacteriological preparations, animal experiments have always played an important role. The experimental models serving this purpose have undergone remarkable development in the past decades, considerably owing to advances in the statistical methods employed.

According to the original model of the active immunity test, all the animals in the experiment were given the same dose of the vaccine to be tested. After a certain time had elapsed, groups were formed of the immunized animals and challenged by the pathogenic agent ; the infecting dose varied in geometrical progression. At the same time control animals were also infected in the same manner. The minimum lethal dose was calculated for both the control and the immunized animals, in order to calculate the protection index. Later this procedure was modified, and instead of minimum lethal dose $LD_{50}$ was calculated. Obviously, this procedure has been meant to realize absolute assessment for determining the immunizing power of a preparation, by expressing the potency of a vaccine in terms reflecting the magnitude of the averted danger. To express the potency of typhoid vaccines some authors endeavoured to find an even more exact absolute standard for defining the immunizing potency of a serum or vaccine. An instance of such endeavours was the mouse unit proposed by the Hungarians Lovrekovich and Rauss, another the TIU, typhoid immunity unit, suggested by the American Luippold.

The question of the active mouse protection test for the titration of typhoid vaccine was tackled by ourselves when a new type of vaccine had to be standardized. The results obtained by the above-outlined model expressed the differences in the immunizing potency of the preparations inaccurately, and did not furnish reproducible results. This absolute standard, which had been intended to express the potency of a serum or vaccine, by talling against how many times the $LD_{50}$ the preparation induced protection, showed wide variations in repeated experiments. The cause of this was partly the difference in the technique of infection, and partly in the marked variability of tolerance shown by individual animals.

The incorrect indication by the old model of the differences between the immunizing capacities of the preparationsin test may be explained chiefly by the fact that the immunizing doses used were too large. The validity of the logarith-

mic relation between the dose administered and the degree of protection is limited : beyond certain limits protection will no longer increase but even tend downwards. Thus overdosage diminishes the differences existing between preparations.

*3.2.* In contrast to former concepts, modern methods of biological standardization are based on the fundamental idea of establishing the potency of the preparation to be tested in relation to some other preparation accepted as standard. Evidently, the fundamental requirement for the use of such a method is the standard preparation itself, which must be efficient and of constant value. In constructing a new model for the active protection test for typhoid vaccine, the first task consisted of providing a standard preparation. DR. TOLNAI succeeded in preparing such a standard. Furthermore, the construction of the new model was opposite to the old one the vaccine doses being graded and the challenge dose constant. This model has provided reproducible results applicable to statistical evaluation [5, 6, 7].

Construction of such a model is far from being easy. Apart from having to secure an appropriate standard preparation, the doses applied for immunization and challenge require careful adjustment. In the case under review, it was another difficulty to carry out infection under standard circumstances. Co-ordination of all the factors required a great many experiments. In this work statistical control played a very important role.

A common requirement for the use of methods of statistical analysis is that the actual distribution of the investigated phenomenon should be complied by the statistical assumption. In this case the process could be reversed. Relying on data of foreign authors, the lognormal tolerance distribution was taken for granted ; we had been, therefore, improving the experimental procedure until a lognormal distribution was, in fact, obtained. Thus the statistical model offered information concerning the adequacy of experimental conditions. Such methods have been several times resorted to at this Institute. The experimental model for testing the efficiency of contact insecticides has been developed on this basis by DR. SZTANKAY at the Department of Parasitology of this Institute [8] although graded intoxication of live insects with contact insecticides is even more tiresome than, for instance, inoculation of mice with typhoid bacteria.

#### 4. Epidemiological investigation of potency of sera and vaccines

*4.1.* The efficiency of biological preparations is ultimately decided by their actual use in man, hence the last word cannot come from the laboratory tests for safety and potency. This must be done by epidemiological investigations. The principles of a field trial of serobacteriological preparations have been

carefully elaborated, but its practical realization involves extensive organization, considerable apparatus, and, consequently, high expenses. In addition, one cannot always guarantee appropriate conditions for such investigations, therefore the results of a field trial are often questionable. The effects of a vaccine must be kept under constant control even when its use has been extended to the whole exposed population. In such cases, evaluation of the effect cannot be carried out forthwith ; it mostly requires long observation and highly specified procedures. In the following I shall give a brief survey on pertussis vaccination in Hungary.

*4.2.* As a result of several years' work, DR. UJHELYI, chief of the Vaccine Research Department of this Institute, had developed an alum-adsorbed vaccine against whooping-cough. After conclusion of the laboratory tests, the vaccine was given a field trial in Budapest, and from 1953 it has been included among the compulsory vaccinations.

For the field trial the above vaccine was combined with the diphtheria vaccine then used for compulsory vaccination. (The combined vaccine had also been prepared by DR. UJHELYI.) In April and May, 1952, the infants born between April and September, 1951, were vaccinated with two inoculations. This half age-group comprised 15 853 infants; of these 11 781, *i.e.* 74.3 per cent, received two inoculations, as prescribed ; 1291, *i.e.* 8.1 per cent, received only one inoculation, and 2781, *i.e.* 17.6 per cent, for some reason or other, were not vaccinated. This kind of field trial is certainly objectionable. In a correctly conducted study the groups of vaccinated and untreated cases should have comprised an approximately equal number of subjects, selection should have been random, and to the controls a placebo should have been administered. In contrast, the infants in the control group were those whose parents had omitted to take their children to the announced vaccination, either from sheer carelessness or because for some reason inoculation had been contraindicated. The group of those who had one inoculation was formed by infants whose parents had missed the first or second inoculation.

After vaccination, the incidence of whooping-cough among the children involved was observed from June, 1952, until April, 1953. Reports on pertussis cases among the children under review were checked by name in the vaccination records ; moreover, control was in most cases completed by the visiting district nurse. During the whole time, 696 cases of whooping-cough occurred in the age group involved ; 187 had been inoculated twice, 122 once, and 387 belonged to the unvaccinated group. In the three groups, the morbidity per 1000 amounted to 1.44, 8.59, and 12.65, respectively. On this basis it was stated that the morbidity among the twice inoculated children amounted only to 11.4 per cent of the rate among unvaccinated ones ; the protection induced by the vaccination was thus estimated to about 90 per cent.

*4.3.* In 1953, vaccination against whooping-cough was made compulsory. The use of a combined diphtheria—pertussis—tetanus vaccine was introduced, with UJHELYI's vaccine as the pertussis component. The inoculations were started in April and May, 1953. Infants 6 to 12 months of age were vaccinated with two inoculations administered at an interval of one month. In September and October of the same year infants in their second half year and children in their fourth half year were immunized in the same manner. Since 1954, the schedule of combined vaccination has been as follows. In April and May the 6 to 11 months old infants are immunized by two inoculations at a four-week interval, and the 18 to 23 months old children are revaccinated with one inoculation. In September and October the groups that in the meantime had completed their half-year age are immunized and also the children six years of age, entering school, are given two inoculations at an interval of four weeks.

The compulsory vaccination of 1954 appeared to produce very slight results ; even doubts began to arise whether it had any effect. In addition, there was a striking discrepancy. While the 1952 field trial that had involved 74 per cent of a half age group in Budapest showed about 90 per cent protection, the incidence of whooping-cough in the same age group did not decrease as compared with the unvaccinated age groups. There was also some suspicion that the inoculation lists were inaccurately checked by the district nurses so that vaccinated infants who had contracted the disease were included in the unvaccinated group. Vaccination was nevertheless continued in 1955, and a new evaluation was initiated in possession of the 1955 data.

*4.4.* If a whole population has been vaccinated, there is no possibility for a precise statistical analysis of epidemiological data. Though compulsory vaccination can never be executed completely, *i.e.* there are always persons to form an unvaccinated group, utilization of such group as a control is practically unfeasible, in view of deficient recording. Thus, comparison of the number of pertussis cases in the light of the register was, in the actual case, impossible. The only way of evaluation was to compare morbidity in the vaccinated group with that in the unvaccinated group. However, when this method is used, it has to be taken into account that morbidity depends on age, so that it will vary in various age groups even where no immunization had taken place. The epidemiological data of the above-mentioned years have been examined with regard to these factors. The changes in the morbidity of the vaccinated age groups were compared with those of the unvaccinated four to six year old children, with due consideration to the differences that had existed in the morbidity of these age groups. In the course of these studies the adaptation of BECKER's plotting method proved to be useful in surveying the inoculations performed [9].

As evidenced by the investigations outlined above, the country-wide vaccination exerted a definite effect. It reduced the morbidity of the eligible children by about 50 per cent. The actual effect must have been more since a certain proportion of eligible children had escaped vaccination. It is noteworthy that no effect of vaccination could be demonstrated in the group of children aged 6 to 7 years. According to DR. ERDŐS' explanation, the amount of antigen per body weight was too little to induce immunity.

*4.5.* In connection with the above work the results of the experimental vaccination carried out by DRS. UJHELYI and FERENCZY in Budapest in 1952 [10] were re-examined. Their records had shown that morbidity among the twice inoculated children was more than 83 per cent, less than in the group of children inoculated only once, thus in a group other than the unvaccinated one. This fact has resolved the suspicion that the data of vaccinated children might have been falsely classed as those of unvaccinated subjects.

It has been established, further, that the vaccinated half year group in 1952 and 1953 — assuming an even distribution of births — must have constituted only 4 to 18.5 per cent of the 0 to 3 year old population; consequently, for the whole population of Budapest, morbidity should have been reduced by no more than 4 to 16 per cent in the involved age groups. The fact that even such a decrease did not ensue, may be explained by two circumstances. One is that the relative morbidity of various age groups changes with time ; the other, that notification of whooping-cough has never been complete. In conjunction with the experimental vaccination, however, attention of the Budapest physicians was called to the importance of accurate notification. The results of this warning might have negatively biased the reduction in incidence due to vaccination. The final analysis showed that vaccination against pertussis had been effective. The question is nevertheless kept open, and further investigations are in progress, also with another type of vaccine.

