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Penicillin Induced Lysis in *Escherichia coli*

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SUMMARY

The paper is a survey of the action of α-aminobenzylpenicillin (ampicillin) on *Escherichia coli*. The rate at which lysis was induced in exponentially growing organisms was studied for different concentrations of ampicillin, using pure D- and L-forms as well as a 6:4 mixture. The interpolated concentration of penicillin which gives lysis in one generation, the 'LIOG value', has been used for characterization of penicillin derivatives with twelve different side chains. These LIOG values were also used for characterization of some penicillin-resistant mutants. The lysis rate has been recorded for cultures of concentrations between 4×10^5 and 10^8 rods/ml. and found to be independent of the population density. The use of five different media showed that for a given ampicillin concentration the time to lysis was proportional to the growth rate. The addition of penicillinase to a culture growing with penicillin rescued the organisms as late as a few minutes before lysis. Synergistic effects on the lysis rates were found with 6-aminopenicillanic acid and two amino acids, which as residues are side chains, in two of the penicillins tested. Different models for penicillin action in *E. coli* are discussed.

INTRODUCTION

Cell walls of Gram-negative bacteria are complex structures containing polysaccharides, lipids and protein-like material with a variety of amino acids some of which are D-isomers (see reviews by Salton, 1960*a*, *b*; Work, 1961). An organism like *Escherichia coli* can therefore be said to make two classes of polymerized amino acids: cell wall material and intracellular proteins. Several studies indicate that penicillin selectively inhibits the synthesis of wall material and that chloramphenicol or puromycin selectively block the synthesis of intracellular proteins (see reviews by Strominger, 1960; Novelli, 1960). Thus there may exist two pathways for the synthesis of polymerized amino acids, with a branching point somewhere between the amino acids and the ribosomal particles. The classical benzylpenicillin, penicillin G, shows a relatively poor antibiotic activity against Gram-negative bacteria such as *E. coli*. Rolinson & Stevens (1961) have, however, recently described some properties of a new penicillin with a D-amino acid residue as side chain, α-aminobenzylpenicillin (Penbritin; ampicillin), and this penicillin derivative has a rather high bactericidal effect against non-penicillinase producing *E. coli*.

The present paper is mainly a survey of the action of ampicillin on *Escherichia coli*, measured as the induction of lysis. Some penicillin-resistant mutants have been isolated and characterized. Resistance to penicillin is in many cases related to the production of the enzyme penicillinase (see review by Pollock, 1959). It is therefore possible that the system will be suitable for combined genetic and biochemical

studies. As an approach to the study of penicillinase action *in vivo* we have added purified penicillinase to sensitive cultures at different stages of penicillin action and can in this way prevent lysis. The recent isolation of 6-aminopenicillanic acid (Batchelor *et al.* 1959) has already led to the synthesis of several thousands of new penicillins (Abraham & Newton, 1961) and this work has opened possibilities for a systematic study of the influence of the side chain of the penicillin molecule. We have in the present paper studied certain aspects of this problem with the co-operation of Dr B. Sjöberg, of the Astra Co. Södertälje, Sweden.

METHODS

Organism. Escherichia coli K12, strain x5, was used in all experiments except where otherwise stated. Other strains used were mutants of strain x5 resistant to different concentrations of penicillin. Strain x5 was an *Hfr* auxotroph requiring thiamine, arginine, and either methionine or cysteine. The parent strain ($3OS_0$ arg 5) was obtained from Dr W. K. Maas. The methionine/cysteine marker was introduced by the conventional Davis–Lederberg technique as described by Lederberg (1950). The generation time of strain x5 in nutrient glucose medium (see below) was 30–40 min.; in minimal medium with arginine or methionine about 60 min.

Medium. The basal medium E according to Vogel & Bonner (1956) was used in all but one experiment. It was normally supplemented with: thiamine, 1 μg./ml.; Difco nutrient broth, 0·8 %; glucose, 0·5 %. In minimal medium, nutrient broth was replaced with L-arginine, 25 μg./ml. and DL-methionine, 25 μg./ml. Casamino acids, agar, and nutrient agar were Difco standard products. In the preparation of plates with ampicillin, care was taken to minimize the time the ampicillin was heated, but some hydrolysis may nevertheless have occurred and given an error in the ampicillin concentration in the plates.

Growth conditions. An overnight culture was grown at 30° without shaking. This culture was diluted in the morning with fresh medium and allowed to grow on a rotatory shaker at 37° for 1½–2 hr.; it was checked that the organisms were growing at the expected rate. A sample (2–4 ml.) of this culture was used as inoculum and at zero time diluted with medium to 20 ml., by using 100 ml. flasks with side tube. Growth was normally followed with a Klett–Summerson photo-electric colorimeter, using filter no. 66.

Materials. All penicillin derivatives were kindly obtained from Astra Co., Södertälje, Sweden. They have been numbered 1–14 and are listed in Table 2, which gives the formula of the side chains, purity data and our determinations for the lytic activity. The aminopenicillins (compounds 1–7) were synthesized as described by Ekström *et al.* (to be published) and compounds 9 and 11 according to Sjöberg & Ekström (to be published). All other penicillin derivatives used were products commercially available, which had been subjected to further purifications by Dr Sjöberg's group. Three forms of ampicillin were used; the pure D- and L-isomers, and a mixture (designated SPC 270), containing about 60 % D-isomer and 40 % L-isomer, resulting from an asymmetric synthesis. Concentrated solutions of penicillins were made up once a week and stored at −18°. Solutions of the L-form of ampicillin were always freshly made. All concentrations given refer to the amount of penicillin analytically determined in each sample. The penicillinase used was from *Bacillus cereus* and is

commercially available ('Neutrapen') from Scheen Pharmaceutical Corp., New York 1, N.Y., U.S.A. One unit of this penicillinase is defined as the amount of penicillinase which would inactivate one unit of penicillin per minute at 25° and pH 7·0 (Levy, 1950).

Experiments with radioactive isotopes. Carrier free ^{35}S sulphate with 0·67 μC./μl. and uniformly labelled ^{14}C arginine with 5·9 mC./m-mole were obtained from The Radiochemical Centre, Amersham, England. DL-methionine-2-^{14}C with 0·57 mC./m-mole was obtained from California Corp. for Biochemical Research, Los Angeles 63, Calif., U.S.A. The sulphur-free medium of Roberts *et al.* (1957) was used, supplemented with glucose (0·5 %), ^{14}C arginine and ^{14}C-methionine (0·06 and 0·12 μmole/ml., respectively) and ^{35}S sulphate (0·67 μC /ml.). At the indicated times, samples (1 ml.) of culture were withdrawn and passed through filter Co 5 from Membranfilter (Göttingen, Germany). Each sample was washed with 10 ml. of a solution containing 0·01 M-HCl, 1 % (w/v) $(NH_4)_2SO_4$ and 0·1 % (w/v) each of unlabelled L-arginine and DL-methionine. The filters were mounted in planchets and counted with a Nuclear Chicago windowless gas flow counter.

RESULTS

Ampicillin-induced lysis was studied with *Escherichia coli* K 12, strain X 5, with a generation time of about 35 min. in broth medium at 37°. An exponentially growing culture was diluted at zero time into three parts with fresh medium. To the first and second parts, ampicillin (SPC 270) was added to final concentrations of 4·2 and 8·3 μg./ml., respectively. The third portion was the control. Growth was continued at 37° and the optical density followed for about two generations. Figure 1 shows that, in the cultures with ampicillin, the cell mass continued to increase, for high concentrations of penicillin, at a rate somewhat lower than the normal growth, and that lysis started rather abruptly. The highest points of the curves for penicillin cultures (filled symbols in Fig. 1) have been defined as 'the moment when lysis starts', and the 'time to lysis' as the period between the addition of penicillin and 'the moment when lysis starts'. The 'time to lysis' can be expressed in terms of normal generations and the 'lysis ratio' is in the following defined as:

$$\text{lysis ratio} = \frac{\text{generation time of control culture (min.)}}{\text{time to lysis of penicillin culture (min.)}}.$$

Figure 1 shows that the lysis ratio varied with the penicillin concentration and with 4·2 and 8·3 μg. ampicillin (SPC 270)/ml. the lysis ratios obtained were 0·77 and 1·0, respectively. The penicillin dependence of the lysis ratio was compared for three forms of ampicillin, pure D- and L- isomers and a mixture of about 60 % D- and 40 % L-isomer (SPC 270). The results are given in Fig. 2 and show that the D-isomer was more active than the L-isomer and that the 6:4 mixture (SPC 270) was only slightly less active than the D-isomer. Drs B. Sjöberg and B. Ekström have recently found that the half-life at 37° and pH 7 is about 100 hr. for the D-isomer and 15 hr. for the L-isomer, and that these times are significantly influenced by the composition of the medium. This difference in stability would affect determinations of the antibiotic activity by viable counts or minimum inhibitory concentration (MIC). However, our determinations of the lysis ratios were completed within a few hours and

a difference in stability cannot explain the differences in the lysis ratios for the D- and L-isomers.

The experimental error in the determination of the lysis ratio may well be of the order of 10 %, and, for ratios higher than 2, even greater. Determinations of curves like those in Fig. 2 are, however, fairly reproducible when 6 to 8 points are taken in each experiment. The extrapolated penicillin concentration which corresponds to lysis in one generation (LIOG value) is used in the sequel as a characteristic of different penicillin derivatives as well as of bacterial strains. The LIOG values obtained from Fig. 2 are for the D-isomer 6 μg./ml., for the L-isomer 22 μg./ml. and for SPC 8 μg./ml.

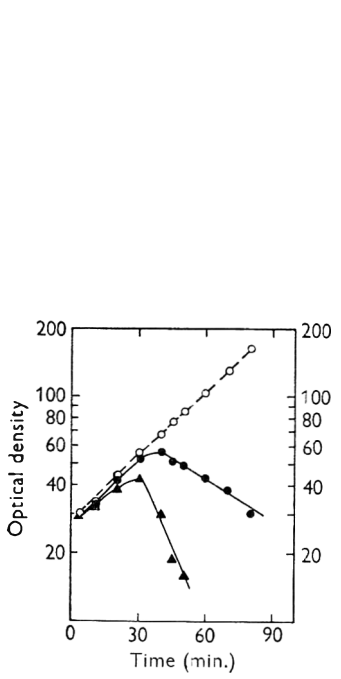


Fig. 1

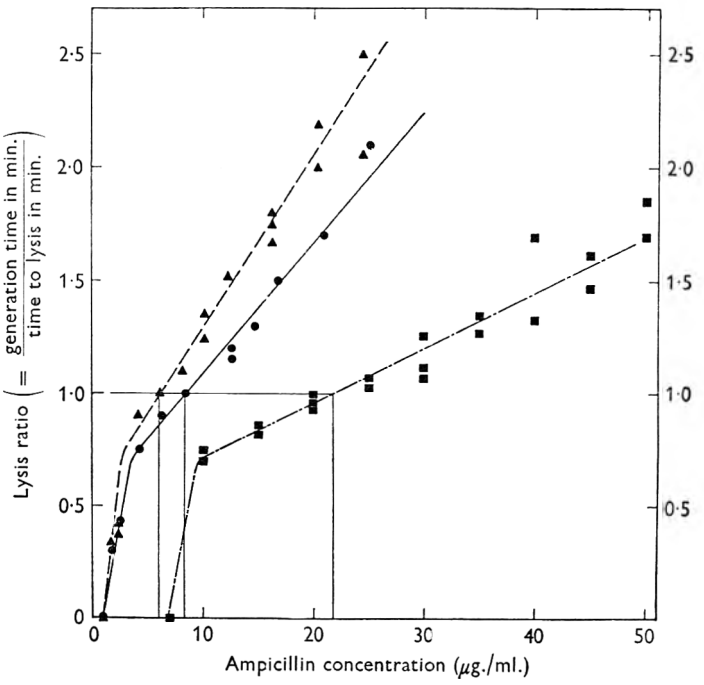


Fig. 2

Fig. 1. Growth curves for three cultures obtained by dilution of an exponentially growing inoculum. Addition at zero time of 4·2 μg./ml. (●—●) and 8·3 μg./ml. (▲—▲) of ampicillin (SPC 270), respectively. No addition (○- -○).

Fig. 2. Lysis rates for different concentrations of three forms of ampicillin (α-aminobenzyl penicillin). Pure D-isomer (▲- -▲), pure L-isomer (■- -■), and the mixture (6:4) SPC 270 (●—●). The LIOG values are defined as concentrations giving lysis ratio = 1.

Independence of lysis rate and concentration of organisms

A well-known characteristic of penicillin G (see the review by Cooper, 1956) is that for non-penicillinase-producing strains the antibiotic effect is independent of the size of the inoculum. This has been confirmed with ampicillin. The Klett photometer used only permitted the measurement of growth curves with a threefold difference in size of inoculum; under these conditions the time to lysis was found to vary by only 0·1 generation, which was considered to be within the experimental error. It was found, however, that growth above 10^5 organisms/ml. could be recorded

in a minimal medium with carrier-free radioactive sulphate and ^{14}C labelled arginine and methionine, simply by following the incorporated radioactivity collected on a bacterial filter. Figure 3 shows the results of such an experiment where a dilute culture after 3 hr. was divided into two parts: to one was added 6·2 μg. ampicillin (SPC 270)/ml., the other portion being kept as control. As reference, viable counts were made at the two points indicated in the figure. It can be seen that the incorporation continued in the penicillin culture for about 1 hr. and that the radioactivity on the filter then decreased for the next 2 hr. The generation time for the control in this experiment was 78 min. and the lysis ratio recorded was 0·86, which is in good agreement with the value 0·79 obtained with about 10^8 organisms/ml. The similarity between the curves obtained by the incorporation of isotopes and the reading on the Klett photometer is also a control on the assumption that the optical density was directly related to cell mass.

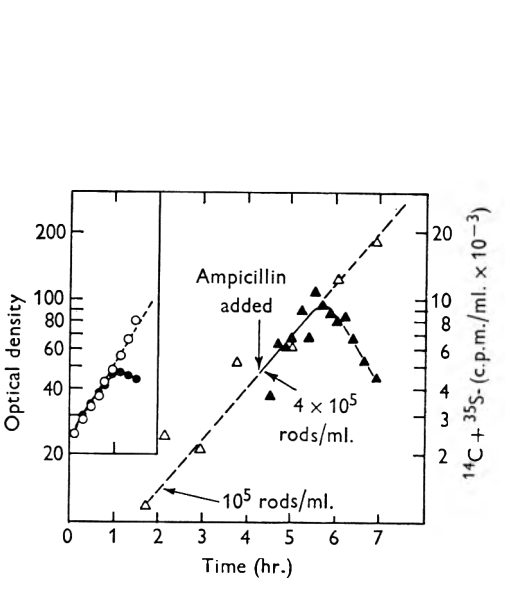


Fig. 3

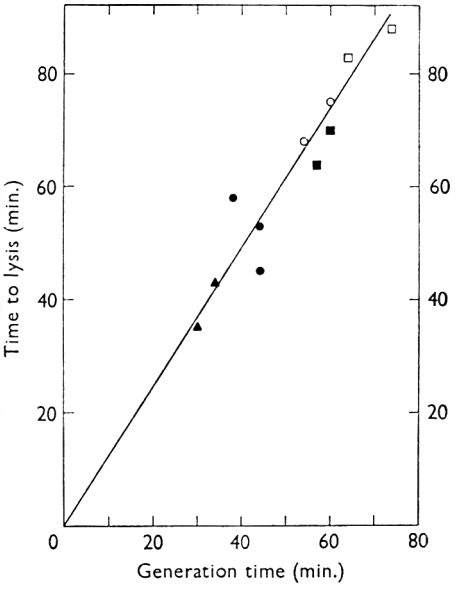


Fig. 4

Fig. 3. Growth curve for a dilute culture followed by recording the radioactivity collected on a bacterial filter (△--△). Minimal medium with carrier free ^{35}S sulphate and ^{14}C arginine and ^{14}C methionine. Arrows indicate time of viable counts and addition of 6·2 μg./ml. of ampicillin (SPC 270) to half of the culture (▲—▲). Left part gives a control experiment with about 10^8 rods/ml.

Fig. 4. The time to lysis for different generation times obtained during balanced growth in different media. Nutrient broth + glucose (▲); casamino acids + glucose (●); arginine + methionine + glucose (■); casamino acids + glycerol (○); arginine + methionine + glycerol (□); ampicillin (SPC 270) conc. 6·2 μg./ml.

The influence of the medium on the time to lysis

Todd (1945) showed that for Gram-positive bacteria 'the most rapid lysis occurs with organisms at the maximal rate of multiplication', but he could not demonstrate this for 'Bacillus coli'. Since then some conflicting data have been reported (see review by Hugo & Russell, 1961), but it is a general rule that penicillins act only

on growing organisms. We studied the time to lysis for *Escherichia coli* strain x 5 in five different media which gave more than a twofold difference in growth rates. The results obtained with 6·2 μg. ampicillin (SPC 270)/ml. are given in Fig. 4 and show that the time to lysis was directly proportional to the generation time. This means that when data for the lysis rates are expressed in terms of generations, they are independent of the growth rate and this makes the LIOG value a more meaningful parameter.

Prestidge & Pardee (1957) showed with *Escherichia coli* that inhibition of protein synthesis by chloramphenicol stopped penicillin action. A change from a rich medium to minimal medium is known to give a lag in growth during which time some protein synthesis occurs (Kjeldgaard, Maaløe & Schachter, 1958; Neidhardt & Magasanik, 1960). In the following experiment we used this lag to study ampicillin action during limited protein synthesis. A culture of *E. coli* strain x 5 (100 ml.) was grown in nutrient glucose medium, centrifuged and the organisms washed once with an equal volume of medium E and then resuspended in minimal medium with 6·2 μg. ampicillin (SPC 270)/ml. Aeration and incubation at 37° were continued and after 15 and 75 min. two 18 ml. samples were transferred to flasks with 2 ml. of 8 % nutrient broth containing 6·2 μg. ampicillin/ml. Figure 5 shows that no lysis occurred during the lag phase, but that lysis started a certain time after growth was resumed. The time to lysis was 54 min. in the first flask (curve *A*) and in the second one 96 min. (curve *B*). Thus some destruction of penicillin would seem to have occurred during this lag phase. It has only been possible to demonstrate this time effect with ampicillin concentrations lower than the LIOG value, a fact which is consistent with the break in the curve for the concentration dependence (see Fig. 2).

Exposure to penicillin for limited times

An excess of penicillinase (Methods) can in a few seconds inactivate all the penicillin of a culture and the enzyme is thus a suitable tool for obtaining pulse doses of penicillin. An exponentially growing culture was inoculated at zero time into several flasks of media containing 6·2 μg. ampicillin (SPC 270)/ml. Incubation was continued and penicillinase was added at different times to a final concentration of 500 units/ml. When penicillinase was added within 0·8 generation growth continued in a normal way. When it was added after 1 generation penicillinase almost completely prevented lysis and the growth curve showed only an inflexion point. When the penicillinase treatment was replaced by centrifugation, and washing and resuspending the organisms in fresh medium without ampicillin the same result was obtained. Figure 6*a* shows the Klett photometer readings from an experiment in which an exponentially growing culture of *E. coli* strain x 5 was divided into three portions: one portion served as control, and the two others received 6·2 μg. ampicillin (SPC 270)/ml. at zero time. Growth conditions were continued and, after 1·3 generations, one of the ampicillin cultures received 500 units penicillinase/ml. About 15 % of the organisms lysed in this culture before growth was resumed. The culture which only received ampicillin gave almost complete lysis. Figure 6*b* gives viable counts for the same three cultures. The ampicillin culture, which received penicillinase at the start of lysis, lost 88 % of its viable units in 75 min. but recovered the starting population of viable organisms about 60 min. later. This result is believed to come from two superimposed effects. First, a fraction of the organisms

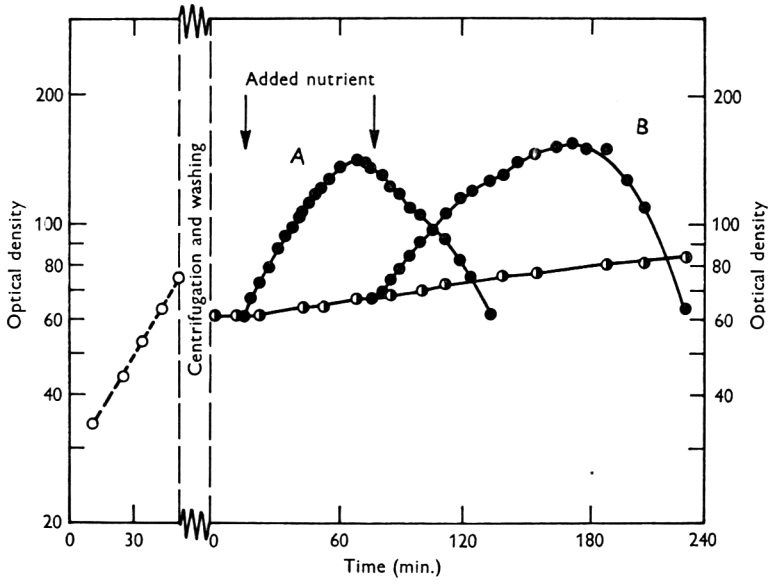


Fig. 5. Ampicillin action during lag phase produced by a shift from rich to minimal medium. Growth in nutrient glucose (○--○); washed cells suspended in minimal medium with 6·2 μg./ml. of ampicillin (SPC 270) (◑—◑); aliquots of culture to which nutrient was added at the times indicated by the arrows (●—●).

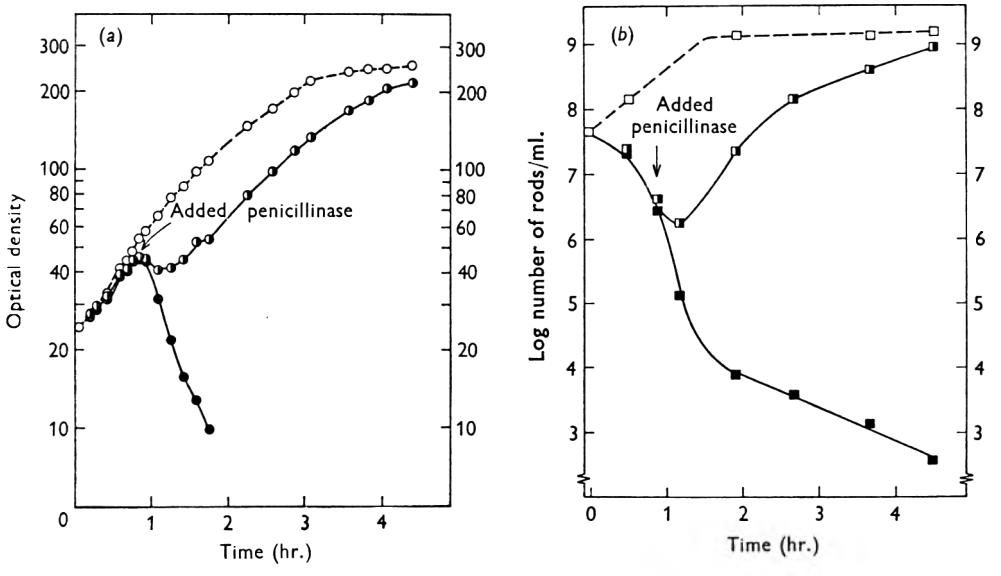


Fig. 6. Influence of penicillinase added to a culture growing in the presence of 6·2 μg./ml. of ampicillin (SPC 270). Part (*a*) gives optical densities (circles) and part (*b*) viable counts (squares) for the same three cultures. Ampicillin added at zero time (filled symbols). Ampicillin added at zero time and penicillinase at time indicated by arrows (half-filled symbols). No additions (open symbols).

is likely to be killed during the viable count determination, which makes the death rate seem faster than it really is. Secondly, penicillin is known to give rise to large elongated organisms (see review by Rogers, 1962) and, if such organisms started to divide, the number of viable units might increase more rapidly than in normal growth.

Mutants resistant to ampicillin

Several penicillin-resistant mutants were derived from *Escherichia coli* strain x 5. They were isolated by the spreading of about 10^9 organisms on nutrient agar plates containing different concentrations of the D-isomer of ampicillin. The spontaneous frequency of mutants resistant to 1 μg. ampicillin/ml. was estimated for *E. coli* strain x 5 to be about 4 in 10^8 organisms and a similar figure was obtained for an *E. coli* strain B. Some of the mutants were characterized by their LIOG and MIC values, and by their growth on plates with ampicillin; the results are summarized in Table 1. For three mutants, LIOG values were determined for ampicillin and for penicillin G; Table 1 shows that the ratio of the LIOG values changed. Preliminary investigations have shown no penicillinase in the wild type but some in the mutants. A further study of penicillin-resistant mutants is planned.

Table 1. *Characterization of some penicillin-resistant mutants of* Escherichia coli *K12 strain x5*

Strain	Parent strain	Ampicillin during isolation (μg./ml.)	Limits of ampicillin on plates (μg./ml.)	MIC ampicillin (24 hr.) (μg./ml.)	LIOG ampicillin (μg./ml.)	LIOG penicillin G (μg./ml.)	LIOG penicillin G / LIOG ampicillin
x 5	—	—	< 1	4	6	54	9·0
x 5–1	x 5	1	4 < 8	8	19	—	—
x 5–4	x 5	1	4 < 8	8	19	—	—
x 5–5	x 5–1	5	4 < 8	8	19	110	5·8
x 5–8	x 5–5	50	120 < 240	64	180	870	4·8

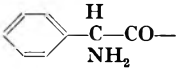
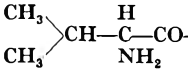
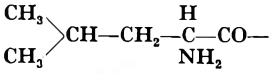
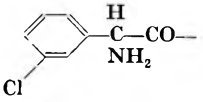
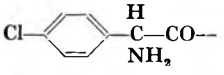
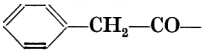
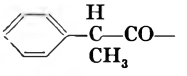
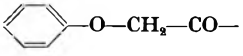
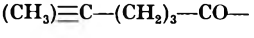
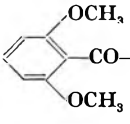
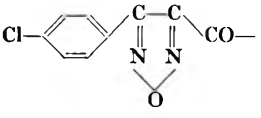
Strain x 5–8 was obtained after ultra-violet radiation, other mutants are spontaneous. The ampicillin used during isolation was pure D-isomer, while other experiments were done with SPC 270.

Influence of different side chains on the penicillin

Most penicillins so far have been found rather inactive against Gram-negative bacteria and relatively few comparisons have therefore been published about the action of different penicillins on *Escherichia coli*; Hugo & Russell (1960) and Russell (1962) reported comparisons of some penicillin derivatives. Rogers & Mandelstam (1962) studied inhibition of mucopeptide formation in *E. coli* and found ampicillin to be about 10 times more active than penicillin G. We have compared the lytic activity (estimated as LIOG values) of several penicillin derivatives, with strain x 5 as test organism; the results are given in Table 2. They show that the side chain of the penicillin molecule has a profound influence on the lysis rate of *E. coli*. This is exemplified by, for instance, the two chloro-substituted isomers of ampicillin, which differ by a factor of 4, the *m* form being more active than the *p* form. A comparison of the structures of the side chains shows that the substituents around the α-carbon atom are of importance. The most striking differences were recorded when a hydrogen in penicillin G was substituted with a methyl group or an amino group (compare

compounds nos. 1–3, 8 and 9). The fact that the D-isomer of ampicillin is almost 4 times more active than the L-isomer (see Fig. 2) shows that the steric configuration around the α-carbon atom is also of importance. It may be noted that penicillins nos. 12 and 13, which are resistant to penicillinase (Doyle *et al.* 1961) and

Table 2. *Penicillin derivatives tested on* Escherichia coli *K 12 strain x 5*

No.	Side chain	Steric form	Per cent penicillin*	LIOG (mμ-mole/ml.)
1		D	81[a]	17
2		L	99[a]	60
3		D + L (6:4)	83[a]	23
4		DL	78[a]	108
5		D	70[a]	55
6		DL	82[a]	36
7		DL	92[a]	130
8		—	99[b]	150
9		DL	91[a]	850
10		—	99[b]	600
11		—	92[a]	970
12		—	94[a]	> 4650
13		—	91[a]	> 3000
14	H—	—	99[a]	1430

* These data were obtained from Dr Sjöberg, Astra. *a* refers to analysis by differential alkalimetric titration; *b* refers to analysis by the hydroxylamine method. The remaining percentages are in most of the penicillins accounted for by moisture.

relatively active against certain Gram-positive bacteria, possess almost no lytic activity for *E. coli*. Cephalosporin N was not available to us in a sufficiently pure form.

We tested certain amino acids (whose residues are side chains in some of the

penicillins) for their effect on the time to lysis with 6-aminopenicillanic acid as well as ampicillin. The results of such an experiment with 6-aminopenicillanic acid and D-phenylglycine is shown in Fig. 7; synergistic effects were found also with valine +6-aminopenicillanic acid. Relatively high concentrations of amino acids were needed, but no influence on the growth curve was obtained in control experiments with the same concentration of amino acid only (compare open circles and open triangles in Fig. 7). The fact that phenylglycine gave synergistic effects with 6-amino penicillanic acid and with ampicillin is most easily explained as a limited hydrolysis and resynthesis of ampicillin, reactions which are both known to occur in *Escherichia coli*.

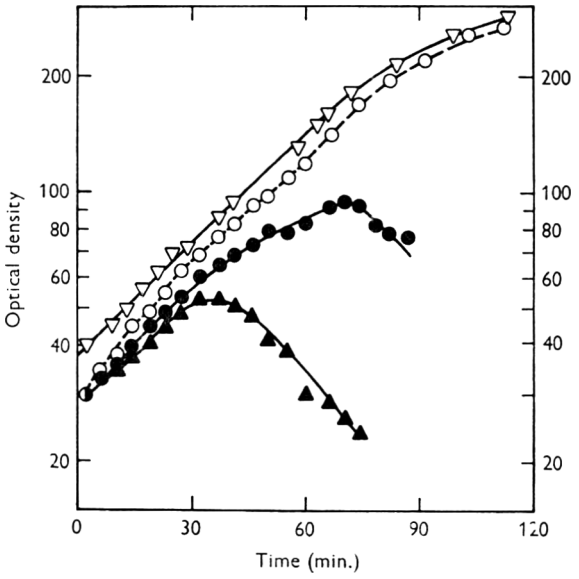


Fig. 7. Growth curves with 6-aminopenicillanic acid (100 μg./ml.) (●—●); 6-amino-penicillanic acid (100 μg./ml.)+D-phenyl glycine (350 μg./ml.) (▲—▲); D-phenyl glycine (350 μg./ml.) (▽—▽); and no addition (○—○).

DISCUSSION

In the extensive literature on penicillin action (see review by Hugo & Russell, 1961) the methods of observation normally used are measurements of viable count, which takes 48 hr., and minimal inhibitory concentration (MIC), which takes 24 hr. These times correspond, with *Escherichia coli* strain x5, to about 72 and 36 generations, respectively; and the two methods have a disadvantage in common in that they record the result of penicillin action rather than the course of the reaction. Prestidge & Pardee (1957) observed that bacteria treated with penicillin excreted a nucleotide-like material which can be used for recording of penicillin action on the bacteria. Optical methods were used by Liebermeister & Kellenberger (1956) for the study of penicillin action on *Proteus vulgaris* and by Fukasawa & Nikaido (1961) for a galactose-induced lysis of certain mutants of *Salmonella enteritidis*. The optical method of studying penicillin action would seem to have the following advantages: (1) To permit a relatively accurate recording of the time to lysis which is a basis for

an analysis of penicillin action. (2) To permit comparative investigations of penicillin derivatives with relatively short half-lives, as exemplified in the study of the D- and the L- isomers of ampicillin. (3) To permit a rapid recording of the penicillin sensitivity of a bacterial strain (expressed as LIOG value), information which sometimes is of clinical interest, and where the time saved (as compared with a determination of M.I.C.) can be of importance. The penicillin dependence of the lysis ratio is also a useful method for the characterization of penicillin-resistant mutants.

The most commonly accepted explanation for penicillin action (see reviews by Strominger, 1960; Rogers, 1962) is that the compound blocks cell-wall synthesis by inhibiting an enzyme which joins the Park & Strominger mucopeptide with the acceptor of the wall structure (Park & Strominger, 1957). Protein synthesis and presumably a large part of the cell metabolism is, however, unaffected by penicillin (Mandelstam & Rogers, 1959; Hancock & Park, 1958) and as the result of continued increase in cell mass the osmotic pressure is increased until lysis occurs. Conflicting statements have previously been made about penicillin action on RNA metabolism. However, we have found that the activity of soluble RNA seems to be affected (to be published) and this is reasonable, since uridylic and cytidylic compounds accumulate during penicillin action (Strominger, 1960; Saukkonen, 1961) and the re-utilization of these mucleotides may thus be blocked. Several investigators (Prestidge & Pardee, 1957; Hugo & Russell, 1961) have suggested that penicillin may have an additional action beside that of the inhibition of cell-wall synthesis.

The results of our work suggest that a mechanism for the action of penicillin on *Escherichia coli* would be consistent with the following three observations: (1) Within a wide range penicillin action is independent of the number of organisms in the culture. (This implies lack of penicillinase or any other enzyme which can cause inactivation of penicillin.) (2) The time to lysis is inversely proportional to the growth rate under balanced growth at 37°. (3) The lysis ratio has a characteristic dependence on the penicillin concentration, and is linear over a certain range. The first of these observations has been interpreted to mean that each rod may be considered as a separate unit and that the amount of penicillin possibly consumed is negligible for the concentration dependence observed during balanced growth.

The second relation, that there is a direct correlation between the growth rate and the time to lysis is consistent with the well-known observation that penicillin only kills growing organisms. In spite of this fact most previous workers have described the course of penicillin action only in time (minutes) and not in terms of generations. By using radioactive penicillin with Gram-positive organisms Cooper, Clowes & Rowley (1954) showed the specific binding of penicillin and they also introduced the concept of the penicillin-binding component (PBC). The biochemical nature of the PBC is yet unknown, but Cooper (1956) seemed to visualize the component as a membrane-bound enzyme; this hypothesis was further discussed by Pollock (1957). It is reasonable to assume that organisms with higher growth rate, in general, would have a higher rate of synthesis of their essential enzymes. Thus, if PBC were an enzyme and if penicillin action were the inactivation of this enzyme, one would expect a higher growth rate to decrease the sensitivity to penicillin; this is contrary to what we have observed. Two alternative explanations seem, however, to account for the fact that the time to lysis decreases with increasing growth rate. (*a*) PBC might

be an enzyme which is not inactivated by penicillin but which converts penicillin into another product which in turn is the real toxic factor for the bacteria. If so, then a higher rate of synthesis of PBC might lead to a higher concentration of the real toxic factor. (*b*) PBC might be an intermediate B in a pathway for the synthesis of cell-wall components. It would normally react with compound A–X to give the essential products B–X and A, and the absence of these compounds would cause lysis. This is not an unreasonable assumption, since it is known that starvation for lysine or diaminopimelic acid causes lysis (Meadow, Hoare & Work, 1957). In the presence of penicillin, intermediate B would instead react with penicillin (Pen) and give the products B–Y and D:

$$A\text{–}X + B \xrightarrow{E} B\text{–}X - A, \quad (1)$$

$$Pen + B \xrightarrow{E} B\text{–}Y + D. \quad (2)$$

If compound B were such an intermediate it might well be that its intracellular concentration would be inversively proportional to the growth rate. Thus, if the absence of B–X and A produced lysis, such a system would account for the fact that the lysis rate is proportional to the growth rate. The time required by the organisms to exhaust their steady state concentration of intermediate B would be recorded as the time to lysis. The penicillin dependence observed would be consistent with arguments about the expected influence that penicillin concentration and pulse doses might have on the rates of reactions (1) and (2).

It was pointed out by Cooper (1956) that the penicillin side chain seems to be of relatively little importance for the activity against Gram-positive organisms. Our results in Table 2 show that the side chain has a marked influence on penicillin action with strain x5 of *Escherichia coli*. A comparison of the LIOG values expressed on molar basis for compounds no. 12–14 show that these penicillin derivatives have a lower activity than 6-aminopenicillanic acid (no. 14) and that bulky and probably unbiological side chains can give lower activity than absence of side chain. Table 2 also shows that amino-acyl side chains in general give highly active penicillins. Since methyl and amino groups are of a similar size it would seem difficult to explain the differences between compounds nos. 3, 8 and 9 in Table 2 in terms of steric hindrance. The drastic influence of the side chain may, however, be accounted for by assuming an interaction, possibly a hydrogen bond, between certain groups on the α-carbon atom and the β-lactam ring.

An additional hypothesis seems to be consistent with our results, namely that the side chain could be transferred to an acceptor by a transpeptidase reaction. The side chains of penicillins nos. 12 and 13 would both be expected to provide steric hindrance for such a reaction and this might explain why these compounds are almost inactive. A transpeptidase reaction may be reasonable in view of the fact that the cell wall of *Escherichia coli* contains at least eleven common amino acids (Salton, 1960*b*; Work, 1961). Some strains of *E. coli* are also known to possess an enzyme which can hydrolyse the bond between the side chain and the 6-amino-penicillanic acid (English, McBride & Huang, 1960).

The reactions discussed above are only alternative ways to account for the action of penicillin. The model is speculative but may be useful in the number of further experiments which it can suggest, especially through the use of different labelled penicillins. It should be emphasized that the discussion concerns *Escherichia coli*.

Rogers & Mandelstam (1962) pointed out that the concentration of penicillin G required to inhibit mucopeptide synthesis is about 500 times higher for *E. coli* than for a sensitive strain of Staphylococcus. This drastic difference as well as recent comparisons of different penicillins on other strains (Knox & Smith, 1961; Rogers & Jeljaszewicz, 1961) may indicate that parts of the mechanism have species specificity.

We have had the advantage of discussing our results with several colleagues and would especially like to thank Drs B. Sjöberg and G. Bertani for helpful suggestions and criticism. We are also indebted to Dr H. J. Rogers for valuable discussions and for making two manuscripts under publication available to us. The work was supported by Astra Co. and by U.S. Public Health Service, grant no. RG-7576. K.G.E. has been holder of a Sven and Lilly Lawski fellowship.

REFERENCES

Abraham, E. P. & Newton, G. G. F. (1961). New penicillins, cephalosporin C and penicillinase. *Endeavour*, **20**, 92.

Batchelor, F. R., Doyle, F. P., Nayler, J. H. C. & Rolinson, G. N. (1959). Synthesis of penicillin: 6-aminopenicillanic acid in penicillin fermentations. *Nature, Lond.* **183**, 257.

Cooper, P. D., Clowes, R. C. & Rowley, D. (1954). A note on the use of radioactive penicillin. *J. gen. Microbiol.* **10**, 246.

Cooper, P. D. (1956). Site of action of radiopenicillin. *Bact. Rev.* **20**, 28.

Doyle, F. P., Long, A. A. W., Nayler, J. H. C. & Stove, E. R. (1961). New penicillins stable towards both acid and penicillinase. *Nature, Lond.* **192**, 1183.

English, A. R., McBride, T. J. & Huang, H. T. (1960). Microbial resistance to penicillin as related to penicillinase or penicillin acylase activity. *Proc. Soc. exp. Biol., N.Y.* **104**, 547.

Fukasawa, T. & Nikaido, H. (1961). Galactose-sensitive mutants of Salmonella. II. Bacteriolysis induced by galactose. *Biochim. biophys. Acta*, **48**, 470.

Hancock, R. & Park, J. T. (1958). Cell-wall synthesis by *Staphylococcus aureus* in the presence of chloramphenicol. *Nature, Lond.* **181**, 1050.

Hugo, W. B. & Russell, A. D. (1960). Action of 6-aminopenicillanic acid on Gram-negative bacteria. *Nature, Lond.* **188**, 875.

Hugo, W. B. & Russell, A. D. (1961). Review article. The mode of action of penicillin. *J. Pharm., Lond.*, **13**, 705.

Kjeldgaard, N. O., Maaløe, O. & Schaechter, M. (1958). The transition between different physiological states during balanced growth of *Salmonella typhimurium*. *J. gen. Microbiol.* **19**, 607.

Knox, R. & Smith, J. T. (1961). The nature of penicillin resistance in staphylococci. *Lancet*, ii, 520.

Lederberg, J. (1950). Isolation and characterization of biochemical mutants of bacteria. *Meth. med. Res.* **3**, 5.

Levy G. B. (1950). A unit of penicillase. *Nature, Lond.* **166**, 740.

Liebermeister, K. & Kellenberger, E. (1956). Studien zur L-Form der Bakterien. Die Umwandlung der bazillären in die globuläre Zellform bei Proteus unter Einfluss von Penicillin. *Naturforschung*, II*b*, 200.

Mandelstam, J. & Rogers, H. J. (1959). The incorporation of amino acids into the cell-wall mucopeptide of staphylococci and the effect of antibiotics on the process. *Biochem. J.* **72**, 654.

Meadow, P., Hoare, D. S. & Work, E. (1957). Interrelationship between lysine and α-, ϵ-diamino pimelic acid and their derivatives and analogues in mutants of *Escherichia coli*. *Biochem. J.* **66**, 270.

NEIDHARDT, F. C. & MAGASANIK, E. (1960). Studies on the role of ribonucleic acid in the growth of bacteria. *Biochim. biophys. Acta*, **42**, 99.

NOVELLI, G. D. (1960). Protein synthesis in microorganisms. *Annu. Rev. Microbiol.* **14**, 65.

PARK, J. T. & STROMINGER, J. L. (1957). Mode of action of penicillin. Biochemical basis for the mechanism of action of penicillin and for its selective toxicity. *Science*, **125**, 99.

POLLOCK, M. R. (1957). The activity and specificity of inducers of penicillinase production in *Bacillus cereus*, strain NRRL 569. *Biochem. J.* **66**, 419.

POLLOCK, M. R. (1959). Induced formation of enzymes. In *The Enzymes*, Ed. by P. D. Boyer, H. Lardy & K. Myrbäck. Vol. 1, p. 619. New York: Academic Press.

PRESTIDGE, L. S. & PARDEE, A. B. (1957). Induction of bacterial lysis by penicillin. *J. Bact.* **74**, 48.

ROBERTS, R. B., ABELSON, P. H., COWIE, D. B., BOLTON, E. T. & BRITTEN, R. J. (1957). Studies of biosynthesis in *Escherichia coli*. Washington, D.C.: Publ. Carneg. Instn. No. 607.

ROGERS, H. J. & JELJASZEWICZ, J. (1961). Inhibition of the biosynthesis of cell-wall mucopeptides by the penicillins. A study of penicillin-sensitive and penicillin-resistant strains of *Staphylococcus aureus*. *Biochem. J.* **81**, 576.

ROGERS, H. J. (1962). Mode of action of the penicillins. In *Resistance of Bacteria to the Penicillins. CIBA Foundation Study Group*, No. 13, p. 25. London: J. and A. Churchill Ltd.

ROGERS, H. J. & MANDELSTAM, J. (1962). Inhibition of cell wall mucopeptide formation in *Escherichia coli* by benzylpenicillin and 6 [D(-)-α-amino-phenylacetamido] penicillanic acid ('ampicillin'). *Biochem. J.* **84**, 299.

ROLINSON, G. N. & STEVENS, S. (1961). Microbiological studies on a new broad-spectrum penicillin, 'Penbritin'. *British med. J.* (22/7), 191.

RUSSELL, A. D. (1962). A note on the activity of six penicillins against *Escherichia coli*. *J. Pharm., Lond.* **14**, 390.

SALTON, M. R. J. (1960*a*). *Microbial Cell Walls*. New York and London: John Wiley and Sons, Inc.

SALTON, M. R. J. (1960*b*). Surface layers of the bacterial cell. In *The Bacteria*, Ed. by I. C. Gunsalus & R. Y. Stainer, Vol. 1, p. 123. New York: Academic Press.

SAUKKONEN, J. J. (1961). Acid-soluble nucleotides of *Staphylococcus aureus*: massive accumulation of a derivative of cytidine diphosphate in the presence of penicillin. *Nature, Lond.* **192**, 816.

STROMINGER, J. L. (1960). Mononucleotide acid anhydrides and related compounds as intermediates in metabolic reactions. *Physiol. Rev.* **40**, 55.

TODD, E. M. (1945). Bacteriolytic action of penicillin. *Lancet*, i, 74.

VOGEL, H. J. & BONNER, D. M. (1956). Acetylornithinase of *Escherichia coli*: partial purification and some properties. *J. biol. Chem.* **218**, 97.

WORK, E. (1961). The mucopeptides of bacterial cell walls. A review. *J. gen. Microbiol.* **25**, 167.

J. gen. Microbiol. (1963), **31**, 353–363
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Suppression by Methionine of Reversions to Adenine Independence in *Schizosaccharomyces pombe*

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SUMMARY

Spontaneous mutations, and those induced by nitrous acid or ultraviolet light, were studied in the haploid fission yeast *Schizosaccharomyces pombe*. Reverse mutations conferring ability to grow on a minimal medium lacking adenine were scored in adenine auxotrophs, and in di-auxotrophs having requirements for both adenine and methionine. The apparently lower mutability of the *ad-1* mutants in the *ad*$^-$*met*$^-$ diauxotrophic strains compared with the *ad*$^-$ strains was shown to be due to the influence of methionine in the plating medium. L-methionine added to the minimal medium at a concentration of 40 μg. per ml. will suppress the appearance of spontaneous and induced revertants of *adenine-1* mutants. A similar effect has been shown for reversions of an adenine mutant at another locus, and for reversions of a leucine auxotroph. Methionine has the effect of decreasing the extent of residual divisions undergone by adenine-requiring cells when plated on a minimal medium.

INTRODUCTION

Several examples have been found in micro-organisms where change of the genetic background markedly alters the ability of a particular mutant to undergo spontaneous and induced reversion. Glover (1956) quoted several cases in *Escherichia coli* where reversions of one particular auxotroph were influenced to a considerable degree by the presence or absence of other auxotrophic or drug-resistance markers in the same strain. Witkin & Theil (1960) found that a particular trytophan auxotroph of *E. coli* which reverted strongly after treatment with u.v. light would no longer do so if a marker for streptomycin-dependence was introduced into the strain. The most dramatic examples of the influence of genetic background upon mutability are found in microbial strains carrying a mutator gene. This latter may have a profound effect on the spontaneous mutability of many other loci (Treffers, Spinelli & Belser, 1954; Miyake, 1960).