### 5. Tests for sensitivity of pathogenic bacteria to antibiotics

*5.1.* It is the general experience that bacteria may become resistant to the antibiotics in common use. Since the year 1953, at the Department of Bacteriology of this Institute the antibiograms of bacteria isolated from the routine material at the Department have been determined with regard to the antibiotics in hospital-use in Hungary. In this antibiogram the strains are distributed in 3 categories ; "sensitive", "moderately sensitive" and "resistant" strains are distinguished. As to the bacteria isolated in the period 1953—1956, it was found that a certain shift had occurred in the bacterial flora itself: the relative incidence of some pathogenic bacteria had increased.

It was furthermore demonstrated that during the period of observation the resistance of Staphylococcus strains to penicillin, of Pneumococcus strains to streptomycin, and *E. coli* and *Ps. pyocyanea* strains to chloramphenicol had considerably increased. Finally, a well-defined positive correlation has been demonstrated between the resistance of the individual strains to various antibiotics [11].

In the above-outlined work bacteria were tested with the antibiotics in hospital-use. When the work was initiated, in 1953, there were only penicillin, streptomycin, and chloramphenicol is use, to which terramycin was added in 1956. For the whole period, however, only the findings concerning the first three antibiotics were available, hence the change in sensitivity could be evaluated only in relation to these. As mentioned above, the sensitivity to antibiotics of Staphylococcus, Pneumococcus, *E. coli* and *Ps. pyocyanea* strains had changed from 1953 to 1956. Since, however, the number of *Ps. pyocyanea* and Pneumococcus strains was comparatively small, and Pneumococcus strains did not show notable resistance, but to streptomycin, we shall only deal with the data concerning Staphylococcus and *E. coli.*

In Tables I and II we present the distribution, according to antibiotic sensitivity, of 2481 Staphylococcus and 2384 *E. coli* strains. To demonstrate the distribution of Staphylococcus strains more or less sensitive to each of the three antibiotics, *Table I* is divided into 3 parts each of which demonstrates distribution by sensitivity to two antibiotics. Since all the *E. coli* strains were resistant to penicillin, their resistance to streptomycin and chloramphenicol was only compared.

Classification of bacterium strains into "sensitive", "moderately sensitive" and "resistant" groups was based on experiments in which the bacterium had been exposed to varying concentrations of antibiotic.

When evaluating the above data, change in sensitivity to antibiotics was demonstrated by the differences in the percentual distribution of strains ; the correlations between the sensitivity of individual strains to various antibiotics were investigated with the $\chi^2$ test. However, this method does not provide any information as regards the degree of correlation. For this purpose the correlation coefficient should be employed. For computation of this parameter, the grades were scaled as follows: the "sensitive" group was marked $-1$, the "moderately sensitive" 0 ; and the "resistant" $+1$. Since the grades represent quantitatively differing groups, this transformation is justified though to some extent arbitrary. At any rate, it suits our purpose.

By the aid of this transformation, the sensitivity to antibiotics of any strain can be expressed in terms of an arithmetic mean ; and the correlation coefficients can be determined. The results of the computation are summarized in *Table III.*

**Table I**

*Sensitivity to antibiotics of the Staphylococcus strains isolated in the period 1953—1956*

| Sensitivity to | | 1953 | 1954 | 1955 | 1956 | 1953—1956 |
|---|---|---|---|---|---|---|
| penicillin | streptomycin | | | | | |
| S | S | 62 | 104 | 182 | 109 | 457 |
| | M | 4 | 18 | 32 | 19 | 73 |
| | R | — | — | 15 | 6 | 21 |
| M | S | 62 | 69 | 80 | 33 | 244 |
| | M | 5 | 9 | 7 | 4 | 25 |
| | R | 15 | 15 | 12 | 5 | 47 |
| R | S | 60 | 189 | 452 | 277 | 978 |
| | M | 13 | 39 | 99 | 45 | 196 |
| | R | 42 | 98 | 180 | 120 | 440 |
| penicillin | chloramphenicol | | | | | |
| S | S | 64 | 121 | 219 | 129 | 533 |
| | M | 2 | 1 | 9 | 5 | 17 |
| | R | — | — | 1 | — | 1 |
| M | S | 67 | 83 | 92 | 36 | 278 |
| | M | 13 | 8 | 5 | 3 | 29 |
| | R | 2 | 2 | 2 | 3 | 9 |
| R | S | 85 | 274 | 598 | 341 | 1298 |
| | M | 20 | 40 | 105 | 54 | 219 |
| | R | 10 | 12 | 28 | 47 | 97 |
| streptomycin | chloramphenicol | | | | | |
| S | S | 172 | 355 | 685 | 394 | 1606 |
| | M | 12 | 5 | 26 | 21 | 64 |
| | R | — | 2 | 3 | 4 | 9 |
| M | S | 16 | 59 | 126 | 59 | 260 |
| | M | 5 | 6 | 12 | 6 | 29 |
| | R | 1 | 1 | — | 3 | 5 |
| R | S | 28 | 64 | 98 | 53 | 243 |
| | M | 18 | 38 | 81 | 35 | 172 |
| | R | 11 | 11 | 28 | 43 | 93 |
| | Totals | 263 | 541 | 1059 | 618 | 2481 |

S = sensitive, M = moderately sensitive, R = resistant

**Table II**

*Sensitivity to antibiotics of the E. coli strains isolated in the period 1953—1956*

| Sensitivity to streptomycin | Sensitivity to chloramphenicol | 1953 | 1954 | 1955 | 1956 | 1953—56 |
|---|---|---|---|---|---|---|
| S | S | 185 | 219 | 473 | 340 | 1217 |
| | M | 8 | 5 | 10 | 11 | 34 |
| | R | 1 | 1 | 4 | 10 | 16 |
| M | S | 19 | 33 | 55 | 63 | 170 |
| | M | 2 | 2 | 9 | 10 | 23 |
| | R | 1 | — | 5 | 9 | 15 |
| R | S | 96 | 139 | 200 | 92 | 527 |
| | M | 44 | 26 | 68 | 7 | 145 |
| | R | 15 | 26 | 26 | 170 | 237 |
| | Totals | 371 | 451 | 850 | 712 | 2384 |

S = sensitive, M = moderately sensitive, R = resistant.

**Table III**

*Parameters referring to the sensitivity to antibiotics of the bacteria under review*

Transformation : S = —1 ; M = 0 ; R = 1

| Bacterium, index | 1953 | 1954 | 1955 | 1956 | 1953—56 altogether |
|---|---|---|---|---|---|
| *Staphylococcus* | | | | | |
| a) average sensitivity to : | | | | | |
| penicillin, $\bar{x}_1$ | 0.19 | 0.38 | 0.47 | 0.50 | 0.43 |
| streptomycin, $\bar{x}_2$ | —0.48 | —0.46 | —0.48 | —0.47 | —0.47 |
| chloramphenicol, $\bar{x}_3$ | —0.78 | —0.86 | —0.83 | —0.74 | —0.81 |
| b) correlation coefficients : | | | | | |
| $r_{12}$ | 0.385 | 0.221 | 0.193 | 0.214 | 0.239 |
| $r_{13}$ | 0.247 | 0.182 | 0.164 | 0.204 | 0.186 |
| $r_{23}$ | 0.505 | 0.470 | 0.505 | 0.554 | 0.502 |
| *E. coli* | | | | | |
| a) average sensitivity to : | | | | | |
| streptomycin, $\bar{x}_2$ | —0.11 | —0.08 | —0.23 | —0.13 | —0.15 |
| chloramphenicol, $\bar{x}_3$ | —0.76 | —0.81 | —0.82 | —0.43 | —0.69 |
| b) correlation coefficient : | | | | | |
| $r_{23}$ | 0.392 | 0.342 | 0.360 | 0.631 | 0.446 |

As shown in *Table III,* resistance of Staphylococcus strains to penicillin greatly increased in the period from 1953 through 1956, while their sensitivity to streptomycin and chloramphenicol remained unchanged. As regards their

sensitivity to various antibiotics, correlations were definitely positive. In the combination of streptomycin and chloramphenicol, this correlation was nearly constant during the four-year period, whereas the correlations of sensitivity to penicillin with that to either of the two antibiotics was markedly decreasing.

Though there occurred some minor fluctuations, the sensitivity of *E. coli* strains to streptomycin did not change during the period of investigation ; their resistance to chloramphenicol, on the other hand, showed a marked increase in 1956. Until the year 1955, the correlation coefficient had displayed practically no change. In 1956, however, it grew suddenly — simultaneously with the increase in resistance to chloramphenicol.

*5.2.* Resistance of pathogenic bacteria to antibiotics is one of the most important and most extensively studied problems of modern medicine, this being the main factor determining the further use of some wide-spectrum preparations. The question is far from being elucidated. Its great importance has drawn our attention to the statistical problems resulting from the above-discussed facts.

Sensitivity of strains of the same species to different antibiotics shows positive correlations. From the correlations regressions can be established. These regressions, however, seem to be fictious and, unlike most regressions, have no direct consequences. It has been found namely that, in general, a rise in the resistance of a bacterium to one antibiotic is not accompanied by such a change in sensitivity to another. Thus the correlation coefficient is subject to changes making regression fictious.

This fortunately means that any antibiotic that has become ineffective in the practice may be replaced by new ones. Biologically, the phenomenon finds an adequate explanation if we assume the existence of a common element in resistance to various antibiotics (perhaps the general capacity of bacteria for developing resistance), and, also, that each antibiotic demands specific adaptation by the pathogenic strain. In the background of both the general element and the factor that brings about resistance to one special antibiotic, there are probably not one, but several — may be variable numbers of — actual causes.

Obviously, the phenomenon should be investigated during its development, for which, unfortunately, no appropriate material is available. Introduction of the time factor as a variable — a step often most instrumental, *e.g.* in the study of social phenomena — is here of no avail, considering that the development of resistance depends not on time generally, but on the special time when the bacterium meets the antibiotic. A final elucidation of these questions requires adequately planned experiments.

The existence of correlations in the sensitivity of bacteria to various antibiotics — as confirmed by the data presented — and the absence of cross-

resistances that might be expected to result on the basis of the correlations, point to the role of a general factor in the development of resistance. This disposition may be an original property of the strain. It is, however, equally possible that development of resistance to one antibiotic gives rise to a general element indespensable for every kind of resistance ; the existence of this property can be detected only during the development of resistance. This peculiar correlation, which lacks the element of regression might probably conceal a factor that by itself does not cause resistance to other antibiotics, but accelerates the process of its development. VÁCZI's current studies strongly support the latter assumption.