In the present work, investigations were made of spontaneous and induced reverse mutations from requirement to non-requirement for adenine (*ad*$^-$ $\rightarrow$ *ad*$^+$) in strains of the haploid fission yeast *Schizosaccharomyces pombe*. The genetics of this organism have been studied extensively by Leupold (1955, 1958, 1961), and reverse mutation experiments were carried out by Heslot (1960, 1962) and Fritz-Niggli (1960). In the work to be described *ad*$^+$ reversions were scored both in strains having adenine as the only requirement, and in strains requiring both adenine and methionine (*ad*$^-$*met*$^-$). The influence of the *met*$^-$ marker on the mutability of the

ad⁻ mutants was investigated. The results obtained led to an explanation at a physiological level of an apparent influence of genetic background on mutability.

METHODS

Strains of Schizosaccharomyces pombe. Ten mutants located at the *adenine-1* locus were kindly provided by Professor U. Leupold. These mutants were produced by u.v. irradiation of a wild-type strain (Leupold, 1955). They are blocked at an early step in the biosynthesis of adenine, before the formation of 5-aminoimidazole ribotide (Leupold, 1961), and do not accumulate a red pigment when grown in limiting concentrations of adenine. These ten *ad-1* mutants are located at ten different sites within the locus (Leupold, Ramirez & Clarke, unpublished results). Most of the experiments were carried out with three mutants, numbers 3, 25 and 40.

Mutants at other loci were provided by Professor U. Leupold, Dr H. Heslot and Mr R. B. Drysdale. Doubly auxotrophic strains requiring both adenine and methionine were isolated from crosses between the singly auxotrophic strains. Except where otherwise stated mutants used in experiments were of the h^- heterothallic mating type.

Complete media. For growth in liquid culture YE medium was used containing 'Difco' Bacto Yeast Extract 5 g., D(+) glucose 30 g., distilled water 1000 ml. For growth of adenine auxotrophs this medium was supplemented with adenine sulphate, 50 mg./l. (YE + Ad). Cultures on solid medium were grown on 'Oxoid' Yeast Extract Agar with the addition of 3% (w/v) glucose, and adenine 50 mg./l. (YEA + Ad).

Minimal medium (MMA). The minimal medium agar used was that of Leupold (1955), but with glucose added to 2% (w/v). 'Ionagar No. 2' (Oxoid) was added to a concentration of 1·6% (w/v).

Buffer (GSVB). Giese's modification of Reader salts solution (Giese, Iverson & Sanders, 1957; Reader, 1927) was used, with the addition of KH_2PO_4 to a concentration of 0·05 M (6·8 g./l.) and of the four vitamins essential for *Schizosaccharomyces pombe*.

Reverse mutations. Cells for mutation experiments were harvested by centrifugation from aliquots of 80 ml. YE + Ad cultures grown with aeration for 48 hr. at 30° in 250 ml. flasks. Cultures were inoculated from colonies on YEA + Ad plates. Aeration was provided by a gentle stream of sterile filtered air.

The cells were resuspended in GSVB, pre-warmed when necessary. For treatment with alkylating agents the cells were resuspended in distilled water plus essential vitamins, and washed after treatment to remove the chemical.

Treatment with nitrous acid was carried out in GSVB suspension pre-warmed to 25° by the addition of a calculated volume of a sterile 5 M-solution of $NaNO_2$. Routinely the final concentration of $NaNO_2$ was 0·1 M, and the treatment was carried out in a 25° water bath.

For u.v. irradiation 5 ml. samples of GSVB suspensions at room temperature were pipetted into sterile 5 cm. diam. Petri dishes. Irradiation was carried out at a distance of 42 cm. from the source, a Phillips 15 w TUV tube. The output of this source in ergs/mm.²/sec. is not known. The suspensions were agitated throughout irradiation by means of a Towers–Gibson vibro shaker. All procedures during and following irradiation were carried out in yellow light to avoid photo-reactivation.

After treatment 0·1 or 0·2 ml. samples of cell suspensions, and of 10^{-1} dilutions, were spread on the surface of MMA plates, supplemented when necessary with growth factors. Plating of the 10^{-1} dilution samples allowed a check to be made for the absence of suppression of revertant colonies by excess auxotrophs (Grigg, 1952). Serial dilutions were made in GSVB and $5 \times 0{\cdot}2$ ml. samples of 10^{-5} dilutions were plated on supplemented MMA to assay viable count. Plates were left at room temperature for 1 hr. to absorb surface moisture, then incubated at 30°. Revertant colonies which approached wild-type in size were scored after 7 days.

RESULTS

In experiments with singly auxotrophic *ad*$^-$ strains and with doubly auxotrophic *ad*$^-$*met*$^-$ strains it was observed that the frequency of appearance of spontaneous *ad*$^+$ revertants was appreciably lower in the *ad*$^-$*met*$^-$ than in the *ad*$^-$ strains. The results for spontaneous reversions of mutants *ad-1*, *3* and *ad-1*, *40* are shown in Fig. 1, unshaded columns A, C, A′ and C′. The lower frequency of spontaneous *ad*$^+$ revertants in *ad*$^-$*met*$^-$ strains compared with *ad*$^-$ strains was found also for induced revertants. Results for reversions of mutant *ad-1*, *40* induced by nitrous acid are shown in Fig. 1, unshaded columns A″ and C″.

Before concluding that the lowered mutability of the *ad-1* mutants in the *ad*$^-$*met*$^-$ strains was due to the influence of the genetic background, it seemed essential to test the effect of plating media upon survival and the expression of reversions. In the singly auxotrophic *ad*$^-$ strains, survival is assayed on minimal medium plus adenine, and *ad*$^+$ revertants are scored on minimal medium. In the *ad*$^-$*met*$^-$ strains, however, survival is assayed on minimal medium plus adenine and methionine, and *ad*$^+$ revertants on minimal medium plus methionine.

Tests were made of the influence of methionine in the plating media upon the survival of the singly auxotrophic *ad*$^-$ cells, and upon the expression of *ad*$^+$ reversions in *ad*$^-$ strains. Methionine added to the plating medium was without obvious influence upon the ability of *ad*$^-$ cells to grow in the presence of adenine. This was true both for untreated cells and for those which had been treated with a mutagen.

Influence of methionine upon revertants of ad$^-$ *strains.* Although methionine in the plating medium exerted no influence on the survival of *ad*$^-$ cells when adenine was also present, there was an obvious effect on the expression of *ad*$^+$ revertants (Clarke, C. H. 1962, *Microbiol. Gen. Bull.* **18**, 7). The numbers of spontaneous and induced *ad*$^+$ revertants were consistently higher on minimal medium than on minimal supplemented with methionine. These results are shown in Fig. 1 and Table 1. The suppressive effect of methionine upon the appearance of *ad*$^+$ colonies is not dependent on the dose of mutagen used as judged by percentage survival of *ad*$^-$ cells.

Delayed addition of methionine. Since methionine added to the minimal medium suppressed the appearance of *ad*$^+$ revertant colonies, experiments were carried out to determine at what stage of the 7-day incubation period methionine exerted its effect. These experiments involved delayed additions of methionine to plates of minimal medium on which untreated or treated cells of *ad*$^-$ mutants had been spread. After known periods of incubation at 30°, concentrated L-methionine solution was pipetted into small wells cut in the agar, sufficient to give a final

concentration of about 40 μg./ml. in the minimal medium. After 1 hr. at room temperature the plates were then re-incubated at 30° until the end of the 7-day period.

Figure 2 shows the influence of delayed addition of methionine on revertants of mutant *ad-1, 40* induced by nitrous acid. It will be seen that the extent of the

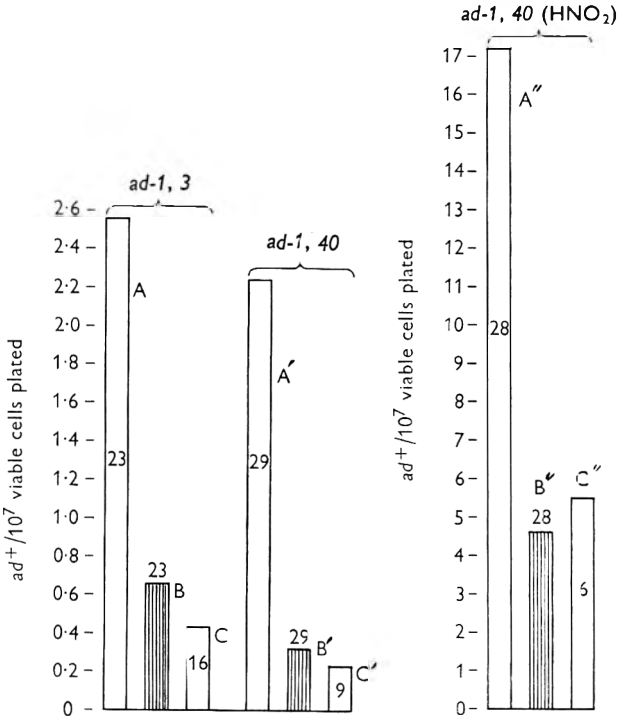

Fig. 1. Reversions of *ad-1, 3* and *ad-1, 40*. Columns A and A′ unshaded. Spontaneous *ad*$^+$ revertants of *ad-1, 3* and *ad-1, 40* respectively, scored on minimal medium (MMA). Column A″ unshaded. Nitrous acid-induced reversions of *ad-1, 40* scored on minimal medium. Mean survival from 28 estimations = 75·2 %. Columns B and B′ shaded. Spontaneous *ad*$^+$ revertants of *ad-1, 3* and *ad-1, 40* scored on minimal medium supplemented with L-methionine at 40 μg./ml. (MMA + Met.) Column B″ shaded. Nitrous acid-induced reversions of *ad-1, 40* scored on MMA + Met medium. Mean survival (28 estimates) = 75·2 %. Columns C and C′ unshaded. Spontaneous revertants of *ad-1, 3* and *ad-1, 40* in *ad*$^-$*met*$^-$ di-auxotrophic strains. *Ad*$^+$ revertants scored on MMA + Met medium. Column C″ unshaded. Nitrous acid-induced reversions of *ad-1, 40* scored in the *ad*$^-$*met*$^-$ di-auxotrophic strain on MMA + Met medium. Mean survival (6 estimates) = 79·5 %. Heights of the columns represent numbers of *ad*$^+$ revertants per 10^7 viable cells plated. Numbers within or directly above columns represent the number of estimates upon which the results are based.

suppressive effect of methionine on *ad*$^+$ revertants decreased with increasing periods of incubation before methionine addition. After about 100 hr. of incubation the addition of methionine has no influence on the numbers of *ad*$^+$ revertants scored.

A similar effect is seen for spontaneous revertants of *ad*$^-$ mutants which arise during the period of incubation on the MMA plates, though the graph is less precise due to the smaller numbers of *ad*$^+$ colonies scored.

Influence of methionine upon colony formation from single cells. Since methionine inhibited the appearance of ad^+ revertant colonies, an investigation was made to determine at what stage of the mutagenic sequence methionine exerted its influence. The last step in the sequence is the growth of a single phenotypically ad^+ cell into a visible colony. Reconstruction experiments were carried out to see if methionine inhibited this last process. Small numbers of wild-type cells, or of ad^+ revertant cells, were mixed with a large known excess of ad^- cells. These mixtures were plated at various cell densities both on minimal medium and on minimal medium supplemented with methionine. The results obtained indicated that methionine has no significant influence on the growth of single phenotypically ad^+ cells into visible colonies, even in the presence of a large excess of ad^- cells. It was concluded that methionine must exert its inhibitory action at some earlier step in the mutagenic sequence than that of colony formation.

Table 1. *Influence of methionine upon reversions to adenine independence*

Strain	No. of esti-mates	Mutagen	Mean survival (%)	Minimal medium (MMA)		Minimal + methionine (MMA + Met)	
				Actual no.* ad^+	$Ad^+/10^7$ viable cells plated	Actual no.* ad^+	$Ad^+/10^7$ viable cells plated
ad-1, 3	23	Spontaneous	—	688	2·55	185	0·65
ad-1, 3.met-4, D19	16	Spontaneous	—	—	—	106	0·43
ad-1, 25	11	HNO_2	77	272	10·6	8	0·41
ad-1, 40	29	Spontaneous	—	417	2·24	55	0·32
ad-1, 40.met-4, D19	9	Spontaneous	—	—	—	16	0·23
ad-1, 40	28	HNO_2	75·2	2106	17·2	632	4·56
ad-1, 40.met-4, D19	6	HNO_2	79·5	—	—	208	5·48
ad-1, 40	13	u.v.	73·2	521	4·04	100	0·69
ad-1, 169	10	HNO_2	91·6	74	1·42	3	0·04
ad-1, 199	19	HNO_2	72·1	922	14·2	735	11·6
ad-1, 199	10	u.v.	72·1	345	4·8	20	0·15

* Uncorrected number of ad^+ colonies scored. If all the plates of a series could not be scored then the actual number scored on the remaining plates is given.

Investigations of events occurring on the plates. Experiments were performed involving observations of the residual divisions undergone by ad^- cells when they were incubated on plates of minimal medium or minimal medium plus methionine. Untreated cells, and cells treated with nitrous acid, were spread at a density of about 5×10^3 cells per plate, incubated at 30° and observations made under $\times 320$ magnification after known periods of time. Initially most of the cells occurred singly, but later divided to form small groups of cells (Bertani, 1951). Figure 3 shows residual divisions of HNO_2-treated cells of mutant *ad-1, 40*. Clearly the presence of methionine in the plating medium greatly inhibits the residual divisions undergone by the ad^- cells.

Actually the agar present in the minimal medium, Ionagar No. 2, contains trace amounts of adenine (M. Luzzati, personal communication), and the residual divisions may be due to this fact. It must be pointed out that residual divisions were followed on plates bearing only some 5×10^3 cells. Revertants are scored on

plates bearing 10^6 or 10^7 cells. Thus the extent of residual divisions actually observed may not be the same as the residual divisions on the plates upon which ad^+ colonies are scored.

If one or two divisions are required after treatment with nitrous acid before an ad^+ reversion is phenotypically expressed (Rudner, 1961) then methionine might well decrease the number of ad^+ revertants scored, simply by inhibiting the residual divisions of ad^- cells.

Differential survival of ad$^-$ *cells in the presence and the absence of methionine in the minimal medium.* Since methionine inhibited the residual divisions undergone by ad^- cells on the minimal medium plates, tests were made to see if such cells remained viable to the same extent both on minimal medium and on minimal plus methionine. Aliquots of 0·2 ml. of 10^{-5} dilutions of suspensions of untreated ad^- cells, and of cells treated with nitrous acid, were spread on plates of minimal

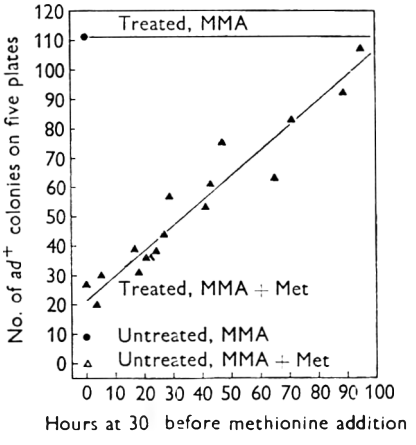

Fig. 2

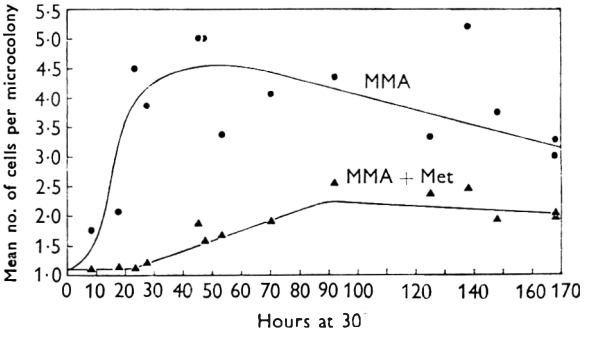

Fig. 3

Fig. 2. The influence of delayed addition of methionine on HNO_2-induced reversions of mutant *ad- 1,40*. Numbers of ad^+ revertant colonies appearing after 7 days' incubation at 30° on five plates are plotted against time in hours before MMA plates received delayed addition of methionine. Untreated series. 1·17 ad^+ reversions per 10^7 viable cells plated on minimal medium (MMA) but no ad^+ reversions per 10^7 viable cells plated on MMA + methionine. Treated series. HNO_2, 98·6 % survival. 14·6 ad^+ reversions per 10^7 viable cells plated on MMA. 3·57 ad^+ reversions per 10^7 viable cells when plated initially on MMA + Met medium. ▲ = MMA plates bearing HNO_2-treated cells which received delayed methionine additions. ● = MMA plates bearing cells which received no delayed addition of methionine. △ = untreated cells on MMA + Met plates, i.e. methionine present throughout the 7-day period of incubation.

Fig. 3. Residual divisions of *ad-1, 40* cells treated with nitrous acid (98·6 % survival) on minimal (MMA) and MMA + methionine plates incubated at 30°. Mean number of cells per microcolony is plotted against hours of incubation at 30°. ▲ = MMA + Met plates. ● = MMA plates.

medium and of minimal medium supplemented with methionine. Plates were incubated at 30° and after known periods of time sets of five minimal and five minimal plus methionine plates received delayed additions of adenine. This was carried out by spraying a sterile aqueous solution of adenine hydrochloride (1 mg./ml.) on to the surface of the plates by means of a chromatography spray. Colonies were scored 7 days after the delayed addition of adenine.

Results for untreated cells of mutant *ad-1, 40*, and for cells treated with nitrous acid, are shown in Fig. 4. From this graph it can be seen that a decrease in viable count is more rapid on minimal medium supplemented with methionine than on plates of unsupplemented minimal medium. This result is probably due to the fact that on minimal medium plus methionine each colony-forming unit contains fewer cells, because of decreased residual divisions, than is the case on minimal medium. It must be pointed out that of necessity these differential survival experiments were carried out at a much lower plating density than is used for the scoring of revertant colonies. On the actual plates on which ad^+ colonies are scored the extent of differential survival may be different from that described above.

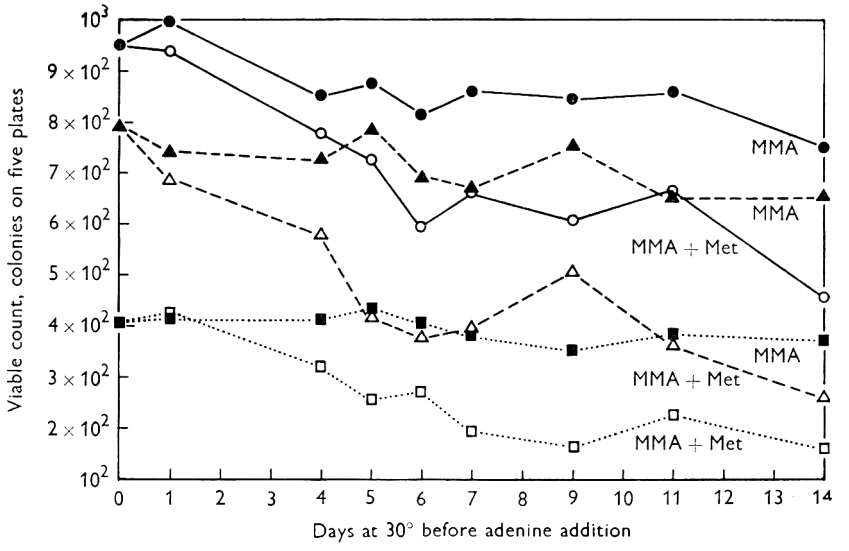

Fig. 4. The differential survival of untreated and HNO_2-treated cells of *ad-1, 40* on minimal medium (MMA) and minimal medium plus methionine (MMA + Met). Cells were plated at a density of about 100–200 viable cells per plate. The plates were incubated at 30° and at intervals received delayed addition of adenine. Colonies were scored 7 days following adenine addition. Viable count is plotted against number of days incubation before delayed adenine addition. ●—● Untreated cells incubated on MMA plates. ○—○ Untreated cells incubated on MMA + Met. ▲- -▲ HNO_2-treated cells (83 % survival) incubated on MMA plates. △- -△ HNO_2-treated cells (83 % survival) incubated on MMA + Met plates. ■····■ HNO_2-treated cells (42 % survival) incubated on MMA plates. □····□ HNO_2-treated cells (42 % survival) incubated on MMA + Met plates.

Influence of methionine upon other revertants. Tests were made to determine whether or not the presence of methionine in the plating medium would suppress the appearance of revertants at loci other than *adenine-1*. An adenine auxotroph, *ad-D2*, which is blocked at one of the first two steps between the formation of 5-aminoimidazole ribotide and inosinic acid (Leupold, 1961), was tested. This mutant accumulates a red pigment when grown in the presence of limiting concentrations of adenine. Spontaneous revertants of *ad-D2* and revertants induced by nitrous acid appeared to be suppressed by the addition of methionine to the minimal medium. The appearance of large colony revertants of the leucine auxotroph *leu-3, 241* induced by nitrous acid was also inhibited when platings were made on minimal medium plus methionine.

Minute colony revertants which are scored after 15 days' incubation at 30° do not appear to be inhibited by the presence of methionine. These minute colony revertants are due to suppressor mutations at loci other than that of the original mutant (Heslot, 1962). Tests were made with spontaneous minute colony revertants, and such revertants induced by treatment with di-ethyl sulphate, ethyl methane sulphonate, and di-epoxybutane. The arginine auxotroph *arg-1, 230*, the leucine auxotroph *leu-3, 241* and the uracil auxotroph *ura-1, 171* were employed in these experiments, some of which were performed with Dr H. Heslot. Methionine apparently had no influence on the expression of minute colony (suppressor) revertants of the above auxotrophs, as is shown in Table 2.

Table 2. *Influence of methionine upon slow growing suppressor reversions (15-day counts)*

			Reversions on MMA*		Reversions on MMA + Met	
Strain	Mutagen	Survival (%)	Actual no. scored*	No./10^7 viable cells plated	Actual no. scored†	No./10^7 viable cells plated
arg-1, 230	Spontaneous	—	84	37·8	70	31·5
	D.E.S.‡	52	769	6620	768	6620
	Spontaneous	—	183	31·0	233	39·5
	D.E.B.	78·5	520	1120	351	950
	E.M.S.	78	704	1520	680	1470
	Spontaneous	—	73	33·4	65	42·5
leu-3, 241	Spontaneous	—	13	0·64	8	0·53
	D.E.S.	63	1984	154	1916	149
	Spontaneous	—	4	0·63	2	0·25
	E.M.S.	62·9	79	15·9	54	10·8
ura-1, 171	Spontaneous	—	27	1·9	24	1·7
	D.E.S.	72·7	113	17	163	16·4
	Spontaneous	—	10	2·4	13	2·5
	D.E.B.	100	25	4·9	28	5·5
	E.M.S.	89	38	8·3	28	6·1
	Spontaneous	—	19	2·0	48	2·5
	HNO_2	81·9	104	6·5	84	5·3

* *MMA* = minimal medium agar. MMA + Met = minimal medium agar + L-methionine, 40 μg./ml.

† Actual numbers of slow growing revertant colonies scored, uncorrected for cases in which all the plates in a series could not be scored.

‡ D.E.S. = di-ethyl sulphate. D.E.B. = di-epoxybutane.
E.M.S. = ethyl methane sulphonate.

Influence of other amino acids on the expression of reversions. A few experiments were performed with ad^- mutants in which platings for ad^+ revertants were made on minimal medium supplemented with L-histidine (40 μg./ml.) or L-leucine (200 μg./ml.). Both these amino acids appeared to have no influence on revertants of either *ad-1, 40* or *ad-D2*. Results are shown in Table 3. Clavilier, Luzzati & Slonimski (1960) likewise found histidine to be without effect on reverse mutations of *ad-3* mutants in haploid strains of *Saccharomyces cerevisiae*.

Table 3. *Influence of histidine and leucine on* ad$^+$ *reversions*

Mutant	Mutagen	Survival (%)	*Ad*$^+$ reversions on MMA* Actual no.† of *ad*$^+$	MMA* *Ad*$^+$/10^7 viable cells plated	MMA + His Actual no.† of *ad*$^+$	MMA + His *Ad*$^+$/10^7 viable cells plated	MMA + Leu Actual no.† of *ad*$^+$	MMA + Leu *Ad*$^+$/10^7 viable cells plated
ad-1, 40	Spontaneous	—	4	0·57	7	1·0	4	0·57
	HNO_2	54·2	11	2·89	17	4·45	10	2·62
	Spontaneous	—	32	3·4	30	3·19	41	4·35
	HNO_2	82	65	8·45	74	9·6	72	9·35
ad-D2	Spontaneous	—	0	0	3	0·63	3	0·63
	HNO_2	41·8	13	8·2	15	7·6	11	5·56
ad-1, 199	Spontaneous	—	9	1·52	8	1·36	—	—
	HNO_2	105	25	4·06	20	3·26	—	—
		101	45	7·57	31	5·21	—	—
		90	74	14·0	44	10·4	—	—
		79	93	20·0	84	18·0	—	—
		102	95	15·8	83	13·8	—	—
		91	93	17·4	82	15·3	—	—
		90	111	21·0	106	20·0	—	—
		84	117	23·7	83	16·8	—	—
		89	122	23·5	108	20·8	—	—

* MMA = minimal medium agar.
His = L-histidine at 40μg./ml. of MMA medium.
Leu = L-leucine at 200μg./ml.
† Uncorrected actual number of *ad*$^+$ revertants counted, even when all the plates of a series could not be scored.

DISCUSSION

Reverse mutation experiments are fraught with difficulties of interpretation because survival and mutations are scored under quite different conditions of medium and plating density. Interpretation of the results of experiments with microbial strains having several markers are equally difficult because the different types of reverse mutation are scored on media supplemented in different ways. The work of Glover (1956) in which he found dramatic effects of the introduction of a new marker upon the mutability of a given mutant was complicated by plating medium differences. The explanation of the loss of u.v. mutability of a tryptophan (*try*$^-$) mutant when a marker for streptomycin dependence (*str-d*) was introduced into the same strain (Witkin & Theil, 1960) is again complicated by differences of plating medium. In the original *try*$^-$ strain *try*$^+$ reversions were scored on supplemented minimal medium. In the *try*$^-$ *str-d* strain, however, *try*$^+$ revertants were scored on supplemented minimal medium plus streptomycin.

Zetterberg (1960), working with *Ophiostoma multiannulatum*, found some evidence for the influence of a *met*$^-$ marker upon mutability of a uracil auxotroph, but was later able to exclude an influence of methionine upon the expression of *ura*$^+$ revertants (Zetterberg, 1962).

Auerbach & Kølmark (1960) were able to show that the different plating media were certainly not mainly responsible for the mutagen specificity found in an adenine-less (*ad*$^-$), inositol-less (*inos*$^-$) di-auxotroph of *Neurospora crassa*. They tested the

influence of inositol on revertants of the singly auxotrophic *ad*$^-$ strain, and the influence of adenine on the *inos*$^-$ strain.

In the present case there was no influence of methionine upon the survival of *ad*$^-$ cells in the presence of adenine, but only upon revertant expression. The extent to which supplementation with L-methionine at 40 μg./ml. of the minimal medium inhibits expression of *ad*$^+$ reversions depends not only on the particular mutant tested, but also upon the mutagen used to induce reversions. For mutant *ad-1, 199* treated with HNO_2 a total of 922 *ad*$^+$ colonies were scored on minimal medium, and 735 on minimal plus methionine. For u.v.-induced revertants of the same mutant, 345 *ad*$^+$ colonies arose on minimal, 20 on minimal plus methionine medium. This difference in suppression of HNO_2- and u.v.-induced reversions of *ad-1, 199* is highly significant at the 1 % level (Clarke, 1962). Similarly there is a difference significant at the 1 % level in the degree of methionine suppression of u.v.-induced revertants of the two mutants *ad-1, 40* and *ad-1, 199*. These results may be due to the fact that mutant 199 is temperature sensitive, being adenine-requiring at 30°, but adenine-independent at 25°.

It is not known if suppression of reversions is a direct consequence of impaired residual divisions, or if depression of revertants and residual divisions are both features of an underlying biochemical effect. Methionine metabolism has been shown to interact with adenine biosynthesis in *Saccharomyces cerevisiae* (Lindegren, 1949; Woodward & Rainbow, 1961). However, in the present experiments reversions induced by nitrous acid treatment of a leucine auxotroph were also depressed in the presence of methionine. Since the slow-growing suppressor revertants of auxotrophs *leu-3, 241, arg-1, 230*, and *ura-1, 171* were not inhibited by methionine, it might be thought that methionine only inhibited the expression of true reverse mutations, but not that of reversions due to suppressor mutations. However, it has recently been found (Clarke, unpublished results) that spontaneous revertants of *ad-1, 40*, susceptible to methionine inhibition, are often due to suppressors. The possibility still remains that methionine only inhibits the expression of revertants showing rapid growth on minimal medium, but not of minute colony, slowly growing revertants.

A somewhat similar case of a plating medium effect involving an amino acid has been described by Luzzati, Clavilier & Slonimski (1959). Histidine was found to exert a depressive effect on non-reciprocal recombination (gene conversion) giving rise to prototrophs at the adenine-histidine (*ad-3*) locus in *Saccharomyces cerevisiae*. The inhibitory effect of histidine was counteracted by very low concentrations of adenine.

It is not known if the inhibitory action of methionine upon *ad*$^+$ reversions in *Schizosaccharomyces pombe* is similarly counteracted by adenine, or if amino acids other than methionine have a similar suppressive effect. The results indicate, however, that plating medium effects may explain some cases of apparent influence of the genetic background on mutability.

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REFERENCES

AUERBACH, C. & KØLMARK, G. (1960). Mutagen specificity and combination treatments in Neurospora. *Microbiol. Gen. Bull.* **17**, 23.

BERTANI, G. (1951). A method for detection of mutations using streptomycin dependence in *Escherichia coli*. *Genetics*, **36**, 598.

CLARKE, C. H. (1962). A case of mutagen specificity attributable to a plating medium effect. *Z. Vererb.-Lehre*, **93**, 435.

CLAVILIER, L., LUZZATI, M. & SLONIMSKI, P. P. (1960). Sur la conversion du gène *ad 3* chez la levure. *C.R. Soc. Biol., Paris*, **154**, 1970.

FRITZ-NIGGLI, H. (1960). Röntgenstrahleninduzierte Rückmutationen (Punktmutationen) bei *Schizosaccharomyces pombe* in Abhängigkeit vom Zellzustand, Bestrahlungsmilieu und Agens der Vorwärtsinduktion. *Naturwissenschaften*, **18**, 429.

GIESE, A. C., IVERSON, R. M. & SANDERS, R. T. (1957). Effect of nutritional state and other conditions on ultra-violet resistance and photoreactivation in yeast. *J. Bact.* **74**, 271.

GLOVER, S. W. (1956). A comparative study of induced reversions in *Escherichia coli*. *Publ. Carneg. Instn*, **612**, 121.

GRIGG, G. W. (1952). Back mutation assay in micro-organisms. *Nature, Lond.* **169**, 98.

HESLOT, H. (1960). *Schizosaccharomyces pombe*: un nouvel organisme pour l'étude de la mutagenèse chimique. *Abh. dtsch. Akad. Wiss. Berl., Klasse Medizin*, **1**, 98.

HESLOT, H. (1962). Etude quantitative de reversions biochimique induites chez la levure *Schizosaccharomyces pombe* par des radiations et des substances radiomimetiques *Abh. dtsch. Akad. Wiss. Berl., Klasse Medizin*, **1**, 193.

LEUPOLD, U. (1955). Versuche zur genetischen Klassifizierung adeninabhängiger Mutanten von *Schizosaccharomyces pombe*. *Arch. Klaus-Stift. VererbForsch.* **30**, 506.

LEUPOLD, U. (1958). Studies on recombination in *Schizosaccharomyces pombe*. *Cold Spr. Harb. Symp. quant. Biol.* **23**, 161.

LEUPOLD, U. (1961). Intragene Rekombination und allele Komplementierung. *Arch. Klaus-Stift. VererbForsch.* **36**, 89.

LINDEGREN, C. C. (1949). *The Yeast Cell: Its Genetics and Cytology*, Chap. 15. St Louis: Educational Publishers Inc.

LUZZATI, M., CLAVILIER, L. & SLONIMSKI, P. P. (1959). Inhibition par l'histidine de la réversion prototrophe chez la levure diploide hétéroallélique pour le locus adénine-histidine. *C.R. Acad. Sci. Paris*, **249**, 1412.

MIYAKE, T. (1960). Mutator factor in *Salmonella typhimurium*. *Genetics*, **45**, 11.

READER, V. (1927). The relation of the growth of certain micro-organisms to the composition of the medium. I. The synthetic culture medium. *Biochem. J.* **21**, 901.

RUDNER, R. (1961). Mutation as an error in base pairing. I. The mutagenicity of base analogues and their incorporation into the DNA of *Salmonella typhimurium*. *Z. Vererb.-Lehre*, **92**, 336.

TREFFERS, H. P., SPINELLI, V. & BELSER, N. O. (1954). A factor (or mutator gene) influencing mutation rates in *Escherichia coli*. *Proc. nat. Acad. Sci., Wash.* **40**, 1064.

WITKIN, E. M. & THEIL, E. C. (1960). The effect of post-treatment with chloramphenicol on various ultraviolet induced mutations in *Escherichia coli*. *Proc. nat. Acad. Sci., Wash.* **46**, 226.

WOODWARD, J. D. & RAINBOW, C. (1961). 5-aminoimidazole and its riboside from biotin-deficient yeast. *J. gen. Microbiol.* **25**, 141.

ZETTERBERG, G. (1960). The mutagenic effect of 8-ethoxycaffein caffein and dimethylsulphate in the Ophiostoma back-mutation test. *Hereditas*, **46**, 279.

ZETTERBERG, G. (1962). Genetic influence on the back-mutation rate in biochemical mutant strains of Ophiostoma. *Exp. Cell Res.* **27**, 560.

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Nutritional Studies on *Desulfovibrio desulfuricans* using Chemically Defined Media

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SUMMARY

A non-precipitating chemically defined medium containing lactate, sulphate and other inorganic salts supported repeated subculture of *Desulfovibrio desulfuricans*, strain Hildenborough; yields of bacteria were comparable with those obtained in media containing yeast extract or peptone. Addition of yeast extract, amino acid mixtures or ATP to the defined medium increased the crop. Growth on other organic substances was poorer than on lactate; amino acids were less efficient nitrogen sources than ammonia. Pyruvate served as electron acceptor for hydrogen uptake by resting organisms but did not support growth in sulphate-free medium.

INTRODUCTION

Numerous strains of sulphate-reducing bacteria representing three or more species have now been isolated, yet in relatively few cases have critical studies of their growth and nutrition been made. Butlin, Adams & Thomas (1949) developed a complex medium (medium C) giving rapid and abundant growth of unnamed strains of sulphate reducers, and demonstrated an iron requirement. Autotrophic growth in a chemically defined medium was claimed. Postgate (1951, 1953) obtained poor growth of *Desulfovibrio desulfuricans* strain Hildenborough in a simple glucose ammonia medium incorporating cysteine; the yield was improved somewhat by adding serine, ornithine and isoleucine, and markedly by adding yeast extract. Cysteine was shown by Grossman & Postgate (1953) to act by poising the redox potential and to be replaceable by Na_2S. A peptone yeast-extract medium supporting massive growth of strain Hildenborough was devised (Postgate, 1951). This strain, one of those used by Butlin *et al.* (1949), was recently proved incapable of autotrophic growth (Mechalas & Rittenberg, 1960; Postgate, 1960), whereas an unnamed strain was stated by Sorokin (1960) to grow autotrophically in a defined medium. Autotrophic incorporation of CO_2 by strain Sylt 3 in presence of yeast extract was claimed by Stüven (1960). Grossman & Postgate (1955), who reviewed the earlier work on utilization of carbon sources, reported growth of strain El Agheila Z on malate, succinate, lactate or pyruvate in a medium containing yeast extract. Carbon sources were also reviewed by Postgate (1959), who pointed out that an unfavourable E_h value, and marginal growth on impurities, may give rise to conflicting observations. Stimulatory effects in defined media by biotin (Wikén & Ghose, 1954) and ATP (Kadota & Miyoshi, 1960) have been reported. Studies on the inorganic nutrition of marine strains have been made by Kimata, Kadota, Hata & Tajima (1955*b*) and Hata (1960*a*, *b*) with a medium containing desalted

peptone; certain strains were unable to utilize inorganic nitrogen compounds (Kimata, Kadota & Hata, 1955*a*). Postgate (1956*b*) found that the halophile El Agheila Z required iron for utilization of sulphate, but not pyruvate, as terminal electron acceptor.

Certain of the contradictory reports on nutrition and metabolism of the sulphate reducers may reflect genuine differences between strains, while others may be attributable to ill-defined conditions of cultivation (e.g. presence of yeast extract or peptone; absence of E_h control). The work reported here was concerned with the development of a non-precipitating chemically defined medium and its use in nutritional studies on Hildenborough, a strain on which several physiological and biochemical studies have been reported.

METHODS

Organism. Desulfovibrio desulfuricans strain Hildenborough (National Collection of Industrial Bacteria, NCIB, no. 8303), purified by Postgate (1953) from an impure culture obtained from Wealden clay at Hildenborough, Kent, was stored in a lyophilized condition. Frequent tests were made on subcultures for anaerobic (Postgate, 1953) and aerobic contaminants.

Preparation of media. All glassware was cleaned in chromic acid (potassium dichromate + sulphuric acid). Media were prepared from analytical (Analar) grade chemicals where available, and Pyrex-distilled water. ATP was obtained from Sigma Chemical Co. (St Louis, U.S.A.). Chemicals of doubtful purity were purified by recrystallization. Lactic acid and yeast extract were incorporated into media before autoclaving at 115° for 20 min.: the fall in pH during autoclaving indicated dissociation of the dimer of lactic acid. All other organic substances were sterilized separately in solution by passing through sterile Oxoid membrane filter disks, grade AP, 4 cm. diam. (Oxo Ltd., London E.C. 4), supported in glass Seitz-type filters. Solutions of ferrous salts formed precipitates on autoclaving or filtering through grade EKS Seitz filter pads. Membrane-filtered solutions in 2·5 mM-H_2SO_4 showed no precipitation during storage for two months at 3°: stock solutions of $FeSO_4$ were therefore prepared in this manner. In all cases media were adjusted to pH 7·2–7·4 with sterile 15% (w/v) NaOH solution after autoclaving, using bromothymol blue indicator.

Cultivation. Lyophilized bacteria were inoculated initially into the medium C of Butlin *et al.* (1949); thereafter subcultures were made weekly into the experimental defined medium and growth experiments inoculated from these stock cultures. During later work stock cultures were maintained in the final version of this medium (the 'standard medium', the development of which is described in Results): lactic acid, 100 mM; KH_2PO_4, 2·5 mM; NH_4Cl, 10 mM; Na_2SO_4, 50 mM; $CaCl_2$, 0·5 mM; $MgSO_4.7H_2O$, 0·25 mM; trace elements B, Co, Cu, Mn, Mo, Zn each 0·05 mg./l. The medium was adjusted to about pH 6·5 with NaOH and autoclaved. When cool, sterile $FeSO_4.7H_2O$ solution was added to 25 μM and the medium adjusted to pH 7·2–7·4.

Both stock and experimental cultures were grown in Pyrex test tubes plugged with cotton-wool and containing 10 ml. medium. Cultures were incubated at 30° in McIntosh & Fildes' anaerobic jars under an atmosphere of 99% H_2 + 1% CO_2 (v/v). Except as indicated below, 1·0 mM-Na_2S was added immediately before

inoculation to poise the redox potential at about −300 mV. Triplicate cultures were used in all experimental treatments.

Estimation of growth. Attempts to observe early exponential growth in studies on nutrient concentrations by haemacytometer counts and optical density readings gave meaningless results. Dry wt. determinations on large samples withdrawn from 500 ml. cultures gave somewhat better results (typical standard deviations of a single determination on each of three cultures were 73·5 % after 24 hr., 35·8 % after 48 hr., 21·4 % after 72 hr., and 6·0 % after 96 hr. of incubation); however, this method was unsuitable for comparing growth in large numbers of cultures. Late exponential and post-exponential growth were therefore estimated by the optical density method described by Postgate (1951) and, when required, equivalent values for dry wt. organism/ml. suspension were read from a calibration curve. Na_2S was omitted from these cultures owing to its darkening effect, and neither cysteine nor ascorbic acid was added since they might serve as nutrients. Instead, a relatively large inoculum (0·2 ml.) from a 4-day-old stock culture was used: this was of sufficient size to prevent failures of growth while not giving excessive carry-over of nutrients, and the maximum standard deviation of the results obtained on days 3–5 (Figs. 1–3) was 7·9 %.

Growth in tubes containing Na_2S was estimated by eye when it appeared to be complete; cultures showing doubtful growth were examined microscopically.

Manometry. Measurement of hydrogenase activity was made at 32° following the procedure of Littlewood & Postgate (1956) except that 15 % (w/v) KOH was used as absorbent for H_2S and CO_2.

RESULTS

Development of the 'standard medium'

A non-precipitating defined medium. Medium C of Butlin *et al.* (1949) had the disadvantages for our purposes that it formed a precipitate when autoclaved, thus losing an uncertain proportion of nutrients (including Fe^{2+}: Dr J. R. Postgate, personal communication), it contained yeast extract, and sodium lactate which is not available in Analar grade, and its content of lactate and sulphate were not optimal (Dr J. R. Postgate, personal communication). The medium was modified in the following ways. The major nutrients were supplied as Analar reagents and their concentrations adjusted to molarities for convenience. Thus 50 mM-lactic acid, 2·5 mM-KH_2PO_4, 0·5 mM-$CaCl_2$ and 25 mM-Na_2SO_4 were used. 10 mM-NH_4Cl was tentatively supplied as sole nitrogen source despite the findings of Kimata *et al.* (1955*a*) that certain strains of *Desulfovibrio desulfuricans* cannot utilize ammonia. To minimize precipitation the divalent cation content was decreased by using 0·25 mM-$MgSO_4.7H_2O$: there appear to be no published results which justify the use of 8·13 mg.-ion Mg^{2+}/l. in medium C. In addition, an arbitrary trace element mixture was incorporated giving a final concentration of 0·05 mg./l. each of B, Co, Cu, Mn, Mo and Zn. This medium, adjusted to about pH 6·5 with NaOH and autoclaved at 115° for 20 min., did not precipitate. After addition of sterile $FeSO_4$ solution to 25 μM and adjustment to pH 7·2, the medium supported growth of strain Hildenborough through serial subculture, giving crops equivalent to about 380 μg. dry wt./ml. suspension.

Effect of concentration of the defined medium. To determine whether the over-all concentration of the defined medium was optimal, growth was observed at $\frac{1}{2}$, 1, 2 and 4 times the original concentration. The results (Fig. 1) show that medium $\times 4$ was inhibitory, while growth in medium $\times 2$, though eventually outstripping that

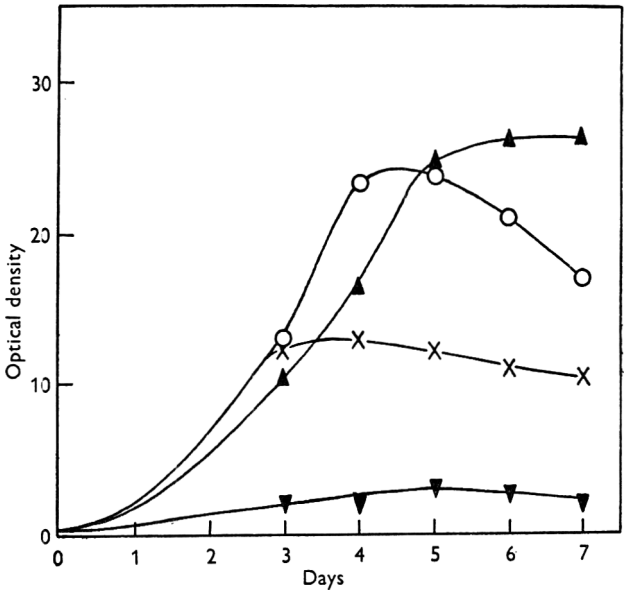

Fig. 1. Growth of *D. desulfuricans* strain Hildenborough in the experimental defined medium at original concentration (○) and at $\frac{1}{2}$ (×), 2 (▲) and 4 (▼) times this concentration.

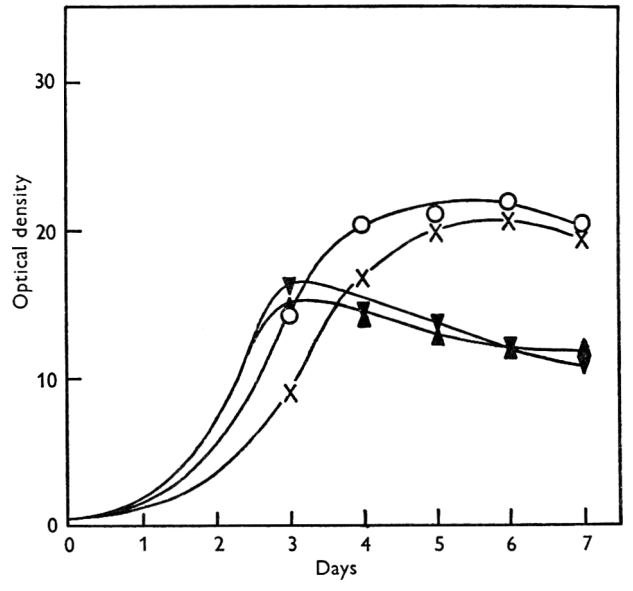

Fig. 2. Effect on growth of *D. desulfuricans* strain Hildenborough of decreasing one component of the defined medium by one-half. ○ = full medium, × = $\frac{1}{2}$-$FeSO_4$, ▲ = $\frac{1}{2}$-lactate, ▼ = $\frac{1}{2}Na_2SO_4$.

in $\times 1$, was slower, suggesting an unfavourably high osmotic pressure or a toxic level of a nutrient. It thus appeared that growth might be more effectively improved by an increase in the concentration of one or two nutrients.

Effect of concentration of individual components. In the experiment illustrated in Fig. 2 one of the major nutrients (lactate, KH_2PO_4, NH_4Cl, $MgSO_4$, $CaCl_2$, Na_2SO_4 or $FeSO_4$) was decreased to one-half of its original concentration, all others being present at full concentration. For the sake of clarity the growth curves for $\frac{1}{2}$-KH_2PO_4, $\frac{1}{2}$-NH_4Cl, $\frac{1}{2}$-$MgSO_4$ and $\frac{1}{2}$-$CaCl_2$ are omitted from the figure; they were all very close to that for full medium. The results suggested that growth in the full medium was limited by both lactate and sulphate. Thus the lactate and sulphate

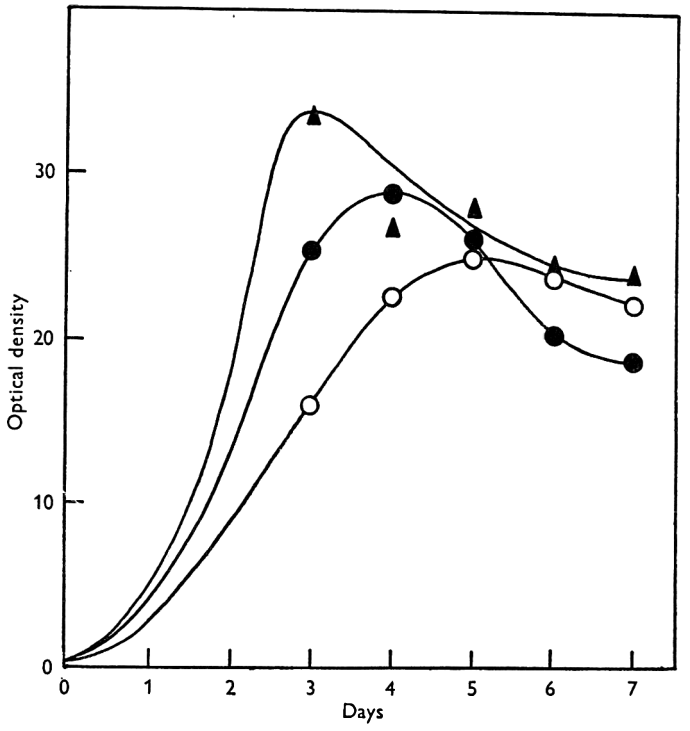

Fig. 3. Stimulation of the growth of *D. desulfuricans* strain Hildenborough in the standard medium by yeast extract. ● = standard medium, ▲ = standard medium + 0·1 % (w/v) yeast extract, ○ = original defined medium.

concentrations were separately increased $1\frac{1}{2}\times$, $2\times$ and $3\times$ in all combinations. It appeared that $2\times$ lactate $+ 2\times$ sulphate produced the most rapid growth; $3\times$ lactate $+ 2\times$ sulphate gave a slightly greater crop but a much lower growth rate. The medium with $2\times$ lactate $+ 2\times$ sulphate was therefore selected as the standard medium for all further experimentation with the Hildenborough strain and for maintenance of stock cultures. Details of preparation are given in Methods.

Yields equivalent to about 480 μg. dry wt. bacteria/ml. suspension are obtained in the standard medium. Soon after the onset of the stationary phase the number of bacteria remaining in suspension appears from microscopic examination greatly to decrease, while mucin containing large numbers of embedded organisms is precipitated, so that optical density determinations become misleading.

Perpetuation of strain Hildenborough in the standard medium. The organism has been serially subcultured more than 50 times in the standard medium without noticeable decrease in rate or abundance of growth. Inoculations of 2 μl. (equivalent to about 0·8 μg. dry wt. organism or a loopful of culture) from young cultures into 50 ml. medium have always in our experience produced normal growth provided that Na_2S, cysteine or ascorbic acid is incorporated. Lyophilized organisms originally grown in the medium can be successfully revived in it.

Effect of nitrogenous supplements in the standard medium

Typical growth in the standard medium and the original defined medium is shown in Fig. 3 which also illustrates the stimulatory effect of adding 1·0 g./l. Difco yeast extract (the concentration in medium C) to the standard medium. Since the lactate and ammonia concentrations in the standard medium were considered to be optimal, it was inferred that certain substances present in yeast extract might constitute more acceptable carbon or nitrogen sources, or serve as growth factors. The effects of the following supplements were therefore examined: Postgate's (1951) mixture of serine + ornithine + isoleucine; the mixture of 18 amino acids employed by Kadota & Miyoshi (1960) as a nitrogen source; 18 amino acids + 0·1 g. ATP/l. (Kadota & Miyoshi, 1960); ATP alone. Table 1 shows that ATP, as well as mixtures of metabolites, markedly improve growth in the standard medium.

Table 1. *Maximum growth of* Desulfovibrio desulfuricans *strain Hildenborough in standard medium with various nitrogenous supplements*

Optical densities were determined daily, and the maximum values converted to dry wt./ml. suspension from a calibration curve.

Supplement	Growth (μg. dry wt. bacteria/ml.)
None	470
Serine + ornithine + isoleucine (each 1·0 g./l.)	500
18 amino acids	620
18 amino acids + ATP 0·1 g./l.)	690
ATP (0·1 g./l.)	630
Yeast extract (1·0 g./l.)	560

Tests of alternative nutrients and electron acceptors

Carbon sources. Substrates to be tested as carbon sources were added to lactate-free standard medium so as to give 150 mg.-atom C/l., no allowance being made for the partial unavailability of DL-forms. Difco yeast extract was tested at 1·0 g./l. Toxicity tests (tubes containing lactate + substrate under trial) were also inoculated; a few amino acids proved inhibitory and their concentration was subsequently halved. Inoculation was with bacteria washed in C-free medium. Despite addition of Na_2S, only pyruvate, the monohydric alcohols and a few sugars supported growth. A 'training' method was therefore tried, in which the media under test were initially supplemented with lactate. Subcultures were made into media without a lactate supplement, and substrates were considered to be utilized when growth continued thereafter at an apparently unimpaired rate through five subcultures. In this way growth was obtained in several more substrates as shown in Table 2, though lags

of up to 5 weeks occurred in some cases before growth appeared in the first subculture into lactate-free medium, and growth was only marginal (denoted ±) in certain others. The first attempt to 'train' the organism to utilize glycerol failed, though at a second attempt fairly heavy growth occurred. No substrate supported such abundant growth as lactate.

Table 2. *Utilization of carbon sources by* Desulfovibrio desulfuricans *strain Hildenborough*

Substrates were at a concentration giving 150 mg.-atom C/l. unless otherwise stated.

Glucose	+	L-aspartic acid	±
Fructose	+ +	L-asparagine	+
Lactose	+	L-citrulline	−
Maltose	+	L-cysteine HCl	−
Sucrose	+ +	L-cystine	±
Methanol	+	L-glutamic acid	+
Ethanol	+	L-glutamine	+
Propanol	+	Glycine	−
Glycerol	+ +	L-histidine HCl	±
Acetate	−	DL-isoleucine*	±
Citrate	−	L-leucine	−
Lactate	+ + +	L-lysine HCl	−
Pyruvate	+ +	DL-methionine*	±
Oxaloacetate	+	L-phenylalanine*	±
Malate	−	L-proline	−
Fumarate	±	DL-serine	−
Succinate	±	DL-threonine*	±
Succinamide	±	DL-tryptophane	±
DL-alanine	−	DL-valine	±
L-arginine HCl	−	Yeast extract (1·0 g./l.)	+

* 75 mg.-atom C/l.

Nitrogen sources. The mixture of 18 amino acids as prescribed by Kadota & Miyoshi (1960), and the individual acids at 5 mg.-atom N/l., were tested as nitrogen sources in ammonia-free standard medium; asparagine, citrulline and cysteine were also tested. Growth in the mixture was about as abundant as in 5 mM-NH_4Cl, while all the individual amino acids except cysteine, glycine, histidine, methionine and tyrosine supported slight growth.

Organic terminal electron acceptors. Pyruvate and fumarate at non-toxic concentration (50 mM), in standard medium without Na_2SO_4 and with one-half of the normal $MgSO_4$ concentration, were tested for ability to serve as terminal electron acceptors. No growth occurred, though the abundant growth of *Escherichia coli* (NCIB, no. 7271) obtained in these media under aerobic conditions indicated that the sulphate concentration was sufficient for assimilatory metabolism. Manometric experiments showed that washed organisms in non-nutrient buffer absorbed hydrogen for the reduction of 15 mM-sodium pyruvate, $-Q_{H_2} = 23{\cdot}5$ µl./mg. dry wt./hr. or about one-tenth of the value obtained with equimolar sulphate as electron acceptor. No detectable hydrogen uptake occurred with sodium fumarate.

DISCUSSION

Kadota & Miyoshi (1960) reported that they were unable to grow certain sulphate-reducing bacteria in defined media unless abnormally large inocula were used or organic substances such as peptone, yeast extract or mixtures of amino acids added. For 'normal' growth *Desulfovibrio desulfuricans* strain Hildenborough required, in a basal ammonia-free medium containing lactate, ATP and mineral salts, the addition of peptones or 18 amino acids: growth was greatly diminished when five of the amino acids were omitted. The effect of adding an ammonium salt was not studied. Our experiments have shown that ammonia is a more acceptable nitrogen source for strain Hildenborough than organic nitrogen compounds. In this respect the organism appears to differ from the marine strains examined by Kimata *et al.* (1955*a*) which were unable to utilize ammonium salts. A lactate medium of suitable composition, containing no other organic substance and with ammonia as sole nitrogen source, supports vigorous growth through indefinite subculture and thus appears to be a complete medium for the Hildenborough strain. Small transfers (2 μl. into 50 ml., the smallest tested by us) grow normally in such a medium poised at a suitable redox potential. The addition of mixtures of metabolites such as yeast extract or an amino acid mixture to the standard defined medium stimulates growth, despite their being poor carbon sources. Nevertheless, the crop in the standard medium without any organic addition (about 480 μg. dry wt. organism/ml. suspension) compares favourably with the value of 300 μg./ml. in medium C and 500–520 μg./ml. in medium C with additional lactate and sulphate (Dr J. R. Postgate, personal communication). The growth of Hildenborough, like that of the halophile Maizuru 1 (Kadota & Miyoshi, 1960) is stimulated by ATP in defined media.

The survey of substrate utilization by strain Hildenborough presented difficulty. Postgate (1959) drew attention to the necessity of poising the redox potential especially of media to which the organism is unaccustomed; even so, in our experience, cases of apparent utilization by the 'training' technique are not always repeatable, or growth dies out after a few subcultures. Growth on most carbon sources is extremely scanty, and may take several weeks to appear, so that marginal growth on impurities cannot always be ruled out. Thus utilization of substrates—other than acetate, the utilization of which by a sulphate reducer would be highly interesting (Selwyn & Postgate, 1959)—does not promise to be a useful method of characterizing these organisms. The growth obtained with 1·0 g. yeast extract/l. as carbon source (up to 120 μg. dry wt./ml.) indicates that reports of substrate utilization or 'autotrophic' growth by *Desulfovibrio desulfuricans* in media incorporating yeast extract should be viewed with caution. Several strains, chiefly halophilic, are able to dismute pyruvate in sulphate-free media (Postgate, 1952, 1956*a*). The Hildenborough strain has been shown to be incapable of growth by this means, in keeping with Postgate's (1952) findings. Neither has it the ability possessed by certain marine strains (Sisler & ZoBell, 1951; Grossman & Postgate, 1955) to reduce fumarate with hydrogen. There are thus some grounds for supposing that the Hildenborough strain may differ from marine strains nutritionally as well as in the ways described by Ochynski & Postgate (1963).

We are indebted to Dr J. R. Postgate, Dr G. H. Booth and our colleagues in the Microbiology Section for valuable discussions. The paper is published by permission of the Director, National Chemical Laboratory.

REFERENCES

Butlin, K. R., Adams, M. E. & Thomas, M. (1949). The isolation and cultivation of sulphate-reducing bacteria. *J. gen. Microbiol.* **3**, 46.

Grossman, J. P. & Postgate, J. R. (1953). The cultivation of sulphate-reducing bacteria. *Nature, Lond.* **171**, 600.

Grossman, J. P. & Postgate, J. R. (1955). The metabolism of malate and certain other compounds by *Desulphovibrio desulphuricans*. *J. gen. Microbiol.* **12**, 429.

Hata, Y. (1960*a*). Inorganic nutrition of marine sulfate-reducing bacteria. *J. Shimonoseki Coll. Fish.* **9**, 347.

Hata, Y. (1960*b*). Influence of heavy metals upon the growth and the activity of the marine sulfate-reducing bacteria. *J. Shimonoseki Coll. Fish.* **9**, 363.

Kadota, H. & Miyoshi, H. (1960). A chemically defined medium for the growth of Desulfovibrio. *Mem. Res. Inst. Fd Sci., Kyoto Univ.* **22**, 20.

Kimata, M., Kadota, H. & Hata, Y. (1955*a*). Studies on the marine sulfate-reducing bacteria. IV. Nutritional requirements of marine sulfate-reducing bacteria (1). *Bull. Jap. Soc. sci. Fish.* **21**, 229.

Kimata, M., Kadota, H., Hata, Y. & Tajima, T. (1955*b*). Studies on the marine sulfate-reducing bacteria. III. Influence of various environmental factors upon the sulfate-reducing activity of marine sulfate-reducing bacteria (2). *Bull. Jap. Soc. sci. Fish.* **21**, 113.

Littlewood, D. & Postgate, J. R. (1956). Substrate inhibition of hydrogenase enhanced by sodium chloride. *Biochim. biophys. Acta*, **20**, 399.

Mechalas, B. J. & Rittenberg, S. C. (1960). Energy coupling in *Desulfovibrio desulfuricans*. *J. Bact.* **80**, 501.

Ochynski, F. W. & Postgate, J. R. (1963). Some biochemical differences between fresh water and salt water strains of sulphate-reducing bacteria. In *Symposium on Marine Microbiology*. Ed. by C. H. Oppenheimer. Springfield, U.S.A.: C. C. Thomas.

Postgate, J. R. (1951). On the nutrition of *Desulphovibrio desulphuricans*. *J. gen. Microbiol.* **5**, 714.

Postgate, J. R. (1952). Growth of sulphate-reducing bacteria in sulphate-free media. *Research*, **5**, 189.

Postgate, J. R. (1953). On the nutrition of *Desulphovibrio desulphuricans*: a correction. *J. gen. Microbiol.* **9**, 440.

Postgate, J. R. (1956*a*). Iron and the synthesis of cytochrome c_3. *J. gen. Microbiol.* **15**, 186.

Postgate, J. R. (1956*b*). Sulphate-reducing bacteria which are deficient in cytochrome. *J. gen. Microbiol.* **15**, viii.

Postgate, J. R. (1959). Sulphate reduction by bacteria. *Annu. Rev. Microbiol.* **13**, 505.

Postgate, J. R. (1960). On the autotrophy of *Desulphovibrio desulphuricans*. *Z. allg. Mikrobiol.* **1**. 53.

Selwyn, S. C. & Postgate, J. R. (1959). A search for the Rubentschikii group of Desulphovibrio. *Antonie van Leeuwenhoek J. Microbiol. Serol.* **25**, 465.

Sisler, F. D. & Zobell, C. E. (1951). Hydrogen utilization by some marine sulfate-reducing bacteria. *J. Bact.* **62**, 117.

Sorokin, Y. I. (1960). Utilization of the carbon in CO_2 by *Vibrio desulfuricans* and certain heterotrophic bacteria. *Doklady Akad. Nauk, U.S.S.R., biol. Sci. Sect.* **132**, 445.

Stüven, K. (1960). Beiträge zur Kenntnis der CO_2- und Lactatassimilation von *Desulfovibrio aestuarii* (van Delden) Kluyver und Van Niel. *Arch. Mikrobiol.* **36**, 31.

Wikén, T. & Ghose, T. K. (1954). Stimulation of bacterial sulphate reduction by (+)-biotin. *Physiol. Plant.* **7**, 713.

J. gen. Microbiol. (1963), **31**, 375–387
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The Fixation of Tetanus Toxin, Strychnine, Serotonin and other Substances by Ganglioside

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SUMMARY

Although tetanus toxin is fixed by brain ganglioside, it is not fixed by a number of substances more or less closely related to brain ganglioside, or by naturally occurring brain ganglioside not containing hexosamine, or by a hexosamine-containing ganglioside from Tay-Sachs brain. Isolated brain gangliosides may vary considerably in their toxin-fixing capacities. In chloroform + methanol extracts of brain a number of gangliosides which differ in ability to fix toxin can be separated chromatographically. Complexes of ganglioside with phrenosine and sphingomyelin have diminished toxin-fixing capacity. Tetanus toxin and ganglioside have a high binding capacity for calcium, but calcium does not affect the fixation of toxin by ganglioside. Ganglioside fixes strychnine, brucine and thebaine, drugs which have the same neurophysiological activity as tetanus toxin. Ganglioside does not fix γ-aminobutyric acid, β-hydroxy-γ-aminobutyric acid, histamine, adrenaline, noradrenaline or dopamine, but it does fix serotonin and a number of related compounds. It has been confirmed that albumin is fixed to a small extent by ganglioside at low salt concentration, but (unlike tetanus toxin) this fixation is practically abolished at physiological salt concentration.

INTRODUCTION

Previous work showed that ganglioside from nervous tissue acts as a specific receptor for tetanus toxin (van Heyningen, 1959*a*, *b*, *c*; van Heyningen & Miller, 1961) The sialic acid residue(s) and its free carboxyl group were shown to be essential for the fixation of the toxin, and a sample of hexosamine-free ganglioside from horse red blood cells was found to be incapable of fixing toxin (van Heyningen & Miller, 1961). This paper reports further studies on the structural requirements in ganglioside for toxin-fixation, on other aspects of the reaction, and on the fixation of a number of other substances.

METHODS

Materials. The tetanus toxin preparation, TD 464 D (van Heyningen, 1959*b*), was supplied by Dr C. G. Pope and Dr R. O. Thomson of the Wellcome Research Laboratories. It contains 40 % of protein, of which 75 % is toxin. Beef brain ganglioside was prepared by the method of van Heyningen & Miller (1961). A number of isolated beef brain gangliosides were supplied by Professor Richard Kuhn of Heidelberg. Normal human brain ganglioside was supplied by Professor Erwin Chargaff of New York. Glycolipid preparations from human, horse, beef, sheep, cat and guinea pig red blood cells; horse, beef and human spleen; human and

horse kidney; and horse lung, were supplied by Professor T. Yamakawa of Tokyo. Samples of hexosamine-free and hexosamine-containing ganglioside from brain were supplied by Professor Ernst Klenk of Cologne; samples of Tay-Sachs ganglioside by Professors Yamakawa, Klenk and Chargaff and by Dr S. Gatt of Jerusalem; colominic acid by Dr G. T. Mills of New York; endotoxin preparations by Professor Otto Westphal of Freiburg; oligosaccharide preparations from milk by Professor Richard Kuhn; phrenosine and sphingomyelin by Professor Herbert E. Carter of Urbana; histone (10 %, w/w, arginine) by Dr L. S. Wolfe of Montreal; γ-aminobutyric acid and β-hydroxy-γ-aminobutyric acid by Professor S. P. Halbert of New York; serotonin creatinine sulphate by Dr D. W. Woolley of New York; dopamine hydrochloride by Dr H. Blaschko of Oxford; adrenaline and noradrenaline were obtained from Burroughs Wellcome, London; tryptamine hydrochloride, histamine dihydrochloride, strychnine sulphate, brucine sulphate, ergometrine acid maleate and reserpine (of which a stock solution was prepared by dissolving 100 mg. in 5 ml. 20 %, w/v, ascorbic acid in water) from British Drug Houses Ltd, London; thebaine from May and Baker, London; lysergic acid diethylamide (LSD-25) from Sandoz Ltd, London; purified bovine plasma albumin Fraction V from Armour Laboratories, London.

Chromatography. Column chromatography was done on Mallinckrodts Analytical Reagent Acid 100 mesh silicic acid (Savory and Moore, London) ground to 300 mesh, and treated according to Hirsch & Ahrens (1958). Silicic acid (10 g.) was suspended in 35 ml. chloroform, poured into tubes, 1·5 cm. diameter, 25 cm. long, and washed with a further 50 ml. chloroform. Ascending paper chromatography was done as described by van Heyningen & Miller (1961).

Analyses. Sialic acid was determined by the resorcinol method of Svennerholm (1957) with methoxy-*N*-acetylneuraminic acid (supplied by Professor Klenk) as standard. Hexosamine was determined by the Procedure A of Svennerholm (1956) with glucosamine hydrochloride as standard. The following substances were analysed by measuring their optical densities at the wavelengths indicated; histamine, 225 mμ; strychnine, 255 mμ; brucine, reserpine, 265 mμ; adrenaline, noradrenaline, dopamine, serotonin, tryptamine, 279 mμ; thebaine, morphine, 285 mμ; lysergic acid diethylamide, 290 mμ; ergometrine, 315 mμ. The absorption by ganglioside at these wavelengths was negligible. When serotonin was estimated in the presence of other substances absorbing in the ultraviolet region the 1-nitroso-2-naphthol method of Udenfriend, Weissbach & Clark (1955) was used. γ-Aminobutyric acid and β-hydroxy-γ-aminobutyric acid were estimated by the ninhydrin reaction.

Ultracentrifugal studies. The Spinco Model E Analytical Ultracentrifuge with RTIC unit and with a phase plate at 60° angle in the schlieren optical system was used. Reactants were dissolved in 0·1 M-phosphate buffer (pH 7·0) and placed in one of the sectors of a double sector cell with buffer in the other. Runs were carried out for 64 min. at 42,040 rev./min. at 18°–21°. Toxin-fixing capacity of ganglioside preparations was measured by the method of van Heyningen & Miller (1961), but expressed as mg. toxin fixed/mg. ganglioside, rather than Receptor Units (RU), since the relation of toxin fixed per unit ganglioside appears on further investigation to be reasonably linear. A value of 300 RU/mg. corresponds to about 1 mg. toxin fixed/mg. ganglioside.

RESULTS

Toxin-fixation by various gangliosides and substances allied to gangliosides

Yamakawa's glycolipids. The samples of glycolipids from red blood cells, spleen, kidney and lung supplied by Professor Yamakawa all contain lignoceric acid, sphingosine and hexose residues, and variable amounts of hexosamine and sialic acid (*N*-glycollylneuraminic acid; cf. *N*-acetylneuraminic acid in brain ganglioside, Yamakawa, Irie & Iwanaga, 1960). Nineteen samples of this material at a final concentration of 1 mg./ml. were tested in the analytical ultracentrifuge by the method of van Heyningen & Miller (1961). Not one had any toxin-fixing capacity.

Endotoxins. The lipopolysaccharide endotoxins supplied by Professor Westphal were tested for toxin-fixing capacity because they resemble gangliosides in so far as they contain lipid and polysaccharide, are water soluble, and have a high sedimentation constant. Six endotoxin preparations were tested at a final concentration of 5 mg./ml. in the analytical ultracentrifuge and only one of these (*Escherichia coli* alk. 1447) fixed toxin. This sample, however, had been treated with alkali and is known to have the capacity to fix proteins indiscriminately (Lüderitz, Westphal, Eichenbeger & Neter, 1958). The mixture of tetanus toxin and this substance did not give rise to a complex of fast-moving peaks in the ultracentrifuge, as is the case with brain ganglioside, but to a single peak which migrated more slowly than either the toxin or the lipopolysaccharide alone.

Colominic acid. Colominic acid is a polymer of *N*-acetylneuraminic acid, with a high sedimentation constant (Barry, 1958). At a final concentration of 5 mg./ml. it did not fix tetanus toxin.

Milk oligosaccharides. Professor Kuhn provided 11 oligosaccharides from human and cow milk; 9 of these were tetrasaccharides containing residues of glucose, galactose, *N*-acetylgalactosamine and *N*-acetylneuraminic acid; one was a tetrasaccharide containing 2 galactose, 1 glucose and 1 *N*-acetylgalactosamine residues; one was a trisaccharide containing galactose, glucose and *N*-acetylgalactosamine. These oligosaccharides thus have a similar constitution to the oligosaccharide moiety of ganglioside, and in at least one of them the sequence of the residues is thought to be the same as in ganglioside (Kuhn, 1960). They were therefore tested for their ability to fix tetanus toxin. Since they had low sedimentation constants they could not be tested in the ultracentrifuge, but since they were diffusible through dialysing membranes, fixation was studied by equilibrium dialysis. Volumes (2 ml.) of 0·1 M-phosphate buffer (pH 7) containing 1·4 mg. oligosaccharide + 2 mg. tetanus toxin/ml., or 1·4 mg. oligosaccharide alone/ml., were dialysed against 2 ml. buffer with continuous agitation of the fluid on either side of the dialysing membrane for 48 hr. at 4°. The sialic acid content was then determined in the dialysis residue and diffusate in all cases except that of the tetrasaccharide which did not contain sialic acid; in this case hexosamine was determined. In all cases the sialic acid (or hexosamine) contents of the residue and diffusate were the same, indicating that the non-diffusible tetanus toxin had not fixed any of the oligosaccharides.

Hexosamine-free ganglioside. Klenk & Gielen (1960) concluded from structural studies that brain ganglioside was a mixture of two gangliosides both containing sialic acid but only one containing hexosamine. They subsequently justified this conclusion by isolating two gangliosides, one with and one without hexosamine

(Klenk & Gielen, 1961). These were tested for toxin-fixing capacity. The hexosamine-containing ganglioside fixed tetanus toxin in the usual way (3·8 mg. toxin/mg.) but the hexosamine-free brain ganglioside appeared to be incapable of fixing toxin.

Kuhn's gangliosides. Kuhn, Wiegandt & Egge (1961) separated from normal human and beef brains 4 distinct crystalline gangliosides (G I to G IV) with decreasing mobilities on paper and silicic acid thin layer chromatography. These gangliosides all contained one residue each of stearic acid, sphingosine, glucose and *N*-acetylgalactosamine, 2 residues each of galactose, but increasing proportions of *N*-acetylneuraminic acid as shown in Table 1. Table 1 also shows that the toxin-fixing capacities of these gangliosides varied considerably, and independently of sialic acid content. The value of about 19 mg. toxin fixed/mg. gangliosides G III and G IV is far higher than those hitherto encountered.

Table 1. *Tetanus-toxin fixation by Kuhn's normal brain gangliosides*

Sample	Residues sialic acid/ molecule	mg. toxin fixed/mg.
G I	1	2·59
G II	2	3·64
G III	2	19·30
G IV	3	19·00

Table 2. *Tetanus toxin-fixing capacity of samples of Tay-Sachs ganglioside, compared with two samples of normal human brain ganglioside*

Source of ganglioside	μmole sialic acid/mg.	mg. toxin fixed/ mg. ganglioside
Yamakawa	0·75	0
Gatt 6	0·73	0
Gatt 2	0·59	0·27
Gatt 4	0·31	0·38
Gatt 3	0·39	0·53
Chargaff	0·52	0·64
Gatt 5	0·55	0·69
Chargaff	0·75	0·90
Gatt 1	0·53	1·33
Klenk	0·42	1·35
Klenk (Normal)*	0·66	1·80
Chargaff (Normal)*	0·89	3·97

* See Table 1, Bernheimer & van Heyningen (1961).

Tay-Sachs gangliosides. Table 2 shows the tetanus toxin-fixing capacities of a number of samples of ganglioside isolated from the brains of infants dying of Tay-Sachs disease. Toxin-fixing capacity was always less than that of normal human brain ganglioside (about 4 mg. toxin fixed/mg. ganglioside) and varied from nil to values quite near those of normal ganglioside. Samples Gatt 6 (0 mg. toxin/mg. ganglioside) and Gatt 2 (0·27 mg. toxin/mg. ganglioside) were obtained after samples Gatt 5 (0·69 mg. toxin/mg. ganglioside) and Gatt 1 (1·33 mg. toxin/mg. ganglioside), respectively, had been further purified by chromatography on silicic acid columns by Dr S. Gatt. Samples of normal brain ganglioside purified by Dr S. Gatt by the same procedure had the usual toxin-fixing capacity (about

4 mg. toxin/mg. ganglioside). The purified samples Gatt 6 contained the usual proportions of sialic acid (0·73 μmole/mg.) and hexosamine (0·71 μmole/mg.). Thus it seems that in Tay-Sachs brains there is a ganglioside, not present in normal brains, containing the usual proportions of sialic acid and hexosamine, but incapable of fixing tetanus toxin. The other samples of Tay-Sachs ganglioside listed in Table 1 were evidently contaminated with different proportions of normal toxin-fixing ganglioside (see Gatt, 1962).

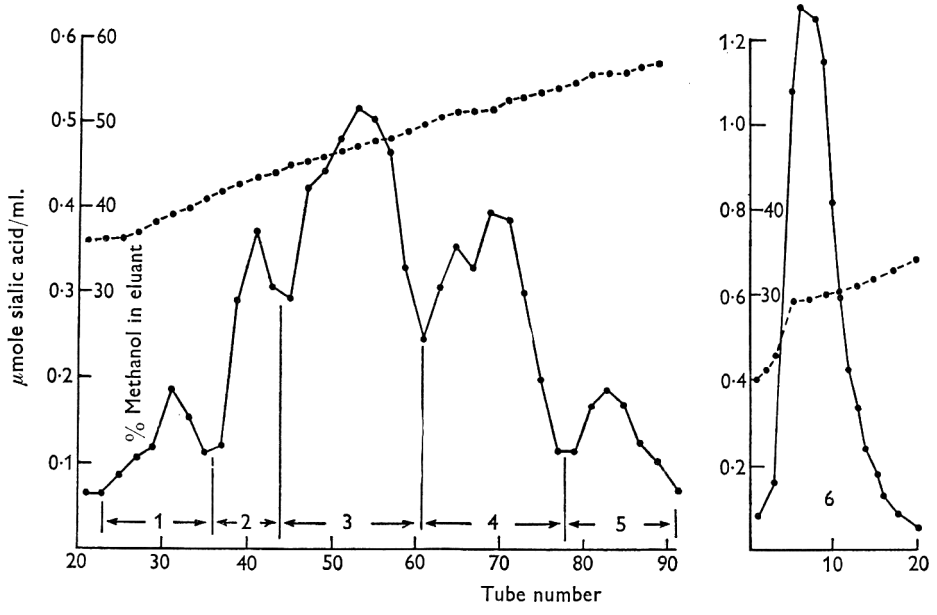

Fig. 1. Peaks 1–5, ganglioside fractions separated from chloroform + methanol extract of brain on silicic acid column. Peak 6, extract partitioned with water and material in water phase chromatographed.

Table 3. *Data on the preparations emerging in the peaks shown in Fig.* 1

Peak	Weight (mg.)	μmole sialic acid/mg.	μmole hexosamine/mg.	Ratio sialic acid/hexosamine	mg. toxin fixed/mg.
1	33	0·19	0·15	1·3	0·84
2	27	0·36	0·18	2·0	1·00
3	48	0·48	0·21	2·3	1·20
4	36	0·65	0·23	2·8	6·60
5	17	0·47	0·16	2·9	5·50
6	160	0·60	0·21	2·9	3·33

Ganglioside in chloroform-methanol extracts of brain. Beef brain was dehydrated by treatment with acetone and each g. dried residue extracted with continuous stirring for 7 hr. with 20 ml. of 67 % (v/v) methanol in chloroform. The solvent of the extract was evaporated off and the residue dissolved in a quarter volume of 20 % methanol in chloroform. An excess of this solution (containing 10 g. extract) was loaded on a column of 10 g. silicic acid suspended in chloroform and the column washed with 20 % methanol in chloroform until no material containing sialic acid appeared in the eluate. The column was then eluted with a chloroform + methanol

gradient ranging from 30 to 60% methanol. The eluate was collected in 10 ml. volumes and the sialic acid content determined in alternate tubes. Over the range 36–57% methanol five distinct sialic acid peaks emerged (Fig. 1). The contents of the tubes were pooled in five fractions as indicated in Fig. 1 and the dry weight, sialic acid and hexosamine contents, and toxin-fixing capacities of the fractions was determined (Table 3). The material in these peaks was water-soluble. Figure 2 shows paper chromatograms of the five fractions, interspersed with samples of a partially purified preparation of ganglioside. Fraction 1 appears to consist almost entirely of 'fast' ganglioside, fractions 2–5 of mixtures of 'fast' and 'slow' ganglioside (van Heyningen & Miller, 1961). None of the fractions appear to be grossly contaminated with other lipids.

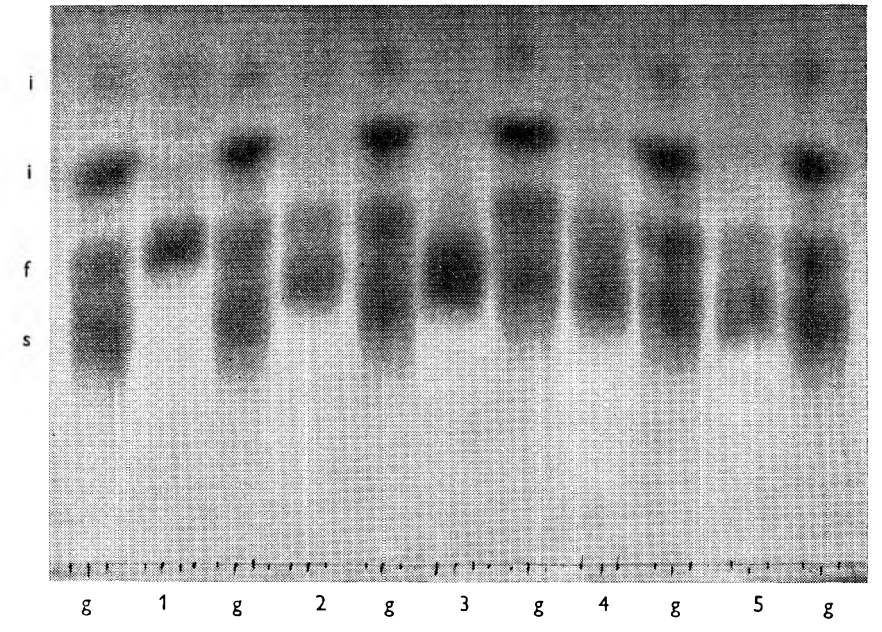

Fig. 2. Ascending paper chromatogram of the material emerging in the five peaks shown in Fig. 1, interspersed with samples of partially purified ganglioside (g). i, impurities; f, 'fast' ganglioside; s, 'slow' ganglioside.

A sample of the original chloroform + methanol extract was subjected to partition-dialysis and, after drying down, the material appearing in the upper aqueous phase was loaded on to a silicic acid column. The column was exhaustively percolated with 10% methanol in chloroform, and then eluted with a chloroform + methanol gradient ranging from 20 to 40% methanol. The eluate was collected in 10 ml. fractions and alternate fractions analysed for sialic acid (peak 6, Fig. 1). The single peak from this preparation emerged at a lower range of methanol concentrations (20–34%) than the five peaks from the unpartitioned chloroform methanol extract (36–57%). The data on the material in this single peak are shown in Table 3. These may be compared with the values of 0·88 μmole sialic acid/mg., 0·28 μmole hexosamine/mg., sialic acid–hexosamine ratio 3·1, 4 mg. toxin fixed/mg. for a purified preparation of 'slow' ganglioside (van Heyningen & Miller, 1961).

Complexes of ganglioside with cerebroside and sphingomyelin. Although ganglioside is water-soluble, it is not extractable from mammalian brain with water. Van Heyningen (1959*c*) showed that ganglioside is complexed with cerebrosides and sphingomyelin in the crude 'protagon' fraction of brain. These complexes are water-insoluble or water-soluble according to the relative proportions of insoluble cerebrosides or sphingomyelin and soluble ganglioside. A study was made in the analytical ultracentrifuge of the water-soluble complexes and their toxin-fixing capacity.

To make the complexes, 10 mg. phrenosine or 10 mg. sphingomyelin + 20 mg. ganglioside were dissolved in 10 ml. 33% methanol in chloroform to give clear solutions. The solvent was evaporated off and the residues dissolved in 4 ml. water to give slightly opalescent solutions. In the ultracentrifuge the ganglioside + phrenosine and ganglioside + sphingomyelin preparations sedimented as single peaks with the areas and sedimentation constants shown in Table 4. Besides the solubilization of phrenosine and sphingomyelin in water, complex formation with ganglioside is indicated by the single peaks of appropriately increased area and sedimentation rate. Similar solutions of the complexes were incubated overnight at 37° with 50 μg. (850,000 LD50) tetanus toxin protein/ml. Table 4 shows that the behaviour of the complexes in the ultracentrifuge was apparently unaffected.

Table 4. *Areas and sedimentation constants of ganglioside, ganglioside + phrenosine and ganglioside + sphingomyelin peaks in the analytical ultracentrifuge before and after treatment with tetanus toxin*

Final concentrations: ganglioside, 5 mg./ml.; phrenosine, 2·5 mg./ml.; sphingomyelin, 2·5 mg./ml. See text for details of treatment with toxin.

	Area of peak* (mm.²)	Sedimentation constant†
Ganglioside	18·8	13·5
Ganglioside + phrenosine	27·4	20·5
Ganglioside + phrenosine (toxin treated)	27·6	20·0
Ganglioside + sphingomyelin	30·0	19·5
Ganglioside + sphingomyelin (toxin treated)	29·3	20·5

* Area on photographic plate after 16 min. sedimentation, uncorrected for sectorial effect.
† At 18° in 0·1 M-phosphate buffer pH 7.

Toxin fixation by these complexes was determined at a final concentration of 0·5 mg. ganglioside/ml. and 0·25 mg. phrenosine or sphingomyelin/ml. The following values were obtained: ganglioside alone, 3·56 mg. toxin/mg. ganglioside; ganglioside + phrenosine, 1·10 mg. toxin/mg. ganglioside; ganglioside + sphingomyelin, 0·93 mg. toxin/mg. ganglioside. Evidently complexing with phrenosine or sphingomyelin decreased the toxin-fixing capacity of ganglioside to about one-third.

Effect of histone on toxin fixation by ganglioside. McIlwain (1961) showed that the metabolic response of brain slices to electrical stimulation was decreased under certain conditions, including the addition of histone to the slices, and that the addition of ganglioside restored the response. He also showed that the addition of histone to brain emulsions decreased the amount of ganglioside that could be

extracted with chloroform + methanol. The interaction of histone and ganglioside, and the effect of histone on the fixation of toxin by ganglioside, was therefore studied in the ultracentrifuge. A sample of histone containing 10% arginine was used. The peak of histone alone at a concentration of 5 mg./ml. barely moved away from the meniscus after 64 min. at 42,040 rev./min. When ganglioside at 2·5 mg./ml. was added the histone peak did not move any further, but was slightly decreased in height, and its leading edge merged with the trailing edge of the ganglioside peak, the sedimentation rate of which appeared to be diminished. For these reasons it was not possible to make a quantitative measure of histone fixation by ganglioside, but a very rough estimate based on the decrease in the height of the histone peak suggests that about 1·5 mg. histone may be fixed by 2·5 mg. ganglioside under these circumstances (compare 4 mg. tetanus toxin/mg. ganglioside). This experiment suggested that histone decreased the sedimentation rate of ganglioside; this was confirmed by the following sedimentation constants (20°, 0·1 M-phosphate buffer, pH 7): ganglioside (14 mg./ml.), 12·9; ganglioside (14 mg./ml.) + histone (2 mg./ml.), 9·0. The ganglioside + histone peak was sharper than that of ganglioside alone. The effect of histone was to increase the toxin-fixing capacity of ganglioside, rather than to diminish it. Under the conditions of the test, 0·5 mg. ganglioside fixed 2 mg. toxin, but when 1 mg. histone was added all the available toxin, 3·75 mg., was fixed. A control experiment showed that histone alone (10 mg./ml.) had no effect on the toxin peak.

Binding of calcium ions by tetanus toxin and ganglioside. For reasons irrelevant to the present paper, the binding of calcium ions by toxin and ganglioside was determined in collaboration with Dr R. Woodman. Toxin and ganglioside were decalcified by dialysis for 2 days against several changes of 0·025 M-ethylene-diamine-tetra-acetic acid followed by 2 days against several changes of water. The dialysed solutions were freeze-dried. To determine calcium binding, 4 ml. volumes containing 0·1 mg. toxin or 0·1 mg. ganglioside/ml. 0·5 mM-$CaCl_2$ in 5 mM-imidazole buffer (pH 7) were dialysed against 4 ml. buffer with continuous agitation for 24 hr. at 3°. The calcium contents of the dialysis diffusate and the dialysis residue were then determined and it was calculated that under these circumstances tetanus toxin bound 1·18 m-mole Ca^{2+}/g. and ganglioside 1·87 m-mole Ca^{2+}/g. This finding that both ganglioside and toxin appeared to have high calcium-binding capacities suggested the possibility that the calcium ion might form a link between the toxin and the carboxyl group of ganglioside during the fixation of toxin by ganglioside. The fixation of decalcified toxin (5 mg./ml.; 0·02 m-mole Ca/g.) by decalcified ganglioside (2·5 mg./ml.; 0·035 m-mole Ca/g.) in 0·01 M-imidazole buffer was measured in the presence and absence of calcium chloride added to a final concentration of 0·05 M. The toxin fixed in the presence and absence of added calcium was, respectively, 3·61 and 3·59 mg. toxin/mg. ganglioside.

Fixation of albumin by ganglioside

Doery & North (1961) claimed that plasma albumin is fixed by ganglioside, and by implication equated the fixation of tetanus toxin and albumin. Their evidence for the fixation of albumin was based on an increase in the sedimentation constant of albumin in the presence of a high concentration (5 mg./ml.) of ganglioside.

During the early stages of the present work (van Heyningen, 1959*b*) it was shown that the crude tetanus toxin receptor also fixed certain other proteins, namely lysozyme, trypsin and plasma albumin, at low salt concentration. However, it was also shown that the fixation of these proteins was different from that of tetanus toxin, since it was strongly diminished, if not abolished, at physiological salt concentration, whereas the fixation of tetanus toxin was unaffected by salt. The fixation of plasma albumin by ganglioside was investigated in the ultracentrifuge. In 0·1 M-phosphate buffer (pH 7) 0·15 mg. albumin was fixed/mg. ganglioside (compare 4 mg. toxin/mg.); in salt-free water the fixation of albumin was doubled and the fixation of toxin unaffected.

The fixation of strychnine and other convulsant drugs by ganglioside

It has long been recognized that strychnine and tetanus toxin have the same pharmacological action (see Sherrington, 1947). In more recent years it has been demonstrated that, like tetanus toxin, strychnine suppresses synaptic inhibition. The convulsant drugs brucine (a dimethoxy derivative of strychnine) and thebaine (a dimethoxy derivative of morphine) have a similar action (see Eccles, 1957). Morphine is a narcotic drug with a convulsant action under certain circumstances (see Goodman & Gilman, 1960). The possible fixation of these drugs by ganglioside was therefore investigated. For these studies the fixed and moving partition cells of the Spinco Model E Analytical Ultracentrifuge were used (see Schachman, 1959). Ganglioside and drug were mixed at a final concentration of 4 mM and centrifuged in the partition cell at 59,780 rev./min. for 1 hr. The rotor was then stopped by a cautious application of the brake (see Schachman, 1959) and the contents of the upper and lower portions of the separation cells analysed for ganglioside and drug. In all cases all the ganglioside was found in the lower portion. The drugs alone did not migrate appreciably. From the data thus obtained the weight of drug fixed by ganglioside could be determined. These values are shown in Table 5. Other studies showed that the fixation of strychnine was more or less directly proportional to the concentrations both of strychnine and ganglioside.

Table 5. *Fixation of convulsant drugs by ganglioside*

	Moles fixed/ mole ganglioside*
Tetanus toxin	0·08
Strychnine	0·28
Brucine	0·24
Thebaine	0·32
Morphine	0·11

* Assuming a molecular weight of 1384 for ganglioside.

When tetanus toxin was added to ganglioside at a final concentration of 5 mg. protein/ml., before strychnine was added, the fixation of strychnine was decreased by about half. This concentration of toxin (5 mg. protein/5 mg. ganglioside) is not nearly enough to 'saturate' the ganglioside, but it was impracticable to use a higher concentration. On the other hand, in a system containing 5 mg. toxin protein/ml.

and 0·5 mg. ganglioside/ml., the addition of 6 mg. strychnine/ml. increased the fixation of toxin from 1·52 mg. toxin/0·5 mg. ganglioside to 2·46 mg. toxin/0·5 mg. ganglioside.

The fixation of serotonin and related drugs

Tetanus toxin acts by suppressing synaptic inhibition, but the nature of the inhibitory transmitter (if any) is not known. A number of substances have at one time or another been suggested, with greater or less emphasis, as possible inhibitory transmitters, including γ-aminobutyric acid, β-hydroxy-γ-aminobutyric acid, histamine, adrenaline, noradrenaline, dopamine and serotonin (see Florey, 1961). The fixation of these substances (at 4 mM) by ganglioside (at 4 mM) was tested in the partition cells of the analytical ultra centrifuge in the same way as was strychnine. The drugs alone did not migrate appreciably. None of the drugs was fixed, except serotonin (see Table 6). Control experiments showed that creatinine sulphate (the serotonin was used as serotonin creatinine sulphate) was not fixed by ganglioside. The fixation of serotonin was proportional to the concentration of serotonin and of ganglioside. The hexosamine-free ganglioside isolated by Klenk

Table 6. *Fixation of serotonin and related drugs by ganglioside*

	Moles fixed/ mole ganglioside*
Serotonin	0·15
Tryptamine	0·18
LSD-25	0·37
Ergometrine	0·21
Reserpine	0
Chlorpromazine	0·76

* Assuming a molecular weight of 1384 for ganglioside.