## 6. Summary

The phenol coefficient has proved in itself insufficient for establishing the efficiency of disinfectants, since the various disinfectants differ in many a relevant property. By means of simple methods, the heat and concentration constants were also determined. As regards the effect of formalin on bacterial spores, the CHICK—WATSON relation could not be confirmed. Instead, a log-arith rectilinear regression of the time of efficient exposure on the concentration has been established, corresponding to WEBER—FECHNER's law.

In the titration of biological preparations, experimental models based on the principles of standardization are more correct indicators of immunity than the methods formerly employed. In the elaboration of new models, the criterion of the adequacy of experimental conditions was a lognormal distribution.

Next, an outline of the epidemiological trial of pertussis vaccine in Hungary and a brief account of our studies concerning the efficacy of compulsory vaccination against whooping-cough has been given. Some of the difficulties which frequently prevent the reliable evaluation of serobacterial preparations by epidemiological investigations have been outlined.

Statistical analysis of data concerning sensitivity to antibiotics revealed no cross-resistance among the bacterial strains obtained from material submitted for routine tests to the State Institute of Hygiene during the years 1953 to 1956. This observation is in agreement with data in the pertaining literature. Nevertheless, a correlation has been found to exist in the sensitivity of pathogenic bacteria to various antibiotics. A change in sensitivity to one antibiotic is not necessarily accompanied by a change in sensitivity to another antibiotic, only by a change in the correlation coefficient. This specific correlation seems to point to the existence of some dispositional factor which might be a general element of every form of resistance, without, however, being capable of arousing it alone. This factor presumably promotes development of resistance against other antibiotics.

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# ACTA MICROBIOLOGICA

## ТОМ VI.

## РЕЗЮМЕ

### МАЛОНОВАЯ КИСЛОТА, КАК ЕДИНСТВЕННЫЙ ИСТОЧНИК УГЛЕРОДА И ЭНЕРГИИ В СЛУЧАЕ БОЛЕЗНЕТВОРНЫХ ГРИБОВ

Е. К. НОВАК

В своих опытах автор исследовал способность отдельных патогенных и непатогенных видов грибов использовать соль малоновой кислоты.

Было установлено, что 20 из 24 исследованных видов грибов были способны использовать малоновую кислоту в качестве единственного источника углерода и энергии.

Среди отдельных видов одного и того же рода наблюдаются отклонения в употреблении малоновой кислоты. Данное отклонение может служить основой для их дифференциации.

Согласно проведенным опытам, употребление соли малоновой кислоты осуществляется в случае вида ***Candida albicans*** другим путем, чем это было выявлено раньше в случае вида ***Botryotinia fuckeliana***.

Разработанная питательная среда с малоновой кислотой пригодна в медицинской микологии для обработки встречающихся в повседневной работе веществ, ибо бактерии на этой питательной среде трудно развиваются и следовательно они не загрязняют культуры грибов.

### КОЛИЧЕСТВЕННЫЙ ПОЛУМИКРОМЕТОД КУЛЬТИВИРОВАНИЯ ТКАНЕЙ И ЕГО ПРИМЕНЕНИЕ В МИКРОБИОЛОГИИ

II. Титрование вируса болезни Ауйеского при помощи полумикрометода культивирования тканей

Ш. ХОРВАТ

Автор исследовал при помощи полумикрометода культивирования тканей условия титрования вируса болезни Ауйеского, как и возможности нейтрализационной пробы. В ходе опытов было установлено, что титрование вирусов можно проводить при лучших условиях в тканевой культуре, чем на животных. В случае нейтрализации вируса болезни Ауйеского рекомендуется в целях достижения меньших возможностей девиации исчислять нейтрализационный титр вместо 100 $\mathbf{ID_{50}}$ (инфективная доза) в 1000 $\mathbf{ID_{50}}$.

### КОЛИЧЕСТВЕННЫЙ ПОЛУМИКРОМЕТОД КУЛЬТИВИРОВАНИЯ ТКАНЕЙ И ЕГО ПРИМЕНЕНИЕ В МИКРОБИОЛОГИИ

III. Исследование действия различных химических веществ и токсинов палочки газового отека на фибробласты сердца цыплят с помощью полумикрометода культивирования тканей

Ш. ХОРВАТ

Разработанный автором количественный полумикрометод культивирования тканей оказался пригодным для определения цитотоксического действия некоторых химических веществ (кристалл-фиолет, бриллиантовая зелень, сулема) и токсинов ***Bac. oedematiens***, ***Bac. perfringens***, как и ***Vibrion septique***.

## НЕКОТОРЫЕ БИОЛОГИЧЕСКИЕ СВОЙСТВА ВЕНГЕРСКОГО ШТАММА ВИРУСА КЛЕЩЕВОГО ЭНЦЕФАЛИТА

### Адаптирование вируса к эмбрионированному яйцу и к тканевой культуре зародышей цыплят

Е. МОЛЬНАР

1. Адаптированный к мозгу мышей штамм вируса с обозначением $\mathbf{KEm_1}$ вызывает в зародышах цыплят характерное изменение, которое можно нейтрализовать гомологичной иммунной сывороткой.

2. Вирулентнось на мыши штамма вируса $\mathbf{KEm_1}$, адаптированного к зародышам цыплят, не изменялась во время 20 пассажей; одновременно же вирулентность в отношении желточного пузыря зародышей цыплят значительно повышалась. К полости аллантоиса вирус трудно адаптируется.

3. Штамм вируса $\mathbf{KEm_1}$ можно размножить и проводить серийный пассаж на культуры тканей зародышей цыплят типа Майтланда, далее на культуры человеческих зародышевых тканевых кусков кожной мышцы, изготовленные методом Эндерса и сотрудников.

4. Вирусный штамм $\mathbf{KEm_1}$ постепенно утрачивает в ходе серийного пассажа на тканевые культуры зародышей цыплят свою вирулентность на мыши, в то же время он сохраняет свою убивающую способность по отношению к зародышам цыплят.

5. Иммунная сыворотка кроликов, полученная при помощи адаптированного к тканевой культуре зародышей цыплят штамма $\mathbf{KEm_1}$ защищает зародышей цыплят, или же мышей против вируса, адаптированного к мозгу мышей.

6. Адаптированный к тканевой культуре зародышей цыплят авирулентный в отношении мышей вариант интерферирует в мозге мышей с адаптированным к мозгу мышей вирусом $\mathbf{KEm_1}$.

## ВЫЗВАННАЯ ПИЛОКАРПИНОМ И ХОЛОДНОЙ ВОДОЙ «ПОДОБНАЯ АНАФИЛАКСИИ» РЕАКЦИЯ

Е. БЕРЕГИ

После 1—4-кратной подачи пилокарпина кроликам, сенсибилизированным нормальной лошадиной сывороткой, и купания животных в холодной воде, у них развивался шок, который по клиническим, как и патологоанатомическим признакам, был подобным анафилаксии. Если же сенсибилизированные животные получали только пилокарпин, или же их только купали в холодной воде, или опыты проводились без сенсибилизации, то никогда не развивались подобные симптомы и патологоанатомические изменения. Предварительной обработкой животных атропином удалось препятствовать появлению подобному анафилаксии шоку, в то время как наркоз евипан-эфиром не имел такого эффекта. Подобным методом удалось вызвать у морских свинок клинически удлиненный шок, а патологоанатомически, соответствующие анафилаксическому шоку изменения. У крыс таким методом не удалось вызывать подобный анафилаксии шок.

## ДЕЙСТВИЕ МЕТИЛЕНОВОЙ СИНЬКИ НА ЧУВСТВИТЕЛЬНОСТЬ К ЖЕЛЕЗУ ФЕРМЕНТАЦИИ STREPTOMYCES RIMOSUS

И. ХОРВАТ, К. МАДЬАР и И. ГАДО

Авторы выявили, что токсическому действию железа на ферментацию ***Streptomyces rimosus*** можно препятствовать примешиванием 30 $\mu$г/мл метиленовой синьки в питательную среду.

# ЗНАЧЕНИЕ ИНТЕРВАЛА МЕЖДУ ОТДЕЛЬНЫМИ ПРИВИВКАМИ В СЛУЧАЕ ПРИВИВОК, ПРОВЕДЕННЫХ ПОЛИВАЛЕНТНОЙ ВАКЦИНОЙ ПРОТИВ ДИЗЕНТЕРИИ И КОМБИНИРОВАННОЙ ТИФО-ДИЗЕНТЕРИЙНО-СТОЛБНЯЧНОЙ ВАКЦИНОЙ

К. РАУШ, И. КЕТЬИ, Л. РЕТИ и И. ЙОО

1. В связи с активной иммунизацией мышей, применяемый после интервала в 2 недели, второй стимул более сигнификантно повысил иммунность, чем стимул, применяемый после интервала в 1 неделю, но он не отличался сигнификантно от иммунности, достигнутой при прививках, проведенных интервалами в 4 недели в случае неадсорбированных дизентерийных вытяжек Бойвина.

2. Согласно исследованиям на людях с дизентерийной поливалентной вакциной, адсорбированной к квасцовому осадку, вакцинация в 4-недельных интервалах привела к сигнификантно лучшему повышению защитного титра мышей, чем применяемая в интервалах в 2 недели вакцина. Защитный титр сыворотки людей, привитых в интервалах в 8 недель, не показал значительных статистических отклонений по сравнению с защитной способностью людей, привитых в 4-недельные интервалы.

3. Компоненты Шига комбинированных вакцин, адсорбированных к гелю $Al(OH)_3$ обладали при повышении интервала прививок от 3 до 6 недель сигнификантно более высокой иммуногенностью, в то время как повышение интервала прививок от 4 до 6 недель уже не имело такого выраженного результата. На иммуногенность тифозного компонента изменение интервала не имело сигнификантного действия.

4. Столбнячный компонент тифо-дизентерийно-столбнячной вакцины дает сигнификантно более высокий средний титр антитоксина, если повысить интервал прививок от 4 до 6, или до 8 недель, причем в то же время устраняется группа с низким иммунитетом.

5. Авторы на основе имеющихся данных обсуждают возможности стойкости иммунитета.