& Gielen (1961) from normal human brain that did not fix tetanus toxin also did not fix serotonin. The hexosamine-containing Tay-Sachs ganglioside (Gatt 6) which did not fix tetanus toxin fixed only one-third as much serotonin as normal brain ganglioside (0·048 mole serotonin/mole Tay-Sachs ganglioside; compare Table 6). Tetanus toxin (5 mg./ml.) did not affect the fixation of serotonin, and serotonin did not affect the fixation of toxin. Several drugs chemically or pharmacologically related to serotonin were also fixed by ganglioside, namely tryptamine, lysergic acid diethylamide (LSD-25) and ergometrine (Table 6); reserpine was not fixed. Harris, Saiffer & Volk (1960) reported that chlorpromazine was fixed by ganglioside in equilibrium dialysis experiments, and Table 6 shows that this observation was confirmed in the ultracentrifuge. (Chlorpromazine is also avidly fixed by the rubber springs of the Yphantis moving partition cell of the ultracentrifuge, and the fixed partition cell had to be used in these experiments.) In the presence of 4 mM-LSD-25 or ergometrine the fixation of serotonin was somewhat decreased (from 0·15 to 0·10 and 0·08 mole/mole ganglioside, respectively).

When serotonin was incubated with 10 million LD50 doses of tetanus toxin/ml. for 6 hr. at 37° there was no decrease in its capacity to yield a chromophore with 1-nitroso-2-naphthol (Udenfriend *et al.* 1955), or in its capacity to stimulate guinea-pig ileum.

DISCUSSION

Almost invariably the word 'ganglioside' should be read as 'a mixture of gangliosides' (see Rapport & Norton, 1962). Klenk & Gielen (1961) showed that there are at least two gangliosides containing one residue of sialic acid per molecule; in one the residues of the oligosaccharide moiety are in a chain, and include hexosamine; in the other the oligosaccharide moiety is branched and hexosamine is replaced by galactose. Kuhn *et al.* (1961) separated four different gangliosides, all containing hexosamine, but containing from 1 to 3 sialic acid residues. The hexosamine-free ganglioside of normal brain does not fix tetanus toxin, but there is a hexosamine-containing ganglioside in Tay-Sachs brain which also does not fix toxin. The sialic acid residue of ganglioside is essential for tetanus toxin fixation, and to some extent toxin fixation is proportional to the sialic acid content of ganglioside (Bernheimer & van Heyningen, 1961). But the experiments with the gangliosides isolated by Kuhn *et al.* (1961) showed that there could also be a considerable variation in toxin-fixing capacity which was independent of sialic acid content.

The separation of a number of crude gangliosides with widely different toxin-fixing capacities from a chloroform + methanol extract of brain on a silicic acid column invites comparison with the four gangliosides of Kuhn *et al.* (1961). Gangliosides are usually obtained from chloroform + methanol extracts of brain after partition into an aqueous phase. The direct application of the crude chloroform + methanol extract to a silicic acid column may prove useful in eliminating this stage and in bringing about a more efficient separation of different gangliosides.

The fixation of tetanus toxin is ganglioside-specific and toxin-specific, and is avid, since some gangliosides fix up to twenty times their weight of tetanus toxin. North & Doery (1961); Doery & North (1961) claimed that other toxins, namely diphtheria and staphylococcal toxins, and proteins, e.g. plasma albumin, are fixed by ganglioside. The fixation of diphtheria and staphylococcal toxins was deduced from the fact that these toxins are inactivated on standing with ganglioside at 37° in the absence of protective colloids. However, it has already been demonstrated that diphtheria toxin is not fixed by ganglioside, and it has been pointed out that the specific fixation of tetanus toxin is unrelated to the non-specific inactivation of a number of toxins (van Heyningen, 1961). The fixation of albumin by crude tetanus toxin receptor was demonstrated some years ago, when it was shown that this fixation was different from that of tetanus toxin since it was abolished by salt, which did not affect the fixation of tetanus toxin (van Heyningen, 1959*b*). In the present paper it has been shown that the fixation of albumin by gangliosides is of a smaller order of magnitude than that of tetanus toxin, and the inhibitory effect of salt has been confirmed. The fixation of strychnine, brucine and thebaine by ganglioside may be significant in view of the fact that these drugs have the same neurophysiological action as tetanus toxin. The fixation of serotonin by other substances besides ganglioside has been detected by other techniques by Dombro, Bradham, Campbell & Woolley (1961).

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REFERENCES

BARRY, G. T. (1958). Colominic acid, a polymer of *N*-acetylneuraminic acid. *J. exp. Med.* **107**, 507.

BERNHEIMER, A. W. & VAN HEYNINGEN, W. E. (1961). The relation between the tetanus toxin-fixing and influenza virus-inhibiting properties of ganglioside. *J. gen. Microbiol.* **24**, 121.

DOERY, H. M. & NORTH, E. A. (1961). The interaction of staphylococcal toxin and ganglioside. 1. Inactivation of the lethal effect of staphylococcal toxin in mice. *Aust. J. exp. Biol. med. Sci.* **39**, 333.

DOMBRO, R. S., BRADHAM, L. S., CAMPBELL, N. K. & WOOLLEY, D. W. (1961). Serotonin receptors. III. The role of salt formation. *Biochim. biophys. Acta*, **54**, 516.

ECCLES, J. C. (1957). *The physiology of nerve cells.* London: Oxford University Press.

FLOREY, E. (editor) (1961). *Nervous inhibition.* Oxford: Pergamon Press.

GATT, S. (1962). Chloroform soluble complexes of gangliosides with lipids and salts. *Bull. Res. Counc. Israel*, **11A**, 90.

GOODMAN, L. S. & GILMAN, A. (1960). *The pharmacological basis of therapeutics.* New York: The Macmillan Company.

HARRIS, A. F., SAIFER, A. & VOLK, B. W. (1960). Interaction of chlorpromazine with strandin. *Proc. Soc. exp. Biol., N.Y.* **104**, 542.

HIRSCH, K. & AHRENS, E. H. (1958). The separation of complex lipide mixtures by the use of silicic acid chromatography. *J. biol. Chem.* **233**, 311.

KLENK, E. & GIELEN, W. (1960). Zur Kenntnis der Ganglioside des Gehirns. *Hoppe-Seyl. Z.* **319**, 283.

KLENK, E. & GIELEN, W. (1961). Untersuchungen über die Konstitution der Ganglioside aus Menschengehirn und die Trennung des Gemisches in die Komponenten. *Hoppe-Seyl. Z.* **326**, 144.

KUHN, R. (1960). Über die Ganglioside des Gehirns. *Angew. Chem.* **72**, 805.

KUHN, R., WIEGANDT, H. & EGGE, H. (1961). Zum Bauplan der Ganglioside. *Angew. Chem.* **73**, 580.

LÜDERITZ, O., WESTPHAL, O., EICHENBERGER, E. & NETER, E. (1958). Über die Komplexbildung von bakteriellen Lipopolysacchariden mit Proteinen und Lipoiden. *Biochem. Z.* **330**, 21.

MCILWAIN, H. (1961). Characterization of naturally occurring materials which restore excitability to isolated cerebral tissues. *Biochem. J.* **78**, 24.

NORTH, E. A. & DOERY, H. M. (1961). Inactivation of staphylococcal, tetanus and diphtheria toxins by ganglioside. *Brit. J. exp. Path.* **42**, 23.

RAPPORT, M. M. & NORTON, W. T. (1962). Chemistry of the lipids. *Annu. Rev. Biochem.* **31**, 103.

SCHACHMAN, H. K. (1959). *Ultracentrifugation in biochemistry.* New York: Academic Press Inc.

SHERRINGTON, C. (1947). *Integrative action of the nervous system.* Cambridge: University Press.

SVENNERHOLM, L. (1956). The determination of hexosamines with special reference to nervous tissue. *Acta Soc. Med. upsalien.* **61**, 287.

SVENNERHOLM, L. (1957). Quantitative estimation of sialic acids. II. A colorimetric resorcinol-hydrochloric acid method. *Biochim. biophys. Acta*, **24**, 604.

UDENFRIEND, S., WEISSBACH, H. & CLARK, C. T. (1955). The estimation of 5-hydroxytryptamine (serotonin) in biological tissue. *J. biol. Chem.* **215**, 337.

VAN HEYNINGEN, W. E. (1959*a*). The fixation of tetanus toxin by nervous tissue. *J. gen. Microbiol.* **20**, 291.

VAN HEYNINGEN, W. E. (1959*b*). Chemical assay of the tetanus toxin receptor in nervous tissue. *J. gen. Microbiol.* **20**, 301.

VAN HEYNINGEN, W. E. (1959*c*). Tentative identification of the tetanus toxin receptor in nervous tissue. *J. gen. Microbiol.* **20**, 310.

VAN HEYNINGEN, W. E. (1961). The relation between the fixation and inactivation of tetanus toxin by ganglioside. *Brit. J. exp. Path.* **42**, 397.

VAN HEYNINGEN, W. E. & MILLER, P. A. (1961). The fixation of tetanus toxin by ganglioside. *J. gen. Microbiol.* **24**, 107.

YAMAKAWA, T., IRIE, R. & IWANAGA, M. (1960). The chemistry of lipid of posthemolytic residue or stroma of erythrocytes. *J. Biochem.* **48**, 490.

J. gen. Microbiol. (1963), **31**, 389–392
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The Fixation of Tetanus Toxin by Frog Brain

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SUMMARY

The apparent non-fixation of tetanus toxin by frog brain emulsion has been re-examined. Frog brain emulsion does fix tetanus toxin, but only with 1/2000th the capacity of mammalian brain emulsion. This low toxin-fixing capacity may be connected with the observation that the ganglioside in frog brain, unlike that in mammalian brain, is extractable with aqueous solvents.

INTRODUCTION

Rowson (1961) showed that when tetanus toxin at a concentration of 1000 mouse LD 50 doses/ml. was mixed with frog brain emulsion at a concentration of 250 mg./ml. there was no measurable fixation of toxin. We have now re-investigated this problem and have found that frog brain does indeed fix tetanus toxin, but that its toxin-fixing capacity is very low as compared with that of mammalian brain. A partial explanation for this low toxin-fixing capacity of frog brain has been found.

METHODS

Frog nervous tissue. Live *Rana temporaria* were obtained at intervals as required from Cornwall or Ireland. They were of both sexes and weighed between 15 and 30 g. They were kept at 4° on moist grass. The frogs were decapitated with large scissors and the brain and spinal cord dissected out, placed in chilled tubes and preserved at −20° when not required immediately. About 20% of the 'brains' consisted of spinal cord. The total yield of nervous tissue from each animal averaged about 100 mg. fresh wt.

Tetanus toxin. The material TD 464 D as described by van Heyningen (1959*b*) was used. It contained about 10 million mouse LD 50 doses/mg., or 25 million LD 50 doses/mg. protein.

Mammalian ganglioside. A partially purified beef-brain ganglioside was used (van Heyningen & Miller, 1961).

Sialic acid. This was assayed by the difference in the resorcinol values (Svennerholm, 1957) before and after hydrolysis with 8 N-HCl for 30 min. at 100° (Long & Staples, 1959).

RESULTS

Fixation of 2 LD 50, 10 LD 50 *and* 20 L+ *units of tetanus toxin.* Determinations were made, on various species of animal, of the least amount of brain tissue that would fix 10 mouse LD 50 doses and 20 L+ units (500,000 LD 50) of tetanus toxin, by the biological assay methods of van Heyningen (1959*a*). The results are shown in Table 1. It was not possible in these tests to use brain tissue at a higher concen-

tration than 250 mg./10 LD50 because the brain suspensions were diluted with an equal volume of test toxin solution, and 0·5 ml. of this mixture injected into the mice. In the case of frog brain, although 250 mg. did not protect mice against the lethal effects of 10 LD50 toxin, it appeared to bring about a slight delay in the onset of toxic symptoms. We therefore decided to test the toxin-fixing capacity of frog brain with 2 LD50 instead of 10 LD50, in the manner referred to above. In each of two experiments 94 mg. fresh wt. of frog brain fixed 2 LD50 of tetanus toxin, and in one experiment 38 mg. frog spinal cord fixed 2 LD50 of toxin. Thus, if it be assumed that 0·05 mg. guinea-pig brain can fix 2 LD50 toxin (since 0·25 mg. fix 10 LD50; Table 1), it appears that guinea-pig brain has about 2000 times the tetanus-toxin-fixing capacity of frog brain.

Table 1. *Tetanus-toxin-fixing capacity of brain tissue from various species*

	mg. fresh wt. of brain fixing	
	10 LD50 doses toxin	20 L+ units (0·5 million LD50 doses toxin)
Guinea-pig	0·25	11
Chicken	0·40	50
Beef	2·50	100
Frog	250 mg. fail	250 mg. fail

Table 2. *Non-diffusible sialic acid contents of guinea-pig and frog nervous tissue (mean of five estimations in each case)*

	μmole/g. fresh wt.
Guinea-pig cerebral cortex	0·61
Frog brain	0·27
Frog spinal cord	0·26

Ganglioside content of frog brain. Since it had been shown previously that ganglioside was responsible for the fixation of tetanus toxin by mammalian brain (van Heyningen, 1959*c*; van Heyningen & Miller, 1961), we investigated the ganglioside content of frog brain. The fresh frog brain was extracted with 19 volumes of chloroform + methanol (2 + 1 by vol.) and the ganglioside transferred from this extract into 0·1 M-KCl in water by the method of Folch, Lees & Sloane Stanley (1957). The aqueous phase was dialysed against distilled water for 18 hr. at 4° and sialic acid determined in suitable samples. It was assumed that these values reflected the ganglioside values. The results in Table 2 show that the relatively low toxin-fixing capacity of frog nervous tissue was not due to a lack of ganglioside.

Toxin-fixation by frog-brain ganglioside. Crude preparations of frog-brain ganglioside (0·17 μmole sialic acid/mg.; compare 0·8 μmole sialic acid/mg. purified beef-brain ganglioside) were made by the methods described for beef-brain ganglioside by van Heyningen & Miller (1961). On thin layer chromatography the frog ganglioside behaved like purified beef-brain ganglioside. The toxin-fixing capacity of crude

frog-brain ganglioside was measured by the analytical ultracentrifugal method of van Heyningen & Miller (1961); 1 mg. of the crude ganglioside preparation fixed 1 mg. tetanus toxin (compare 4 mg. toxin fixed/mg. purified beef-brain ganglioside). Thus the toxin-fixing capacity of frog-brain ganglioside appears to be similar to that of mammalian brain ganglioside.

Distribution of ganglioside in frog brain. The ganglioside in mammalian brain cannot be extracted with aqueous solvents (see Folch, Arsove & Meath, 1951; van Heyningen, 1959*c*). However, we found that the ganglioside of frog brain is readily extractable with water, 0·1 M-phosphate buffer (pH 7) and frog Ringer solution. Frog brain was homogenized in 5 volumes of each of these solvents for 20 min. and centrifuged at 5000 *g* for 1 hr. The aqueous supernatant fluid was dialysed against three changes each of 5 l. distilled water during 24 hr. and the non-diffusible sialic acid determined. The results are shown in Table 3. It appears that all the ganglioside in the frog brain was extractable with aqueous solvents.

Table 3. *Non-diffusible sialic acid in aqueous extracts and residues of frog brain*

	μmole sialic acid/g. fresh wt.		
	Water	0·1 M-phosphate buffer (pH 7)	'Frog Ringer'
Extract:	0·27, 0·33, 0·17, 0·27, 0·25	0·17, 0·27, 0·09*, 0·10*	0·11*, 0·11*
Residue:	0, 0, 0·2, 0, 0	0, 0, 0*, 0*	0*, 0*

* These values obtained in July, remainder in April/May.

Tetanus-toxin-fixation at low toxin concentration. In the experiments on the fixation of tetanus toxin by frog brain, the frog ganglioside would have been in solution and would not have been sedimented in the centrifugal field (5000 *g*) used. The experiment with 10 LD50 of tetanus toxin was therefore repeated, but this time the frog brain was centrifuged at 198,000 *g* (50,000 rev./min. in the Spinco L 50) for 2 hr. Again it was observed that 250 mg. frog brain did not fix 10 LD50 of toxin, although all the gangl oside was recovered from the sedimented residue. However, we had reason to believe that isolated ganglioside (as distinct from ganglioside *in situ* in the brain) would have a very low toxin-fixing capacity in dilute toxin solutions (20 LD50/ml.) although it has a very high toxin-fixing capacity in concentrated toxin solutions (namely, 4 mg. toxin fixed/mg. ganglioside at 5 mg. toxin/ml., or 100 million LD50 fixed/mg. ganglioside at 125 million LD50 toxin/ml.; van Heyningen & Miller, 1961).

It had previously been observed (van Heyningen, 1959*a*) that at low toxin concentrations the toxin receptor activity of the 'protagon' fraction of mammalian brain (i.e. crude water-insoluble ganglioside + cerebroside complex) was not greater than that of whole brain, whereas at high toxin concentration it was much greater. This was confirmed in an experiment in which beef-brain ganglioside at 0·5 mg./20 LD50/ml. 0·1 M-phosphate buffer (pH 7), was centrifuged at 198,000 *g* for 2 hr. Less than 20 LD50 toxin/ml. were centrifuged down, whereas the same concentration of ganglioside in the presence of 125 million LD50 toxin/ml. would have centrifuged 50 million LD50 toxin/ml. (van Heyningen & Miller, 1961).

DISCUSSION

The susceptibility of the frog to tetanus toxin increases with temperature; at 26° the frog is about 3000 times as resistant as the mouse (Rowson, 1961). It is not known what the relative susceptibilities of these two species would be at the same temperature, but a very rough extrapolation of Rowson's data (the temperature effect curve flattens out above 23°) suggests that if it were possible to maintain frogs at 37° the increase in susceptibility over that at 26° would be less than tenfold. We have shown that mammalian (guinea-pig) brain has about 2000 times the toxin-fixing capacity of frog brain, and perhaps the greater resistance of the frog to tetanus toxin is due to the smaller capacity of its nervous tissue to fix the toxin. This low toxin-fixing capacity of frog brain is not due to a lack of ganglioside, or to the inability of the isolated ganglioside to fix toxin under the same conditions as does mammalian brain ganglioside. It appears to be connected with our observation that frog-brain ganglioside, unlike mammalian brain ganglioside, is extractable with aqueous solvents. In the test system for measuring tetanus toxin-fixation by brain tissue, the frog-brain ganglioside is therefore in solution and in a different physical state from that in emulsified mammalian brain (or in the intact frog brain?). At very high toxin concentrations (125 million LD50/ml.) free ganglioside in solution has a far greater toxin-fixing capacity than brain emulsion (namely, 100 million LD50/mg. ganglioside, as compared with 5000 LD50/mg. fresh brain tissue; van Heyningen, 1959*a*; van Heyningen & Miller, 1961); but at low toxin concentration (20 LD50/ml.) mammalian brain emulsion has a greater toxin-fixing capacity than free ganglioside in solution (namely, 40 LD50/mg. fresh brain tissue, as compared with < 40 LD50/mg. free ganglioside). This anomalous behaviour has been observed before, when it was suggested that it might be due to slight differences in the curvatures of the adsorption isotherms for brain emulsion and isolated receptor at low concentrations of adsorbate (van Heyningen, 1959*a*).

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REFERENCES

FOLCH, J., ARSOVE, S. & MEATH, J. A. (1951). Isolation of brain strandin, a new type of large molecule tissue component. *J. biol. Chem.* **191**, 819.

FOLCH, J., LEES, M. & SLOANE STANLEY, G. H. (1957). A simple method for the isolation and purification of total lipides from animal tissues. *J. biol. Chem.* **226**, 497.

LONG, C. & STAPLES, D. A. (1959). Determination of neuraminic acid in crude brain lipids. *Biochem. J.* **73**, 385.

ROWSON, K. E. K. (1961). The action of tetanus toxin in frogs. *J. gen. Microbiol.* **25**, 315.

SVENNERHOLM, L. (1957). Quantitative estimation of sialic acids. II. A colorimetric resorcinol-hydrochloric acid method. *Biochem. biophys. Acta*, **24**, 604.

VAN HEYNINGEN, W. E. (1959*a*). The fixation of tetanus toxin by nervous tissue. *J. gen. Microbiol.* **20**, 291.

VAN HEYNINGEN, W. E. (1959*b*). Chemical assay of the tetanus toxin receptor in nervous tissue. *J. gen. Microbiol.* **20**, 301.

VAN HEYNINGEN, W. E. (1959*c*). Tentative identification of the tetanus toxin receptor in nervous tissue. *J. gen. Microbiol.* **20**, 310.

VAN HEYNINGEN, W. E. & MILLER, P. A. (1961). The fixation of tetanus toxin by ganglioside. *J. gen. Microbiol.* **24**, 107.

J. gen. Microbiol. (1963), **31**, 393–404
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The Single Mitochondrion, Fine Structure, and Germination of the Spore of *Blastocladiella emersonii*

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SUMMARY

The motile spore of the water fungus, *Blastocladiella emersonii*, contains a single, large, posterior, eccentrically disposed mitochondrion; some 6–12 prominent, strongly osmiophilic, lipid-like organelles, bordered by a double membrane, occur along its outer edge. A single flagellum with the classical 9-plus-2 fibrillar structure is attached by at least one banded rootlet to the mitochondrion. The nuclear cap (a package of ribosomes) overlies the nucleus and is separated from it by a double membrane. The cytoplasm is somewhat granular, contains structures believed to be organelles (previously described as γ particles), but is devoid of any obvious cytoplasmic reticulum. Before spore germination, the flagellum undergoes a series of characteristic movements. Following this, the nuclear apparatus rotates through some 270°, but the spore itself does not turn. The flagellum is then retracted into the cell. Subsequently, the spore germinates by formation of a germ tube and the nuclear cap disintegrates.

INTRODUCTION

The motile, posteriorly uniflagellate, uninucleate spore of the water mould, *Blastocladiella emersonii*, is about $7 \times 9\mu$; the flagellum is about 20μ long. It was described (Cantino & Hyatt, 1953; Cantino & Horenstein, 1956) as possessing: (*a*) a typically Blastocladiaceous (Sparrow, 1960), crescent-shaped nuclear cap (the so-called 'food body') intimately in contact with and partially covering the nucleus; (*b*) a large, posteriorly placed, irregularly shaped 'side body' bearing about half a dozen lipid-like granules, the side body being inserted to the side of the nuclear apparatus and near the point of entry of the flagellum; and (*c*) small organelles labelled γ particles, about $0{\cdot}5\mu$ in diameter and randomly distributed in the cytoplasm with a frequency of about 13 per spore (when derived from 'ordinary colourless' or OC cells). It was shown, finally, that the flagellum was not simply shed or somehow lost, but actually retracted prior to germination of the spore.

The 'side body' has been found in spores of other species of Blastocladiella (Sparrow, 1960). Stüben (1939) reported its presence (his 'Seitenkörper') in a Blastocladiella-like organism, and the occurrence of this organelle was used as a partial basis for creating a new genus, *Sphaerocladia*. Later, Couch & Whiffen (1942) showed that such 'side bodies' (*a*) were demonstrable in osmic-fixed spores of several species of Blastocladiella (for this and other reasons, Stüben's Sphaerocladia was transferred to the genus *Blastocladiella*); (*b*) often possessed attached fat-like bodies;

and (*c*) in some instances appeared to be intimately associated with the 'cilium' (flagellum) of the spore.

In this report, we shall show that in *Blastocladiella emersonii* the 'side body' is a single mitochondrion with which the flagellum is intimately associated, that relative to cell volume it is a giant one, and that no other mitochondria are detectable in the spore.

METHODS

Preparation of spores

Single generation populations of OC cells were grown on Petri plates of PYG agar (0·125 % peptone, 0·125 % yeast extract, 0·3 % glucose, and 2·0 % agar; Difco Laboratories, Detroit, Michigan). As soon as discharge of spores began *in situ* (the exact time depending upon temperature and population density; see Results), plates were flooded with distilled water to accelerate spore liberation (for general procedures and discussion, see McCurdy & Cantino, 1960; Cantino & Lovett, 1963). Such suspensions of swimming spores were either used directly for light microscopy or centrifuged for electron microscopy, for which exceptionally dense spore populations were needed and obtained as follows: a Petri plate of mature plants was flooded with about 20 ml. of water; the spore suspension thus obtained was used to flood a second plate of *Blastocladiella emersonii*; the new spore suspension, now much denser, was used to flood a third plate of the fungus, and so on until an adequate concentration was obtained.

Light microscopy

The flagellum, the nuclear cap, and the prominent refractile granules adjacent to the side body were always clearly distinguishable in unstained spores. When desired, additional contrast was obtained with a vital stain (Trypan blue, up to 1 % solution) and a filter with maximum transmission at 580 mμ. Spore suspensions were either examined directly in hanging drop mounts, or mounted (alive) in thin agar blocks as follows:

A drop of a thick spore suspension was preheated to 40° and added to a drop of molten PYG agar, also at 40°; the two were vigorously mixed and then promptly transferred on to a flat sheet of glass at room temperature, where the suspension solidified into a thin layer immediately. A block with the desired dimensions was cut out, placed on to a coverslip, inverted over a depression slide, and sealed with silicone grease.

Electron microscopy

For examination of flagellated spores, thick suspensions were centrifuged at about 1800 ***g*** for one min. in 50 ml. conical tubes, the supernatant was removed immediately, fixative was added to the loose pellet, and the mixture was shaken vigorously. Pellets were fixed in Zetterqvist's fixative (Pease, 1960) at pH 5·6 and 7·5. Some were fixed first in Zetterqvist's fixative (2–12 hr.) and then in a solution containing 2·5 % $KMnO_4$ and 2·9 % NaCl (2–12 hr.); others were fixed only in the $KMnO_4$-NaCl reagent. Time of fixation ranged from 2 hr. to 24 hr., the latter yielding better results. After fixation, pellets were washed and then dehydrated in 30, 60, 80, 95 %, and absolute ethanol, 15–30 min. in each, for methacrylate embedding. Material fixed in $KMnO_4$ was stained with 2 % phosphotungstic acid in absolute ethanol. When pellets were

to be embedded in vestopal-W, they were dehydrated with acetone and then treated according to Ryter & Kellenberger (1958) and Zellickson & Hartmann (1960). For embeddings in epon and araldite, spores were processed according to Luft (1961) and Glauert & Glauert (1958).

For examination of spores which had retracted their flagella, pellets were prepared as above and then allowed to remain at about 25° for 5 min. before addition of fixative; during this time about half of the spores retracted flagella. An alternative approach made use of thick spore suspensions in solidified agar blocks as employed for light microscopy; these were cut into small pieces at least 1 mm. thick and fixed after different time intervals up to 10 min., but such specimens were not as well preserved as those obtained from pellets.

Sections were made with a diamond knife (Fernandez-Moran, 1956) and glass knives on a Leitz ultra-microtome, mounted on 100- and 200-mesh copper grids covered with a parlodion film, and coated with a thin film of carbon (Pease, 1960). Sections were stained in uranyl acetate or lead hydroxide (Watson, 1958; Dalton & Zeigel, 1960) and observed with a Phillips 100B electron microscope.

Thick sections (3–5 μ) were cut adjacent to the thin ones (25–45 mμ) and examined with a phase contrast microscope, in order to determine the exact orientation of the section through the spores.

RESULTS

Optimal conditions for preparation of spore suspensions from OC cells

Blastocladiella emersonii produces four kinds of plants, distinguishable from one another by their morphology, colour, and rates of growth (Cantino & Hyatt, 1953; Cantino, 1961). At maturity, each type of thallus liberates motile uniflagellate

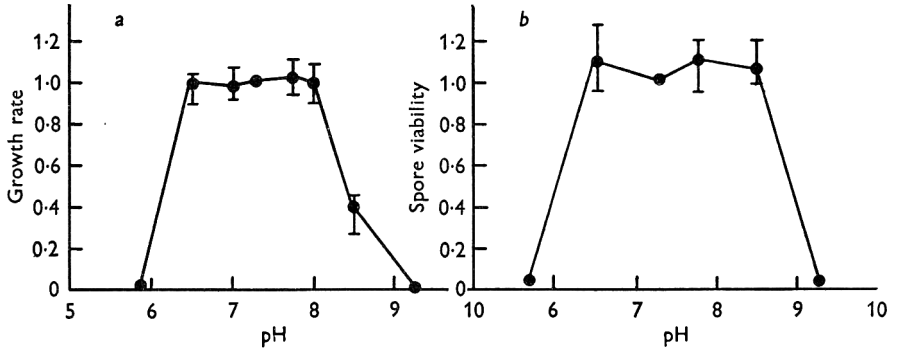

Fig. 1. *a*, The relative growth rate of OC cells at 20° on PYG medium adjusted to different pH levels. Length and width of several dozen randomly selected cells were measured at intervals from about 5 to 18 hr.; the average increase in size per hour at pH 7·3 was set at 1·0, and the data obtained at other pH levels were related to it. *b*, The relative viability of spores (derived from OC cells) on medium PYG at the different pH values used above. The total number of OC cells produced from these spores at pH 7·3 was set at 1·0, and the number of OC cells derived from an equal quantity of spores inoculated on PYG at the other pH levels were related to it.

spores and these, too, may differ from one another in viability, size, colour, and the number of certain cytoplasmic organelles (Cantino & Horenstein, 1956). It was essential to do electron microscopy with homogeneous populations of only one kind

of swarmer at a time; some of the conditions necessary to achieve this are described below. On solid media, growth of *B. emersonii* is roughly the same between pH 6 and 8 (Fig. 1*a*). Populations of first generation thalli consist almost wholly of OC cells; RS ('resistant sporangial') cells are not produced, while the appearance of 'orange' and 'late-colourless' cells is delayed until almost all OC cells have reached maturity (Cantino & Hyatt, 1953). By flooding populations with water when OC cells are ready to discharge swarmers, selection of one class of spores is almost automatically assured. It was also desirable to select conditions where spores exhibit maximum viability and therefore, presumably, maximum uniformity of internal structure. Spore viability (Fig. 1*b*) more or less parallels the growth responses

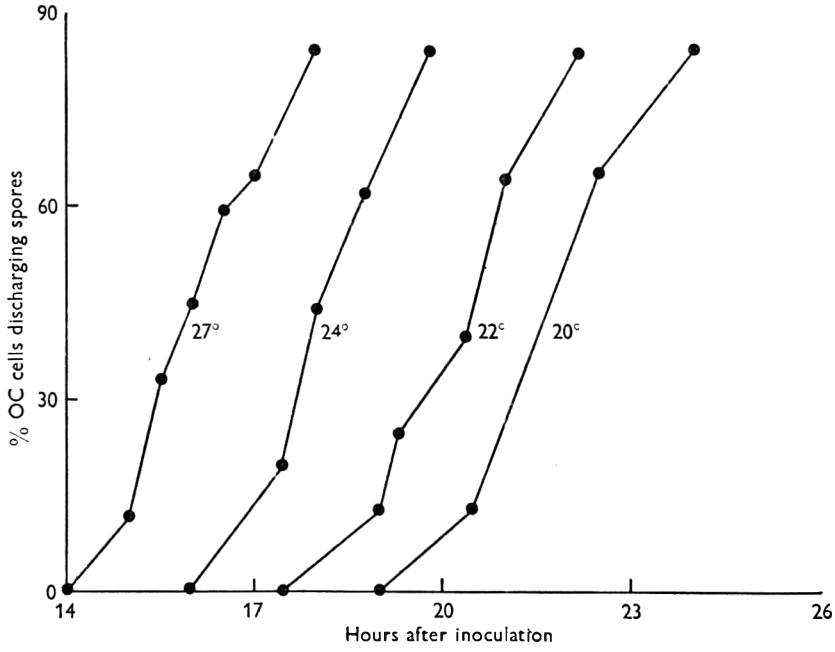

Fig. 2. The effect of temperature on the rate at which a population of OC cells (about 1000/plate) discharges spores *in situ* when grown on PYG medium, pH 6·8. Cumulative data are plotted on the vertical axis.

of the parent cells (Fig. 1*a*). Consequently, medium PYG at pH 6·8 was selected for growth of OC cells used in electron microscopy. Finally, the rate at which spores were discharged from individual thalli in a population of about 1000 OC cells per Petri plate was established at 20°, 22°, 24°, and 27°. At 20° (Fig. 2), OC cells begin to release their spores at 20 hr.; some 4 hr. later about 85 % of the population has discharged. At 27°, on the other hand, the process begins at about 15 hr. and accordingly it ends earlier. With this information, it is possible to arrange combinations of temperature and time so that cultures can be started and harvested at convenient intervals.

Fine structure of the flagellated spore

Our interpretation of the essential features and fine structures of the spore, as deduced by electron microscopy, is illustrated in Fig. 3. Micrographs which

illustrate many of these things are shown in the plates and will be referred to in their appropriate places below.

The nuclear cap (Pl. 1, fig. 1) is a striking feature of the spore. As in Allomyces (Turian & Kellenberger, 1956; Blondel & Turian, 1960), the cap in Blastocladiella is a massive, granular structure which partially encloses the anterior end of the nucleus. Recently, such nuclear caps were isolated (Lovett, to be published) essentially free from nuclei and nucleoli, and in sufficient quantity to permit quantitative analyses; the data revealed that this organelle consists of ribonucleoprotein particles —presumably, ribosomes. Their possible origin in the developing cell has been discussed elsewhere (Cantino & Lovett, 1963). The nucleus extends into the nuclear cap

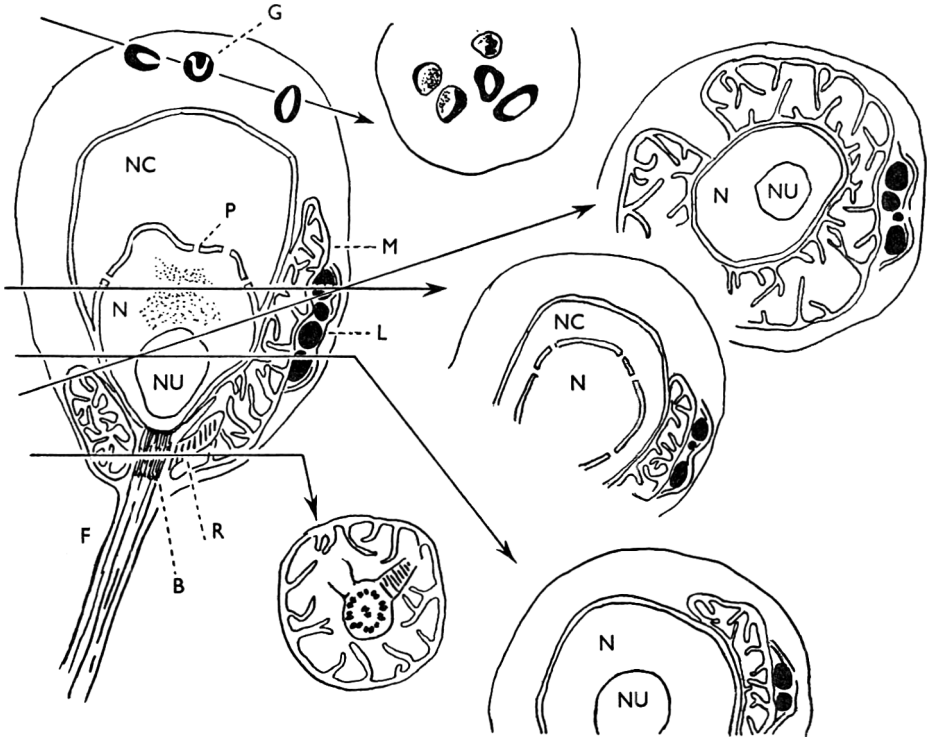

Fig. 3. Interpretive sketch of the details and fine structure of a spore derived from an OC cell (G, γ particle; NC, nuclear cap; N, nucleus; NU, nucleolus; M, mitochondrion; L, lipid body; F, flagellum; P, pore; B, basal structure; R, banded rootlet).

(Pl. 1, fig. 1; Pl. 3, fig. 6). The cap is separated from the nucleus by a double membrane, which contains round pits of about 1000 Å. diameter; these are visible in both cross-section and tangential section on Pl. 2, fig. 2. A cross-section through the top of a nucleus (Pl. 2, fig. 3) reveals how numerous the pores may be in certain areas. These holes are similar in dimension to those described for Allomyces (about 950 Å. diameter; Turian & Kellenberger, 1956). A certain amount of granular material is apparently distributed unevenly in the nucleus, and a nucleolus is embedded in it near the posterior end of the spore (Pl. 1, fig. 1).

The single mitochondrion of the spore

In *Blastocladiella emersonii*, there is a single mitochondrion which, aside from its enormous size (Pls. 1 and 3) relative to cell volume, possesses a classical structure with numerous cristae arising by invagination of the inner component of a double membrane (Pl. 4). Without question, it corresponds to the 'side body' seen in the past with the light microscope. This situation contrasts markedly with that described for motile cells—albeit gametes—of Allomyces, wherein many small mitochondria occur (Turian & Kellenberger, 1956). In *B. emersonii*, the mitochondrion completely surrounds the base of the flagellum (Pls. 4; Pl. 6, fig. 13); it extends upward along one side almost to the anterior end of the nuclear apparatus, while on the opposite side it extends upward for a short distance only (Pl. 1, fig. 1; Pl. 3, fig. 4). Fibres of the flagellum enter the spore through a central opening in the mitochondrion (Pls. 1 and 4). Extending from this cavity, there is at least one lateral canal—and a second one is often visible (Pls. 4 and 5)—which may be lobed (Pl. 6, fig. 14) and which penetrates for some distance through the body of the mitochondrion. Whenever a banded structure has been detected, it has always been found in such a canal (see later).

The refractive granules, seen by light microscopy to be associated loosely with the 'side body', are immediately evident in electron micrographs (Pl. 3, fig. 5). They lie in one to several layers, number roughly 6–12, and are intimately adjacent to the mitochondrion but clearly separated from the cytoplasm by a double membrane. Judging from their refractiveness in the light microscope and their strongly osmiophilic character in the electron microscope, they are in all probability lipoidal bodies.

The flagellum and its point of attachment

The flagellum contains 9 outer and 2 inner fibres, each of them a double structure (Pl. 6, fig. 15), and is attached to the posterior end of the spore. The flagellar sheath is confluent (Pl. 1, fig. 1) with the outer membrane of the cell, while the fibres end abruptly at the nuclear membrane (Pl. 1, fig. 1). At this point, the aggregate of fibres is clearly differentiated into a sort of basal structure (Pl. 3, fig. 4; Pl. 5, fig. 10) which appears to be anchored by non-banded, rootlet-like appendages extending upward between the nucleus and the mitochondrion (Pl. 5, fig. 9). A second structure which also originates near the base of the flagellum appears quite regularly; whether it is an individual fibre or consists of 2 or more fibrils is not yet clear. It lies in a lateral canal in the mitochondrion and consists of a series of transverse, electron-dense plates with an average diameter of 210 Å. (Pl. 5, fig. 10; Pl. 6, figs. 11, 12); presumably, it is a 'rootlet' (see Discussion). We have never seen more than one of these stranded structures in any single picture, although two lateral canals in the mitochondrion (Pl. 4, fig. 7; Pl. 6, figs. 11, 14) are frequently visible simultaneously. In every instance where we have seen it, the 'rootlet' extends only into the long arm of the mitochondrion.

The cytoplasm and inclusions

The cytoplasm, although somewhat granular, is conspicuously devoid of an obvious endoplasmic reticulum. The only well defined inclusions in Blastocladiella

are some strongly osmiophilic organelles bound by a single membrane (Pl. 6, fig. 16); clearly, these should not be confused with the lobed osmiophilic granules of different shape found in the cytoplasm of Allomyces (Blondel & Turian, 1960). We believe that they consist of a spherical body incompletely girdled by a band of strongly osmiophilic material.

Retraction of the flagellum

When viewed in the light microscope, motile spores of *Blastocladiella emersonii* are similar in most respects to those figured by Couch & Whiffen (1942) for *B. simplex*; however, they do not possess any detectable structure which would clearly correspond to a rhizoplast (unless the basal structure at the flagellar terminus is

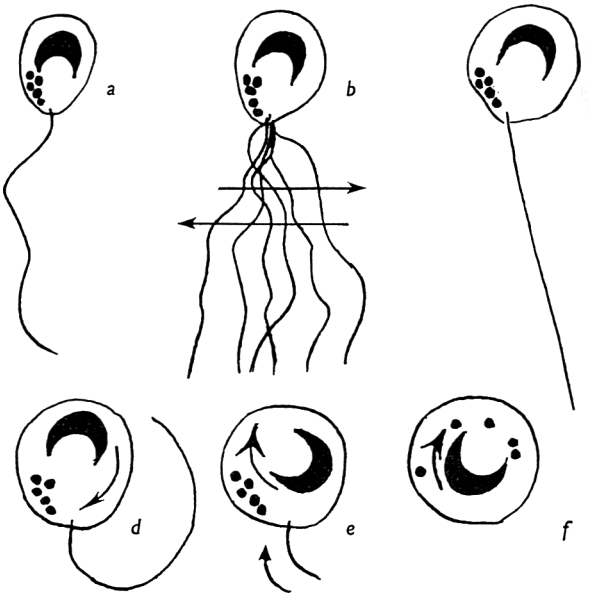

Fig. 4. The sequence of events involved in retraction of the flagellum by a spore from an OC cell (see text).

likened to one). In its active swimming state, the spore is roughly ovoid and is propelled by a whiplash flagellum in which the terminal region tapers gradually (Cantino & Hyatt, 1953). Often, the spore stops swimming and begins to move about in amoeboid fashion, only to begin swimming once again at a later time; the flagellum is never lost under these conditions. In hanging drop mounts, the time at which the spore rounds up and finally retracts its flagellum depends upon environmental conditions; at 25°, it commonly occurs about 30 min. after cultures are set up.

The first indication that retraction of the flagellum may begin occurs when it stops its propulsive whiplash beat (Fig. 4*a*) and, instead, begins to vibrate rapidly back and forth (Fig. 4*b*). This type of fibrillation, which lasts for some 5–10 sec., apparently has no propulsive force, because the cell settles down and then remains

quite stationary. When this activity has ceased, the spore does one of two things; either it resumes its active whip-lash beat (Fig. 4*a*), or its flagellum becomes very still for about 5 sec. (Fig. 4*b*). After the latter has occurred, the spore either moves about slowly in amoeboid fashion for a while and then goes through the cycle shown in Fig. 4*a–c*, or it immediately begins retracting its flagellum as follows: following the stage shown in Fig. 4*c*, the cell becomes irregularly spherical. The flagellum sweeps around in a wide arc, and then it becomes stationary once more (Fig. 4*d*). At this stage the nuclear apparatus (as reflected by the position of the cap) begins to turn within the cell in a direction opposite to that taken by the flagellum; generally, it rotates through about 270° (Fig. 4*e*). Simultaneously, the flagellum gets shorter and quickly vanishes, apparently within the body of the cell; however, the flagellum has never been seen inside the cell. During this whole process, which takes some 30–40 sec. (Fig. 4*d–f*), the spore itself does not rotate. While the nuclear apparatus is turning, the disposition of the refractive granules attached to the 'side body'—that is, the mitochondrion—does not change. But, when the nuclear cap has nearly completed its turn, the constellation of granules does become disorganized; the granules rearrange themselves in a more-or-less curved line throughout the spore body (Fig. 4*f*). Simultaneously, the cell, which was irregularly spherical at the beginning of retraction, now becomes an almost perfect sphere; it remains this way until a rhizoid is produced some 10–30 min. later.

When spores are embedded in agar rather than suspended freely in hanging drops, flagellar retraction becomes more closely synchronized; it begins by about the 2nd min. after embedding and is completed by the 5th. Under these conditions, the spore rounds up by the time it is examined microscopically, and the viscosity of the system tends to interfere with flagellar movements (as in Fig. 4*c*, *d*). Retraction does occur, however, and each spore subsequently produces a germling.

Spore structure after flagellar retraction

Attempts were made to ascertain with both light and electron microscopy what happened to the flagellum upon retraction. All that can be said with certainty is that no trace of the 9-plus-2 flagellar structure was ever found. But, some 20 hr. later, when a germinating spore has given rise to a new thallus, hundreds of second-generation spores are cleaved out again from the multinucleate protoplast within the plant (Pl. 7, fig. 17); each of these, upon discharge, possesses a new flagellum and the cycle begins anew.

DISCUSSION

Fine structure

Cytological investigations, notably those of Cotner (1930), Matthews (1937), Couch & Whiffen (1942), and Koch (1961) have been made on spores of aquatic fungi, but early workers were handicapped by the inadequate resolution of the light microscope. Turian & Kellenberger (1956) and Blondel & Turian (1958) made the first, detailed electron-microscopic studies of the fine structure of motile cells in the water mould Allomyces.

The present investigation of fine structure in the spore of *Blastocladiella emersonii* reveals an anatomy similar to that reported for Allomyces but by no means identical with it. The similarity lies in the structure and mutual disposition of the nucleus,

nucleolus, and nuclear cap. Turian (1956) showed cytochemically that the cap in Allomyces appears to contain RNA; Lovett's (to be published) chemical analyses of isolated caps in *B. emersonii* reveal that this organelle is, indeed, a tightly packed aggregate of ribosomes. There are, however, three main points of difference between these two fungi:

(1) There are many mitochondria distributed throughout the body of the motile gamete of Allomyces (no report is available on its spores); in the spore of Blastocladiella there is only one, very large, eccentrically oriented mitochondrion, which surrounds the basal part of the flagellum. We do not know of any comparable structure described in other fungi. Perhaps the nearest thing to it is the way in which mitochondria surround the tail in sperm cells; for example, the arrangement of four large mitochondria around the flagellar base in the Oyster (Galstoff & Philpott, 1960), and in the annelid worm Hydroides (Colwin & Colwin, 1961).

(2) The small cytoplasmic organelles (Fig. 3) in Blastocladiella are different from those in Allomyces. Since it is unlikely that the difference in shape resulted from variations in technique, we conclude that this distinction is real. Furthermore, since no other clear-cut cytoplasmic particles are present, these organelles are almost certainly the γ particles seen with the light microscope (Cantino & Horenstein, 1956) and implicated in the development of *B. emersonii* (Cantino & Horenstein, 1954).

(3) The banded apparatus (Fig. 3) associated with the basal part of the flagellum in Blastocladiella does not occur, apparently, in Allomyces; indeed, it does not appear to be quite the same as some of the fibrous structures found in comparable locations within other organisms. For example, bands have not been found in the flagellum roots of Euglena (Gibbs, 1960), the rootlet fibres in the zoospore of a brown alga (Manton, 1957), nor the rootlet fibres of the ciliate, Nyctotherus (King *et al.* 1961). In other instances, however, bands occur, i.e. in the fused double rootlet fibres of Pleurobrachia (Bradfield, 1955), the anterior fibrilla of Colpidium (Pitelka, 1961), and especially the rootlet fibres illustrated by Fawcett & Porter (1954) for the ciliated epithelium of a frog and a mollusc. It is tempting to consider the latter and the rootlets of *B. emersonii* as homologous structures.

In Blastocladiella, the banded body extends deeply into a lateral canal in the mitochondrion; indeed, it appears to be in intimate contact with it. Whether or not the banded structure is consistently separated by a double membrane from the interior of the mitochondrion has not been decided. Photographic evidence for the connexion between the basal region of the flagellum and a mitochondrion in Oyster sperm (Galstoff & Philpott, 1960) demonstrates that such a situation is not impossible; pictures of the sperm of Hydroides (Colwin & Colwin, 1961) and the protozoon Leishmania (Pyne, 1958) suggest the same thing.

Flagellar retraction

A beginning has been made in answering a central question: what is the mechanism of flagellar retraction in *Blastocladiella emersonii*? Observations with the light microscope revealed two things: (*a*) that an intriguing sequence of events is associated with movement of the tail; and in particular (*b*) that, in the brief interval during which the flagellum is retracted, the nuclear apparatus turns through some 270°, while the giant mitochondrion—using its associated lipid granules as markers—remains intact and essentially stationary until the flagellum is almost completely

absorbed. Only during the very last stage of retraction does the 'side body' appear to begin to change in aspect in that the fat-like granules originally attached to it become distributed throughout the cell. Electron micrographs of cells fixed 5 min. after retraction confirmed that the lipid-like bodies are no longer associated with it. But in no instance has a 9-plus-2 flagellar structure ever been found within such cells; we can only conclude that the flagellum is digested or otherwise dispersed within a few sec. after it enters the cell. Finally, as to mode of entry, we have entertained the thought that rotation of the nuclear apparatus might represent a mechanism for 'winding in' the flagellum, so to speak; however, there is not enough evidence to support or refute this notion.

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REFERENCES

BLONDEL, B. & TURIAN, G. (1958). Étude sur le champignon *Allomyces macrogynus* Em. *Proc. 4th Int. Conf. Electron Microscopy*, p. 507.

BLONDEL, B. & TURIAN, G. (1960). Relation between basophilia and fine structure of cytoplasm in the fungus *Allomyces macrogynus* Em. *J. biophys. biochem. Cytol.* **7**, 127.

BRADFIELD, J. R. G. (1955). Fibre patterns in animal flagella and cilia. *Soc. exp. Biol.* **9**, 306.

CANTINO, E. C. (1961). The relation between biochemical and morphological differentiation in non-filamentous aquatic fungi. *Symp. Soc. gen. Microbiol.* **11**, 243.

CANTINO, E. C. & HORENSTEIN, E. A. (1954). Cytoplasmic exchange without gametic copulation in the water mold *Blastocladiella emersonii*. *Amer. Nat.* **88**, 143.

CANTINO, E. C. & HORENSTEIN, E. A. (1956). Gamma and the cytoplasmic control of differentiation in Blastocladiella. *Mycologia*, **48**, 443.

CANTINO, E. C. & HYATT, M. T. (1953). Phenotypic 'sex' determination in the life history of a new species of Blastocladiella, *B. emersonii*. *Leeuwenhoek ned. Tijdschr.* **19**, 25.

CANTINO, E. C. & LOVETT, J. S. (1963). Non filamentous aquatic fungi; model systems for biochemical studies of morphological differentiation. *Advances in Morphogenesis*, Vol. 3 (in the Press).

COLWIN, A. J. & COLWIN, L. H. (1961). Fine structure of the spermatozoon of *Hydroides hexogonus* (Annelida) with special reference to the acrosomal region. *J. biophys. biochem. Cytol.* **10**, 211.

COTNER, F. G. (1930). Cytological study of the zoospores of Blastocladia. *Bot. Gaz.* **89**, 295.

COUCH, J. N. & WHIFFEN, A. J. (1942). Observations on the genus *Blastocladiella*. *Amer. J. Bot.* **29**, 582.

DALTON, A. J. & ZEIGEL, R. F. (1960). A simplified method of staining biological material with lead hydroxide. *J. biochem. biophys. Cytol.* **7**, 409.

FAWCETT, D. W. & PORTER, K. R. (1954). A study of the fine structure of ciliated epithelia. *J. Morphol.* **94**, 221.

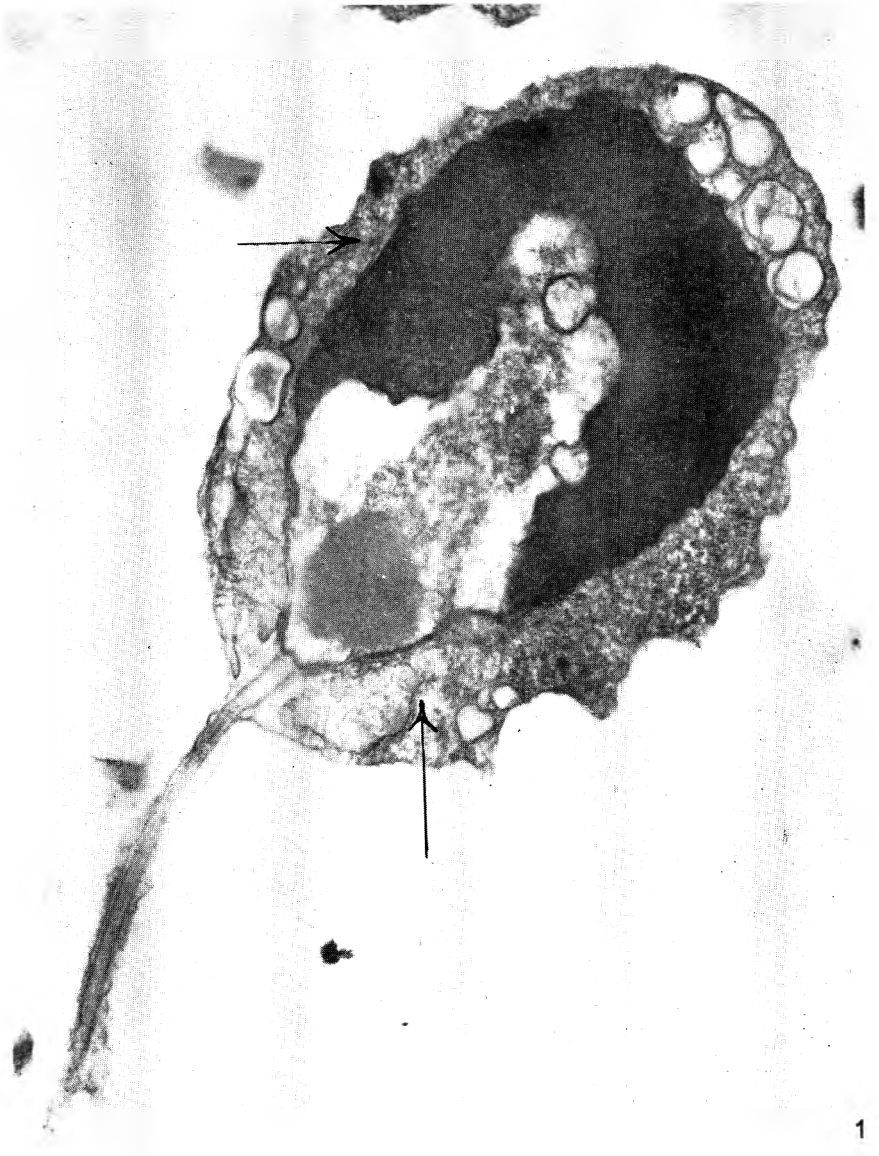
FERNANDEZ-MORAN, H. (1956). A new microtome with diamond knife. *Industr. Diam. Rev.* **16**, 128.

GALSTOFF, P. S. & PHILPOTT, D. E. (1960). Ultrastructure of the spermatozoon of the oyster *Cranostrea virginica*. *J. ultrastruct. Res.* **3**, 261.

GIBBS, S. P. (1960). The fine structure of *Euglena gracilis* with special reference to the chloroplasts and pyrenoids. *J. ultrastruct. Res.* **4**, 127.

GLAUERT, A. M. & GLAUERT R. H. (1958). Araldite as an embedding medium for electron microscopy. *J. biochem. biophys. Cytol.* **4**, 191.

KING, R. L., BEAMS, H. W., TAHMISIAN, T. N. & DEVINE, R. L. (1961). The ciliature and ultraciliature of *Nyctotherus ovalis*. *J. Protozool.* **8**, 98.



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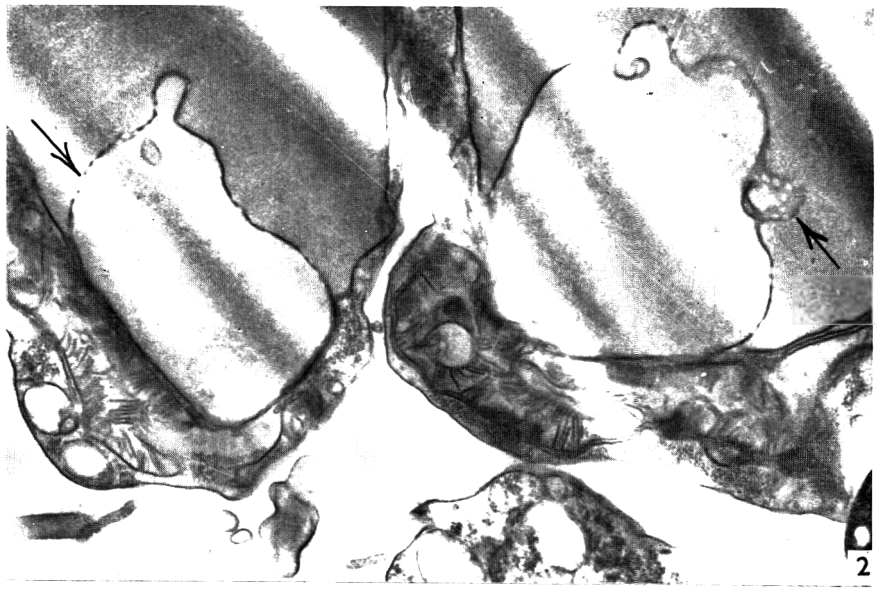
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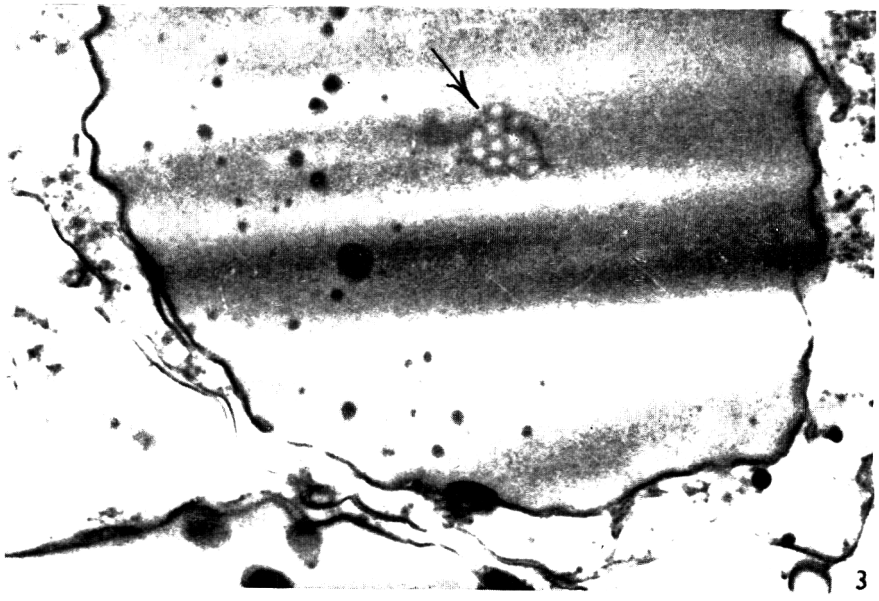
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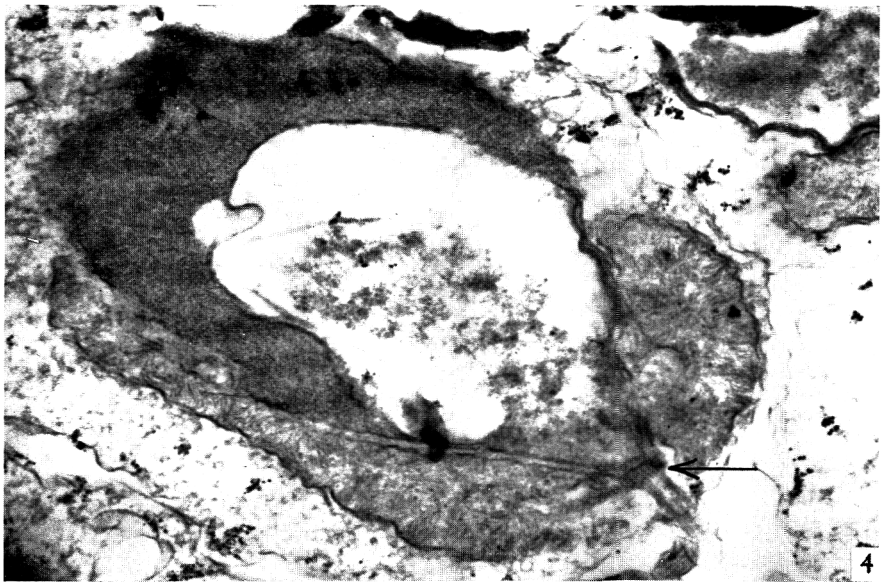
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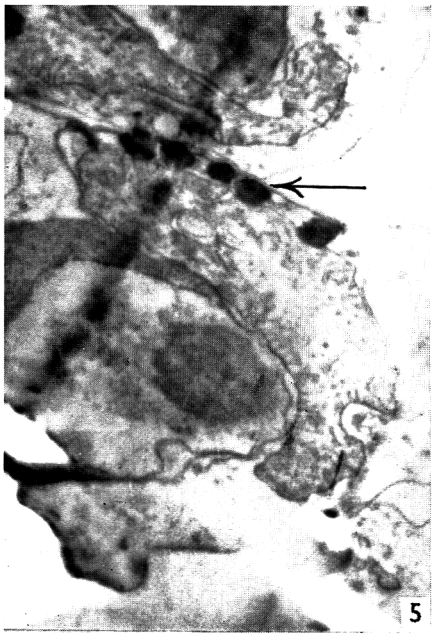
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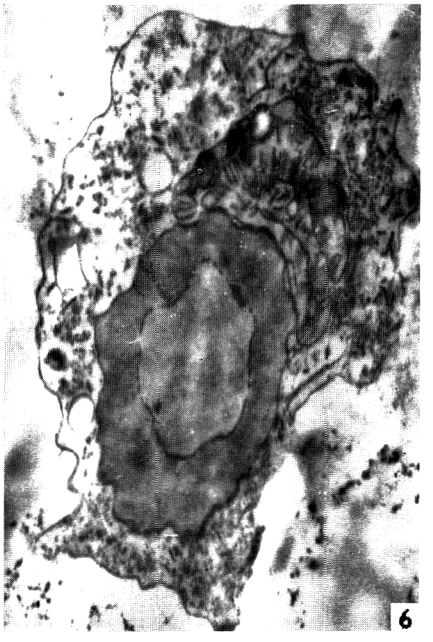
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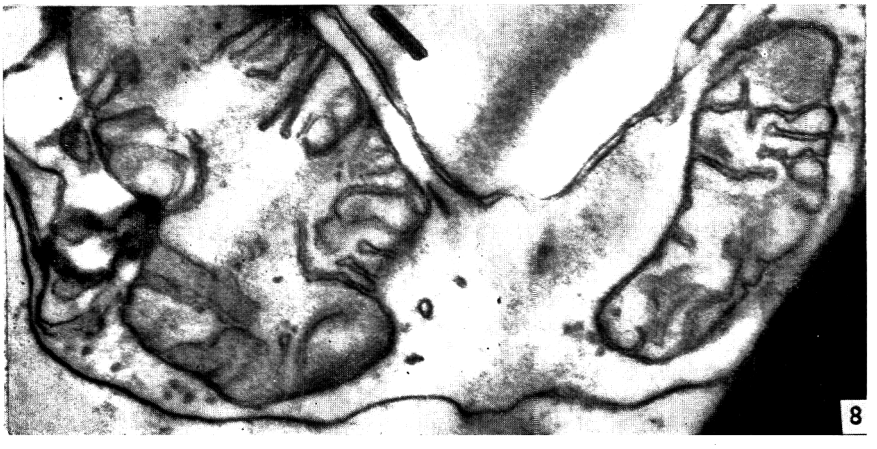
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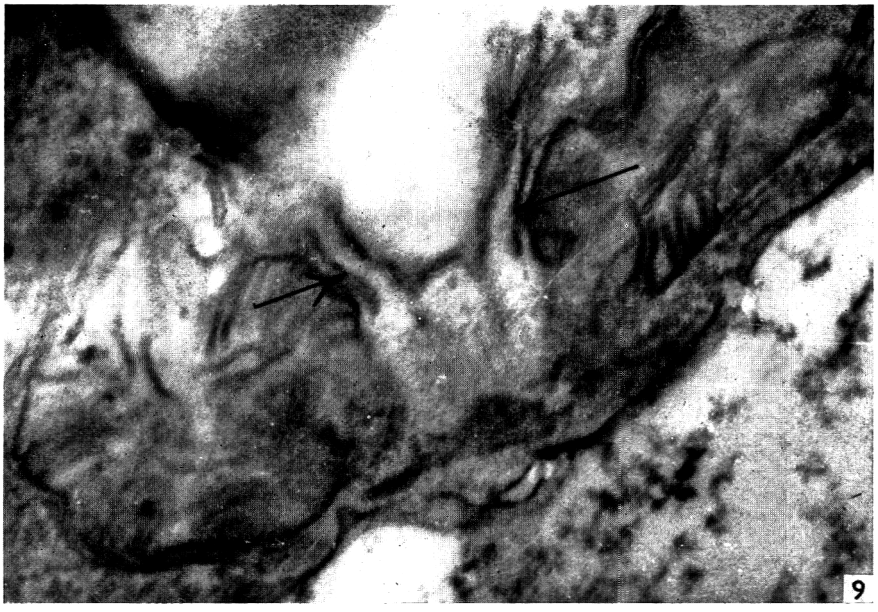
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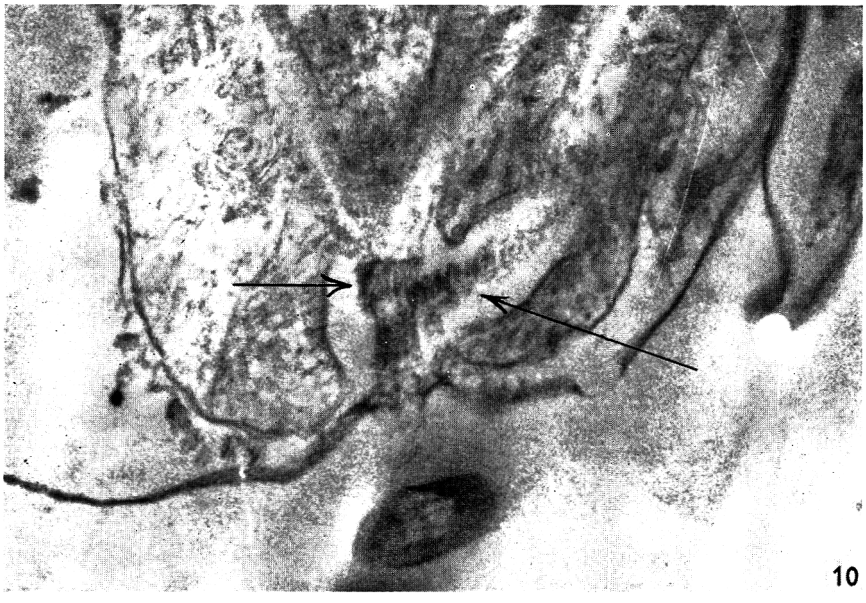
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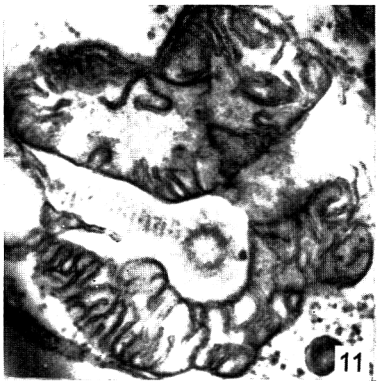
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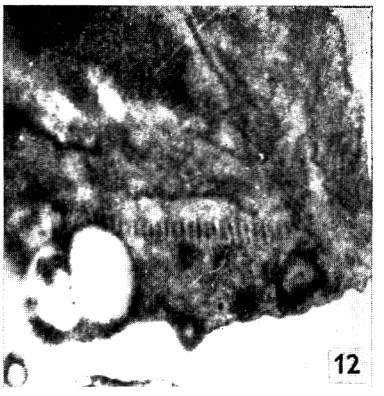
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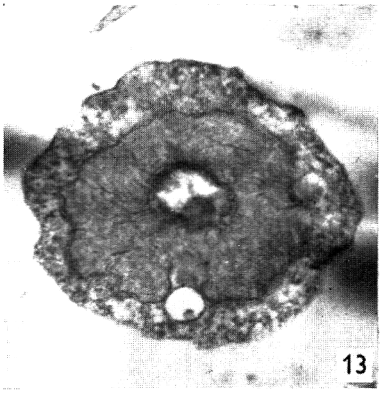
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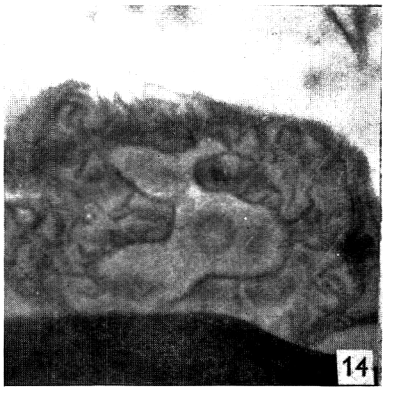
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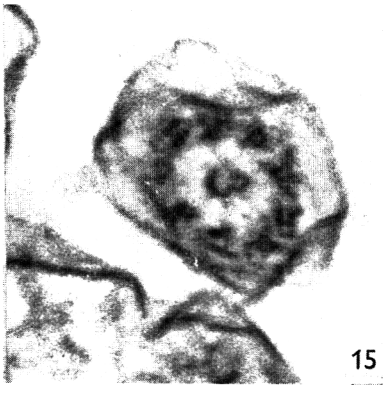
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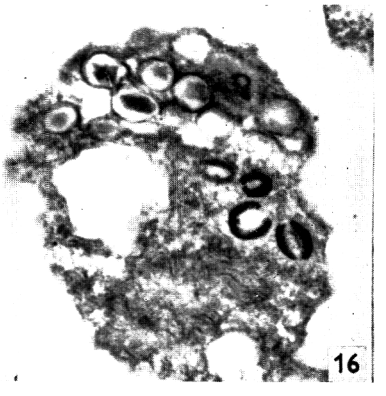
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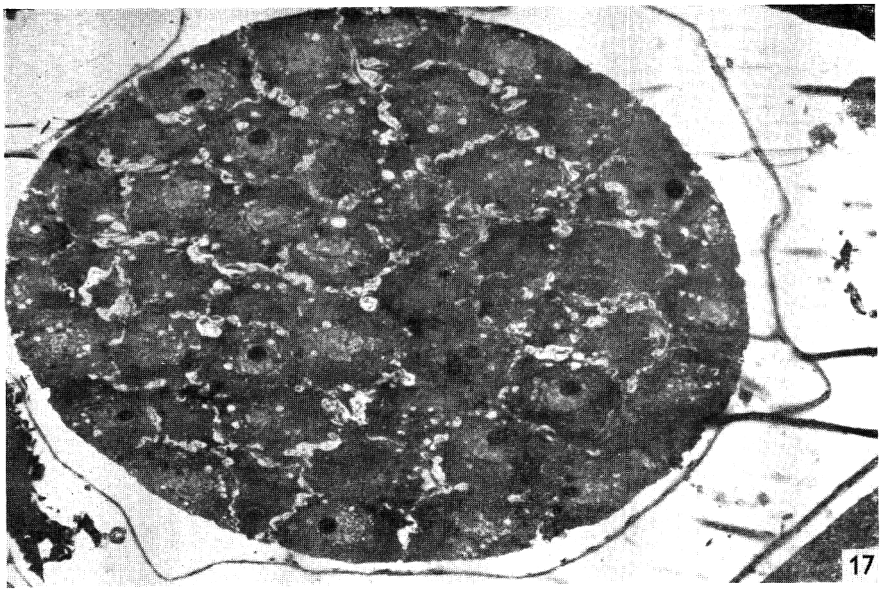
17

KOCH, W. J. (1961). Studies of the motile cells of chytrids. III. Major types. *Amer. J. Bot.* **48**, 786.

LUFT, J. H. (1961). Improvements in epoxy resin embedding methods. *J. biochem. biophys. Cytol.* **9**, 409.

MANTON, I. (1957). Observations with the electron microscope on the internal structure of the zoospore of a brown alga. *J. exp. Bot.* **8**, 294.

MATTHEWS, V. D. (1937). A new genus of the Blastocladiaceae. *J. Elisha Mitchell Sci. Soc.* **53**, 191.

MCCURDY, H. D., JUN. & CANTINO, E. C. (1960). Isocitritase, glycine-alanine transaminase, and development in *Blastocladiella emersonii*. *Plant Physiol.* **35**, 463.

PEASE, D. C. (1960). *Histological Techniques for Electron Microscopy.* New York: Academic Press.

PITELKA, D. R. (1961). Fine structure of the silverline and fibrillar systems of three tetrahymenid ciliates. *J. Protozool.* **8**, 75.

PYNE, C. K. (1958). Electron microscope investigations on the leptomonad form of *Leishmania donovani*. *Exp. Cell Res.* **14**, 388.

RYTER, A. & KELLENBERGER, E. (1958). L'inclusion au polyester pour l'ultramicrotomie. *J. ultrastruct. Res.* **2**, 200.

SPARROW, F. K., JUN. (1960). *Aquatic Phycomycetes.* Ann. Arbor, Michigan, U.S.A.: Univ. Mich. Press.

STÜBEN, H. (1939). Über Entwicklungsgeschichte und Ernährungsphysiologie eines neuen niederen Phycomyceten mit Generationswechsel. '*Planta*', **30**, 353.

TURIAN, G. (1956). Le corps paranucléaire des gamètes géants d'*Allomyces javanicus* traité à l'acide borique. *Protoplasma*, **47**, 135.

TURIAN, G. & KELLENBERGER, E. (1956). Ultrastructure du corps paranucléaire, des mitochondries et de la membrane nucléaire des gamètes d'*Allomyces macrogynus*. *Exp. Cell Res.* **11**, 417.

WATSON, M. L. (1958). Staining of tissue sections for electron microscopy with heavy metals. II. Application of solutions containing lead and barium. *J. biochem. biophys. Cytol.* **4**, 727.

ZELLICKSON, A. S. & HARTMANN, J. R. (1960). Vestopal W, a superior embedding medium for electron microscope study of human epidermis. *J. invest. Derm.* **35**, 315.

EXPLANATION OF PLATES

Plates 1–6. Electron micrographs of thin sections of the spore of *Blastocladiella emersonii*. Plate 7. Electron micrograph of a section through a mature OC plant of *B. emersonii* undergoing protoplasmic cleavage into spores.

PLATE 1

Fig. 1. Longitudinal section through a spore. The flagellar sheath is continuous with the spore membrane, and a core of flagellar fibres extends through the channel in the mitochondrion and terminates at the nuclear membrane. The single mitochondrion is eccentrically situated in the spore, with its short end at right (arrow) and its long end at left extending upward to a region (arrow) opposite the massive nuclear cap. The nucleus sometimes invaginates into the nuclear cap as seen here, but in other instances it does not do so; whether or not this variability is a property of the spore or an artifact produced in preparation has not been decided. × 17,000. Fixative, Zetterqvist, pH 7, followed by $KMnO_4$-NaCl.

PLATE 2

Fig. 2. Section through the nucleus in the basal regions of two spores. At left, the pores in the nuclear membrane (arrow) separating nucleus and nuclear cap are shown in longitudinal section; at right, tangential sections through such pores are seen (arrow). The mitochondrion below the nucleus is particularly obvious once again. × 14,200. Fixative, $KMnO_4$-NaCl.

Fig. 3. A cross-section through the nuclear cap at a region just below the point where the nucleus makes contact with the cap. Only the tip of the nucleus is visible, and the pores in the membrane (arrow) separating nucleus and nuclear cap are particularly evident. × 23,500. Fixative, $KMnO_4$-NaCl.

PLATE 3

Fig. 4. Longitudinal section through a spore. Note size of the eccentrically placed mitochondrion in relation to nucleus and nuclear cap, and the differentiated region (arrow) of the base of the flagellum. × 16,600. Fixative, Zetterqvist, pH 5.

Fig. 5. Longitudinal section through basal part of spore showing five of the lipid granules (arrow) always associated with the long end of the mitochondrion and retained by a double membrane. × 13,000. Fixative, Zetterqvist, pH 7.

Fig. 6. A cross-section through a spore above the region of the nucleolus and the short arm of the mitochondrion, but through the nucleus, nuclear cap, and the long arm of the mitochondrion. Note double membrane around lipid granules. × 12,000. Fixative, $KMnO_4$.

PLATE 4

Fig. 7. A cross-section of the mitochondrion at the base of the spore showing the central flagellar channel and two lateral canals attached thereto. Note extensive cristae. × 37,200. Fixative, $KMnO_4$-NaCl.

Fig. 8. A longitudinal section through the base of a spore illustrating the structure of the cristae and the central channel through the mitochondrion. × 37,200. Fixative, $KMnO_4$-NaCl.

PLATE 5

Fig. 9. Longitudinal section through the base of a spore illustrating the non-banded appendages (arrows) extending up along the nucleus, by which the flagellum appears to be partially anchored. × 38,500. Fixative, $KMnO_4$-NaCl.

Fig. 10. A longitudinal section through the base of a spore showing the differentiated region of the flagellar structure at the point where it ends adjacent to the nucleus (arrow), and a banded 'rootlet' attached thereto and extending into one of the lateral canals (arrow) in the mitochondrion. × 29,500. Fixative, Zetterqvist, pH 7.

PLATE 6

Fig. 11. A cross-section through the mitochondrion of the spore showing its central channel, the core of flagellar fibres, the banded rootlet attached thereto extending into the lateral mitochondrial canal (left), and a second lateral canal (right) devoid of a rootlet. × 16,700. Fixative, $KMnO_4$-NaCl.

Fig. 12. A longitudinal section through part of the basal region of the spore illustrating a rootlet with particularly regular bands extending into a lateral canal of the mitochondrion. × 17,500. Fixative, Zetterqvist, pH 7.

Fig. 13. A cross-section through a spore below the nuclear region showing how the mitochondrion completely surrounds the channel through which the flagellar apparatus enters the cell. × 13,600. Fixative, Zetterqvist, pH 7, followed by $KMnO_4$-NaCl.

Fig. 14. Cross-section through a mitochondrion at the base of the spore showing the central channel through which the flagellar structure (note ring of fibres) enters the cell, and two lateral canals with one obviously lobed. × 18,900. Fixative, $KMnO_4$-NaCl.

Fig. 15. Cross-section of a flagellum showing the double nature of the nine outer fibres and the two central ones. × 144,000. Fixative, Zetterqvist, pH 5.

Fig. 16. Cross-section through a cytoplasmic region of the spore showing the cytoplasmic organelles believed to correspond to γ particles. × 13,000. Fixative, Zetterqvist, pH 7, followed by $KMnO_4$-NaCl.

PLATE 7

Fig. 17. A cross-section through a mature OC plant which has cleaved out its protoplast into spores. The small, dark, spherical structures surrounded by light areas are nucleoli and nuclei, respectively. The spores are separated from one another by intercellular spaces (white irregular lines). Note that the total protoplast of the plant has been used for spore production. × 3100. Fixative, Zetterqvist, pH 7, followed by $KMnO_4$-NaCl.

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Effect of Chemical Modifiers on Inactivation and Mutation-Induction by Gamma Radiation in *Escherichia coli*

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SUMMARY

The effect of chemical sensitizing and protective agents on the rate of mutation induction by gamma radiation in strain WP2 of *Escherichia coli* B/r was studied. This organism will not grow in the absence of tryptophan but it mutates spontaneously at a very low rate, and under the influence of radiation at a much higher rate, to stable forms which can grow in the absence of tryptophan. Both inactivation and mutation-induction were apparently related to radiation dose by an exponential function in the absence of modifying agents; in their presence this was not always the case. The sensitizing agents oxygen and *N*-ethylmaleimide, and the protective agents cysteine, glycerol, dimethyl sulphoxide and thiourea, affected both the inactivating and mutation-inducing actions of radiation though not always to the same extent.

INTRODUCTION

Although there are several reports of conditions before, during, and after irradiation which affect the yield of mutations with higher organisms (see, for example, Burdette, 1961), there are few in which the number of mutations induced in bacteria has been shown to be altered by chemical agents present during irradiation. Anderson (1957) showed that oxygen enhanced the rate at which mutations at two different loci in *Escherichia coli* were induced by X-rays. A dose-modifying factor (see statistical section) of 3 was found both for reversions to purine independence and for inactivation with one strain. With a different streptomycin-dependent strain the dose-modifying factor for reversions to independence was only 1·3, whereas for lethality it was 2·5. There is a report (Hollaender, Billen & Doudney, 1956) that cysteamine protected against mutation induction, but as no experimental details were given the possibility that oxygen-depletion was responsible cannot be ruled out.

Following the demonstration of sensitization by *N*-ethylmaleimide (Bridges, 1960, 1961, 1962*a*) and of protection by dimethyl sulphoxide (Bridges, 1962*b*) and thiourea (Bridges, 1963) when inactivation of colony-forming ability was the criterion of radiation damage, the influence of these compounds, together with the familiar modifiers oxygen, glycerol and cysteine, was investigated using an additional criterion of radiation effect, namely mutation.

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METHODS

Experimental

Reversions to tryptophan independence in strain WP 2 of *Escherichia coli* B/r (kindly given by Dr C. O. Doudney) were studied. It is possible to use the same plating medium for total counts and for reversion counts with this organism. This is desirable because the survival of *E. coli* B/r and its response to chemical modifiers is known to be markedly influenced by the post-irradiation plating medium (Stapleton, Sbarra & Hollaender, 1955; Alper & Gillies, 1958; Freeman & Bridges, 1960). The medium used was that of Haas & Doudney (1957) which was essentially an inorganic salts solution with ammonium ions as nitrogen source and glucose as carbon source. To this was added a small amount of Oxoid no. 1 dehydrated nutrient broth (final concentration: 0·625 g./l.). A surface plating technique was used. At a suitable dilution the tryptophan-dependent organisms produced small but visible colonies after 2 days' incubation at 30°. When undiluted suspension was spread over the plate the tryptophan-dependent organisms quickly formed a background smear of growth and revertants appeared later as typical coliform colonies. Mutants were counted after 5 days' incubation at 30°. Stationary phase organisms were used for these experiments. They were washed off a 20 hr. culture on nutrient agar (Oxoid) with 10 ml. 0·067 M-phosphate buffer (pH 7), centrifuged and resuspended in 15 ml. buffer with or without the chemical modifier. Irradiations were begun 6–10 min. after resuspension and were given in five or six fractions, samples being removed for mutant and viable counts after each dose fraction. Suspensions with cysteine were left for 20 min. before irradiation. ^{60}Co γ-radiation was given at room temperature in a glass vessel at the rate of 4500 rad/min.; air or oxygen-free nitrogen was bubbled through the suspension for 6 min. before and throughout irradiation.

Statistical

The number of induced mutants per 10^8 survivors (G) for a given dose is given by

$$G = \frac{M}{N} - \frac{M_0}{N_0} \times 10^3,$$

where M is the number of mutants/ml. in the irradiated suspension, M_0 the number of spontaneous mutants/ml. in the unirradiated suspension, N the number of survivors/ml. in the irradiated suspension and N_0 the number of viable organisms/ml. in the unirradiated suspension (Anderson, 1957). The expression corrects for pre-existing spontaneous mutants and also for those produced spontaneously during growth on the low concentration of organic nutrients on the plates (Kada, Brun & Marcovich, 1960). The number of surviving organisms (N) was found to be related to radiation dose (x) by an exponential equation $N = Ae^{-Bx}$ except in the presence of the protective agents. By fitting a line to the observed survival data using this equation it was possible to obtain a calculated mean value for the number of survivors (N) at each dose. This mean value is less subject to variation than the corresponding observed value at any particular dose and was therefore used in calculating the mutation rate G (except with the protective agents).

In the type of experiment described above it is impossible to separate the

mutagenic from the lethal action of radiation; in some of the experiments reported here, up to 96% of the bacteria were killed. In consequence, when it is desired to correlate mutation-induction with dose, it is necessary to correct the observed number of mutants to allow for those killed. The assumption is frequently made that the newly induced mutants have the same radiation sensitivity as the parent strain. When this assumption is valid a plot of G against dose is a real measure of the rate of mutation-induction. When the assumption is invalid, a plot of G against dose represents an unknown function of the true mutation rate.

The relation of G with respect to dose (x) was not linear in these experiments. Three mathematical relations were applied. The first, $G = ax^n$ is implicit in the way that Kada, Brun & Marcovich (1960) plotted their data but has no theoretical basis. The second $G = ax+bx^2$ is what might be expected if there are separate 'one-hit' and 'two-hit' components. The third, $G = ae^{bx}$ represents an exponential relation with dose. Analyses were performed with all three models; the third expression although having no theoretical basis was found to fit the data most accurately and consistently and was used for testing significance. None of the relations, however, was very satisfactory.

Where the dose-response data for inactivation and mutation-induction could be fitted with exponential curves, the effectiveness of the modifier (expressed as an enhancement or protection ratio) was given by the ratio of the slope of the fitted exponential curve for the modified state to that for the anoxic control, the largest figure being taken as the numerator. Where exponential curves could not be fitted, enhancement or protection ratios were the ratios of the doses of radiation needed to produce a stated effect under modified and control (anoxic) conditions. When the modified dose-response curve can be made to fit exactly over the control curve merely by multiplying the doses by a constant factor, the effect of an agent is said to be dose-modifying.

RESULTS

Sensitizing agents

Measurements of lethality and mutation-induction were performed simultaneously on the same suspensions and with the same plating medium. It was suspected that *N*-ethylmaleimide (NEM) might affect the mutation rate by changing the post-irradiation development and expression of mutants which are known to be dependent upon metabolism (Kada *et al.* 1960; Kada, Doudney & Haas, 1961). NEM inhibits certain energy-giving reactions and, with the technique used, was bound to be carried over to some extent on to the plating medium. Experiments in which NEM was added immediately after anoxic irradiation, however, did not show any significant change in the induced-mutation rate. It may therefore be assumed that any change in mutation rate observed with NEM is a result of its being present during irradiation.

Three experiments in which the effects of oxygen and NEM were investigated are combined in Fig. 1, where the surviving fraction and G are plotted against dose of radiation. The fitted lines represent an exponential function of dose.

Neither NEM nor oxygen had any effect on the spontaneous mutation rate. NEM and oxygen enhanced both radiation-induced mutation and inactivation, and the enhancement ratios are shown in Table 1. For inactivation the enhancement

Table 1. *Enhancement ratios for the effects of oxygen and N-ethylmaleimide on* Escherichia coli *WP2*

N-Ethylmaleimide (NEM) was used at 0·0005 M, oxygen at the concentration of air-saturated solution.

	Enhancement ratio		
	For inactivation	For mutation-induction	
	Based on slopes	Based on slopes	Based on doses for which $G = 300$
Oxygen	3·2	2·4	2·1
NEM	2·1	1·8	1·7
Oxygen + NEM	3·1	2·4	1·9

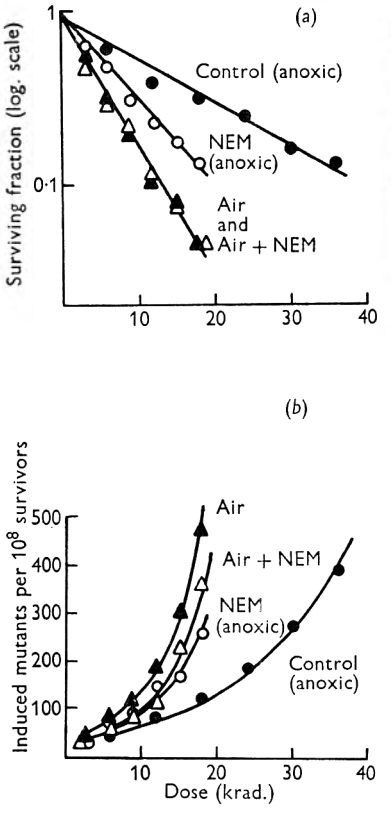

Fig. 1

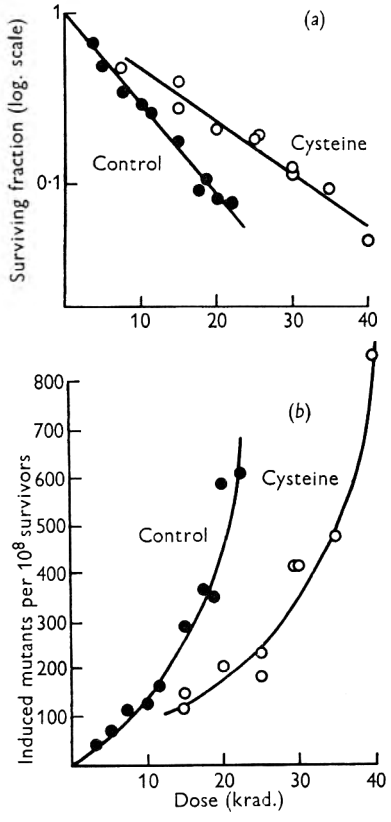

Fig. 2

Fig. 1. Irradiation of *Escherichia coli* WP2: effect of oxygen (air-saturated solution) and 0·0005 M-*N*-ethylmaleimide (*a*) on inactivation, (*b*) on mutation-induction. The lines represent fitted exponential functions.

Fig. 2. Irradiation of *Escherichia coli* WP2 under anoxia: effect of 0·1 M-cysteine (*a*) on inactivation, (*b*) on mutation-induction. The lines have been fitted by eye.

ratios, based on slopes of survival curves, were greater for oxygen and for NEM + oxygen (which were identical) than for NEM alone. A similar pattern was shown for mutation induction when the enhancement ratios were based on the slopes of the fitted exponential functions except that the ratios were smaller, indicating that neither oxygen nor NEM enhanced mutation-induction to the same extent as inactivation. When, on the other hand, the enhancement ratios were based on the doses needed to produce a given effect (e.g. $G = 300$) an additional fact emerged, namely that the mutation enhancement ratio for oxygen + NEM was significantly less than that for oxygen alone. Examination of the data reveals that treatment with NEM + oxygen is not strictly dose-modifying. One interpretation of the data is that a constant small proportion of mutants is not expressed when NEM is present under aerobic conditions.

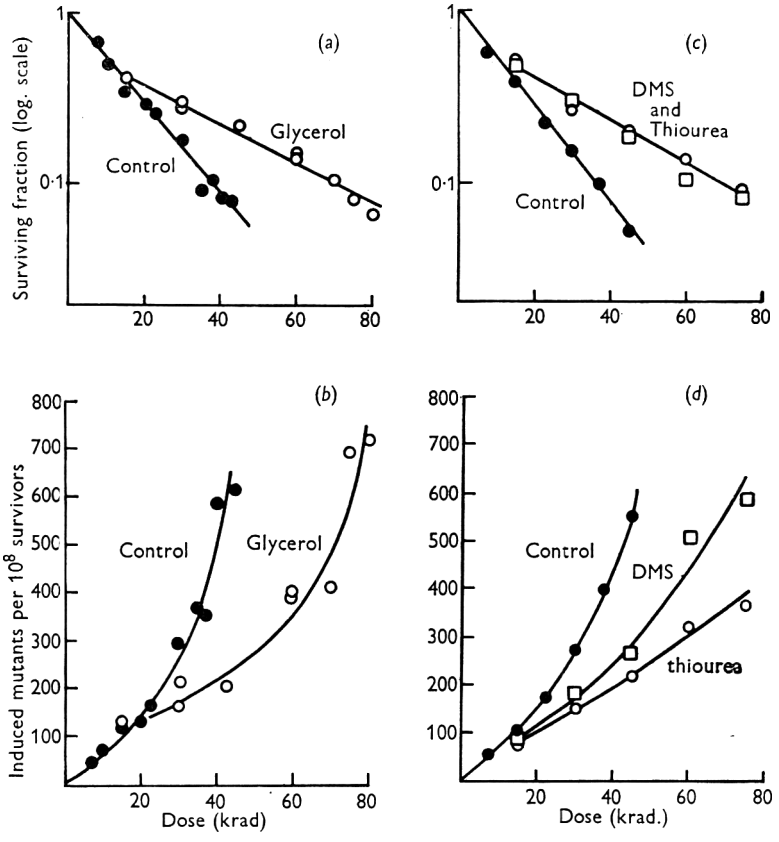

Fig. 3. Irradiation of *Escherichia coli* WP2 under anoxia: effect of M-glycerol, 0·2 M-thiourea and 2 M-dimethyl sulphoxide (DMS) (*a*, *c*) on inactivation, (*b*, *d*) on mutation induction. The lines have been fitted by eye.