# ИССЛЕДОВАНИЕ СПОРОЦИДНОГО ДЕЙСТВИЯ НЕКОТОРЫХ ДЕЗИНФИЦИРУЮЩИХ СРЕДСТВ

Й. СИТА и Г. БАРШИ

Авторы исследовали спороцидное действие некоторых имеющихся в продаже в Венгрии дезинфицирующих средств.

1. Среди исследованных дезинфицирующих средств совершенно не удалось в течение 24 часов при 20° С выявить спороцидного действия стерогенола, фамосепта и сулемы.

2. Фенол + 12% **NaCl** показал в случае ***Cl. Sporogenes*** и ***B. anthracis*** селективное спороцидное действие, а на остальные споры он оказался при 20° С неэффективным.

3. 2%-ый неомагнол и 5%-ый формалин имеют выраженное спороцидное действие. В случае неомагнола данное действие зависит от величины pH ; в целях выяснения этого действия были поставлены специальные эксперименты, которые еще не закончены.

# ЗАВИСИМОСТЬ БАКТЕРИЦИДНОГО ИЛИ ЖЕ СПОРОЦИДНОГО ДЕЙСТВИЯ ФОРМАЛИНА ОТ ИЗМЕНЕНИЙ ТЕМПЕРАТУРЫ И КОНЦЕНТРАЦИИ

Й. СИТА и Г. БАРШИ

Авторы исследовали и сопоставляли те взаимосвязи, которые наблюдаются между бактерицидным и спороцидным действиями формалина и изменениями температуры и концентрации.

1. В случае формалина изменение температуры меняет спороцидное действие при тех же закономерностях, которые действительны в общем для всех дезинфицирующих средств в случае вегетативных форм. Температурный коэффициент формалина в отношении спор ***Cl. sordellii*** в два раза больше, чем температурный коэффициент этого же дезинфицирующего вещества в отношении ***S. typhi.*** Это означает, что изменение температуры имеет большее влияние на спорцидное, чем на бактерицидное действие формалина.

2. В отношении зависимости спороцидного действия от изменения концентрации формалина не удалось доказать зависимости *Чик-Уатсона*, а вместо ней авторы установили простую линейную зависимость. Это означает, что в случае повышения концентрации, согласно кратной прогрессии, время воздействия уменьшается линейно.

## ВОДНАЯ ЭПИДЕМИЯ, ВЫЗВАННАЯ СЕРОТИПОМ ESCHERICHIA COLI 124 : 72 : 32

Б. ЛАНЬИ, Й. СИТА, Б. РИНГЕЛХАНН и К. КОВАЧ

1. Авторы описывают эпидемию водного происхождения, сопровожденную 255 случаями воспаления слизистой оболочки кишок. Болезнетворный агент они изолировали из кала больных.
2. Вызывающим эпидемию штаммом оказался серотип *E. coli* 124 : 72 : 32.
3. На основе анализа антигена штамм оказался серологически идентичным с изолированным Юингом (*Ewing*) штаммом 227.
4. В короткое время это уже третья эпидемия водного происхождения в Венгрии, вызванная штаммом *E. coli* 0 : 124.
5. Вызванная этим штаммом подобная дизентерии картина болезни делает необходимым в случае его выделения принимать такие же санитарно-противоэпидемиологические меры, как при дизентерии и салмонеллёза.

## ЦИТОМОРФОЛОГИЧЕСКИЕ ИЗМЕНЕНИЯ В ТКАНЕВЫХ КУЛЬТУРАХ ЧЕЛОВЕЧЕСКИХ ЗАРОДЫШЕВЫХ ПОЧЕК, ЗАРАЖЕННЫХ ШТАММАМИ АДЕНОВИРУСА ТИПА 5

И. НАС и М. ТОТ

Авторы исследовали на изготовленных методом трипсинизации тканевых культурах человеческих зародышевых почек дегенеративные изменения, вызванные в отдельных клетках штаммами, относящимися к аденовирусу типа 5, как и штаммами аденовируса, изолированными ими раньше.

Обнаруженные изменения находились — в полном согласии с литературными данными — прежде всего в ядре зараженных клеток. В одной части изменений кажется на основе хронологического появления и постепенного образования типичных морфологических форм обоснованным предположение определенного процесса развития. Сперва, по истечении прибл. 16—30 часов после заражения, появляется эозинофильная зернистость клеточных ядер, которая диффузно распределяется в ядре, причем около ядрышек часто наблюдается поредение, или полное отсутствие зернистости. Уже эта стадия хорошо отличима от картины, наблюдаемой в ядре контрольных клеток. В отдельных случаях зернистость появляется в форме более или менее больших, круглых, или овальных, скоплений зернышек, а у одной части скоплений наблюдается круговая структура. Зараженные клеточные ядра в большинстве случаев сильно набухают. 30—45 часов после заражения зернышки ориентируются в пучки. В этой стадии еще видно, что пучки состоят из зернышек, а позже, после 40—50 часов зернышки все больше уплотняются в центре клеточного ядра, пока еще в продольной, или пучковой форме. У одной части еще видно, что центральный пучек тоже зернистой структуры и можно различать также и ядрышко. Окрашивание по большей части еще эозинофильное, но встречается уже много базофильных образований. 50—60 часов после заражения зернистые пучки все более скопляются в центре ядер в виде центральной массы, причем их эозинофильное окрашивание превращается в базофильное. Находящаяся вне центральной массы часть клеточных ядер превращается в стекловидный, гладкий, блестящий фон. В дальнейшем наступает уменьшение плазмы, сморщивание всей клетки, усиливается базофильное окрашивание. Наконец вся клетка округляется, становится сильно базофильной, и в окрашенном препарате больше нельзя различать отдельных компонентов.

Авторы наблюдали несколько таких изменений, которых они не могли включить в предположенный ход дегенеративного процесса. Они обнаружили, например, такие клеточные ядра, которые содержали в большем, или меньшем количестве эозинофильных телец, величиной гораздо больше зернышек, и окруженные иногда базофильным кольцом. Встречались также такие клеточные ядра, которые содержали 2—3 палочковидных, кристаллоидных образований.

## ИССЛЕДОВАНИЕ ИММУННОГО ЭФФЕКТА ДИЗЕНТЕРИЙНОГО (ШИГА) И СТОЛБНЯЧНОГО АНТИГЕНОВ, КОМБИНИРОВАННЫХ С АНТИГЕНАМИ «НАПОМИНАЮЩЕГО ДЕЙСТВИЯ»

Л. РЕТИ, К. РАУШ, И. КЕТИ и Я. МАРОЦИ

1. Авторы исследовали эффект «Барра» в связи с прививками тифо-дизентерийно-столбнячного и дифтерийно-столбнячного-коклюшно-дизентерийного вакцин детям, предварительно не получившим тифо-столбнячных, далее дифтерийных и дифтерийно-столбнячно-коклюшных прививок.

2. Иммуногенность «напоминающего действия» антигенов дизентерии Шига у детей, обладающих основным иммунитетом в отношении тифа и столбняка, или дифтерии, столбняка и коклюша статистически не показала более низких величин чем у детей, предварительно не иммунизированных.

3. Иммунный ответ токсоида, комбинированного антигеном тифа, в случае основного иммунитета против тифа, не отличается статистически сигнификантно от иммунитета, вызванного у предварительно не привитых против тифа лиц.

4. Согласно авторам примененные вакцины, благодаря правильному выбору соотношения между антигеном и адсорбентом, обеспечивают максимальный иммунитет антигенов. Авторы приписывают этому обстоятельству, что в их экспериментальных условиях вообще не состоялся феномена Барра.

## ДЕЙСТВИЕ АНТИБИОТИКОВ НА SALMONELLA TYPHI В ЧЕЛОВЕЧЕСКОЙ ЖЕЛЧИ

Э. Т. ГЛАЗ

Автор исследовал бактерицидное действие антибиотиков на *Salmonella typhi* в полученной различным путем человеческой желчи. Сопоставлением полученных *in vitro* величин торможения с описанными различными авторами желчной концентрации этих средств, автор установил, что для стерилизации желчной жидкости *in vivo* можно применять в расчет только пенициллин, но и это, по всей вероятности, лишь при комбинировании подачи пенициллина с желчегонным средством. Лечение пенициллином может быть успешным только у бациллоносителей, не проявляющих, или проявляющих только в незначительной степени хронических изменений желчного пузыря.

## О СОДЕРЖАНИИ СВЯЗЫВАЮЩИХ КОМПЛЕМЕНТ АНТИТЕЛ ПРОТИВ ВИРУСА РУБАРТА В ЧЕЛОВЕЧЕСКИХ СЫВОРОТКАХ

П. ОЛА

Автор исследовал содержание связывающих комплемент антител против вируса Рубарта в 308 пробах нормальных человеческих сывороток и получил в 28% проб положительный результат. Процентное соотношение положительных сывороток было до известной степени вариирующим, в зависимости от возраста и происхождения сывороток. Восприимчивость человека в отношении вируса Рубарта не кажется вероятной, если учесть, что в пробе крови 6 лиц, занимавшихся длительное время с этим вирусом, совершенно не удалось выявить связывающих комплемент антител. В крови лиц, переболевших гепатитом, встречаемость антител против вируса Рубарта была одинаковой со встречаемостью, установленной в крови здоровых людей.

## УПОТРЕБЛЕНИЕ СОЛИ МАЛОНОВОЙ КИСЛОТЫ У CANDIDA ALBICANS

Е. К. НОВАК

Исследуя употребление соли малоновой кислоты у *Candida albicans*, автор выявил первенство окисления по сравнению с декарбоксилированием. Он установил, что расход кислорода наступает быстрее, чем производство углекислого газа в присутствии малоновой кислоты. Отдельными ингибиторами можно одновременно задерживать расход $O_2$,

как и производство $CO_2$, в то время как другие ингибиторы задерживают производство $CO_2$ прежде чем они задерживают расход $O_2$, что указывает на независимость расхода $O_2$. Вторичное производство $CO_2$ доказывается его зависимостью от задержки расхода $O_2$. Доказательством первичного окисления является отсутствие производства углекислого газа при анаэробных условиях. Из величин дыхательного коэффициента можно сделать заключения о промежуточном образовании глиоксиловой кислоты и в результате последующего дальнейшего окисления об образовании конечного продукта.