Protective agents

All the protective agents were tested under conditions of strict anoxia, thus eliminating oxygen-depletion as a cause of apparent protection. None of the chemicals tested affected the spontaneous mutation rate. The data are plotted in

Figs. 2 and 3 and the protection ratios are given in Table 2. In Fig. 3 (*c*, *d*) the results of two experiments with dimethyl sulphoxide and thiourea are combined. Two points emerge from these data. With the possible exception of cysteine, the treatments were not dose-modifying; protection was shown only at doses greater than 10–15 krads. With glycerol, for example, this 'threshold' effect was shown whether the compound was added 6 or 26 min. before irradiation, indicating that the threshold is not due to a requirement for pre-incubation with the compound. The second point is that glycerol, cysteine and thiourea protected against mutation-induction and inactivation to the same extent, whereas dimethyl sulphoxide was appreciably less efficient at protecting against mutation-induction than against inactivation.

Table 2. *Protection ratios for the effects of various compounds on* Escherichia coli *WP2*

All irradiations were under anoxic conditions.

	Protection ratio	
	For inactivation. Based on doses for 10 % survival	For mutation-induction. Based on doses for which $G = 400$
Cysteine (0·1 M)	1·7	1·7
Glycerol (1 M)	1·9	1·8
Thiourea (0·2 M)	1·9	2·0
Dimethyl sulphoxide (2 M)	1·9	1·5

DISCUSSION

Sensitizing agents. In previous publications (Bridges, 1961, 1962*a*) it was shown that *N*-ethylmaleimide (NEM) enhances the lethal action of radiation only when present during irradiation; in this and other respects it resembles oxygen. It was suggested that NEM and oxygen are both able to combine with reactive sites which appear during irradiation on important biological molecules. In the absence of NEM and oxygen these sites are assumed to be restorable so that the molecule does not lose its biological integrity. The sites which react with oxygen are not known but it was suggested that those which react with NEM might be –SH groups or –S free radicals. If this hypothesis be correct, proteins must be intimately involved in sensitization, and hence in mutation, since they are the only large sulphur-containing molecules of importance to the cell.

The present results are somewhat difficult to reconcile with this hypothesis if mutations arise as a result of changes in deoxyribonucleic acid (DNA). Hill (1962) studied ultraviolet-induced reversions to prototrophy in *Escherichia coli* B/r (WP2) and concluded that the change from tryptophan dependence to independence was frequently unstable and might be due to the detachment of an episome from a chromosomal suppressor locus. Such a possibility illustrates one of the problems involved in the study of mutagenesis, namely the difficulty of knowing whether the mutations observed are chromosomal mutations or episomal changes. As systems in which mutants are easily induced tend to be chosen for investigation, it is possible

that they may be atypical. Biochemical reversions to prototrophy are certainly atypical in that they are beneficial under almost all conditions of growth. It is not known what episomes are composed of, apart from DNA, so that it is difficult to interpret results in terms of chemical changes.

There are several lines of evidence which indicate that chemical sensitization depends on the nature of the target molecule and is not a uniform and general phenomenon, for example: the effects of oxygen and NEM vary in extent from one species to another (Bridges, 1961; Cromroy & Adler, 1962); the magnitude of the oxygen effect can be markedly influenced by treatments after irradiation (Alper & Gillies, 1958); the enhancing effects of NEM and oxygen are different in magnitude when inactivation or mutation-induction are taken as the end results; and NEM enhances only 'type 3' (protein?) damage in *Serratia marcescens* (Dewey, 1963). These facts support the above hypothesis about the mechanism of sensitization since only in those molecules containing –S–S– bonds or masked –SH groups would radiation damage be enhanced by NEM. The importance to the cell of such molecules might be expected to differ in different organisms.

Protective agents. Four types of compound are known to protect cells from radiation damage by means other than oxygen depletion; aliphatic alcohols, dimethyl sulphoxide, thiourea and the cysteine-cysteamine group. Their mechanism of action is not known, but under anoxic conditions they all appear to protect largely against the same component of damage (Bridges, 1963). In contrast to sensitization the available evidence seems to indicate that chemical protection is a fairly uniform and general phenomenon (Cromroy & Adler, 1962; Bridges, 1963) and largely independent of the nature of the targets (Dewey, 1962). The present results with thiourea, glycerol and cysteine would seem to confirm this idea. With dimethyl sulphoxide, however, significantly less protection was afforded against mutation-induction than against inactivation. It is possible that dimethyl sulphoxide has difficulty in penetrating sufficiently near to the locus which determines tryptophan-dependence.

I am greatly indebted to Mr T. Hobson (Theoretical Physics Division, A.E.R.E., Harwell) for a considerable amount of statistical help.

REFERENCES

ALPER, T. & GILLIES, N. E. (1958). Dependence of the observed oxygen effect on the post-irradiation treatment of micro-organisms. *Nature, Lond.* **181**, 961.

ANDERSON, E. H. (1957). The effect of oxygen on mutation induction by X-rays. *Proc. nat. Acad. Sci., Wash.* **37**, 340.

BRIDGES, B. A. (1960). Sensitization of *Escherichia coli* to gamma radiation by *N*-ethylmaleimide. *Nature, Lond.* **188**, 415.

BRIDGES, B. A. (1961). The effect of *N*-ethylmaleimide on the radiation sensitivity of bacteria. *J. gen. Microbiol.* **26**, 467.

BRIDGES, B. A. (1962*a*). The chemical sensitization of Pseudomonas species to ionizing radiation. *Radiation Res.* **16**, 232.

BRIDGES, B. A. (1962*b*). Protection of Pseudomonas sp. against gamma radiation by dimethyl sulphoxide. *Int. J. radiation Biol.* **5**, 101.

BRIDGES, B. A. (1962). The chemical protection of Pseudomonas species against ionizing radiation. *Radiation Res.* **17**, 801.

BURDETTE, W. J. (1961). Influence of penicillin on frequency of induced mutations. *Proc. nat. Acad. Sci., Wash.* **47**, 1813.

CROMROY, H. L. & ADLER, H. I. (1962). Influences of β-mercaptoethylamine and oxygen removal on the X-ray sensitivity of four strains of *Escherichia coli*. *J. gen. Microbiol.* **28**, 431.

DEWEY, D. L. (1962). X-ray inactivation of inducible enzyme synthesis and the effect of oxygen and glycerol. *Nature, Lond.* **194**, 158.

DEWEY, D. L. (1963). The X-ray sensitivity of *Serratia marcescens*. *Radiation Res.* (in the Press).

FREEMAN, B. M. & BRIDGES, B. A. (1960). Suitability of various plating media for counting bacteria after exposure to gamma radiation. *Int. J. appl. Radiation and Isotopes*, **8**, 136.

HAAS, F. L. & DOUDNEY, C. O. (1957). A relation of nucleic acid synthesis to radiation-induced mutation frequency in bacteria. *Proc. nat. Acad. Sci., Wash.* **43**, 871.

HILL, R. F. (1962). Ultraviolet-induced reversions from auxotrophy in *Escherichia coli*. *J. gen. Microbiol.* (in the Press).

HOLLAENDER, A., BILLEN, D. & DOUDNEY, C. O. (1956). The modification of X-ray-induced mutations in *Escherichia coli* by pre- and post-treatment. *Radiation Res.* **5**, 235.

KADA, T., BRUN, E. & MARCOVICH, H. (1960). Comparaison de l'induction de mutants prototrophes par les rayons X et u.v. chez *Escherichia coli* B/r Try^-. *Ann. Inst. Pasteur*, **99**, 547.

KADA, T., DOUDNEY, C. O. & HAAS, F. L. (1961). Some biochemical factors in X-ray-induced mutation in bacteria. *Genetics*, **46**, 683.

STAPLETON, G. E., SBARRA, A. J. & HOLLAENDER, A. (1955). Some nutritional aspects of bacterial recovery from ionizing radiations. *J. Bact.* **70**, 7.

J. gen. Microbiol. (1963), **31**, 413–420
Printed in Great Britain

Repression of Inducible Tartrate Dehydratases in Pseudomonas Strains

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SUMMARY

The effect of various growth substrates on the synthesis of three inducible enzymes, the stereo-specific tartrate dehydratases, has been followed in strains of Pseudomonas. Four organic acids (succinate, malate, acetate, *meso*-tartrate) inhibited induction of some or of all of the tartrate dehydratases, while three (pyruvate, *l*-tartrate, and *d*-tartrate) did not inhibit. Whenever inhibition occurred, synthesis of *d*- and *l*-tartrate dehydratase was repressed much more strongly than synthesis of *meso*-tartrate dehydratase. Pre-induction of the bacteria did not prevent subsequent repression and a degree of correlation was found between the growth rate supported by substrates and the extent of repression. The significance of the different degrees of repression given by substrates feeding into closely related sites of the Krebs cycle and of the differences in repression exerted on enzymes acting on closely related compounds is discussed.

INTRODUCTION

Several structurally unrelated metabolizable substrates have been shown to inhibit the synthesis of certain enzymes in bacteria (Monod, 1942; Mandelstam, 1961; Magasanik, 1961). To explain the inhibition, it has been suggested that these substrates give rise to metabolic intermediates which act as enzyme repressors. Thus the effect, which has been known for many years as diauxie or the 'glucose effect', has recently been termed catabolite repression (Mandelstam, 1961; Magasanik, 1961). While little is known about the nature or the specificity of the substances responsible for catabolite repression (Engelsberg, Watson & Hoffe, 1961; Magasanik, 1961; Mandelstam, 1962), studies on the relation between substrate metabolism and concentration of constitutive enzymes in *Escherichia coli* and yeasts have indicated that compounds other than the specific repressors considered responsible for the induction phenomenon are involved (Brown & Monod, 1961; Mandelstam, 1962; McQuillan & Halvorson, 1962).

In the experiments to be reported, aspects of catabolite repression have been studied in strains of Pseudomonas which metabolize inductively two or all three of the optical isomers of tartaric acid. Induction to tartrate utilization in these strains involves the synthesis of dehydratases which catalyse the conversion of tartrate isomers to oxaloacetate. Three tartrate dehydratases, each specific for one of the optical isomers, are known; these are induced only by their particular substrates (Shilo, 1957; Shilo & Stanier, 1957). Catabolite repression of tartrate dehydratase synthesis by organic acids has been reported (Rosenberger & Shilo, 1961). In the

present work attempts have been made to study the specificity of repression against the three dehydratases and to determine, by the use of substrates which enter metabolism at identical or closely related points of the Krebs cycle, which metabolic steps are critical for the accumulation of enzyme repressors. Substrates such as the three tartrate isomers, succinate, malate, acetate and pyruvate share all or nearly all of their catabolic pathways and differences in the degree of repression should be traceable to differences in the rates of the small number of metabolic steps not common to each or to specific unshared intermediates.

METHODS

Organisms. The strains of Pseudomonas used were described by Shilo & Stanier (1957).

Media and growth conditions. The basal medium used contained per l. distilled H_2O: $(NH_4)_2SO_4$, 1 g.; KH_2PO_4, 1·5 g.; Na_2HPO_4, 0·25 g.; $MgSO_4.7H_2O$, 50 mg.; $(NH_4)_2SO_4.FeSO_4.6H_2O$, 5 mg.; Yeast extract (Difco), 25 mg.; pH 6. Carbon sources were added to a final concentration of 0·3% (w/v) or as indicated in the text. Carbon source and basal medium were sterilized together by autoclaving (20 min. at 121°) except in the case of sodium pyruvate which was sterilized by filtration. Cultures were grown in Erlenmeyer flasks on a rotary shaker at 30°.

Enzyme assays. For *d*-tartrate dehydratase estimations organisms were washed once in 50 mM-tris (2-amino-2 hydroxymethylpropane-1,3-diol) buffer (pH 8·7), re-suspended in the same buffer and lysed by the addition of 4 μmole ethylenediaminetetra-acetic acid (EDTA) and 20 μg. lysozyme/ml. (Repaske, 1958). The lysates were incubated for 10 min. at 30° with 12·5 mM-*d*-tartrate, 4 mM-EDTA and 50 mM-tris buffer (pH 8·7). In the presence of dehydratase and EDTA, oxaloacetate accumulated without further enzymic breakdown (Shilo, 1957). The reaction was stopped by the addition of trichloroacetic acid, and, after centrifugation to remove precipitated proteins, the keto acids formed were estimated by the method of Friedman & Haugen (1943).

Meso-tartrate dehydratase was assayed as above, but with the substitution of 20 mM-phosphate buffer (pH 7·2) for tris buffer and 12·5 mM-*meso*-tartrate for *d*-tartrate. For both enzymes under the above conditions, keto-acid accumulation proceeded at a linear rate for at least 15 min., and was proportional to the enzyme concentration. One dehydratase unit has been taken as the amount forming 1 μmole keto acid/hour.

Oxygen uptake with various substrates. Ability to oxidize substrates with or without lag was measured by the conventional Warburg technique at 30°.

Estimation of bacterial dry weight and protein. Bacterial dry weights were determined by turbidity measurements in a Klett–Summerson colorimeter by comparison with a calibration curve. Protein was estimated by the method of Lowry, *et al.* (1951) with bovine serum albumin as standard.

Estimation of d-*tartrate* was by the method of Matchett *et al.* (1944). When the kinetics of *d*-tartrate utilization by washed suspensions were followed, samples were boiled for 2 min. to stop further breakdown, solids removed by centrifugation and assays performed on the supernatant fluid.

RESULTS

Repression of tartrate dehydratase synthesis by organic acids

Organisms were grown with the substrate under test as sole source of carbon and energy and, when the logarithmic phase of growth was reached, one of the tartrate isomers was added as inducer. Cultures had a density of 0·1–0·2 mg. bacterial dry weight/ml. at the time of inducer addition and induction was followed for about two generations. When induction occurred under the above conditions, plots of the amounts of *d*- or *meso*-tartrate dehydratase formed against total bacterial protein had the form of a straight line. The slope of this line, the differential rate of synthesis, was used to compare the rates of inductive enzyme formation. As no quantitative assay methods for *l*-tartrate dehydratase are available, adaptation to *l*-tartrate utilization was determined manometrically. Organisms were harvested approximately two generations after inducer addition, and their ability to oxidize *l*-tartrate without lag determined.

Table 1. *Rate of* d-*tartrate dehydratase synthesis on inducer addition to Pseudomonas strains growing with various organic acids*

Pseudomonas strain	Differential rate of enzyme synthesis* during growth on following substrates plus inducer†						
	d-tartrate	*l*-tartrate	*meso*-tartrate	Succinate	*dl*-malate	Acetate	Pyruvate
MD9	28·3	25·9	< 2	< 2	< 2	n.g.	42·7
LML	26·9	32·2	< 2	< 2	< 2	< 2	35·1
MD	32·6	n.g.	< 2	< 2	< 2	< 2	41·7
12	16·5	11·6	n.g.	< 2	< 2	< 2	6·4

* *d*-tartrate dehydratase units formed/mg. total protein synthesized; † 0·3 % (w/v) substrate + 0·1 % (w/v) inducer; n.g., no growth with substrate as sole carbon source.

Table 2. *Rate of* meso-*tartrate dehydratase synthesis on inducer addition to Pseudomonas strains growing with various organic acids*

Pseudomonas strain	Differential rate of enzyme synthesis* during growth on following substrates plus inducer†					
	meso-tartrate	*d*-tartrate	*l*-tartrate	succinate	*dl*-malate	acetate
MD9	147	81	117	19	29	n.g.
MD	114	121	n.g.	54	53	46
LML	83	196	135	17	74	28

* *meso*-tartrate dehydratase units formed/mg. total protein synthesized; † 0·3 % (w/v) substrate + 0·1 % (w/v) inducer; n.g. = no growth with substrate as sole carbon source.

The results obtained with four strains and seven organic acids are summarized in Tables 1, 2 and 3. Induction of *d*- or *l*-tartrate dehydratase was completely inhibited by intermediates of the Krebs cycle and by *meso*-tartrate. With both these dehydratases, pyruvate and the non-inducing optically active tartrate isomer allowed synthesis to proceed at rates comparable to those with inducer

alone. *Meso*-tartrate dehydratase synthesis was not inhibited by the other tartrate isomers and was in no case completely repressed. Pyruvate stood out from the other Krebs cycle intermediates tested, since in its presence synthesis of any of the three inducible dehydratases proceeded at rates equal to those with inducer alone.

Table 3. *Induction to* l-*tartrate oxidation after inducer addition to Pseudomonas strains growing with various organic acids*

Pseudomonas strain	O_2 uptake* (0–20 min.; substrate *l*-tartrate) of washed organisms harvested after growth on following substrates plus inducer†			
	d-tartrate	*meso*-tartrate	succinate	pyruvate
MD9	76	4	16	70
LML	63	10	15	61
12	80	n.g.	16	64

* μl. O_2/mg. dry wt., bacteria corrected for endogenous respiration; † 0·3 % (w/v) substrate + 0·1 (w/v) inducer; n.g. = no. growth of strain with substrate as sole carbon source.

Table 4. Meso-*tartrate dehydratase synthesis in washed suspensions of Pseudomonas strain* MD 9

Time	Treatment and substrates added	Rate of *meso*-tartrate dehydratase synthesis (units/mg. protein/hr.) in washed organisms* previously grown on succinate
0–30 min.	succinate (20 mM)	—
30–120 min.	*meso*-tartrate (6·6 mM) succinate (20 mM)	3·4
120–180 min.	Organisms centrifuged, re-suspended in buffer + 6·6 mM *meso*-tartrate	10·6

* Organisms washed once in 22 mM phosphate buffer (pH 7·2), resuspended in same buffer.

Repression of meso-*tartrate dehydratase synthesis in washed suspensions*

Synthesis of *meso*-tartrate dehydratase was inhibited to a rather variable extent during growth on Krebs cycle intermediates, but was never completely repressed (Table 2). Since catabolite repression of certain enzymes has been shown to be more severe in washed suspensions than in growing cultures (Mandelstam, 1961), the effect of succinate on induction by *meso*-tartrate in washed suspensions was examined (Table 4). While succinate decreased the rate of dehydratase formation, induction did take place. *Meso*-tartrate dehydratase thus appeared to be more resistant to repression by metabolic intermediates than most other catabolic enzymes (Magasanik, 1961).

Effect of meso-*tartrate on induction by gluconate*

As growth on *meso*-tartrate inhibited induction of the *d*- or *l*-tartrate dehydratases, it seemed of interest to determine its effect on a third inducible system.

Organisms of Pseudomonas strain MD9 grown on *meso*-tartrate oxidized gluconate only after a pronounced lag, showing the inducible nature of this system. Addition of gluconate to organisms growing on *meso*-tartrate yielded organisms fully induced to gluconate oxidation. The tartrate isomer thus did not repress induction by gluconate.

Influence of inducer and substrate concentrations on repression

Pseudomonas strain md_9 was grown on various concentrations of *meso*- or *d*-tartrate and graded concentrations of *d*- or *meso*-tartrate were added as inducer. With *meso*-tartrate as substrate and the *d*-tartrate as inducer, the highest inducer/substrate ratio tried (6:1) did not induce. In the reverse case, with *d*-tartrate as substrate and *meso*-tartrate as inducer, the lowest inducer/substrate ratio tried (1:6) yielded induced organisms (Fig. 1).

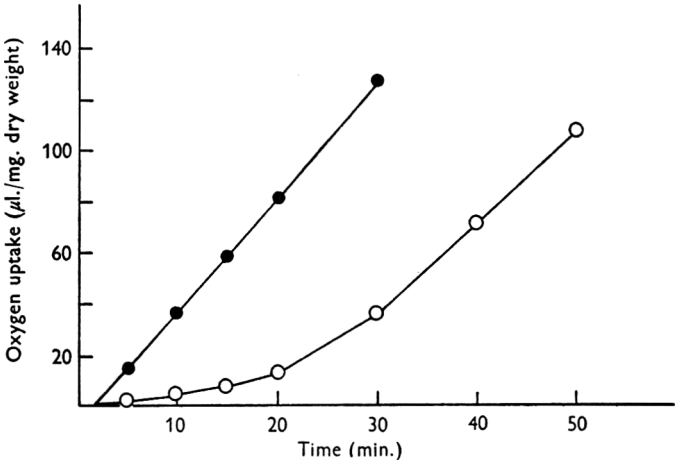

Fig. 1. Effect of differing ratios of inducer to substrate on induction in strain md_9. Cells were grown on $3{\cdot}3 \times 10^{-3}$ M *meso*-tartrate with subsequent addition of 2×10^{-2} M *d*-tartrate (○—○) or on 2×10^{-2} M *d*-tartrate with subsequent addition of $3{\cdot}3 \times 10^{-3}$ M *meso*-tartrate (●—●). Approximately 2 generations after inducer addition cells were harvested and ability to oxidize *meso*- or *d*-tartrate without lag examined by the conventional Warburg technique.

Effect of organic acids on inducer entry

Some of the organic acids which repressed *d*-tartrate dehydratase induction are structurally related to the inducer and it seemed possible that they inhibited by competing with the inducer for its entry-site into the cell. To test this possibility, the rate of *d*-tartrate metabolism of resting suspensions of organisms fully induced to *d*-tartrate was measured in the presence and absence of *dl*-malate and *meso*-tartrate. *d*-Tartrate alone was utilized at a rate of 5·08 µmole/hr./mg. dry weight of organisms, while in the presence of threefold greater concentrations of *dl*-malate or *meso*-tartrate the rates were 4·22 and 3·54 µmole/hr./mg. dry weight respectively. These inhibitions (17–30%) indicate that competition at the site of entry must be relatively slight.

Repression in pre-induced organisms

Cohn & Horibata (1959) reported that glucose completely repressed β-galactosidase induction in uninduced *Escherichia coli*, but that organisms actively forming β-galactosidase continued to do so at an unchanged rate for many generations in the presence of glucose plus inducer.

Effects of pre-induction were tested in Pseudomonas strain MD9 growing on *d*- or *l*-tartrate. Cultures were transferred to medium containing the same isomer together with another organic acid in equimolar proportions. Transfers to fresh medium containing the same two substrates were made approximately every five generations to ensure the continued presence of high concentrations of unchanged substrates. After 15–23 generations, organisms were harvested and tested for ability to oxidize *d*- or *l*-tartrate without lag. Pre-induction did not prevent repression of the *d*- or *l*-tartrate oxidizing system by *meso*-tartrate or succinate (Table 5). Organisms grown on a mixture of *d*- and *l*-tartrate were induced for the oxidation of both isomers, irrespective of the state of adaptation of the inoculum.

Table 5. *Repression of tartrate dehydratase synthesis in pre-induced Pseudomonas strain* MD9

Growth substrate of inoculum*	Substrates in cultures (each 10^{-2} M)	No. of generations before harvest	O_2 uptake on addition of substrate 0–20 min. (μl./mg. dry weight organism)			
			d-tartrate	*meso*-tartrate	*l*-tartrate	succinate
d-tartrate	*d*-tartrate + *meso*-tartrate	20	4	113	n.t.	n.t.
d-tartrate	*d*-tartrate + succinate	23	3	n.t.	n.t.	86
d-tartrate	*d*-tartrate + *l*-tartrate	15	71	n.t.	60	n.t.
l-tartrate	*l*-tartrate + *meso*-tartrate	15	n.t.	79	6	n.t.
l-tartrate	*l*-tartrate + *d*-tartrate	15	68	n.t.	53	n.t.

* Substrate conc. 0·3 % (w/v); n.t. = not tested.

Table 6. *Average generation times of Pseudomonas strains* MD9, *md and lml during growth on organic acids*

Pseudomonas strain	Substrate			
	succinate	*meso*-tartrate	*d*-tartrate	pyruvate
	generation time (min.)			
MD9	50	50	65	75
MD	54	53	85	72
LML	58	59	65	93

Correlation between growth rate in batch culture and repression

Neidhardt & Magasanik (1957) and Mandelstam (1962) showed that the degree of enzyme repression by various substrates tended to be related to the maximal growth rate supported by them. To determine whether a similar correlation exists in organisms which form tartrate dehydratases, the growth rate of three Pseudomonas strains in batch culture was compared (Table 7). In all cases growth was slower with pyruvate or *d*- tartrate than with succinate or *meso*-tartrate, though with strain lml the difference was no greater than 10%. These results are in keeping with those of the above mentioned authors, who found that carbon sources which supported a low growth rate repressed less effectively.

DISCUSSION

One of the aims of the present work was to compare the degree of repression of the three tartrate dehydratases in the same strain and under the same growth conditions and thus to evaluate the specificity of the inhibition. When repression occurred in any of the four strains examined, inhibition of the *d*- and *l*-tartrate dehydratases was always much greater than inhibition of the *meso*-tartrate enzyme. This was most strikingly shown during growth on *meso*-tartrate itself, where the *meso*-tartrate dehydratase was formed at high rates while induction of the enzymes attacking *d*- or *l*-tartrate was completely repressed. Similarly, Krebs cycle intermediates completely prevented induction by *d*- or *l*-tartrate, while *meso*-tartrate induced formation of its dehydratase, although at a decreased rate. It may be noted that growth on *meso*-tartrate inhibited induction by the other two tartrate isomers, but did not prevent induction to gluconate oxidation.

The literature contains few reports of catabolite repression specific for particular enzymes. Neidhardt & Magasanik (1957) described differential repression of histidase and inositol dehydrogenase in a strain of Aerobacter growing with glucose and histidine as sole nitrogen source. Both of these inducible enzymes were normally repressed by glucose, but under the above conditions histidase was formed while induction of inositol dehydrogenase remained inhibited. From these results the authors concluded that two different repressors acted on the synthesis of the enzymes (Magasanik, 1961). In the case of the tartrate dehydratases, however, differential repression could be equally well explained by different inhibitors or by a widely varying sensitivity to the same repressor.

Four of the seven substrates tested in the present work were effective repressors of induction by *d*- or *l*-tartrate, while three (pyruvate, *d*-tartrate, *l*-tartrate) allowed induction to proceed at rates equivalent to those with inducer alone. It has been known for several years that some substrates repress enzyme induction much more strongly than others and Neidhardt (1960), Mandelstam (1961, 1962) and Magasanik (1961) have related these differences to differences in the relative rates of substrate metabolism and total cell synthesis. The compounds tested in the present work all enter cell metabolism via the Krebs cycle and at sites in the cycle close to each other. Thus, *meso*-tartrate and *l*-tartrate are both converted to oxaloacetate in a single enzymic step, and therefore give rise to identical metabolic intermediates, yet they have very different effects on induction by

d-tartrate. It would appear therefore that the only metabolic differences which make for stronger or weaker repression are the rates of the first steps in substrate metabolism. Thus *d*- and *l*-tartrate are ineffective as repressors because of relatively slow rates of conversion to oxaloacetate and pyruvate, due to relatively slow rates of entry or carboxylation to oxaloacetate. The suggestion that the initial steps of pyruvate, *d*-tartrate and *l*-tartrate metabolism are slower than those of the other compounds tested is in keeping with the somewhat slower growth rates supported by these three substrates. It is more directly supported by the finding that the amounts of *d*-tartrate dehydratase during growth on inducer alone were three to five times lower than those of *meso*-tartrate dehydratase. Such low amounts of *d*-tartrate dehydratase (and by analogy of *l*-tartrate dehydratase) during growth with inducer as sole carbon source may in fact be a corollary of the sensitivity of this enzyme to repression by its own catabolic products. Higher dehydratase concentrations would lead to repressor pools of the size formed during growth on *meso*-tartrate or Krebs cycle intermediates, and these would be accompanied by inhibition of enzyme synthesis and return to a low dehydratase content.

REFERENCES

BROWN, D. D. & MONOD, J. (1961). Carbon source repression of β galactosidase in *Escherichia coli*. *Fed. Proc.* **20**, 222.

COHN, M. & HORIBATA, K. (1959). Physiology of the inhibition by glucose of the induced synthesis of the β galactosidase enzyme system in *Escherichia coli*. *J. Bact.* **78**, 624.

ENGELSBERG, E., WATSON, J. A. & HOFFE, P. A. (1961). The glucose effect and the relationship between glucose permease, acid phosphatase and glucose resistance. *Cold. Spr. Harb. Symp. quant. Biol.* **26**, 261.

FRIEDMAN, T. E. & HAUGEN, G. E. (1943). Pyruvic acid. II. The determination of keto acids in blood and urine. *J. biol. Chem.* **147**, 415.

LOWRY, O. H., ROSEBROUGH, N. J., FARR, A. L. & RANDALL, A. J. (1951). Protein measurement with the Folin phenol reagent. *J. biol. Chem.* **193**, 265.

MACQUILLAN, A. M. & HALVORSON, H. O. (1962). Metabolic control of β galactosidase synthesis in yeasts. *J. Bact.* **84**, 23.

MAGASANIK, B. (1961). Catabolite repression. *Cold. Spr. Harb. Symp. quant. Biol.* **26**, 249.

MANDELSTAM, J. (1961) Induction and repression of β-galactosidase in non-growing *Escherichia coli*. *Biochem. J.* **79**, 489.

MANDELSTAM, J. (1962). The repression of constitutive β-galactosidase in *Escherichia coli* by glucose and other carbon sources. *Biochem. J.* **82**, 489.

MATCHETT, J. R., LEGAULT, R. R., NIMMO, C. C. & NOTTER, G. K. (1944). Tartrates from grape wastes. *Industr. Engng Chem.* (*Industr.*) **36**, 851.

MONOD, J. (1942). *Recherches sur la croissance des cultures bactérienne*. Paris: Hermann et Cie.

NEIDHARDT, F. C. & MAGASANIK, B. (1957). Reversal of the glucose inhibition of histidase biosynthesis in *Aerobacter aerogenes*. *J. Bact.* **73**, 253.

NEIDHARDT, F. C. (1960). Mutants of *Aerobacter aerogenes* lacking glucose repression. *J. Bact.* **80**, 536.

REPASKE, R. (1958). Lysis of gram negative organisms and the role of versene. *Biochim. biophys. Acta*, **30**, 225.

ROSENBERGER, R. F. & SHILO, M. (1961). Diauxie in tartrate utilizing strains of Pseudomonas and its control by oxaloacetate. *Biochem. biophys. Res. Comm.* **4**, 414.

SHILO, M. (1957). The enzymic conversion of the tartaric acids to oxaloacetic acid. *J. gen. Microbiol.* **16**, 472.

SHILO, M. & STANIER, R. Y. (1957). The utilization of the tartaric acids by Pseudomonas. *J. gen. Microbiol.* **16**, 482.

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A New Genus of the Actinomycetales: *Microellobosporia* gen.nov.

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SUMMARY

Two aerobic mesophilic species of a new genus belonging to the family Actinoplanaceae are described under the name *Microellobosporia* (*M. cinerea* type species). The new genus is characterized by the production of small club-shaped sporangia on the aerial mycelium. Similar stuctures are also formed on the substrate mycelium. The non-motile sporangiospores are few in number and arranged in a single straight row inside the sporangium.

INTRODUCTION

Since the publication of the 1957 edition of *Bergey's Manual*, Rothwell (1957) has published detailed studies on an organism, previously found by Karling (1954), which could be placed in the family Actinoplanaceae Couch. There has been some speculation as to the relationship between these 'Karling's organisms' and members of the genus *Streptomyces* (Hesseltine, 1960) following the work of several investigators on the mode of spore formation in this latter genus (Krassilnikov, 1949; Vernon, 1955; Glauert & Hopwood, 1961). The present paper reports the isolation and description of three aerobic actinomycete strains which form sporangia containing one short single row of sporangiospores. The strains differ significantly from the previously described genera in the family Actinoplanaceae and it is proposed to include them in a new genus *Microellobosporia*, the type species being *M. cinerea*. A culture has been deposited in the culture collection of the Institute of Microbiology, Rutgers, The State University, New Brunswick, New Jersey (No. 3855) as well as two strains of a second species *M. flavea* (Nos. 3857 and 3858). Because no qualitative difference was found in the microscopic properties of these three strains, morphologic properties of members of this genus were illustrated when suitable, in Plate 1 with photographs of the strains of *M. flavea*.

METHODS

Isolation. Samples of soil, diluted in pH 7·0 phosphate buffer or tap water, were spread on tap-water agar, the defined agar medium of Lechevalier, Solotorovsky & McDurmont (1961) or glucose asparagine agar (Waksman, 1950) and incubated at 26° or 36°. Plates not overgrown with bacteria and fungi were examined at weekly intervals.

Culture media. The isolates were subcultured onto slopes of potato + carrot agar, and freeze dried. The composition of the media used in their characterization and for which there is no reference in the text are given below.

Potato + carrot agar. Diced potato 150 g., diced carrot 30 g., tap water 1000 ml., steamed 30 min., filtered, the volume adjusted to 1000 ml., agar 20 g., pH 6·5.

Soya + glucose agar. Soya-bean meal 10 g., glucose 10 g., $CaCO_3$ 0·75 g., water 250 ml., pH 5·5–5·8. Autoclave at 15 lb. for 15 min., decant liquid and dilute 1/8 with water. Adjust pH to 7·9–8·1, add 17 g. agar/l.

XSM agar. Malt extract 1 g., yeast extract 1 g., liver extract 1 g., corn steep liquor 1 g., glucose 5 g., sucrose 2 g., pH to 7·0, agar 20 g., distilled water 1 l.

Corn meal + salts agar. Coarse ground maize meal 50 g., tap water 1000 ml., steamed 30 min. and filtered. To the filtrate were added Na_2HPO_4 1·15 g., KH_2PO_4 0·25 g., KCl 0·2 g., $MgSO_4.7H_2O$ 0·2 g., agar 20 g. and the volume adjusted to 1000 ml., pH 6·8.

Oatflour agar. Oatflour 25 g., $CaCO_3$ 20 g., glucose 5 g., distilled water 1 l., pH 6·8, agar 20 g.

Photographs. Photomicrographs were taken using either a Watson 'Bactil 60' binocular microscope with a Watson Eyepiece Camera and Ilford Pan. F film or a trinocular American Optical 'Microstar' microscope with a 35 mm. photography attachment and Kodak High Contrast Copy film. Electron photomicrographs were taken with an RCA EMU-3D electron microscope. Collodion films mounted on grids were touched onto 21 day cultures grown at 30° on potato + carrot agar, the mycelium becoming attached to the collodion membrane.

Examination of spore germination

Spores, suspended in pH 7·0 phosphate buffer and filtered through fine nylon net, were diluted and spread on the surface of 1·8% water agar in Petri dishes. The plates were incubated at 30° and periodically examined directly with the microscope for the appearance of germ tubes. The method of Lechevalier *et al.* (1961) was also used.

RESULTS

Description of Microellobosporia *Cross, Lechevalier and Lechevalier gen.nov.*

(from ellobos—enclosed in a pod)

Morphology. Fine mycelium (about 1·0 μ in diameter) which is differentiated into: (1) a substrate (primary) mycelium which grows into and forms a compact layer on top of the agar, (2) an aerial (secondary) mycelium which arises from the substrate mycelium and grows in the air away from the agar surface. The aerial hyphae bear sporangia on short sporangiophores. The sporangia contain a single row of non-motile sporangiospores, usually numbering 2–5. The substrate mycelium produces similar structures which were likewise interpreted as being sporangia containing a single row of non-motile spores. Three strains were isolated and these have been separated into two species.

Type species. Microellobosporia cinerea *Cross, Lechevalier and Lechevalier sp.nov.*

Substrate mycelium. About 1 μ in diam. Hyphae long, branching, penetrating the agar medium and forming compact colonies which are at first colourless becoming

pink to red-purple depending upon the medium and pH of that medium. Sporangia on short sporangiophores are borne on the surface of the agar and very likely in the agar (Pl. 1, fig. 3). Technical difficulties concerned with the staining of the sporangial wall inside the agar did not permit establishment of the sporangial nature of these sporogenic structures with absolute certainty. Photographs (Pl. 1, fig. 3) show a light aureole around the spores which might be caused by a sporangial wall. On some agar media, where the production of aerial mycelium is limited, the surface of the colonies are covered with sporangia.

Aerial mycelium. About 1 μ in diam. Long-branching hyphae grow from the colony and also, to a lesser extent, from the substrate mycelium ramifying in the agar around the colony. Some autolysis occurs on a variety of media after lengthy incubation.

Sporangia. Sporangia appear singly and laterally on the aerial hyphae after 3–4 days incubation (Pl. 1, figs. 1, 2). Later they are borne in greater numbers and also occur terminally. When mature, the sporangia vary in length from 2 to 9 μ and in diam. from 1·5 to 3·6 μ depending upon the number and size of the contained spores. Usually the sporangia contain 2 to 5 spores; single-spored sporangia occur infrequently and then most often on the short aerial hyphae growing from the substrate mycelium encircling the colony. Occasionally long sporangia containing 6 or 7 spores can be seen. Sporogenic structures borne on the substrate hyphae are associated with areas of colonial growth which appear somewhat darker in colour than neighbouring, non-sporogenic hyphae. The sporangial wall is thin and wrinkled and does not appear to bear any appendages (Pl. 1, figs. 4, 5 and 10). It originates from the apex of the sporangiophore which is swollen. The spores vary in size, even within a single sporangium, from 1·5 to 3·5 μ (average 2·5 μ), and are round to oval. A short sporangiophore which tapers towards the main hypha is usually present and this can occasionally produce a lateral branch which matures into a sporangium (Pl. 1, fig. 2). Phase-contrast examination of the sporangia, borne on the substrate mycelium, suggests a structure similar to that of the aerial sporangia, though in this case the majority of the sporangia contain only two spores (Pl. 1, fig. 3). Mature spores are easily separated from one another on release from the sporangium, but can remain attached and germinate in pairs. During germination one to three germ tubes are produced which quickly show branching.

Sporangium formation. A lateral bud on a hypha grows to form a short lateral club-shaped initial structure. The contents divide up into spores which swell slightly to give the sporangium a beaded appearance. This process is illustrated for strain No. 3857 (Pl. 1, figs. 6–9). These sporangia, at first sight, resemble the chains of conidia produced by Waksmania and Micropolyspora. In some cases the tip of the sporangial wall can be seen, with the light microscope, projecting as a cap beyond the terminal spore. In water mounts stained with dilute methylene blue, the sporangial wall can be seen ruptured and liberating the contained spores (Pl. 1, figs. 4 and 5).

Appearance on various media. Cultures were incubated in the dark at 30° for 2 weeks. When first isolated the species showed good pigment production as described below. Repeated subculturing reduced the intensity of the pigments.

Czapek solution agar (Raper & Thom, 1949). Excellent growth. Colonies large, convex, often with an apical depression and deep radial fissures. Reverse colours

light brown to purple brown. Aerial mycelium slow in forming and then only thinly covering the colony, white to off white. Soluble pigment purple brown.

Synthetic agar (Lindenbein, 1952). Growth good, colonies pulvinate, smooth and entire, reverse colour bright red. Aerial mycelium thin and mealy, white. Both the colonies and soluble pigment show indicator properties: when flooded with dilute NaOH they become purplish pink, and with dilute HCl yellow to orange-pink.

Inorganic salts + starch agar (Pridham *et al.* 1957). Growth good, colonies coloured pale pink brown. Aerial mycelium thin, cottony white to pale grey. Soluble pigment pale yellow.

Oatmeal agar (Küster, 1959). Growth good, reverse colour light grey brown becoming dark grey brown when lysis of the aerial mycelium occurs. Aerial mycelium thin, light grey, with abundant sporangia. No soluble pigment.

Oatflour agar. Excellent growth, very large convex colonies (up to 15 mm. in diameter), bright red in colour. Aerial mycelium pale grey to medium grey. Soluble pigment red to wine red.

Soya + glucose agar. Growth moderate, colonies pale brown to light purple brown, aerial mycelium sparse, light grey. Colonies lacking aerial mycelium show abundant production of sporangia on their surface. No soluble pigment.

Rice extract agar (Lechevalier & Lechevalier, 1957). Growth moderate, colonies pale brown to pale purple. Aerial mycelium pale grey. Little soluble pigment, pale purple.

Potato + carrot agar. Growth good, colonies umbonate with undulate margin. Reverse colour brown to purple brown. Aerial mycelium good, velvety, pale grey with abundant sporangia. Soluble pigment slight, red orange to red brown.

Corn meal – salts agar. Growth good, colonies pulvinate, smooth, reverse colour pale pink. Aerial mycelium thin, pale grey, abundant sporangia. Soluble pigment none to very pale pink.

Dextrose + tryptone agar (Burkholder, Sun, Anderson & Ehrlich, 1955). Growth very good, colonies pulvinate with central depression and few radial fissures. Reverse colour reddish orange to purple red. Aerial mycelium variable, good on some colonies but only sparse on others, white. Soluble pigment strong reddish orange.

XSM agar. Growth very good, reverse colony colour pale purple brown to strong brown purple. Aerial mycelium good, velvety, pale grey, some lysis. Soluble pigment purple brown.

Glycerol + asparagine agar (Burkholder *et al.* 1955). Growth excellent, large colonies with central depression and radial fissures, pink to red in colour. Abundant sporangia produced on substrate mycelium. Aerial mycelium moderate, light grey. Soluble pigment pink.

Yeast extract agar (Waksman, 1950). Pink to red, moist growth, wrinkled. No aerial hyphae.

Sabouraud's medium—with 2% glucose (Segretain, Drouhet & Mariat, 1958). Excellent pink to reddish, moist and wrinkled growth. No aerial hyphae.

Defined medium (Lechevalier *et al.* 1961). Excellent light orange tan vegetative growth. Soluble yellow pigment. No aerial mycelium.

Beef extract + starch agar (Society of American Bacteriologists, 1957). Deep rose-pink vegetative growth. Soluble brownish pigment; no aerial mycelium.

Potato plug. Pink to rose convoluted growth. No aerial hyphae.

Production of melanoid pigment. No melanoid pigment was produced when the isolate was grown on tyrosine + casein + nitrate agar (Menzies & Dade, 1959), peptone + iron + yeast extract agar (Tresner & Danga, 1958) or the cystine-bismuth agar of Hunter & Crecelius (1938).

Gelatine hydrolysis (Society of American Bacteriologists, 1957). Gelatine was hydrolysed but not as strongly as by the two strains of *M. flavea.*

Starch hydrolysis (Society of American Bacteriologists, 1957). Starch was strongly hydrolysed.

Nitrate reduction (Society of American Bacteriologists, 1957). No nitrite was detected.

Litmus milk (*Difco Manual,* 1953). Growth took place with peptonization and alkalinization but no coagulation.

Effect of pH and temperature on growth. The effect of temperature on growth was investigated by incubating inoculated slants of potato + carrot agar at 22°, 28°, 37° and 47°. Growth was good at the three lower temperatures and null at 47°. In addition, the effects of pH and temperature on growth of the isolate were simultaneously investigated using the buffered glucose + asparagine medium of Taber (1960). The results are given in Table 1.

Table 1. *Growth of* Microellobosporia cinerea *on glucose + asparagine agar buffered at different pH levels and incubated at different temperatures*

Temp. (°C.)	pH 8·5	6·9	6·7	6·6	6·3	6·1
42	−	−	−	−	−	−
37	−	−	−	−	−	−
30	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +
25	+ + +	+ + +	+ +	+	+	+
22	−	−	−	−	−	−

− No growth, + little growth, + + moderate growth, + + + good growth.

Growth occurs in the temperature range 25°–30°, with the optimum temperature for growth and sporangium production being about 30°. The culture appears more sensitive to an acid pH when incubated at a lower temperature. This result was interesting for it suggested that the pH sensitivity of a culture could be influenced by the incubation temperature and that Taber's findings on the acid sensitivity of actinomycetes might have to be modified if a range of temperatures were used in the study.

Antibiotic properties. Microellobosporia cinerea 3855 was tested for antibiotic activity by cross-streak test on six different solid media. Zones of inhibition were produced with Gram-positive bacteria and a Mycobacterium, no inhibition of Gram-negative bacteria and fungi was observed. Shake-flask cultures showed weak activity against *Staphylococcus aureus* but no activity against a range of Gram-negative bacteria, yeasts and fungi using a cup-plate assay method.

Sensitivity to antibiotics. On nutrient agar with glucose (Lechevalier *et al.* 1961), at pH 6·3, the growth of the culture was not affected by 50 μg./ml. of either amphotericin B or pimaricin. Growth was completely inhibited by 30 μg. chloramphenicol ml./ or 5 μg. neomycin/ml. On potato + glucose agar (Waksman, 1950), at pH 6·0, the antifungal antibiotics were also without effect, whereas 20 μg. chloramphenicol/ml. and 5 μg. neomycin/ml. were completely inhibitory.

Source. Isolated from a soil sample collected at Princess Margaret Glacis, Pilta, Malta.

Description of Microellobosporia flavea, *Cross, Lechevalier and Lechevalier sp.nov.*

The two strains of this species showed the same microscopical properties previously described for the type species. The aerial mycelium was pale to light grey, and, whereas the type species was a pinkish organism, *M. flavea* produced a yellow soluble pigment, the colour of which was not affected by pH.

Appearance on various media. Cultures were incubated in the dark at 30 or 37° for 2 weeks. This was due to differences in the temperature optima of the two strains. Isolate 3857 grew best at 30°, whereas 3858 sporulated more abundantly at 37°. Both strains grew well at the same temperatures as the strain of the type species. The two strains of *M. flavea*, like that of the type species vigorously hydrolysed gelatine and starch. On litmus milk and nitrate medium, there was also no difference in the behaviour of the representatives of the two species.

Czapek solution agar. Good, off-white, smooth, leathery growth. No aerial hyphae. 3857 had a slight orange tint; 3858 a slight tan tint.

Synthetic agar (Lindenbein). Growth good, reverse colony colour orange. Aerial mycelium white, abundant (3857) or scant and powdery (3858). Soluble pigment orange yellow.

Defined medium (Lechevalier). Yellow-cream, convoluted, vegetative growth. No aerial mycelium. No soluble pigment.

Corn meal + salts agar. Good, off white vegetative growth. Aerial mycelium white to ash grey, thin (3857) or good (3858). No soluble pigment.

Beef extract + starch agar. Colourless vegetative growth; no soluble pigment. No aerial hyphae.

Yeast extract agar. Light tan, moist, wrinkled growth. No aerial hyphae.

Potato plug. Convoluted growth without aerial hyphae. 3857 was brownish-cream coloured whereas 3858 was cream coloured without a brownish tint.

Potato + carrot agar. Off-white vegetative mycelium, reverse of culture greenish. Abundant woolly grey aerial mycelium (3857) or scanty grey aerial mycelium (3858). Abundant sporangial formation.

Rice extract agar. 3857: off-white vegetative growth; light charcoal grey aerial mycelium; no soluble pigment. 3858: white vegetative growth; sparse white aerial mycelium; soluble yellow pigment.

Pablum agar (Lechevalier & Lechevalier, 1957). White to tan vegetative growth; light charcoal grey aerial mycelium. Soluble yellow pigment. Abundant formation of sporangia.

Sabouraud's medium with glucose. Moist wrinkled light tan vegetative mycelium. No aerial hyphae.

Antibiotic properties. By cross-streak test, strain 3857 showed no antibiotic activity and strain 3858 was only faintly active against Gram-positive bacteria.

Sensitivity to antibiotics. The two strains of *M. flavea* were resistant and sensitive to the same antibiotics as *M. cinerea*. They were somewhat more sensitive to the action of the antibacterial antibiotics than the representative of type species.

Source. Strain 3857 was isolated from a soil sample collected at the base of a tree in Orly Airport, France, and strain 3858 from grassland soil collected in Les Andelys, Eure, France.

DISCUSSION

The characteristic production of sporangia containing few linearly arranged spores, on both the aerial and substrate mycelium in these cultures differentiates them from previously described species and justifies the erection of a new genus in the family Actinoplanaceae Couch, 1955. As a consequence, the following revision of the existing key to the family is proposed:

1. *Aerial mycelium usually not formed*; conidia lacking; sporangia, usually spherical, large (8–22 μ), containing motile spores.
Actinoplanes Couch, 1950

2. *Aerial mycelium abundant*; conidia as well as sporangia are formed in some species; sporangia spherical, large (7–19 μ) containing non-motile spores.
Streptosporangium Couch, 1955

3. *Aerial mycelium abundant*; conidia lacking; small sporangia, produced on both substrate and aerial mycelium, club shaped (2–9 $\mu \times 1{\cdot}5$–$3{\cdot}6\,\mu$) containing a short single row of non-motile spores.
Microellobosporia. gen.nov.

Spore formation can be seen to take place inside the wall of the initial sporangial structures and the development of the spores and their relation to the wall appears quite different from that described by Glauert & Hopwood (1961) for *Streptomyces violaceoruber*. The sporangial wall originates from the swollen tip of the sporangiophore and remains intact and separate from the spores during development. After spore release the sporangial wall persists as an open sac. It would be tempting to extend the relationships suggested by Hesseltine (1960) between the genera *Actinoplanes* and *Streptosporangium* on the one hand, and *Streptomyces* on the other, to include *Microellobosporia*. Here the spores are few in number, they are aligned in a single straight row and exceed somewhat the spore size typical of the Streptomyces. But until more detailed work on the walls of the mycelium and sporangium, and their relation to the developing spore have been carried out it would be unwise at this stage to propose any evolutionary relationships.

The fact that two related strains (Nos. 3857 and 3858) were isolated quite soon after the initial isolation of *Microellobosporia cinerea* would suggest that members of this genus are not rare soil-inhabiting organisms. They are not exacting in their nutritional or temperature requirements, and can be isolated readily using media conventionally used for the isolation of Streptomyces. When growing on agar they closely resemble Streptomyces in gross appearance and one must assume they have been isolated in the past and mistaken for these organisms.

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REFERENCES

Bergey's Manual of Determinative Bacteriology (1957). 7th ed., ed. by R. S. Breed, E. G. D. Murray & N. R. Smith. Baltimore: The Williams and Wilkins Co.

BURKHOLDER, P. R., SUN, S. H., ANDERSON, L. E. & EHRLICH, J. (1955). The identity of viomycin producing cultures of Streptomyces. *Bull. Torrey Bot. Club.* **82**, 108.

Difco Manual of Dehydrated Culture Media and Reagents for Microbiological and Clinical Laboratory Procedures (1953). 9th ed. Detroit: Difco Laboratories.

GLAUERT, A. M. & HOPWOOD, D. A. (1961). The fine structure of *Streptomyces violaceoruber* (*S. coelicolor*). III. The walls of the mycelium and spores. *J. biophys. biochem. Cytol.* **10**, 505.

HESSELTINE, C. W. (1960). Relationships of the Actinomycetales. *Mycologia*, **52**, 460.

HUNTER, C. A. & CRECELIUS, A. G. (1938). Hydrogen sulphide studies. I. Detection of hydrogen sulphide in cultures. *J. Bact.* **35**, 185.

KARLING, J. S. (1954). An unusual Keratinophilic microorganism. *Proc. Indiana Acad. Sci.* **63**, 83.

KRASSILNIKOV, N. A. (1949). *Guide to the Identification of Bacteria and Actinomycetes.* English edition edited by J. B. Routien. New York: Chas. Pfizer and Co.

KÜSTER, E. (1959). Outline of a comparative study of criteria used in characterization of the Actinomycetes. *Int. Bull. bact. Nomen.* **9**, 97.

LECHEVALIER, H. A., SOLOTOROVSKY, M. & MCDURMONT, C. I. (1961). A new genus of the Actinomycetales: *Micropolyspora* gen.nov. *J. gen. Microbiol.* **26**, 11.

LECHEVALIER, M. P. & LECHEVALIER, H. A. (1957). A new genus of Actinomycetales: *Waksmania* gen.nov. *J. gen. Microbiol.* **17**, 104.

LINDENBEIN, W. (1952). Über einige chemisch interessante Aktinomyceten Stämme und ihre Klassifizierung. *Arch. Mikrobiol.* **17**, 361.

MENZIES, J. D. & DADE, C. E. (1959). A selective indicator medium for isolating *Streptomyces scabies* from potato tubers or soil. *Phytopathol.* **49**, 457.

PRIDHAM, T. G., ANDERSEN, P., FOLEY, C., LINDENFELSER, L. A., HESSELTINE, C. W. & BENEDICT, R. G. (1957). A selection of media for maintenance and taxonomic study of Streptomyces. *Antibiotics Ann.*, 1956/57, p. 947.

RAPER, K. B. & THOM, C. (1949). *Manual of the Penicillia*, p. 64. Baltimore: The Williams and Wilkins Co.

ROTHWELL, F. M. (1957). A further study of Karling's Keratinophilic organism. *Mycologia*, **49**, 68.

SEGRETAIN, G., DROUHET, E. & MARIAT, F. (1958). *Diagnostic de Laboratoire en Mycologie Médicale.* St.-Mandé, Seine: Editions de la Tourelle.

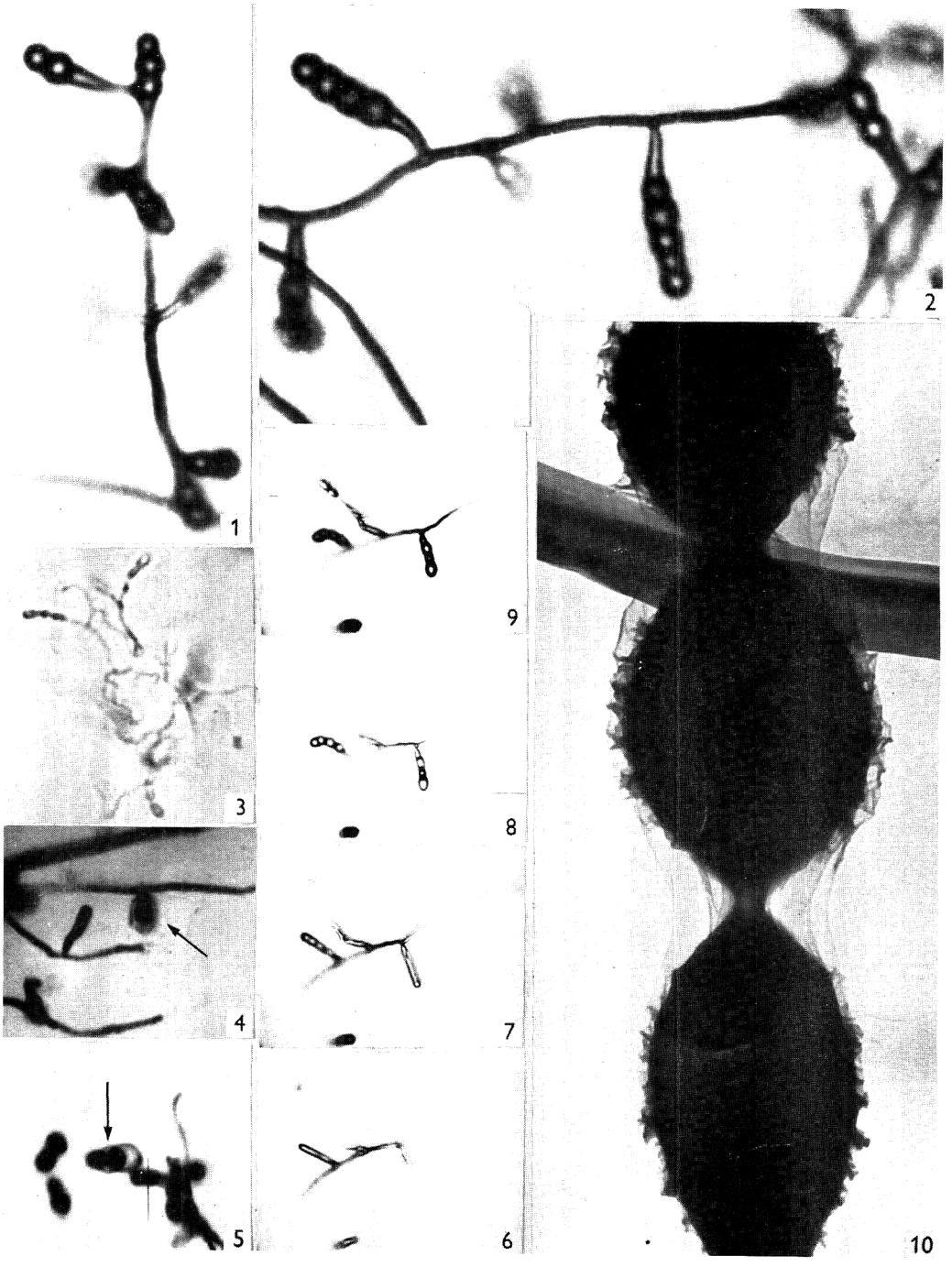
Society of American Bacteriologists (1957). Manual of Microbiological Methods. New York: McGraw-Hill, Inc.

TABER, W. A. (1960). Evidence for the existence of acid-sensitive actinomycetes in soil. *Canad. J. Microbiol.* **6**, 503.

TRESNER, H. D. & DANGA, F. (1958). Hydrogen sulphide production by Streptomyces as a criterion for species differentiation. *J. Bact.* **76**, 239.

VERNON, T. R. (1955). Spore formation in the genus *Streptomyces*. *Nature, Lond.* **176**, 935.

WAKSMAN, S. A. (1950). *The Actinomycetes—their Nature, Occurrence, Activities and Importance.* Waltham, Mass.: Chronica Botanica Co.



T. CROSS, M. P. LECHEVALIER AND H. LECHEVALIER

(*Facing p.* 429)

EXPLANATION OF PLATE 1

Fig. 1. *M. cinerea* 3855; mature sporangia on aerial mycelium (corn meal + salts agar, 7-day culture). × 1400.

Fig. 2. *M. cinerea* 3855; aerial mycelium showing proliferation of lateral sporangiophore from base of a sporangium (potato + carrot agar, 10-day culture). × 1400.

Fig. 3. *M. cinerea* 3855; chains of spores on substrate mycelium seen 100 μ below the surface of the agar (potato + carrot agar, 10-day culture). × 940.

Fig. 4. *M. flavea* 3858; ruptured sporangium from aerial mycelium, stained with methylene blue; note sporangial wall. × 2500.

Fig. 5. *M. flavea* 3857; disruptured sporangia from aerial mycelium, stained with methylene blue; sporangial wall is easily seen, open as if to permit release of spores. × 2500.

Fig. 6. *M. flavea* 3857; aerial mycelium showing growing lateral club-shaped structure (potato + carrot agar, 8 days). × 540.

Fig. 7. Same culture, same place, half a day later; sporangiospores are forming in the left club-shaped structure. × 540.

Fig. 8. Same culture, same place, 9 days; sporangiospores have been formed during the night inside the right branch; the left sporangium is fully formed. × 540.

Fig. 9. Same culture, same place, half a day later; the right sporangium is fully formed; two spores have been formed inside the tip of the middle branch. × 540.

Fig. 10. *M. flavea* 3857; electron microphotograph of part of a sporangium (potato + carrot agar, 21 days). × 23,000.

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The Serological Grouping of Three Strains of *Streptococcus equinus*

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SUMMARY

Group D antisera were prepared from three culture-collection strains of *Streptococcus equinus*. Reciprocal absorption tests showed that they were Group D streptococci.

INTRODUCTION

It is now well established that the streptococci are one of the predominant groups of organisms in the intestine of the pig (Raibaud & Caulet, 1957; Fuller *et al.* 1960). Consequently the classification of streptococci from this source has recently attracted much attention and many different species have been described as predominating in the pig gut. The type of organism isolated from mixed populations is always in some measure dependent on the medium used. Since at least 30 different media have been used by various workers (see Raibaud *et al.* 1961) it is not surprising that conflicting claims have been made. However, apart from the variation which can be accounted for in this way, there is the problem of classifying organisms which bear resemblance to more than one species. The lactose non-fermenting streptococci isolated from the pig gut are an example of this latter group of organisms. In previous work (Fuller *et al.* 1960) numerous lactose non-fermenting Group D streptococci were isolated from pig faeces; because of their inability to ferment lactose or to hydrolyse starch they were classified as *Streptococcus equinus*, although other workers had classified similar strains as *S. bovis* (Raibaud & Caulet, 1957). An objection to the classification of these lactose non-fermenting isolates as *S. equinus* was that they were of serological Group D, whereas no group specific antigen had so far been demonstrated for this species (*Bergey's Manual*, 1957). It was thus essential to know whether accepted strains of *S. equinus* were of serological Group D.

METHODS

Organisms examined. Streptococcus faecalis NCTC 775, *S. faecalis* var. *liquefaciens* (Elv. 2025) NCDO 588, *S. faecalis* var. *zymogenes* NCDO 586, *S. faecium* NCTC 7171, *S. durans* (98D) NCDO, 596, *S. bovis* (Pearl 11) NCDO 597, *S. equinus* ATCC 9812, NCDO 1090 and 1091. The three strains of *Streptococcus equinus* all conformed to the description given in *Bergey's Manual* (1957).

Media. For the preparation of vaccines and extracts organisms were grown for 24 hr. at 37° in glucose Lemco broth (%, w/v; Evan's peptone, 1; Lab. Lemco, 1; NaCl (Analar), 0·5; glucose, 1).

Serological methods

Preparation of antisera. Antisera were prepared by using the three strains of *Streptococcus equinus* and the strain of *S. bovis*. Vaccines were prepared by the method of Shattock (1949). Saline suspensions of organisms were shaken in a tissue disintegrator (Mickle, 1948) for 90 min. One ml. was injected into the ear vein of rabbits at 3- to 4-day intervals, fresh vaccine preparations being used for each injection. Eleven doses of *S. equinus* and nine of *S. bovis* were given. Test bleedings were made after six injections and antisera were tested by the ring precipitin test against HCl extracts of streptococci of serological groups A–O.

Antigen extracts. These were prepared by the method of Lancefield (1933) and when necessary were concentrated by precipitation with ethanol as described by Shattock (1949).

Absorption of antisera. The method of Shattock (1949) was used.

RESULTS

In preliminary tests after six injections, the three *Streptococcus equinus* antisera gave no precipitation with any of the extracts of groups A to O streptococci whilst the *S. bovis* antiserum reacted weakly with the group D extract. At the end of the series of injections all four antisera reacted strongly with the extract prepared from *S. faecalis* 775, but when the sera were tested against extracts of the six different group D streptococci shown in Table 1 precipitation did not occur in all cases.

Table 1. *Precipitin reactions of* Streptococcus equinus *and* S. bovis *group antisera with normal and concentrated extracts of group D streptococci*

	HCl-extracts of											
	S. faecium		*S. faecalis*		*S. faecalis* var. *liquefaciens*		*S. faecalis* var. *zymogenes*		*S. durans*		*S. bovis* 597	
Antisera	N	C	N	C	N	C	N	C	N	C	N	C
S. equinus												
1090	−	+	+	+	+	+	−	+	−	+	−	+
1091	−	+	+	+	+	+	+	+	−	+	−	+
9812	−	+	+	+	−	+	−	+	−	+	−	+
S. bovis												
597	+	+	+	+	+	+	+	+	+	+	−	+

N, HCl extracts; C, concentrated HCl extracts.

However, when the extracts were concentrated the four antisera reacted with all the group D extracts but were still negative to extracts of the other serological groups. Therefore, in subsequent tests concentrated extracts were used.

To confirm that *Streptococcus equinus* was group D, reciprocal absorption tests were made (Tables 2, 3). When the group D antiserum prepared from *S. bovis* was absorbed with suspensions of *S. equinus* two absorptions were necessary to remove all the group D antibodies. After this treatment the only precipitin reaction which occurred was the type reaction between the *S. bovis* antiserum and the homo-

logous extract. One absorption was sufficient to remove the group D antibodies from the *S. equinus* antisera by using *S. faecalis* 775. The only remaining reactions were those between homologous systems. There was no evidence from either of these tests of any shared type antigens, no cross precipitation occurring between the three strains of *S. equinus* or between these and *S. bovis*

Table 2. *Absorption of group D antiserum by* Streptococcus equinus *suspensions*

	Group D serum (*S. bovis* 597)				
		Absorbed with			
Antigen; concentrated extract from	Unabsorbed	*S. equinus* 9812	*S. equinus* 1090	*S. equinus* 1091	*S. bovis* 597
S. faecium	+	−	−	−	−
S. faecalis	+	−	−	−	−
var. *liquefaciens*	+	−	−	−	−
var. *zymogenes*	+	−	−	−	−
S. durans	+	−	−	−	−
S. bovis 597	+	+	+	+	−
S. equinus 9812	+	−	−	−	−
S. equinus 1090	+	−	−	−	−
S. equinus 1091	+	−	−	−	−

Table 3. *Absorption of* Streptococcus equinus *and* S. bovis *group D antisera with* S. faecalis 775

	Antisera							
	S. equinus 9812		*S. equinus* 1090		*S. equinus* 1091		*S. bovis* 597	
Antigen; concentrated extract from	Unabs.	Abs.	Unabs.	Abs.	Unabs.	Abs.	Unabs.	Abs.
S. faecium	+	−	+	−	+	−	+	−
S. faecalis	+	−	+	−	+	−	+	−
var. *liquefaciens*	+	−	+	−	+	−	+	−
var. *zymogenes*	+	−	+	−	+	−	+	−
S. durans	+	−	+	−	+	−	+	−
S. bovis 597	+	−	+	−	+	−	+	+
S. equinus 9812	+	+	+	−	+	−	+	−
S. equinus 1090	+	−	+	+	+	−	+	−
S. equinus 1091	+	−	+	−	+	+	+	−

DISCUSSION

By using three culture collection strains of *Streptococcus equinus* we have prepared specific group D antisera thereby showing that *S. equinus* belongs to serological group D. It seems that the group D antigen is either serologically inaccessible or present in relatively small amounts as shown by the necessity to concentrate some extracts to get precipitation with *Streptococcus equinus* group antisera and the necessity to absorb twice with *S. equinus* suspensions to remove group D antibodies from a *S. bovis* group antiserum. This agrees with our previous findings (Fuller *et al.* 1960) that *S. equinus* strains isolated from pig faeces were difficult to group unless concentrated extracts were used. It may also explain past failures to detect a group specific antigen.

Since this work was completed Smith & Shattock (1962) have reported on a

collection of streptococci isolated from horse faeces and corresponding to the description of *Streptococcus equinus* (Andrewes & Horder, 1906). These strains all produced the group D antigen and the preparation of a group D antiserum from one of these strains confirmed their opinion that *S. equinus* belongs to group D.

Group D is at present comprised of *Streptococcus faecalis* and its two variants *liquefaciens* and *zymogenes*, *S. durans*, *S. faecium* and *S. bovis*. These organisms are all of intestinal origin and since *S. equinus* is the predominant streptococcus in horse faeces (Andrewes & Horder, 1906) it is perhaps not surprising that, if it belongs to any of the existing groups, it should belong to group D. The similarity of the physiological characters of *S. equinus* and *S. bovis* (Sherman, 1937) supports the inclusion of *S. equinus* in group D. Seeley & Dain (1960) stated that aside from the inability of *S. equinus* to ferment lactose, no significant differences exist in our present definitions and knowledge of the species. This close relationship to *S. bovis* coupled with a reluctance to place group D organisms in a species for which no group antigen was known may have led to the classification of *S. equinus* isolates as *S. bovis*. The acceptance of *S. equinus* as a member of serological group D removes the latter difficulty. More recently Smith & Shattock (1962) have re-examined the relationship between the two species and suggest that when the physiological characters of each species are viewed as a whole, then a satisfactory separation can be made. We also believe that these two species can be separated on the basis of physiological tests and would propose that the species *S. equinus* be retained and in future included in group D.

We are indebted to Mr D. G. Smith and Dr P. M. Frances Shattock (Microbiology Department, University of Reading) for allowing us to quote from their paper before it was published.

REFERENCES

ANDREWES, F. W. & HORDER, T. J. (1906). A study of the streptococci pathogenic for man. *Lancet*, ii, 708.

Bergey's Manual of Determinative Bacteriology (1957). 7th ed. Ed. by R. S. Breed, E. G. D. Murray & N. R. Smith. London: Ballière, Tindall and Cox Ltd.

FULLER, R., NEWLAND, L. G. M., BRIGGS, C. A. E., BRAUDE, R. & MITCHELL, K. G. (1960). The normal intestinal flora of the pig. IV. The effect of dietary supplements of penicillin, chlortetracycline or copper sulphate on the faecal flora. *J. appl. Bact.* **23**, 195.

LANCEFIELD, R. C. (1933). A serological differentiation of human and other groups of haemolytic streptococci. *J. exp. Med.* **57**, 571.

MICKLE, H. (1948). Tissue disintegration *J. Roy. Micros. Soc.* **68**, 10.

RAIBAUD, P. & CAULET, M. (1957). Les streptocoques dominants dans le tube digestif du porc adulte. *C.R. Acad. Sci., Paris*, **244**, 1291.

RAIBAUD, P., CAULET, M., GALPIN, J. V. & MOCQUOT, G. (1961). Studies on the bacterial flora of the alimentary tract of pigs. II. Streptococci. Selective enumeration and differentiation of the dominant group. *J. appl. Bact.* **24**, 285.

SEELEY, H. W. & DAIN, J. A. (1960). Starch hydrolysing streptococci. *J. Bact.* **79**, 230.

SHATTOCK, P. M. F. (1949). The streptococci of group D; the serological grouping of *Streptococcus bovis* and observations on serologically refractory group D strains. *J. gen. Microbiol.* **3**. 80.

SHERMAN, J. M. (1937). The streptococci. *Bact. Rev.* **1**, 3.

SMITH, D. G. & SHATTOCK, P. M. F. (1962). The serological grouping of *S. equinus*. *J. gen. Microbiol.* (in the Press).

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The Structure of Coliphages

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SUMMARY

The detailed morphology of several new coliphages is compared with that of the more familiar types; the resulting information is considered for its taxonomic value. Some were examined at molecular level and new information on the tail structure of the 'T even' phages is described. It is shown that the so-called tail fibres are, in fact, the remains of a network which surrounds the extended sheath. From this information it is possible to propose the changes in molecular packing accompanying sheath contraction.

A new phage with an octahedral head and contractile tail is described. It is also shown that phage T3 almost certainly has an octahedral head. Electron micrographs of ϕR (a ϕX174 type phage) show that it consists of two parts: an icosahedral protein shell, and morphological subunits attached to each apex.

INTRODUCTION

Bacteriophages specific to *Escherichia coli* include a number of different morphological types. These are mostly restricted to the T group and relatively few others have been described. In the present work, some new coliphages which have been isolated from sewage-contaminated water are illustrated; some of them are morphologically distinct from those in the T group. There are three morphological types within the T group (Bradley & Kay, 1960): the 'T even' phages with contractile sheaths, T1 and T5 with long tails without contractile sheaths, and T3 and T7 with very short tails. The very small phages ϕX174 (Sinsheimer, 1959; Tromans & Horne, 1961) and ϕR (Bradley, 1961; Kay, 1962; Kay & Bradley, 1962) constitute another group. The phages described here add three more morphological types; each group is studied in some detail. In the cases of phages T2 and T4 the molecular changes in the tail which lead to nucleic acid injection can be deduced.

METHODS

Sources of bacteriophages used. The following kindly supplied the bacteriophages mentioned: Dr D. Kay (Sir William Dunn School of Pathology, University of Oxford), phages T2, T3, T5, ϕR, 66t$^-$ and also *Escherichia coli* strains B and C2; Dr and Mrs K. G. Lark (Saint Louis University School of Medicine, U.S.A.) phages T4 and T5 and also *E. coli* strain F.

Phage C1 was obtained by growing a typhoid phage (ϕ1, Fildes & Kay, 1955) on *Escherichia coli* C2 and phage F1 from a second typhoid phage (ϕ2, Bradley & Kay, 1960) grown on *E. coli* F, both original phages being sent by Dr Kay.

Phage E1 was isolated from a water sample from near Aldermaston, Berkshire, and phages α1, α3, β4 and γ6 from water samples (rivers, streams, reservoirs) taken

within a 50-mile radius of Edinburgh. The host in all these cases was *Escherichia coli* c2, save for phage E1 which was grown on *E. coli* B.

Isolation, growth and purification of phages. Escherichia coli c2 was used to isolate phages from water samples. The following procedure was used. An overnight slope culture of *E. coli* c2 was suspended in 1 ml. water. One ml. of the water sample which contained 0·1 M-ammonium acetate and thymol was then added to the bacterial suspension; 1 ml. of this mixture was plated out by the double agar layer method (Adams, 1950). After incubation, the plaques formed were picked and suspended in 0·1 M-ammonium acetate solution. Plaques were selected by their morphology, and the resulting phage suspension was further purified by plating and plaque picking. In general the phage count for water samples varied from 1 to 50 plaque-forming particles specific to *E. coli* c2/ml. water. Samples from sewage-contaminated rivers contained more phage particles than did samples from reservoirs or ponds.

Phages were grown in broth cultures (in the cases of the T phages and phage ϕR a defined medium was used) or as follows. Sufficient phage particles were plated out by the double agar layer method to cause the lysis of all the host bacteria. Phages were eluted by pouring 2–3 ml. of 0·1 M-ammonium acetate onto the plate after incubation, and allowing it to stand for some hours. The decanted ammonium acetate solution usually contained about 10^{11} particles from each plate. Phage suspensions were purified by several cycles of centrifugation. Ammonium acetate solution (0·1 M) was used as a suspending medium in most cases because phages are very stable in it. With phage ϕR a special purification procedure was used (Kay, 1962).

Electron microscope specimen preparation. The negative contrast method of Brenner & Horne (1959*a*) was used throughout. The embedding chemicals were potassium phosphotungstate, sodium molybdate and uranyl acetate. Shadowing with platinum metal or platinum + carbon (Bradley, 1959) was also used.

RESULTS

'*T even*' *type phages*

Plate 1, fig. 1 shows phage T4 which was suspended in 0·1 M-ammonium carbonate solution and embedded in sodium molybdate as a negative stain. What is best described as a collar can be clearly seen between the base of the head and the top of the tail sheath. In addition, two parallel lines join the base-plate of the tail and the collar outside the sheath. At two points (arrowed) the lines split into a network. The phage thus appears to possess an external structure surrounding the tail sheath. The nature of this assembly is illustrated where the negative staining material has caused the disruption of the sheath (Pl. 1, fig. 2). Here, the centre portion of the sheath is missing, but the top and bottom remain intact; two pairs of fibres join the portions. The appearance is consistent with the presence of six fibres, two of which are invisible being obscured by the tail core. This is a logical number since it is known that the base-like plate is hexagonal (Brenner & Horne, 1959*b*), and one might expect a fibre to be attached at each corner. Plate 1, fig. 3 also shows clearly that the structure is fibrous in nature; it has been broken up and its appearance is remarkably like the often observed tail fibres of phage T2. From these three

micrographs it can be seen that the sheath is surrounded by a fibrous network. As will become apparent, results with phage T2 show that the tail fibres are, in fact, the broken network.

The newly isolated phage $\alpha 1$, which is morphologically similar to phage T4, has some structure in the collar (Pl. 1, fig. 4), which is likely to possess sixfold radial symmetry like the rest of the tail. A typhoid phage 66t$^-$ (Pl. 1, fig. 5) also has a fibrous outer network and this and the collar are shown even more clearly and consistently than in phage T4.

Phage T2 is illustrated in Pl. 2, figs. 7–10. A few particles possessed a fibrous network (Pl. 2, figs. 7, 8), but no tail fibres. Others (Pl. 1, figs. 9, 10) had tail fibres, but no network. No obvious collar was found as in the other 'T even' type phages.

Plate 2, fig. 10 shows a number of different components: two intact particles, kinked tail fibres (*f*), a core and base-plate (*c*), and a contracted sheath (*s*). The latter has twelve distinct cross-striations with a spacing of about 33 Å. Similar periodicities were found on the contracted sheaths of phages T4 and 66t$^-$ (Pl. 3, fig. 17).

In a very few micrographs, the negative staining and preservation were sufficiently good to show the actual subunits of the extended sheath in phages T2 and T4. They are illustrated, together with a possible model, in Pl. 3, fig. 11. Their arrangement will be discussed below.

Phage E1. Plate 3, figs. 12 and 13 illustrate two intact particles of phage E1 with superimposed models of octahedra for comparison with the head shape. The tail sheaths are extended and exhibit a subunit structure. There are also about twenty-six cross-striations with a 45 Å spacing. There is an indistinct base-plate. A phage with a contracted sheath is shown in Pl. 3, fig. 15. This has distinct parallel longitudinal striations in addition to rather indistinct cross-striations numbering 12 or 13. The shadowed phage (Pl. 3, fig. 16) clearly shows four tail fibres, only faintly visible in the negatively stained preparations.

Phage C1. This phage has a large (900 Å) head and a long tail. The head shape is consistent with an octahedron and is shown in Pl. 4, fig. 18. The tail has cross-striations and a very fine point at the tip (Pl. 4, figs. 19, 22).

Phage F1. Phage F1 is very similar to phage C1 though it has not been possible to establish the head shape. The tail is different and is shown in the drawing in Pl. 4, fig. 21, which may be compared with the micrographs in Pl. 4, fig. 20.

Phage T5. Platinum + carbon shadowing was used to establish the head shape. In Pl. 4, figs. 23 and 24 the shadows are cast by heads in orientations differing by about 60° and are characteristic of an icosahedron. In Pl. 4, fig. 25, some facets of the head are visible and can be compared with the superimposed model of an icosahedron. Uranyl acetate preparations (Pl. 5, fig. 28), show marked cross-striations in the tail, caused presumably by the protein subunits of which it is built. There are forty striations in the whole length of the tail.

Phages $\beta 4$ and $\gamma 2$. Phage $\beta 4$ is shown in Pl. 5, fig. 29 and phage $\gamma 2$ in Pl. 5, fig. 30. They are similar in appearance and size (heads 600 Å, tails 1500 Å). The heads exhibit a hexagonal outline and the tails cross-striations. In the case of $\gamma 2$ there is a small group of fibres at the tail tip (arrowed) and the tails of $\beta 4$ have curled up, possibly due to the method of preparation.

Phage T3. The appearance of this phage is familiar, but micrographs shown here (Pl. 5, figs. 31, 32) reveal two points of interest. In Pl. 5, fig. 31, a small fibre (arrowed) is attached to the tail, and both this micrograph and Pl. 5, fig. 32, strongly suggest that the head is an octahedron.

Small 'tailless' phages. Phages ϕX 174 and ϕR have been described in some detail, but one or two additional observations are mentioned here. Pl. 5, fig. 33, shows phage ϕR in phosphotungstate; the apical subunits of the icosahedron are clearly visible. In Pl. 5, fig. 34, an empty particle exhibits a continuous hexagonal outline and as in Pl. 5, fig. 33, some of the subunits are apparently divided into two parts. The icosahedron is lying on its twofold symmetry axis. Phage α3 is a morphologically similar phage isolated from a stream in Edinburgh, but is somewhat smaller (Pl. 5, fig. 35).

DISCUSSION

The structure of phages T2 and T4. In recent years since the detailed basic work of Brenner *et al.* (1959) there has been a good deal of discussion and speculation concerning the tail structure of these phages. Anderson (1960) observed a thin disk or collar on phage T4 between the base of the head and the top of the tail sheath. In addition to this, Daems, Van de Pol & Cohen (1961) observed an outer jacket around the tail sheath and occasionally a helical structure superimposed on the well-known cross-striations. Apart from the fact that these components are definitely known to exist, no detailed information is available about them.

The nature of these features is considerably clarified by the micrographs shown here. The collar of phage T4 is particularly clear in Pl. 1, fig. 1, and a micrograph of phage α1 (Pl. 1, fig. 4) suggests that its collar has a sixfold radial symmetry. This fits into the general symmetry pattern of the whole tail assembly. The collar is extremely delicate and has not yet been viewed end-on or isolated. Until this is done the most likely shape is considered to be a hexagonal plate resembling the base-plate (Brenner & Horne, 1959*b*).

The so-called 'outer jacket' of Daems *et al.* (1961) is, in fact, a network or cage of fibres. It is difficult to discern the exact arrangement, but the micrograph of phage T4 (Pl. 1, fig. 1) suggests that the fibres branch some two-thirds of the way up the tail, each branch being attached to alternate apices of the collar.

The whole interrelated assembly of collar, fibrous network and base-plate is illustrated in the model in Pl. 1, fig. 6. While this may not be exactly correct, it is considered that the proposed structure will closely resemble the true arrangement of the components.

The micrographs of phage T2 provide complementary information. As observed by Daems *et al.* (1961) and originally shown by Brenner *et al.* (1959) phage T2 appears to possess no collar. However, Dr Horne (personal communication) and Dr Almeida (personal communication) have observed a distinct collar on their samples of this phage. Plate 2, figs. 7, 8, reveal a fibrous network, which is undoubtedly delicate and easily disrupted to form the so-called 'tail fibres'. The intact network appears to be attached to the head in the absence of a collar. In Pl. 2, fig. 9, the network has been partly disrupted and at the arrowed points the fibres appear to branch, as is consistent with the arrangement for phage T4. Plate 2, fig. 10*f*, shows the characteristically kinked 'tail fibres'; it seems most likely that the kink occurs

where the fibres of the intact network branch, confirming that the 'tail fibres' are the broken network.

The coarse helical structure found on the phage T4 extended sheath by Daems *et al.* (1961) was not seen clearly on this phage but was occasionally visible on phage T2. It is shown by the arrows *C* in Pl. 3, fig. 11*a* and can be seen to be caused by a difference in density of some of the subunits, the arrangement being shown in the model (Pl. 3, fig. 11*b*). The arrowed subunits seem to be slightly bigger than the others, and since it is unlikely that the tail sheath contains two types of protein, they presumably represent the same subunits in a different orientation.

Plate 3, fig. 11 also suggests a reasonable arrangement for the subunits of the extended sheaths of phages T2 and T4, which appear to be similar. In Pl. 3, fig. 11*a*, the top row of four units is very clear, two more being out of sight. In the next two rows, sufficient are visible to allow the preparation of the model, which seems to agree reasonably well with the phage T4 sheath (Pl. 3, fig. 11*c*). Only the portions of the sheaths arrowed at *A* and *B* show the subunit arrangement clearly. Elsewhere the structure is indistinct due to disorganization. This accounts for departures from the model which can be detected in some places. The arrangement shown is the most likely on the basis of the micrographs, but a single primary helix cannot be ruled out.

In the model, the subunits are related by screw symmetry and are in a series of annuli of sixfold radial symmetry. It is difficult to count the number of annuli exactly because in negatively stained preparations, the bottom few rows near the base-plate are often disorganized; however, there appear to be 24 or 25, probably the former. The number is the same on all the T2-type phages. This gives a total of 144 or 150 subunits, the diameter of the visible profile being 30 Å (compare Brenner *et al.* 1959, who suggested 200 subunits from molecular weight determinations).

In the case of the contracted sheath, all the T2-type phages mentioned here have twelve cross-striations (half the number in the extended sheath) with a spacing of about 33 Å. Brenner *et al.* (1959) showed that there was, in addition, a coarse helix.

From this information it is possible to make a plausible picture of the changes in molecular packing which accompany sheath contraction and the puncture of the cell wall. For the sheath to contract to half its length, the simplest mechanism would be to double the number of subunits from six to twelve per annulus. Thus, referring to the model (Pl. 3, fig. 11*b*) each ring of subunits would slip up into the interstices of the one above. If this were the case, one would expect axial striations to appear on the contracted sheath. In fact, the coarse helix found by Brenner *et al.* (1959) approaches axial striations, which have been found on at least three other phages with contractile tails (P1, Anderson, 1960; typhoid ϕ2, Bradley & Kay, 1960; phage E1, present communication). In addition one would expect to find cross-striations slightly coarser than those in the extended sheath. Those observed were spaced 33 Å as compared with 40 Å for the extended sheath; they numbered, however, half those found on the extended sheath. Though these observations appear to be inconsistent, the presence of the correct number of striations on a contracted sheath is evidence in favour of the change in packing suggested. The likely explanation for the unexpected spacing difference is that the subunits are asymmetrical and probably rotate to some extent as well as changing their packing. This would also account for the fact that in the contracted sheath of the T2-type phages

there is a coarse helix instead of axial striations. These results indicate that the tail sheath is much more than a collection of simply packed spherical subunits.

The function of the collar and fibrous network assembly is not known. One might expect it to have something to contribute towards the infective process. It is interesting to note that a collar is not always found on phage T2; the feature is doubtless more delicate than on other similar phages and liable to destruction during the preparation process.

The octahedral coliphages. Bradley & Kay (1960) described a small phage with a contractile tail and a head which was probably an octahedron. Though it was a typhoid phage (ϕ2), several apparently identical phages have been isolated from the Edinburgh area and Aldermaston on *Escherichia coli* C2. Their structure is important mainly because of the unusual head shape, but they also have an interesting tail assembly.

The initial appearance of ϕ2 heads in phosphotungstate suggested that they were octahedra; empty heads exhibited two characteristic superimposed triangles. It was found by using wire models, however, that a similar effect could be produced by an icosahedron when a number of its edges were rendered invisible, a possibility with negative staining. This is illustrated in Pl. 3, fig. 14. But in the case of the icosahedron (Pl. 3, fig. 14*c*) two of the sides of the triangle are kinked, while in the phage (Pl. 3, fig. 14*b*) and the octahedron (Pl. 3, fig. 14*c*) all the sides are straight. Further confirmation of the head shape was obtained with the morphologically identical phage E1, the actual faces of the octahedron being clearly visible in Pl. 3, figs. 12, 13. The heads of these phages therefore possess 4:3:2 symmetry.

The tail structure was studied on phage ϕ2 by Bradley & Kay (1960) but a few additional features are shown here. The extended sheaths in Pl. 3, figs. 12, 13 have a structure which reflects the helical nature of the arrangement of the subunits, but it is not sufficiently clear to construct a model as with phages T2 and T4. Since the contracted sheaths have both longitudinal and transverse striations, it seems likely that the mechanism of sheath contraction is basically similar to that of the T2-type phages.

At the end of the extended sheath there is a very small base plate, and four short tail fibres can be clearly seen attached to it in the shadowed phage (Pl. 3, fig. 16). This is consistent with a fourfold radial symmetry of the tail assembly, lying as it does on the fourfold axis of the octahedral head. Bearing in mind the fibrous network of the T2 type phages, the micrographs may not show the tail fibres in their true positions. In a very few cases (negatively stained) they seemed to be folded up the outside of the extended sheath.

Morphologically, this is a unique group of phages there being apparently no variants within it; it is common in sewage. Two Pseudomonas phages of very similar appearance have been found (Bradley, unpublished).

Large phages without contractile tails. Two phages are included in this group, C1 and F1. They are characterized by large heads and long tails. Phage C1 has an octahedral head. It has not been possible to establish the shape of the head of phage F1 because the heads usually collapse, but some micrographs also suggest an octahedron. The two phages are biologically different since they have different host ranges in strains of *Escherichia coli*, and the difference in tail structure confirms that they are taxonomically distinct.

Smaller phages without contractile tails. Phage T5 has been studied in some detail as a representative of a large group where the members are often similar morphologically. The evidence presented strongly suggests that phage T5 has an icosahedral head, but it is felt that an octahedron cannot be ruled out until better electron micrographs have been taken, perhaps by improved negative staining. The head shape is important in deducing the distribution of the protein subunits in the tail. It is possible by considering the radial and axial symmetry of the tail, its length, and also the size of the subunits, to build up a picture of the molecular structure.

The radial symmetry of the tail could not be observed directly on the few micrographs obtained of short lengths in section (Pl. 4, fig. 27*s*), but there is a possibility of deducing the symmetry from the geometrical form of the head. It is known that the T2-type phages possess a tail attached to their sixfold symmetry axis; its molecular structure is built up on a basis of sixfold radial symmetry. The four fibres of the octahedral phages, whose tail is attached to the fourfold symmetry axis, also indicate that it too possesses fourfold radial symmetry. Considering these precedents, it seems likely that other phage tails, including that of phage T5, also have the same radial symmetry as that of the axis to which they are attached. Phages, like many other types of virus, are clearly built on a strictly geometric basis and should therefore obey rules of symmetry. The tail of phage T5 is attached to a fivefold symmetry axis of the head, and, on the basis of the above remarks, should have fivefold radial symmetry. The axial arrangement of the subunits can be clearly seen in the tail (arrowed) in Pl. 5, fig. 28. They lie opposite one another, suggesting a series of annuli. The subunits appear to consist of a number of short tubes which can be seen end-on in the disorganized tail (Pl. 4, fig. 27, arrowed).

There are about 40 annuli per tail and with five subunits per annulus, based on the fivefold radial symmetry already discussed, there are some 200 subunits in all. The overall picture of the phage obtained by these results and deductions is illustrated in the model in Pl. 4, fig. 26, which is based on an icosahedron.

Phages $\beta 4$ and $\gamma 2$ represent a group resembling the SBL type of typhoid phage (Bradley & Kay, 1960). They have fairly small heads (about 600 Å), long tails (1500 Å) and look generally similar to phage T5. Both of those shown here exhibit hexagonal outline of the head and cross-striations of the tail (Pl. 5, figs. 29, 30). Their sizes are the same and also their general morphology. Since they differ somewhat in host range, a structural difference might be expected, and, after the study of phages C1 and F1, this might be in the appendage at the tail tip. Such an appendage has been found only in phage $\gamma 2$ and consists of a small group of fibres (Pl. 5, fig. 30, arrowed). In the case of phage $\beta 4$ the tails had curled up. This was noted with several similar phages and may well be due to the method of preparation. This will be the most difficult group to fit into any scheme of classification; morphologically they are very similar. Any differences will be very small and perhaps limited to tail appendages which are difficult to preserve and examine. The phages are very common.

Phages with very short tails. This morphological group is best represented by phage T3. It is mentioned here because a few examples have been found in water samples, but it is relatively uncommon. It is interesting to note that several similar morphological types have been found associated with other bacteria, for example

Brucella abortus (Morgan, Kay & Bradley, 1960) and also species of Pseudomonas (Bradley, unpublished).

The small 'tailless' phages. Phages ϕR174 (Sinsheimer, 1959; Tromans & Horne, 1961) and ϕR (Bradley, 1961; Kay, 1962; Kay & Bradley, 1962) have been described in detail and their unique form is of interest. This morphological type is fairly common in sewage-contaminated waters and more than eight new isolates have recently been obtained (Bradley, 1962). One of them, phage α3, has been described above.

Phages ϕX174 and ϕR are known to consist of single-stranded DNA within an icosahedral protein coat. It seems very likely that the new isolates follow this pattern. Morphological subunits have been found at the apices of the icosahedron, and in the case of phage ϕR they have been resolved into two clearly defined portions (Bradley, 1961), shown in Pl. 5, figs. 33, 34 (arrowed). Their appearance is consistent with that of a tube on its side, but in Pl. 5, fig. 34, where an empty particle is shown in another orientation, they still appear as definitely separated dots. This indicates that the subunits are really in the form of dimers and are not, in fact, short tubes. The protein container thus consists of two distinct parts, the continuous shell containing the DNA, and the apical subunits. The most important fact to discover now is whether these subunits play any part in the infective process, and if not, the mode of DNA injection used by the phage.

Phage taxonomy

It can be seen from the above results that there is a reasonable possibility of classifying phages on a basis of their morphology. Adams (1950) stated that there are three main criteria which should be applied in any system of phage classification: morphology, serological properties, host range characters. Let us consider first how purely morphological characters could be used to classify the coliphages studied here. The phages split into well defined groups: those with contractile tails (T2, E1), those with non-contractile tails (F1, T5), and those with short tails or no tails (T3, ϕR). This might seem to provide a reasonable basis for preliminary division, but within each group another important morphological character, namely, the shape of the head, can be different. In the first group there is the bipyramidal hexagonal prism of phage T2 and the octahedron of phage E1; in the second and third groups there are octahedra and icosahedra.

Such a grouping would clearly lead to confusion, and it is considered that it would be better to use a system of basic morphological types; some of these are listed in Table 1. It will be found that almost all the coliphages fit into one or other of these types. The exceptions are the F1 types and the smaller T5 types; here, the tail structures which would have to be used to differentiate them have not yet been adequately examined. Such a system raises the question as to whether one phage is the same as another because it looks identical in spite of the fact that it may have a different host. Within group of phages specific to one species of bacterium this would raise no problem since the question of host range would only arise at strain level; it seems unreasonable to use strain differences in hosts to differentiate between what are effectively phage species. The greatest problem would be, for example, the case of phage T3; identical morphological types grow on Brucella and Pseudomonas organisms.