## О ЗНАЧЕНИИ СТАНДАРТИЗАЦИИ ПРИ ОПРЕДЕЛЕНИИ ТИТРА АНТИСТРЕПТОЛИЗИНА 0

Й. БЕСЕРМЕНЬИ, Ш. БОЖОКИ и Г. БАРШИ

Авторы определили содержание антистрептолизина 0 в сыворотке 100 лиц. Титрование проводилось в двух различных лабораториях различными методами, применяя в качестве контроля один и тот же стандартный препарат антистрептолизина 0. Величины параллельных определений отклонялись в среднем лишь на 10,2%. Это служит доказательством того, что применением международного антистрептолизина 0, проводимые при различных условиях определения, можно приводить к одному знаменателю.

## NOCARDIA UNIFORMIS NOV. SPEC., — НОВЫЙ ВИД ЛУЧИСТЫХ ГРИБОВ ИЗ СОЛОНЦЕВАТОЙ ПОЧВЫ

М. МАРТОН и И. САБО

В ходе своих исследований авторы выделили из деградированной солонцеватой почвы штаммы нового вида **Nocardia** (Хортобадь, Восточная Венгрия, солончак). Для этих штаммов характерно, что их спектр утилизации источника C весьма узкий, самый бедный из описанных до сих пор видов **Nocardia**. Данный вид встречается в почве только в более глубоких слоях горизонта $B_1$.

## ДАННЫЕ К ЭПИДЕМИОЛОГИИ ЭПИДЕМИЧЕСКОГО ГЕПАТИТА В ВЕНГРИИ

А. ПЕТРИЛЛА, К. ШОЛТ и И. ВЕДРЕШ

Эпидемический гепатит регистрируется в Венгрии лишь с 1950-го года. Количество зарегистрированных больных в последние 2 года приблизительно 20 000 в год. Интенсивные показатели заболеваемости в столице значительно превосходят таковых на периферии. Случаи заболевания показывают выраженное сезонное колебание. Сезонное колебание в столице и на периферии идет в основном параллельно, причем только в зимные месяцы, т. к. в это время, в столице, после октябрского максимума снижается количество заболеваний, а на периферии наблюдается дальнейший подъем до декабря месяца. Сезонное колебание менее выраженное, чем в случаях других кишечных инфекций, пик заболеваний достигается в последнем квартале, т. е. отличается от того, к чему мы привикли в случаях кишечных инфекций. Большинство случаев заболеваний находятся среди дошкольников и учащихся младших классов общеобразовательной школы. Среди более старших возрастных групп интенсивные показатели постепенно снижаются, таким образом интенсивные показатели самые высокие в 3—10 летнем возрасте. Кривая по возрастному заболеванию постепенно снижается, не появляются подъемы в заболеваемости тех возрастных групп, у которых проводится большое количество прививок против разных инфекционных болезней. Кривая заболеваемости по возрастным группам подобна ко кривой заболеваемости тех инфекционных болезней, при которых низкие цифры интенсивных показателей заболеваемости у взрослых объясняются перенесением болезни уже в детском возрасте. Кривая заболеваемости детей, младше 3-х лет и взрослых, старше 30-и лет идет довольно параллельно в течение круглого года, без сезональных колебаний, может быть, значительная часть этих случаев является инокуляционным гепатитом. В возрастных группах 3—14 лет, наоборот, наблюдается сильное, выраженное сезональное колебание. Интенсивные показатели заболеваемости среди медицинских работников

выше в 6—8 раз, чем среди остального населения того же возраста. Смертность в среднем около 1%, в том числе в весенние месяцы в 3 раза выше, чем в зимних. Смертность самая высокая в грудном возрасте и у старых людей, т. е. в основном идет параллельно с кривой общей смертности.

## ПРОИЗВОДСТВО АНТИБИОТИКА ТРИХОТЕЦИН В ФЕРМЕНТЕРАХ

Й. ВЕРЕШ и И. САБО

Авторы получали антибиотик трихотецин во встряхиваемых культурах и ферментерах. Продолжительность ферментации в ферментерах 88—112 часов, а при поверхностном выращивании культур необходимо 25—30 дней. Максимальное содержание активного начала, достижимое при поверхностном выращивании культвур 40—80 $\mu$г/мл, а при глубинном выращивании 200—250 $\mu$г/мл.

Для определения трихотецина авторы применяли диффузионно-биологический метод с тест-микробом *Saccharomyces carlsbergensis*.

Исследовалась производительная способность 7 выделенных из различных источников штаммов *Trichothecium roseum* как и особей, на которые авторы воздействовали ультрафиолетовыми лучами и культивированием на содержащей трихотецин питательной среде. Наибольший выход трихотецина был получен от облученной ультрафиолетовыми лучами особое штамма III. 189.

Из исследованных в ходе опытов около 40 питательных сред различного состава, самыми подходящими оказались питательные среды с содержанием солодовой вытяжки, виннокислого аммония, сахарозы, кукурузного повидла и дрожжевого экстракта. В таких питательных средах можно было получить максимальный выход 200—250 (в исключительных случаях 300) $\mu$г/мл трихотецина. На описанной Фримэнном и Моррисоном питательной среде вышеназванный штамм дал максимальное содержание активного начала только в 80 $\mu$г/мл.

В 10 литровых стеклянных и 40 литровых железных ферментерах продукция активного начала была одинаковой с продукцией на параллельных поверхностных культурах. Для извлечения трихотецина авторы считают экстрагирование 20%-ым дихлорэтаном более подходящим способом, чем извлечение путем взбалтывания хлороформом при таком же соотношении. В целях очистки активного начала авторы применяли $Al_2O_3$ хроматографию, выделение сложным этиловым эфиром, а затем перекристаллизацию из нормального гексана.

## НАХОЖДЕНИЕ МИКРСФИЛАРИЕВ В ОДНОСЛОЙНЫХ ТКАНЕВЫХ КУЛЬТУРАХ ПОЧЕК ОБЕЗЬЯН

П. РУЗИЧКА

Автор сообщает об обнаружении живых микрофилариев в тканевой культуре, изготовленной из эпителиальных клеток почки обезьяны, показывающей слабую зараженность паразитами.

Наличие микрофилариев в первичных тканевых культурах не влияло на рост клеток, ни на цитопатогенное действие вируса полиомиелита.

Зараженные микрофиляриями, полученные из бутылей Ру путем обработки верзеном вторичные культуры в три дня перерождались, несмотря на то, что они лишь спорадически содержали микрофилариев.

В первичных культурах, содержанных в течение 30 дней путем еженедельной смены питательной жидкости, микрофиларии оставались в живых 2 недели.

## НОВЫЕ ДАННЫЕ О РАСПРОСТРАНЕНИИ В ВЕНГРИИ ЗАРАЖЕНИЙ БИЧЬЕГО ТИПА ПРИ ЧЕЛОВЕЧЕСКОМ ВНЕЛЕГОЧНОМ ТУБЕРКУЛЕЗЕ

И. САБО и Н. КЕРТАИ

В 1958 году авторы сообщили результат исследования типа штаммов *Mycobacterium tuberculosis*, происходящих из внелегочных заболеваний и определили роль палочек туберкулеза бичьего типа в этих заболеваниях. Тип 248 штаммов *Mycobacterium*

авторы определили прививкой животным, методом внутрикожной прививки кроликам, разработанным за новейшее время.

Исследованные, чувствительные к лекарствам и обладающие каталазной активностью штаммы были в 62,5% человеческого, и в 35,4% случаев — бичьего типов. Смешанную человеческую и бичью популяцию (смешанная инфекция) авторы нашли в 2,1% случаев. Палочка туберкулеза бичьего типа играла патогенную роль при урогенитальном туберкулезе в 29%, при заболеваниях, сопровожденных гнойными процессами в 40%, а при заболеваниях женских половых органов — в почти 50% случаев.

## МЕГАЦИНОГЕНЕЗ, ИНДУКЦИЯ СИНТЕЗА НОВОГО ИММУНОСПЕЦИФИЧЕСКОГО ВЕЩЕСТВА В ОТДЕЛЬНЫХ ШТАММАХ BACILLUS MEGATERIUM

Г. ИВАНОВИЧ, Э. НАДЬ и Л. АЛФЁЛЬДИ

Облученная ультрафиолетовыми лучами культура штамма *Bacillus megaterium* № 216 в ходе дальнейшего культивирования растворяется. Возникающие лизаты содержат антибактериальное начало — мегацин, возникновение которого сопровождено растворением клеток. Такие лизаты можно получить также из культур, изготовленных на синтетической питательной среде. Путем соответствующей переработки последних можно получить фракционированно очищенные концентраты с высоким титром. Авторы исследовали с помощью таких концентратов свойства мегацина.

Они установили, что мегацин обладает исключительно большой чувствительностью и уже свыше pH 7.5 быстро инактивируется. В противоположность этому он в кислой и нейтральной среде является довольно устойчивым веществом. При такой реакции на температуре в 37° C действие концентратов не меняется, даже в течение 4 часов. Однако, неустойчивость в отношении тепла показывает, что при температуре 80° C титр концентратов быстро снижается. Активное начало инактивируется также двукратно кристаллизованным пепсином (1 $\mu$г/мл) и химотрипсином. В противоположность этому мегацин при реакции pH 7.2 оказался устойчивым в отношении двукратно кристаллизованного трипсина (100 $\mu$г/мл). За белковую природу мегацина говорит также его антигенное свойство. Сыворотка крови привитых концентратами кроликов нейтрализирует бактерицидное действие мегацина. Мегацин дает со своим специфическим антителом преципитацию. Серологическим исследованием удалось доказать, что клетки исследованного штамма первоначально не содержали вещества с такой серологической специфичностью, и что синтез последнего наступает лишь после облучения ультрафиолетовыми лучами.

## ПРИМЕНЕНИЕ ВОССТАНОВИТЕЛЬНЫХ КРАСИТЕЛЕЙ ДЛЯ БЫСТРОГО ОПРЕДЕЛЕНИЯ РАЗВИТИЯ STREPTOMYCES RIMOSUS В ГЛУБИННЫХ КУЛЬТУРАХ

Л. НЬИРИ и З. ЛЕНДЬЕЛ

Авторы исследовали в аппарате Варбурга дыхание гифов штамма *Streptomyces rimosus* BS—21, выращенного на жидкой, аэрированной глубинной культуре, далее активность окислительно-восстановительной ферментной системы, растворами метиленовой синьки и трифенилтетразолхлорида. Они получили кривые окислительно-восстановительной ферментативной активности и дыхания, характерные для типичных ферментаций с хорошим выходом окситетрациклина. В случае отравленных ионами железа или сильно щелочных ферментаций, форма кривых интенсивности дыхания и ферментативной активности своеобразно изменялась, отклоняясь от типичных ферментаций. Способность продукции окситетрациклина этих культур была незначительной.

Цветные реакции, полученные растворами метиленовой синьки и трифенилтетразолхлорида, можно рассматривать результатом функции биологической окислительно-восстановительной ферментной системы.

В знании вышеописанных связей, применением растворов трифенилтетразолхлорида, но в частности метиленовой синьки, можно быстро и надежно определить физиологическое состояние глубинных культур.

## НОВЫЙ СПОСОБ ДЛЯ ИССЛЕДОВАНИЯ КУЛЬТУР SH. SONNEI

Б. ШЕРЕНЬ

1. Автор дает описание нового способа исследования, сущность которого сводится к тому, что морфология хорошо изолированных бактериальных колоний исследуется с помощью ручных луп, причем колонии освещаются источником света, лучи которого падают на колонии под различным углом.

2. Для колонии *Sh. sonnei S* характерен рисунок колонии, названный «звездовидным»; в таких колониях центральная часть, соответствующая звезде, представляет *Sh. sonnei S*, а окраинная часть агглютинирует в противосыворотке фазы II.

3. Автор излагает морфологию колонии фазы II и варианта *R Sh. sonnei.*

4. В случае коньюнктивального заражения звездовидные колонии вирулентны, а оптически гомогенные, средне преломляющие свет колонии оказались авирулентными.

5. Преобладающая часть колоний свежевыделенных культур *Sh. sonnei* состоит из звездовидных колоний, благодаря чему описанный авторами метод пригоден для отожествления и для определения вирулентности палочки Зонне в ходе рутинной бактериологической диагностической работы.

## БАКТЕРИАЛЬНЫЕ БОЛЕЗНЕТВОРНЫЕ АГЕНТЫ ФАСОЛИ И ИХ РАСПРОСТРАНЕНИЕ В ВЕНГРИИ

З. КЛЕМЕНТ

В Венгрии бактериальные заболевания фасоли были известны только с точки зрения признаков болезни. Автор впервые сообщает о встречаемости в Венгрии ***Corynebacterium flaccumfaciens*, *Xanthomonas phaseoli var. fuscans*, *X. phaseoli*, *Pseudomonas phaseoli-cola*** **(medicaginis f. sp. phaseolicola)**. В ходе наблюдений за период 1954—1957 гг. с точки зрения вреда и распространения самым значительным был ***C. flaccumfaciens***, встречаемость которого составляла, по сравнению с другими бактериальными заболеваниями, 87,5%. За ним следуют ***X. p aseoli var. fuscans*** (9%), а затем ***X. phaseoli*** и ***Ps. phaseolicola*** (2—1,5%). Первые два болезнетворных агента распространены по всей стране, в то время как последние два были изолированы только в междуречье Дуная и Тисы. Большое распространение ***C. flaccumfaciens*** следует приписать специальным климатическим условиям Венгрии.

## ДЕЙСТВИЕ ОБЛУЧЕНИЯ УЛЬТРАФИОЛЕТОВЫМИ ЛУЧАМИ НА СПОСОБНОСТЬ ASPERGILLUS NIGER ОКИСЛЯТЬ СТЕРОИДЫ

Г. ВИКС, М. НАТОНЕК и М. КОВАЧ

Авторы получили путем облучения ультрафиолетовыми лучами из штамма 47 ***Aspergillus niger***, образующего из прогестерона 11 α-оксипрогестерон, 11-эпикортикостерон и два более полярных вещества, такой морфологический мутант, который из прогестерона образовал лишь два продукта окисления: по большей части 11 α-оксипрогестерон и в следах одно более полярное вещество. Значит мутант потерял свою 21-гидроксигеназную ферментную систему.

## ВЫЯВЛЕНИЕ СВЯЗЫВАЮЩИХ КОМПЛЕМЕНТ АНТИТЕЛ ПРОТИВ АДЕНОВИРУСОВ В КРОВИ РАЗЛИЧНЫХ ЧЕЛОВЕЧЕСКИХ ГРУПП

И. НАС и М. ТОТ

Авторы исследовали реакцией связывания комплемента содержание антител против аденовирусов в сыворотке крови 426 лиц, четырех возрастных групп, отчасти здоровых, а отчасти находящихся на врачебном лечении. В I группе (переболевшие полиомиелитом и получающие последующее лечение дети, по большой части от 0—10 лет) авторы получили в 48,2% случаев, а во II группе (находящиеся на лечении больные туберкулезом, от 10—20 лет) в 24,5% положительные результаты. В III группе (здоровые молодые люди от 18—20 лет) положительная реакция была получена в 14,7%, а в IV

группе (здоровые взрослые старше 20 лет) — в 13,6% случаев. Титр антител, связывающих комплемент, в группах I—II—IV составлял 1 : 4 — 1 : 32, а в группе III — 1 : 4 — 1 : 8. Средняя величина титров в I группе была 1 : 8,8, во II группе — 1 : 11,6, в III группе — 1 : 4,8, а в IV группе — 1 : 11,4.

## НОВЫЕ ДАННЫЕ К СТОЙКОМУ ГЕТЕРОКАРИОЗУ STREPTOMYCES

Я. ХОРВАТ

Подобный *Streptomyces fasciculus* неспоровый гетерокарионтый вид, находящийся уже восемь лет в коллекции кафедры, автор растирал стеклянными бусами, а иной раз подвергал облучению ультрафиолетовыми лучами. При помощи обоих методов удалось выделить три хорошо дифференцируемых вида. Два из выделенных трех видов стали спорообразующими. Первый вид отличался от исходного вида прямым, а второй — спиральным спорангием, третий вид отличался только по своему цвету. Два споровых вида в течение года подвергались обратному развитию и стали подобными исходному виду, однако, после нового растирания вновь удалось получить из них в большем проценте споровый вид, и в меньшем также остальные два вида. Значит, различные изолированные виды и дальше оставались гетерокарионтами. У двух споровых видов обратное развитие происходит предположительно на действие цитоплазмы.

## КУЛЬТУРЫ SH. SONNEI ФЕРМЕНТИРУЮЩИЕ САЛИЦИН

Б. ШЕРЕНЬ

1. Из 69 штаммов *Sh. sonnei* отожествленных современными морфологическими, биохимическими и серологическими методами исследования, 8 культур ферментировали поздно (после 4—26 дневнего латентного периода) и непоследовательно салицин (2 оксибензиловый спирт-$\beta$-*d*-глюкопираноза).
2. У культур *Sh. dys.* 2. и *Sh. flexneri* автор не наблюдал расщепления салицина.
3. Автор подверг подробному исследованию один из штаммов, расщепляющих салицин, и установил, что наступление ферментации нельзя объяснить методологическими ошибками, а это впервые распознанное свойство данного штамма.
4. Результаты исследований показывают, что было бы ошибочным исключить салицин-положительные штаммы из рода *Shigella*.

## ДЕЙСТВИЕ ВЫРАЩИВАНИЯ НА КУЛЬТУРЕ STREPTOMYCES AUREOFACIENS ДРУГИХ STREPTOMYCES НА ИХ ПРОИЗВОДСТВО АНТИБИОТИКОВ

Я. ХОРВАТ и Ф. БУДАИ

На культуре *Streptomyces aureofaciens* выращенной на наклонном агаре, по истечении определенного времени (как правило начиная с 5-го дня) можно культивировать другие *Streptomyces.* Антибиотический спектр выращенных на культуре *Streptomyces aureofaciens* видов *Str. griseus, Str. globisporus, Str. flaveolus, Str. Xjl, Str. R3, Str. R5, Str, J.* повышается до прибли. 10 пассажов, а затем их антибиотический спектр сокращается. У отдельных видов после многократного пассирования через культуру *Streptomyces aureofaciens* производство антибиотиков даже прекращается. Такой же результат авторы получили в случае обработки вида *Streptomyces globisporus,* дезоксирибонуклеиновой кислотой, изолированной из *Streptomyces aureofaciens,* также после многократного пассажа этого вида. Производство антибиотиков по причине прекращения трансформирующего действия, авторы рассматривают как наследственную ферментную недостаточность.

## БИОХИМИЧЕСКИЕ И АНТИГЕННЫЕ СВОЙСТВА PROTEUS MORGANII

К. РАУШ и Ш. ВЁРЁШ

1. Авторы проводили биохимическое и антигенно-структурное исследование 222 штаммов ***Proteus morganii.***

2. С биохимической точки зрения ***P. morganii*** хорошо можно определить и дифференцировать от остальных членов группы ***Proteus.***

3. Авторы распределили 222 штамма на 29 групп — 3 подгруппы — и с помощью 19 Н антигена — на 57 типов.

4. Определенный неустойчивый к температуре антиген авторам удалось выявить с уверенностью только в одном единственном типе.

5. На основе своих исследований авторы составили антигенную схему ***P. morganii.***

6. Обсуждалась проблема таксономического положения ***P. morganii.***

## СВЯЗЬ МЕЖДУ ПОВЕРХНОСТНЫМИ СВОЙСТВАМИ ШТАММОВ STAPHYLOCOCCUS AUREUS И ИХ РЕЗИСТЕНТНОСТИ К АНТИБИОТИКАМ

### I. Фаготипизация полирезистентных штаммов

Л. ВАЦИ, Э. ЙЕНЕЙ и Л. ГЕДЕР

Авторы исследовали связь между чувствительностью к антибиотикам и фаготипам 355 патогенных штаммов ***Staphylococcus aureus,*** выделенных из больных грудных детей или же взрослых больных.

240 штаммов ***Staphylococcus aureus*** происходили от взрослых больных, а 115 из отделения для грудных детей.

Из исследованных штаммов ***Staphylococcus aureus*** 34 штамма оказались чувствительными к антибиотикам. Резистентными к одному антибиотику были 182 штамма, к двум антибиотикам — 50 штаммов, к трем или же четырем антибиотикам — 24 штамма, а полирезистентными оказались 65 штаммов.

Из исследованных штаммов 35,3% входили в I фаговую группу. 9,5% во II фаговую группу, 11% в III фаговую группу, 0,6% в IV фаговую группу, перекрестную реакцию дали 13,2% штаммов, а типизация 30,4% исследованных штаммов — т. е. всего 107 штаммов ***Staphylococcus aureus*** не была возможной.

В зависимости от расширения спектра резистентности штаммов, возможность определения их типа постепенно уменьшалось, причем 88% резистентных к 5—6 антибиотикам — так наз. полирезистентных — штаммов ***Staphylococcus aureus*** уже нельзя было типизировать фагами.

Число штаммов, типизации которых нельзя было провести, составляло в группе чувствительных к антибиотикам штаммов 11,8%, в группе штаммов, резистентных к двум антибиотикам — 12%, в группе штаммов, резистентных к трем и четырем антибиотикам — 50%, а в группе полирезистентных штаммов — 88%.

Быть может, что у полирезистентных штаммов ***Staphylococcus aureus*** состав поверхности клеток, отклоняющийся от состава чувствительных штаммов, есть причиной явления фаговой адсорбции — и следовательно и возможности типизации — или же проникновения антибиотиков — т. е. резистентности.

## ВИРОЛОГИЧЕСКИЕ НАБЛЮДЕНИЯ ВО ВРЕМЯ ЭПИДЕМИИ ПОЛИОМИЕЛИТА В ВЕНГРИИ В 1957 ГОДУ

П. ФЁЛДЕШ, И. СЕРИ и Ж. БАНОШ

Авторы во время эпидемии полиомиелита в Венгрии в 1957 г. изолировали 21 цитопатогенный штамм вируса. Из них 13 штаммов оказались вирусами полиомиелита 1. типа, один штамм — 2. типа, три штамма 11. типа, а один штамм 7. типа вируса ECHO. Определения типа трех штаммов до сих пор еще не удалось. Из штаммов типа ECHO 11, два были идентичными с прототипом, однако, они обладали до некоторой степени отклоняющейся от прототипа антигенной структурой.

Авторы проводили серологические исследования со сывороткой 214 реконвалесцентов, взятой у больных, переболевших полиомиелитом, и находящихся на ортопедическом последующем лечении. Ими не была выявлена проба сыворотки, которая не содержала бы антитела против одного из типа вируса полиомиелита.

## ДАННЫЕ К БИОСИНТЕЗУ ЦИАНОКОБАЛАМИНА В КУЛЬТУРАХ PROPIONIBACTERIUM SHERMANII

А. ШИМОН

Автор исследовал обмен веществ цианокобаламина в культурах *Propionibacterium shermanii* и установил следующее:

1. 48 часовые культуры в течение 2 часов способны связывать 1,6—1,7 $\mu$г/мл цианокобаламина. По истечении более длительного срока связывающая способность культур еще повышается.

2. Культуры не расщепляют присутствующий в излишнем количестве витамин $B_{12}$ и добавленный витамин $B_{12}$ не влияет в значительной степени на производство культур витамин $B_{12}$-активности.

3. Формалин в количестве 1—2% убивает культуру. Такая культура утрачивает свою способность синтетизировать витамин $B_{12}$, но она сохраняет свою способность связывать добавленный к раствору цианокобаламин и преобразовывать фактор В в витамин $B_{12}$ (в присутствии прекурзора).

4. Сильно аэрированные (встряхиваемые) культуры, в сущности, утрачивают свою способность синтетизировать витамин $B_{12}$, но они сохраняют свою способность преобразовывать фактор В в витамин $B_{12}$. Культура даже при условиях сильной аэрации не способна расщеплять цианокобаламин.

5. Засеваемая из встряхиваемых культур глубинная культура производит такое же количество витамин $B_{12}$-активности, и показывает в общем такое же поведение, как и глубинная культура, при пересеве из глубинной культуры.

## ИССЛЕДОВАНИЯ С ФАГОМ CL. PERFRINGENS

Г. ГАШПАР и Г. ТОЛНАЙ

Авторы разработали метод для титрования фага, растворяющего штамм *Cl. perfringens*. Количество фагов определяется на плотной питательной среде подсчетом плак (**plaque**). Применение описанной авторами питательной среды предоставляет возможность для инкубации пластинок, служащих для титрования фагов без особого анаэробного устройства, благодаря чему устраняются затруднения анаэробного культивирования.

Авторы далее изготовили такой прибор, который пригоден для исследования размножения фагов анаэробных бактерий и для снятия так наз. «одношаговых кривых размножения».

Чувствительность фагов к теплу показала в случае аэробных фагов, как правило, обычные величины. При внутривенном впрыскивании кроликам авторы наблюдали эффективную продукцию антифагов.

Авторы снимали «одношаговую кривую», обозначающую размножение фагов. Опыт проводился в условиях однократной инфекции. Латентный период составлял 45—55 минут, а повышающийся период — 20 минут. Средний выход фагов — 459. Бросается в глаза, ненаблюдаемое в случае аэробных фагов своеобразие кривых выражающееся в слабо повышающихся величинах латентного периода. Авторы не могут дать объяснения для этого явления и желают продолжать свои исследования в этом направлении.

## ДЕЙСТВИЕ УДАЛЕНИЯ ДОСТУПНОЙ ВОДЫ НА ФОРМУ КЛЕТОК И НА КИНЕТИКУ РАЗМНОЖЕНИЯ ШТАММА SACCH. CEREVISIAE VAR. ELLIPSOIDEUS

К. ВАШ и Г. ПРОСТ

Авторы исследовали распределение частоты объема клеток, происходящих из одноклеточной культуры. (Объем исчислялся в знании короткого и длинного диаметров 120 клеток формулой объема эллипсоида вращения). Авторы выявили, что данное распреде-

ление — ненормальное. Почти все кривые распределения частоты были ассимметричными и ввиду присутствия клеток, гораздо более больших среднего объема, показали сильно положительную кривизну. Авторы исследовали тип этих распределений частоты пробит-трансформацией и $\chi^2$-пробой прилегания первоначальных и пробитданных к нормальным кривым. Они пришли к тому заключению, что распределение частоты объема клеток в большинстве случаев имел log нормальный характер. Авторы того мнения, что данное установление предоставляет возможность критического пересмотра таксономии дрожжей, основанной на размерах клеток, далее для статистической оценки циторритического, сморщивающего действия отдельных средств, осмотически действующих на клетки.

При взвешивании клеток в растворах поваренной соли или же глицерина различной концентрации, средняя величина клеточных объемов довольно внезапно снизилась, в осмотическом отношении до концентраций, соответствующих приблизительно 1% хлористому натрию. В случае более высоких концентраций снижение объема было меньшего размера. В противоположность этому, скорость размножения и общий рост показали, начиная с наименьших концентраций, беспрерывное, равномерное уменьшение.

Значит, повышение концентрации вначале вызывает сравнительно сильные изменения в степени набухания клеточной плазмы, повидимому без того, чтобы повлиять на клеточное строение и на размножение. Однако, после достижения снижения набухания определенной степени (что соответствует прибл. 15%-ому среднему уменьшению объема), дальнейшее удаление воды уже сравнительно сильно снижает интенсивность жизненных процессов. Данное критическое сморщивание клеток может сопровождаться более сильным снижением гидратуры клеток, и последнее явление может быть непосредственной причиной замедления размножения клеток.

Два плазмолитических вещества, столь различных по своей химической природе и по своим вещественным свойствам — хлористый натрий и глицерин — оказали подобное действие на клетки. Оба вещества действовали повидимому только путем повышения осмотического давления. Специфических действий (например действия соли) не удалось выявить.

## СВЯЗЬ МЕЖДУ ПОВЕРХНОСТНЫМИ СВОЙСТВАМИ ШТАММОВ STAPHYLOCOCCUS AUREUS И ИХ РЕЗИСТЕНТНОСТИ К АНТИБИОТИКАМ

### II. Фагоцитоз чувствительных и устойчивых к антибиотикам штаммов Staphylococcus aureus

Л. ВАЦИ, ДЬ. ХАДХАЗИ и М. КАТОНА

Авторы исследовали фагоцитоз лейкоцитами крыс чувствительных, и резистентных к нескольким антибиотикам, так наз. полирезистентных штаммов *Staphylococcus aureus*. Было установлено, что

1. полирезистентные штаммы менее подвергаются фагоцитозу, чем чувствительные (как правило 34%),
2. фагоцитоз штаммов, устойчивость которых была вызвана in vitro оказался по сравнению с чувствительными штаммами на 71% выше,
3. трипсиновое переваривание стафилококков не меняет существенно их пожираемости,
4. на действие предварительной обработки периодатом калия пожираемость чувствительных, как и устойчивых стафилококков значительно повышается (69, или же 53%),
5. на действие анионактивного вещества (дуодецилбензолсульфокислый натрий) фагоцитоз резистентных штаммов *Staphylococcus aureus* значительно повышается (+82%), в то время как фагоцитоз чувствительных штаммов остается неизменным,
6. повышающее фагоцитоз действие дуодецилбензолсульфокислого натрия на резистентные клетки оказалось соразмерным концентрации активного вещества,
7. предварительная обработка катион-активным веществом не изменяет фагоцитоз ни чувствительных, ни резистентных клеток,
8. после предварительной обработки устойчивых клеток липазой поджелудочной железы, повышающее фагоцитоз действие анион-активного вещества не имеет место, а у обработанных инактивизированной липазой клеток это действие сохраняется,

9. авторы указывают на значение веществ, входящих в состав клеточной поверхности стафилококков, в частности на значение липоидов, в отношении биологических функций, и свойств клеток (пожираемость, чувствительность к антибиотикам).

## ИССЛЕДОВАНИЕ СВЯЗИ МЕЖДУ ИММУННЫМИ ВЕЩЕСТВАМИ И БЕЛКОВЫМИ ФРАКЦИЯМИ

Л. ГОРЕЦКИ

Относительно связи между иммунными веществами и белковыми фракциями проявляются у отдельных авторов противоречия в отношении роли глобулинов, как носителей антител, перемещающихся со скоростью выше скорости перемещения гамма-глобулина. Причину расхождений следует искать в количестве применяемой сыворотки.

Автор проводил опыты, в которых он при совершенно одинаковых экспериментальных условиях менял количество применяемой для электрофореза сыворотки. В качестве экспериментального метода он применял комбинацию разработанного раньше метода бумажного электрофореза с агарной диффузией. В случае гемолизинов, применяя количество в 40, 60 или же 80 $\mu$l было установлено, что параллельно с повышением количества сыворотки повышается также распространение иммунных веществ на компоненты с большей скоростью перемещения. Относительно антистрептолизинов было при электрофорезе 20, 40 и 60 $\mu$l сыворотки то же самое установлено, однако, действие иммунного вещества в этом случае не распространялось в такой степени на компоненты большей скорости перемещения, чем в случае гемолизинов. В то время как при применении больших количеств сыворотки гемолизинов, действие иммунного вещества показалось даже в альфа-глобулинах, то антистрептолизины были выявлены только в бета-глобулинах. Таким образом, первоначально локализованное только на гамма-глобулины действие иммунного вещества проявлялось в случае применения больших количеств также и вне последних.

На взаимосвязь распределения иммунных веществ в фракциях и количества применяемой сыворотки указывают также те дальнейшие эксперименты, в которых автор после однократной элюции из фильтровальной бумаги нанесенной сыворотки проводил с последней второе извлечение. В данном случае расстилающиеся при первой элюции иммунные вещества проявлялись при второй элюции только в гамма-глобулинах. Вышеописанные опыты дали совпадающие результаты, как в случае применения сывороток более низкого, так и более высокого титров. Принимая степень растворения соразмерной с первоначальной концентрацией антител, то и из этого можно сделать выводы относительно максимума антител в ферограммах. Итак, очевидно, что носителем иммунных веществ был в случае исследованных автором гемолизинов и антистрептолизинов — гамма-глобулин.

При исследовании локализации антител, согласно результатам опытов, следует применять только такое количество сыворотки, при котором распределение не распространяется на несколько фракций. Наблюдаемое в случае применения большого количества сыворотки расстилание иммунного вещества приводит к неправильным выводам относительно локализации.

Проведенные эксперименты делают понятным имеющиеся в литературе противоречия, которые по всей вероятности обусловливались неправильным подбором количества сыворотки. При данном методе следует применять по возможности меньшее количество, благодаря чему эти противоречия устраняются. При исследовании локализации иммунных веществ следует стремиться проводить эксперименты при низкой белковой концентрации.

## КОМБИНИРОВАННАЯ ФЕРМЕНТАЦИЯ: I. ФЕРМЕНТАЦИЯ TRICHOTHECIUM-PENICILLIUM

Б. ЙОХАН, И. САБО и И. КЕРЕСТЕШИ

Авторы выявили, что при засевании штамма ***Penicillium sp.*** ($P_2$) на встряхиваемую культуру штамма ***Trichothecium roseum*** (Tr) (комбинированная ферментация) количество полученного антибиотика трихотецин в 2—5 раза больше, чем в случае ферментации только штаммом **Tr**. Штамм **Tr** сам по себе дал выход трихотецина в среднем 200—

300 г/л. Штамм $P_2$ принадлежит к группе **assymmetrica-velutina** и продуцирует только мало пенициллина. Если постепенно повысить количество $P_2$, засеянного на культуру **Tr**, то выход трихотецина также постепенно повышается, вплоть до достижения критической точки. Если повысить количество $P_2$ выше этой точки, то производство трихотецина прекращается. Чтобы получить максимальный выход трихотецина, количество $P_2$, засеваемого на культуру **Tr** должно быть в определенной пропорции с количеством посева **Tr**. В случае соблюдения этого соотношения посева **Tr**/$P_2$ при повышении или снижении количества обоих посевов выход трихотецина остался неизменным. Вегетативный посев $P_2$ следует засеить на 24 часовую, а споровый посев на О часовую культуру **Tr**. Засеваемый на 42 час или позже вегетативный посев $P_2$ оказался неэффективным.

Другие, применяемые в производстве пенициллина штаммы **Penicillium** также вызывают вышеописанное действие. Убитыми культурами **Penicillium** или экстрактом мицелия, как и пенициллином не удалось повысить выхода трихотецина.

Штамм $P_2$ задерживал на пластинке агара рост **Tr** но не убивал его. **Tr** не задержал роста $P_2$. В ферментациях, дающих высокий выход трихотецина, нити **Tr** показали на действие $P_2$ своеобразное морфологическое изменение. Полученный при комбинированной **Tr**—$P_2$ ферментации антибиотик в отношении биологического и бумажнохроматографического поведения, кристаллической формы и точки плавления совпадал со свойствами трихотецина, полученного из штамма **Tr**.

Вышеописанное явление нельзя приписать действию, возможно, вызванного штаммами **Penicillium** «фактора роста». В комбинированных ферментациях — по сравнению с контролями — при более высоком выходе трихотецина вес мицелия был меньшим. Быть может, что наблюдаемый в таких случаях повышенных выход трихотецина является следствием патологического метаболизма, связанного с наблюдаемым в микроскопе патологическим изменением нитей гриба **Tr**. Однако, пока нельзя исключить возможность, что **Penicillium** продуцирует какой-нибудь прекурзор, или что он с точки зрения производства трихотецина играет роль «фактора активности».

## О МЕГАЦИНАХ

Э. НАДЬ, Л. АЛФЁЛЬДИ и ДЬ. ИВАНОВИЧ

Антибактериальные начала с изо-антигонистическими свойствами различных штаммов *Bacillus megaterium* представляют из себя подобные, или подобного действия вещества. На основе сказанного вполне обосновано обозначение этих веществ бактериоцидной природы общим названием мегацины. Далее это обосновывается также совершенно узким, почти одинаковым спектром их антибактериального действия. Их действие направляется против мембраны цитоплазмы чувствительных бактерий, разрушая последнюю, уничтожая этим осмотический барьер бактерий. Несмотря на эти идентичные свойства, между мегацинами отдельных штаммов наблюдаются также иммуннобиологические разницы, проявляющиеся в их антигенной природе и иммунноспецифичности.

## ЛИЗОГЕНЕЗ И БАКТЕРИОЦИНОГЕНЕЗ B. MEGATERIUM

Э. НАДЬ

Автор исследовал применением метода «**replica-plating**» 100 различных, недавно изолированных штаммов *B. megaterium* с точки зрения их лизогенного и мегациногенного свойства. Было установлено, что 34% штаммов лизогенны, а 53% — мегациногенны. Два летальных биосинтеза встречались совместно в случае 19 штаммов. На основе полученных результатов не удалось доказать генетической связи между двумя явлениями. Наличие двух свойств повидимому совершенно независимо друг от друга. Не удалось выявить перекрестного иммунитета между двумя летальными биосинтезами.

## ПРОЕКТИРОВАНИЕ И ОЦЕНКА ИММУНОЛОГИЧЕСКИХ ЭКСПЕРИМЕНТОВ

Д. Й. ФИННИ

Автор дает краткое описание важнейших статистических показателей иммунологических оценок, при которых сила действия двух вакцин сравнивается на той основе, до какой степени они защищают подопытных животных против стандартной дозы заражающего организма. Для статистического анализа и определения относительной эффективности было предложено много различных методов исчисления, среди которых автор приводит более важные методы на конкретном примере. Среди этих методов ныне уже большинство нельзя предлагать и некоторые — например популярный метод Рид—Мюнха — даже следует отвергнуть.

Для быстрого приближенного определения относительной эффективности наиболее удовлетворяющим кажется метод Шпирман—Кербера и еще несколько графических методов. Первый применим прежде всего в случаях, когда определение было поставлено на одинаковом количестве подопытного материала и различными дозами, причем эти дозы должны быть близкими друг другу и создать геометрический ряд, но важнее всего, чтобы применяемые дозы распространялись от уверенно положительного до уверенно отрицательного ответа.

Эти требования неблагоприятны с точки зрения проведения опыта, если учесть, что быть может предварительная информация делает возможным применять только несколько доз, близких к 50% ответу. Поэтому метод Шпирман—Кербера для анализа правильно поставленного опыта не является достаточно эффективным и надежным.

В общем оценка «**maximum likelihood**» является лучше всего осуществимым методом, либо путем **NED** («**normal equivalent deviate**»), либо путем **logit** трансформации. На практике редко оправдывается придавать преимущество одному из этих методов, перед другими. Вместо метода «**maximum likelihood**» можно применять также метод «**minimum** $\chi^2$» хотя последний, быть может, является менее удовлетворяющим.

## НЕКОТОРЫЕ ПРАКТИЧЕСКИЕ НАБЛЮДЕНИЯ ПРИ БИОЛОГИЧЕСКОЙ ОЦЕНКЕ

Г. БАРШИ

Автор дает обзор о проведенных за последние годы в Государственном институте гигиены более важных работ по биологической оценке.

В связи с испытанием новых четвертичных соединений аммония проводились биологические исследования дезинфицирующих средств. Было установлено, что для определения эффективности дезинфицирующих средств коэффициент фенола не является достаточным, ибо много свойств отдельных дезинфицирующих средств отклоняются друг от друга. С помощью простых средств были кроме коэффициента фенола также установлены коэффициенты температуры и концентрации дезинфицирующих средств. В случае бактериальных спор не удалось на основе действия формалина доказать зависимости Чик-Уатсона, вместо этого, между концентрацией и успешным временем проникновения была установлена простая лог-арит-линейная связь.

В целях исследования эффективности вакцин поставленные согласно принципам стандартизации экспериментальные модели показали более точно защитные величины, чем применяемые до сих пор модели. При разработке новых моделей правильность экспериментальных условий проверялась на основе осуществления ими log-нормального распределения.

Автор дает краткое описание эпидемиологического испытания противококлюшной вакцины, далее исследования эффективности обязательной вакцинации против коклюша, и он вкратце излагает те трудности, которые часто препятствуют однозначной оценке эффективности вакцин при эпидемиологическом исследовании.

Согласно статистическому исследованию данных о чувствительности к антибиотикам бактериальных штаммов, происходящих из рутинных исследований 1953—56 гг., нельзя наблюдать перекрестной резистенции. В противоположность этому была установлена связь между чувствительностью болезнетворных агентов к различным антибиотикам.

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