Clearly, a purely morphological classification cannot be fully justified at present; serological characters must also be taken into account, and the difficulties of determining the serological relationships of a large number of phages are obvious. The use of a Linnæan type of classification would be premature, but it is felt that phages should be named in an informative manner, not merely by the bottle or batch number of the laboratory in which they were isolated, as this is inevitably completely meaningless on its own. In any original description of a phage the following could be mentioned: the reference number, the morphological type and the host genus or perhaps species. Thus the phage $\alpha 1$ described in this paper would be called $\alpha 1$–T2 (coli) indicating that it was similar in appearance with phage T2 and isolated on *Escherichia coli*. Such a system omits one extremely important piece of information, namely, the nature of the phage nucleic acid. This criterion provides an ideal way of dividing phages into large natural groups. Phage nucleic acid may be double-stranded DNA, single-stranded DNA, or RNA. A system of nomenclature of this kind seems essential if the present confusion is to be removed. It is hoped that the above suggestions will arouse the active interest of those concerned.

Table 1. *Basic morphological types of coliphages*

Phage morphological type*	Characters	
	Head shape	Tail type, etc.
T2	Bipyramidal hexagonal prism	Contractile sheath
$\phi 2$	Octahedron	Contractile sheath
C1	Octahedron. Large	Non-contractile sheath; pointed tip
F1	Octahedron. Large	Non-contractile sheath; forked tip
T5	Icosahedron	Non-contractile sheath; tip obscure
T3	Octahedron	Very short
ϕX174	Icosahedron	Tailless, apical subunits

* The types mentioned are restricted to the coliphages and the names are those given when the phages were first described.

Conclusion

It is clear from the foregoing that the electron microscope can provide more information about phage structure than was originally expected. Straightforward observation has permitted something of the nature of the T2/T4 injection mechanism to be understood at molecular level, and there seems no reason why similar success should not be obtained with other phages with contractile tails. The main problem of phage infection is, however, the manner in which the nucleic acid is transferred down the narrow tube of the tail and into the host and it is difficult to see just how the electron microscope can help in this respect. It does show, however, that almost every phage with a tail has some appendage at the tip and this presumably reacts with specific receptor sites on adsorption. The problem of the method of nucleic

acid transfer used by phages of the ϕX174 type is unique since these do not seem to have conventional tails. It seems likely, however, that the apical subunits are in some way involved in the infective process.

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REFERENCES

ADAMS, M. H. (1950). *Bacteriophages.* New York and London: Interscience Publishers.

ANDERSON, T. F. (1960). On the fine structure of the temperate bacteriophages P1, P2 and P22. *Proc. Eur. Reg. Conf. electron microscopy, Delft*, 1960 **2**, 1008.

BRADLEY, D. E. (1959). High-resolution shadow-casting technique for the electron microscope using the simultaneous evaporation of platinum and carbon. *Brit. J. appl. Phys.* **10**, 198.

BRADLEY, D. E. (1961). Negative staining of Bacteriophage ϕR at various pH values. *Virology*, **15**, 203.

BRADLEY, D. E. (1962). Some new small bacteriophages (ϕX174 type). *Nature, Lond.* **195**, 622.

BRADLEY, D. E. & KAY, D. (1960). The fine structure of bacteriophages. *J. gen. Microbiol.* **23**, 553.

BRENNER, S. & HORNE, R. W. (1959*a*). A negative staining method for high resolution electron microscopy of viruses. *Biochim. biophys. Acta*, **34**, 103.

BRENNER, S. & HORNE, R. W. (1959*b*). The structure of bacteriophage. *The Times Science Review*, London, Autumn, 1959.

BRENNER, S., STREISINGER, G., HORNE, R. W., CHAMPE, S. P., BARNETT, L., BENZER, S. & REES, M. W. (1959). Structural components of bacteriophage. *J. mol. Biol.* **1**, 281.

DAEMS, W. TH., VAN DE POL, J. H. & COHEN, J. A. (1961). Some remarks on the morphology of bacteriophage T4B. *J. mol. Biol.* **3**, 225.

FILDES, P. & KAY, D. (1955). The rate of adsorption of bacteriophage by rough and smooth strains of *Salmonella typhi*. *Brit. J. exp. Path.* **36**, 534.

KAY, D. (1962) The nucleic acid composition of bacteriophage ϕR. *J. gen. Microbiol.* **27**, 201.

KAY, D. & BRADLEY, D. E. (1962). The structure of bacteriophage ϕR. *J. gen. Microbiol.* **27**. 195.

MORGAN, W. J. B., KAY, D. & BRADLEY, D. E. (1960). *Brucella bacteriophage. Nature Lond.* **188**, 74.

SINSHEIMER, R. L. (1959). Purification and properties of bacteriophage ϕX174. *J. mol. biol.* **1**, 37.

TROMANS, W. J. & HORNE, R. W. (1961). The structure of bacteriophage ϕX174. *Virology*, **15**, 1.

EXPLANATION OF PLATES 1–5

PLATE 1

Fig. 1. Phage T4 in sodium molybdate, × 333,000 (by courtesy of Academic Press Inc.).

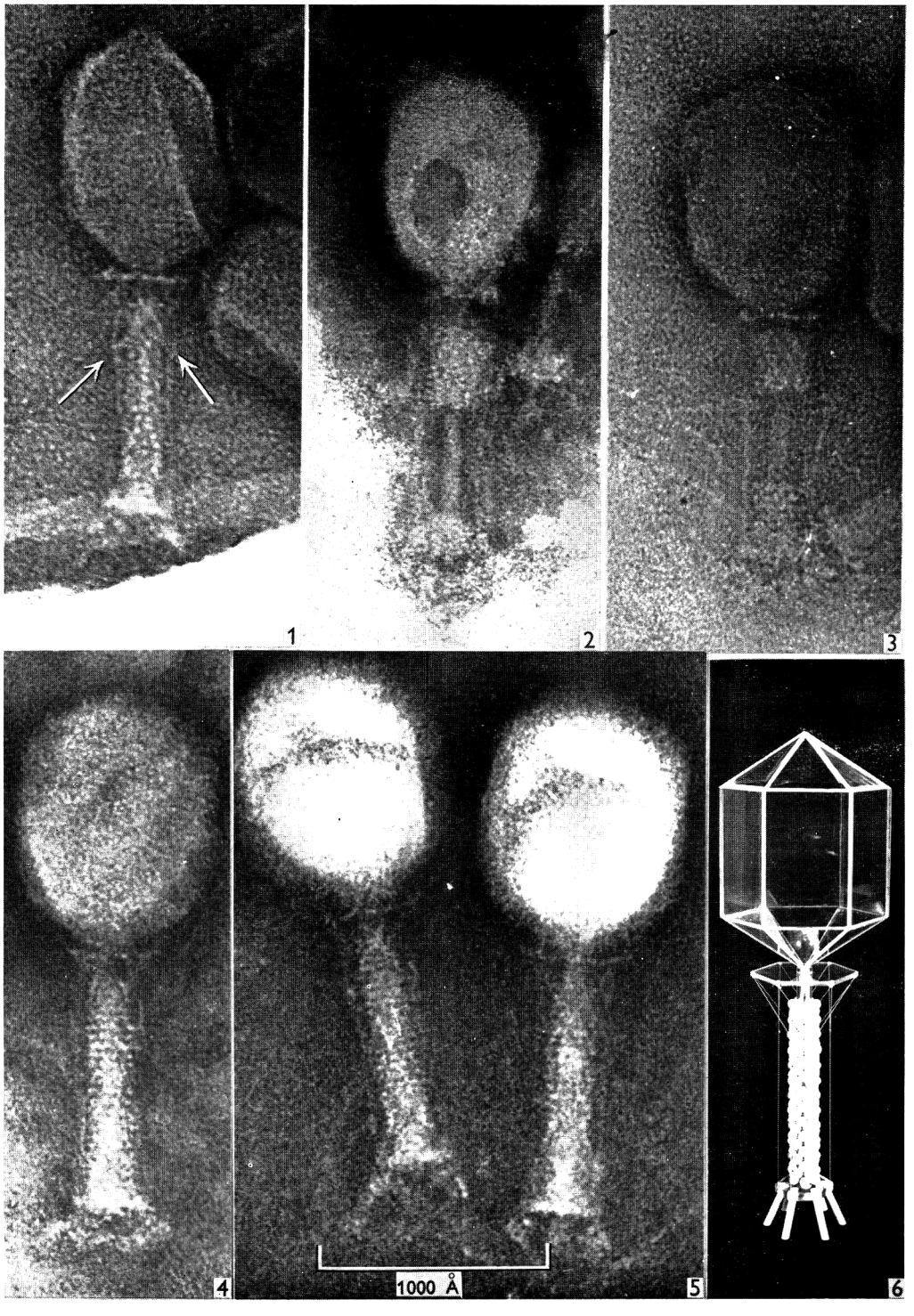
Fig. 2. Phage T4 in phosphotungstate, × 333,000.

Fig. 3. Phage T4 in sodium molybdate, × 333,000.

Fig. 4. Phage α1 in phosphotungstate, × 333,000.

Fig. 5. Phage 66t$^-$ in phosphotungstate, × 333,000.

Fig. 6. Model of phage T4, × 300,000.



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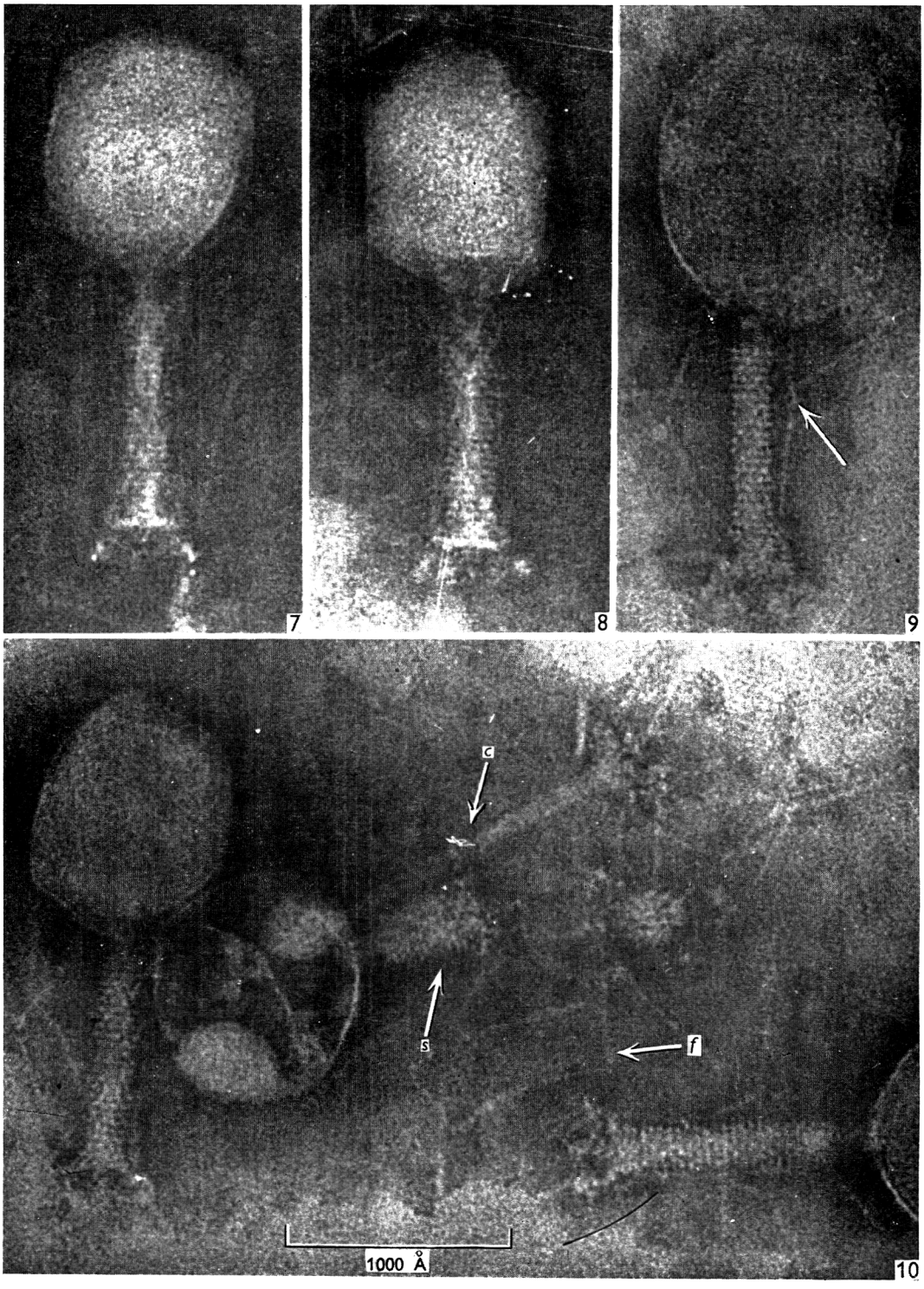

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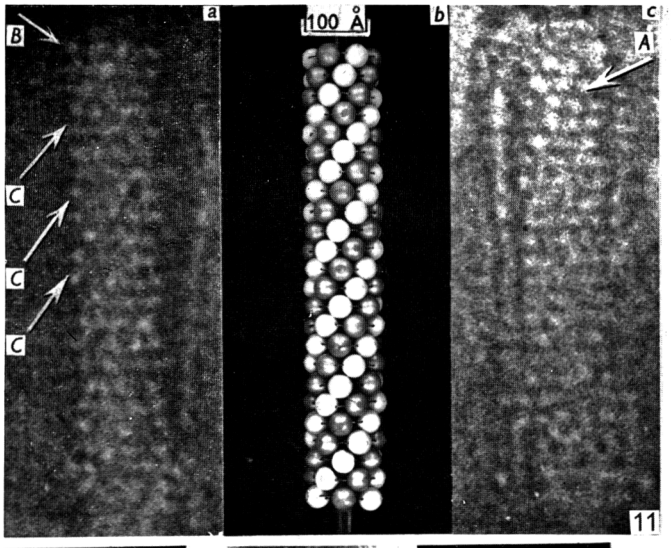
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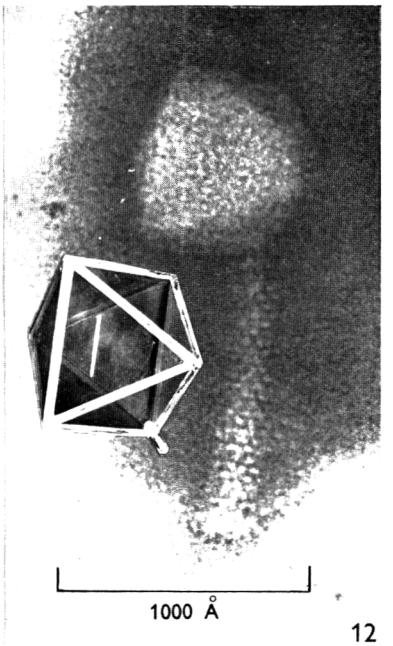
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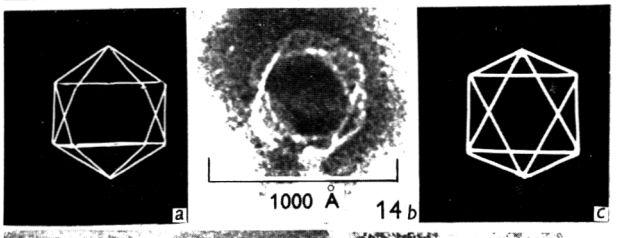
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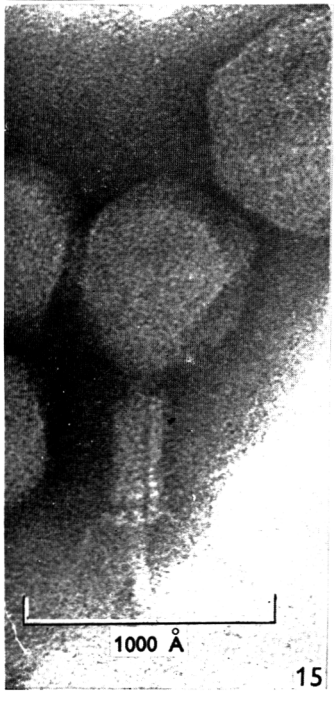
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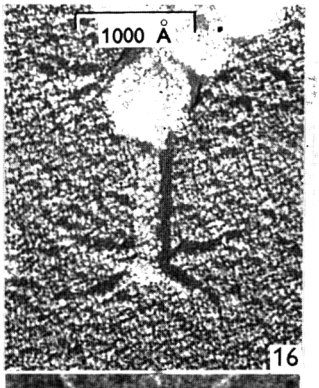
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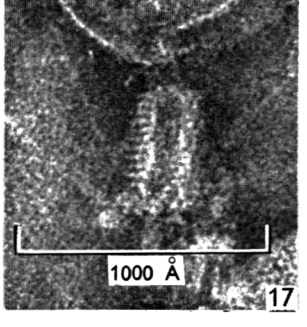
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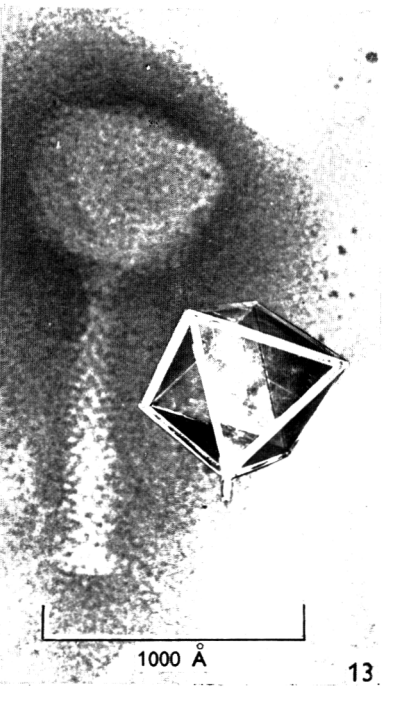
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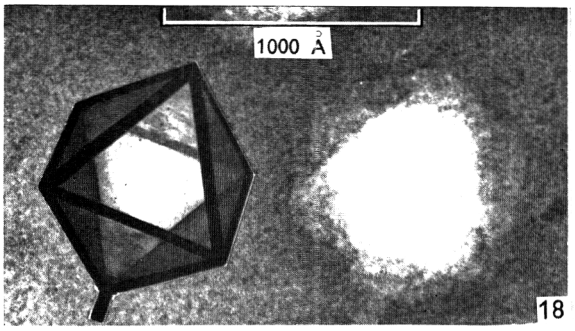
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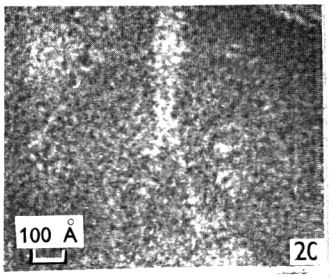
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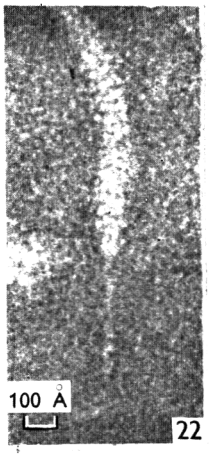
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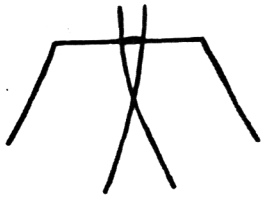
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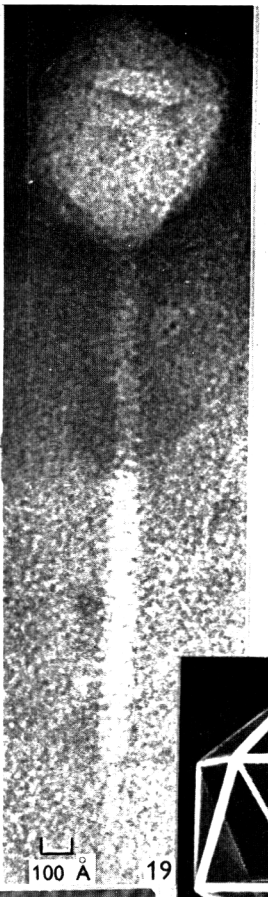
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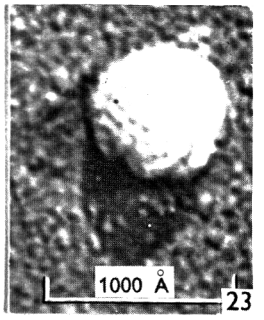
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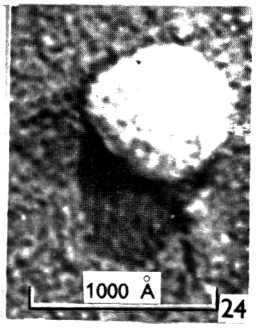
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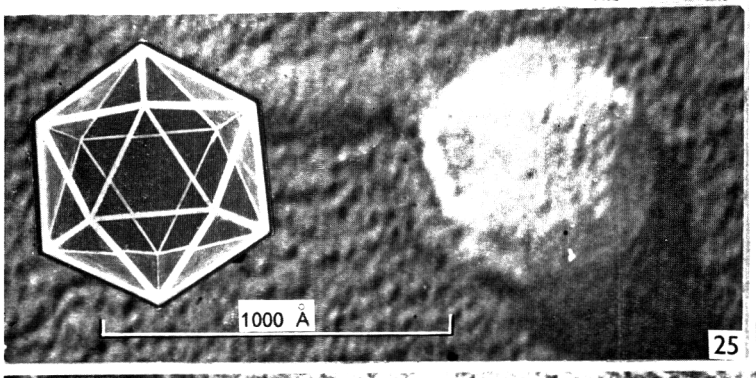
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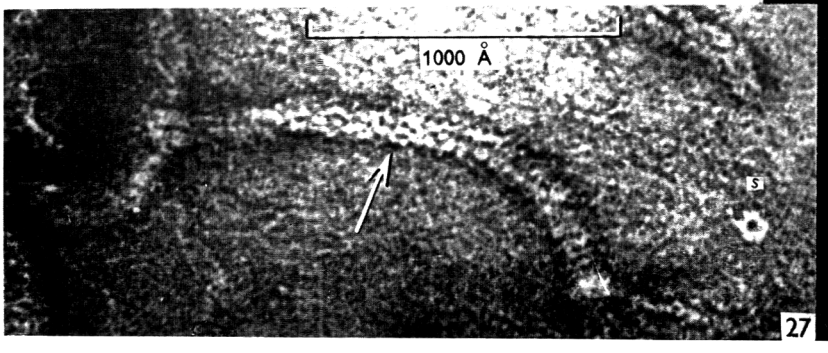
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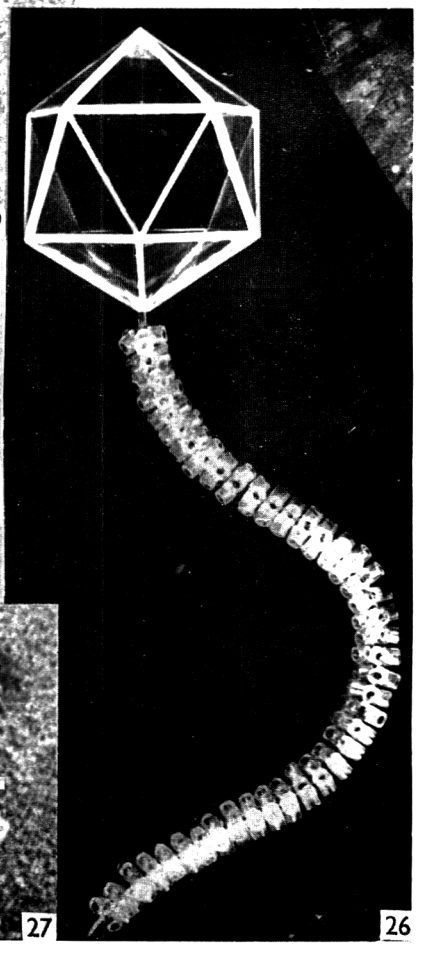
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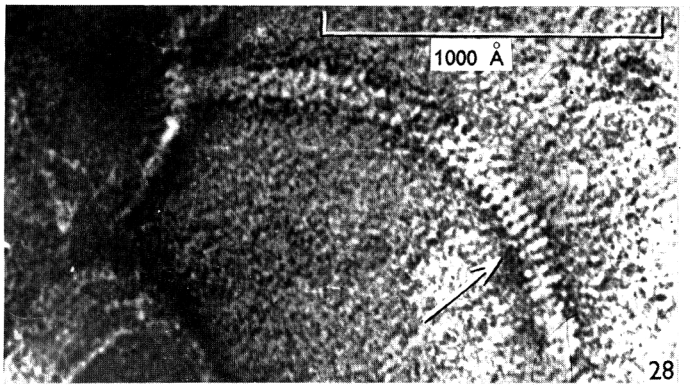
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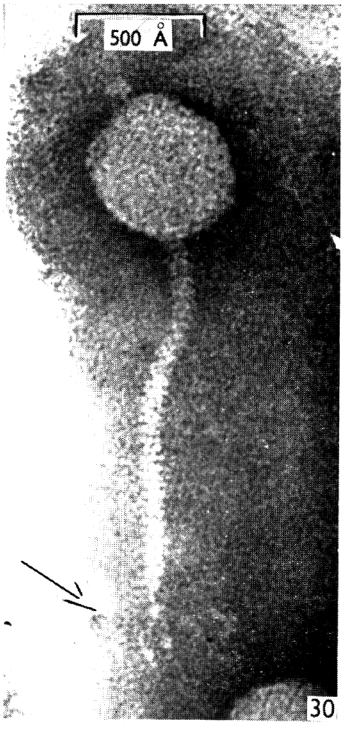
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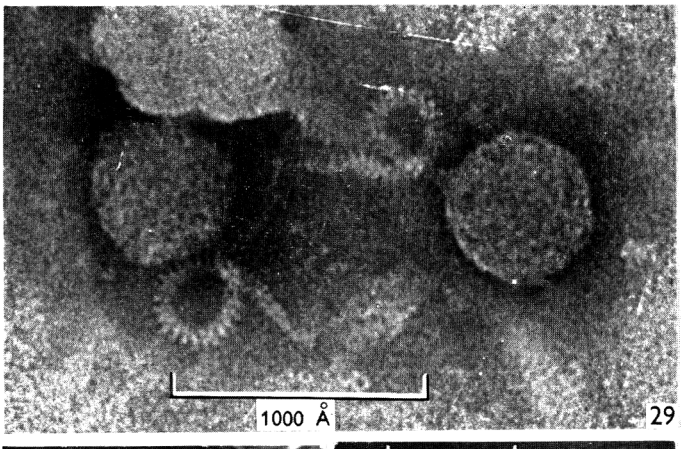
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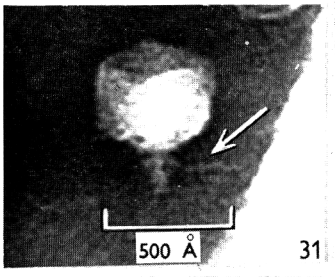
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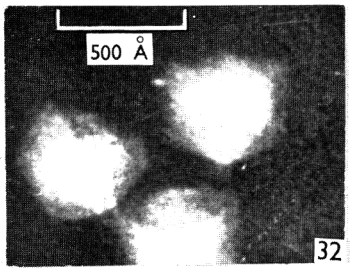
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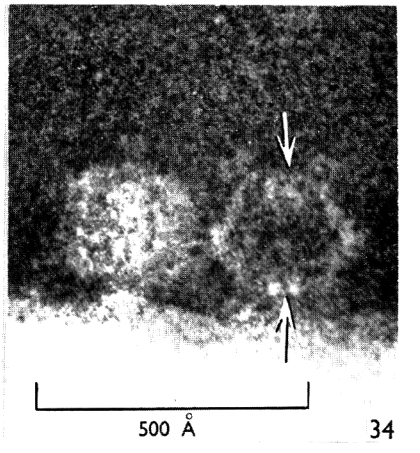
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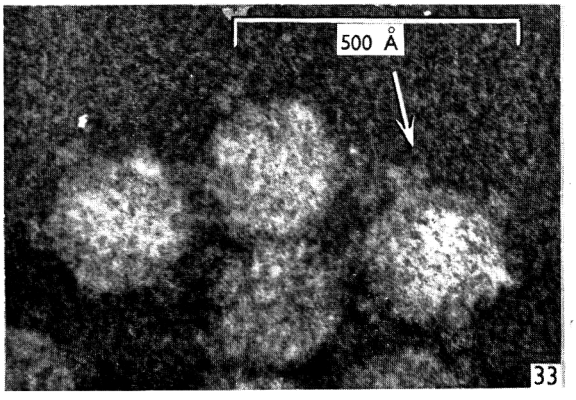
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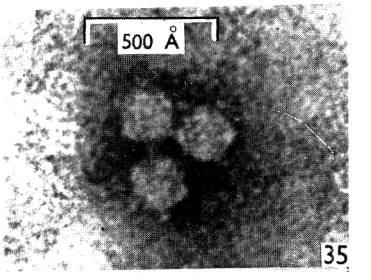
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PLATE 2

Figs. 7–9. Phage T2 in phosphotungstate, ×333,000 (fig. 8 by courtesy of Academic Press Inc.).

Fig. 10. Phage T2 in phosphotungstate, ×333,000; *c*, core; *s*, contracted sheath; *f*, tail fibres (by courtesy of Academic Press Inc.).

PLATE 3

Fig. 11. Extended sheaths, ×700,000, of *a*, T2; *b*, model; *c*, T4; (by courtesy of Academic Press Inc.).

Figs. 12, 13. Phage E1 in phosphotungstate, ×333,000.

Fig. 14. Head of phage ϕ2, ×250,000; *a*, wire model of icosahedron; *b*, empty phage head in phosphotungstate; *c*, wire model of octahedron.

Fig. 15. Phage E1 with contracted sheath (phosphotungstate) ×333,000.

Fig. 16. Phage E1 shadowed 2:1 with platinum, ×100,000.

Fig. 17. Contracted sheath of phage 66t$^-$, ×333,000.

PLATE 4

Figs. 18, 19. Phage C1 in phosphotungstate, ×333,000.

Fig. 20. Tail tip of phage F1, ×333,000.

Fig. 21. Drawing of tail tip of phage F1, ×333,000.

Fig. 22. Tail tip of phage C1, ×333,000.

Figs. 23, 24. Heads of phage T5 platinum/carbon shadowed at 2:1, ×250,000.

Fig. 25. Head of phage T5 platinum/carbon shadowed at 2:1, ×450,000.

Fig. 26. Model of phage T5.

Fig. 27. Phage T5 in uranyl acetate, ×375,000.

PLATE 5

Fig. 28. Phage T5 in uranyl acetate, ×375,000.

Fig. 29. Phage β4 in phosphotungstate, ×333,000.

Fig. 30. Phage γ2 in phosphotungstate, ×333,000.

Figs. 31, 32. Phage T3 in phosphotungstate, ×333,000.

Figs. 33, 34. Phage ϕR in phosphotungstate, ×750,000, fig. 34 by courtesy of *Nature, Lond.*

Fig. 35. Phage α3 in phosphotungstate, ×333,000, by courtesy of *Nature, Lond.*

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With 1 *plate*
Printed in Great Britain

Systematic Position of *Gluconobacter liquefaciens*

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(*Received* 17 *October* 1962)

SUMMARY

A strain of *Gluconobacter liquefaciens* was found to possess peritrichous flagella. Because of this and its biochemical similarity to *Acetobacter aceti*, the organism should be transferred to the genus *Acetobacter* as restricted by Leifson.

INTRODUCTION

Taxonomists prefer to divide the acetic acid bacteria, i.e. the genus *Acetobacter* as used by Beijerinck, into two genera. The organisms of one genus, *Acetobacter*, as restricted by Leifson, characteristically oxidize ethanol to carbon dioxide and water, via acetic acid; these organisms, when motile, possess peritrichous flagella. The other genus, *Acetomonas* Leifson (syn. *Gluconobacter* Asai), comprises those organisms which oxidize ethanol to acetic acid and no further (the suboxydans group of Frateur's 1950 classification) and which, when motile, possess polar flagella. The evidence for this division, on the basis of a correlation between type of flagellation and biochemical properties, may be seen in the papers of Leifson (1954) and Shimwell (1958). The position regarding the classification of these organisms has recently been reviewed by Carr & Shimwell (1961).

Gluconobacter liquefaciens Asai (1934) is one of three organisms reported by Asai & Shoda (1958) as possessing polar flagella and the ability to oxidize lactate and acetate to carbonate—a combination of characters intermediate between *Acetobacter* and *Acetomonas* as defined above. (Asai & Shoda used the generic name *Gluconobacter* as an approximate synonym for *Acetomonas* on the basis of earlier work by Asai, but it does not appear to have been either accurately defined or validly published.) The other two organisms were examined by Shimwell & Carr (1959) and found to possess not polar but peritrichous flagella. Stouthamer (1960) reported *G. liquefaciens* to possess peritrichous flagella but his photograph did not convince De Ley (1961). The organism has been used in several studies (De Ley & Dochy, 1960; De Ley, 1961; Stouthamer, 1961, 1962), in at least one of which (Joubert, Bayens & De Ley, 1961), its position appears to be anomalous. If indeed the flagellation is polar, the classification into *Acetobacter* and *Acetomonas* might be questioned. It seemed worth while to re-examine *G. liquefaciens* to see whether it possesses polar flagella, as reported by Asai & Shoda, or peritrichous flagella, as reported by Stouthamer. If it is in fact polarly flagellate it is intermediate between *Acetomonas* and *Acetobacter* and its existence would put in question the value of Leifson's (1954) classification of the acetic acid bacteria.

METHODS

Organism. A culture of the *Gluconobacter liquefaciens* strain studied by Asai (De Ley, 1961) was obtained from the laboratory of Professor De Ley through the kindness of Dr J. Schell. The culture was classified biochemically by using the scheme and methods of Frateur (1950).

Examination for flagella. Samples were prepared for examination in the light microscope by using Gray's flagella stain and for the electron microscope by shadowing with gold + palladium and viewing in an A.E.I. Electron Microscope EM6.

RESULTS

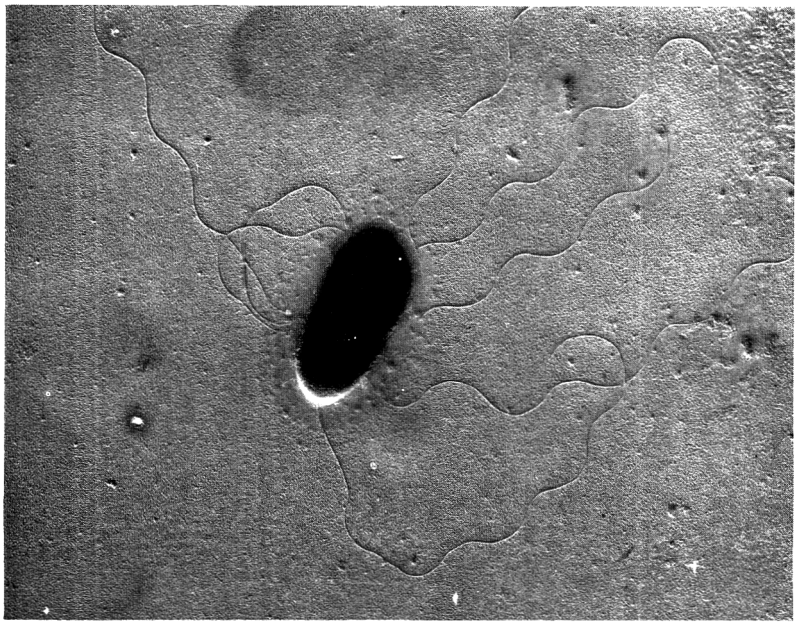
The organism examined was a Gram-negative rod showing a moderate degree of pleomorphism. It oxidized ethanol to acetic acid and thence to carbon dioxide and water. 'Irisation' was produced when the organism was grown on agar plates of Difco yeast extract + calcium lactate. It grew in Hoyer's medium, was catalase positive, produced acid from glucose and dihydroxyacetone from glycerol (ketogenic ability). The organism also produced a brown-black pigment when grown on agar slopes of Difco yeast extract + glucose + $CaCO_3$.

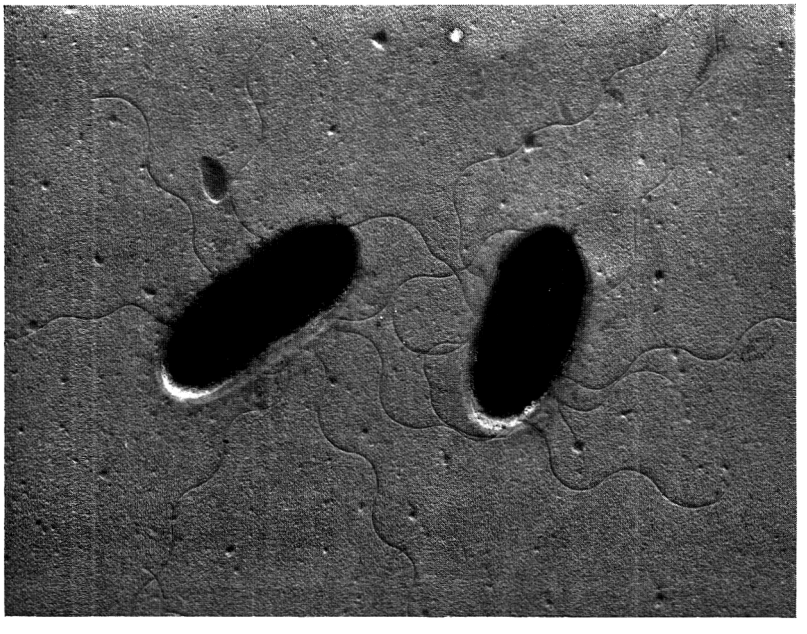
Visual and electron microscopy showed clearly the organism to possess peritrichous flagella; two of the electron micrographs are shown in the plate.

DISCUSSION

This work shows that the strain of *Gluconobacter liquefaciens* examined possesses both peritrichous flagella and the ability to oxidize ethanol beyond acetate, and it clearly belongs to the genus *Acetobacter* as restricted by Leifson. According to Frateur's classification it would be placed in the species *A. aceti*. However, it differs from *A. aceti* as defined by Frateur in producing a brown-black pigment, when grown on glucose + $CaCO_3$. The production of a pigment is characteristic of some *Acetomonas* strains. What taxonomic significance should be given to pigment formation is not known. There have been other reports of acetobacters (as defined above) as possessing polar flagella; but those which have been investigated have, as in the present case, been found to be incorrect. It is perhaps significant that there have been no reports of acetomonads which possess peritrichous flagella. The explanation might be that the rather few lateral flagella of *Acetobacter* may easily become detached and so convert a peritrichously flagellate organism into one which appears to be polarly flagellate. Although the possibility that an organism might possess both polar flagella and the ability to oxidize alcohol to completion cannot be ruled out, no such organism is known.

We are greatly indebted to Mr C. C. Newton of the Department of Electron Physics, University of Birmingham, for his technical assistance. One of the authors (P.J. le B.W.) would like to record his thanks to the Department of Scientific and Industrial Research for financial assistance.





REFERENCES

ASAI, T. (1934). Taxonomic studies of acetic acid bacteria and allied oxidative bacteria in fruits and a new classification of oxidative bacteria. *J. agric. chem. Soc. Japan*, **10**, 621, 731, 932, 1124. (In Japanese.)

ASAI, T. & SHODA, K. (1958). The taxonomy of *Acetobacter* and allied oxidative bacteria. *J. gen. appl. Microbiol. Japan*, **4**, 289.

CARR, J. G. & SHIMWELL, J. L. (1961). The acetic acid bacteria, 1941–1961. A critical review. *Antonie van Leeuwenhoek J. Microbiol. Serol.* **27**, 386.

DE LEY, J. & DOCHY, R. (1960). On the localisation of oxidase systems in *Acetobacter* cells. *Biochim. biophys. Acta*, **40**, 277.

DE LEY, J. (1961). Comparative carbohydrate metabolism and a proposal for a phylogenetic relationship of the acetic acid bacteria. *J. gen. Microbiol.* **24**, 31.

FRATEUR, J. (1950). Essai sur la Systématique des Acetobacters. *La Cellule*, **53**, 287.

JOUBERT, J. J., BAYENS, W. & DE LEY, J. (1961). The catabolism of amino acids by acetic acid bacteria. *Antonie van Leeuwenhoek J. Microbiol. Serol.* **27**, 151.

LEIFSON, E. (1954). The flagellation and taxonomy of species of *Acetobacter*. *Antonie van Leeuwenhoek J. Microbiol. Serol.* **20**, 102.

SHIMWELL, J. L. & CARR, J. G. (1959). The genus *Acetomonas*. *Antonie van Leeuwenhoek J. Microbiol. Serol.* **25**, 353.

SHIMWELL, J. L. (1958). Flagellation and taxonomy of *Acetobacter* and *Acetomonas*. *Antonie van Leeuwenhoek J. Microbiol. Serol.* **24**, 187.

STOUTHAMER, A. H. (1960). Koolhydraatstofwisseling van de Azijnzuubacteriën. Thesis, Utrecht.

STOUTHAMER, A. H. (1961). Glucose and galactose metabolism in *Gluconobacter liquefaciens*. *Biochim. biophys. Acta*, **48**, 484.

STOUTHAMER, A. H. (1962). Energy production in *Gluconobacter liquefaciens*. *Biochim. biophys. Acta*, **56**, 19.

EXPLANATION OF PLATE

Gluconobacter liquefaciens. Electron micrographs showing peritrichous flagella. × 10,000.

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The Effect of Water Activity, Solutes and Temperature on the Viability and Heat Resistance of Freeze-dried Bacterial Spores

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(*Received* 29 *October* 1962)

SUMMARY

Freeze-dried spores of *Bacillus megaterium*, *B. stearothermophilus*, *Clostridium bifermentans* and *C. botulinum* type E suffered little or no loss in viability after storage at 25° at water activity (a_w) values between 0·2 and 0·8. When stored over P_2O_5 (0·00 a_w) the spores of all four species showed a marked loss in viability. The above results were similar for spores whether stored in air or in vacuum. With spores stored over distilled water (1·00 a_w) the Bacillus spores underwent a large loss of viability in vacuum, but not in air; for spores of the clostridia the reverse was true. The addition of DL-glyceraldehyde, diacetyl or ribose (0·05 M) to the spore suspensions before drying caused increased death during storage at 0·50 a_w and to a lesser extent at 0·20 a_w. Death was greater at 30° than at 10°. The addition of sucrose, glutamate or semi-carbazide did not decrease the viability. When the dried spores were resuspended in dilute phosphate buffer after storage for 2–6 years their resistance to heating was greatest after storage at a_w values of 0·4, 0·6 and 0·8.

INTRODUCTION

Information about the retention of viability and heat resistance of spores under various storage conditions is important in culture preservation, in food and medical microbiology, and to increase our knowledge of the bacterial spore and its role in microbial ecology. It is known that spores retain well their viability and heat resistance under many conditions, both in the dry and wet state, but data about precise conditions for storage over extended periods are meagre and in many cases not very critical. Evans & Curran (1960), who made a comprehensive study of the effects of preheating, storage pH values and storage temperature on viability of spores in dilute phosphate buffer reviewed the early literature. In their studies Evans & Curran observed considerable loss in viability at pH 6·0 or less and at 30° and 37° by spores of aerobic species. Bullock & Lightbown (1947) studied the effects of moisture on the viability of *Bacillus subtilis* spores in dried peptone powder. In 32 days at a_w values of 0·0 and 0·32, no decrease in viability occurred. Further work (Bullock & Tallentire, 1952) showed that the spores stored at 0·0 a_w and 20° for 2 years retained 100% viability and did not begin germination on rehydration. At 0·66 a_w the spores still retained their viability after 250 days and did not begin germination on rehydration. At 0·78 a_w 80% of the spores in the rehydrated suspension began germination and were still viable. With storage at higher a_w values

the spores began germination within 20 days and became non-viable in peptone (0·90 and 1·00 a_w). Under very moist conditions, and depending on the media, the partially germinated spores multiplied (in peptone + lactose powder), died (in lactose powder) or remained viable (kaolin powder). Hawrylewicz, Gowdy & Ehrlich (1962) observed that spores of *Clostridium botulinum*, freeze-dried in the presence of crushed lava, showed greater decreases in viability in air and in vacuum than in a nitrogen atmosphere. The effect of storage conditions on the retention of the original heat resistance of spores (not the change to the heat-labile state as studied by Bullock & Tallentire (1952)) is not clear. Drying and then storage in the dry state or in liquid media have been reported either to increase or to decrease the heat resistance of the spores (Magoon, 1926; Williams, 1929). Magoon (1926) stored thin layers of sporulated cultures of *Bacillus mycoides* for up to 180 days at 0, 0·50 and 1·00 a_w at 10, 20 and 30°. Up to four-fold increases in heat resistance were observed during storage at 20° at these three a_w values. Sometimes this increase occurred within 30 days, but on continued storage the heat resistance decreased. The high degree of heat resistance was retained best at 20° at the 0·5 and 1·0 a_w values. In the present work the viability of freeze-dried spores of four organisms stored for periods up to 6 years in air or in vacuum at a_w values from 0·00 to 1·00 has been followed. The opportunity was also taken to determine in several cases the degree of heat resistance of the spores after rehydration.

METHODS

The following organisms were used: *Bacillus megaterium* strain c1 (Knaysi), *B. stearothermophilus* ATCC 7953, *Clostridium bifermentans* Weinberg 226, *C. botulinum* Type E, ATCC 9564.

Spores of *Bacillus megaterium* were grown in potato infusion (Robinow, 1951) in shake flasks at 30°, and those of *B. stearothermophilus* at 50° in this medium at half-strength supplemented with a salts mixture (Ohye & Murrell, 1962). Spores of *Clostridium bifermentans* were grown at 30° in cooked meat medium (Dubovsky & Meyer, 1922), and *C. botulinum* type E at 25° in a mixture of 4 vol. papain meat digest broth (Asheshov, 1941) + 1 vol. of peptic meat digest (Dubovsky & Meyer, 1922) + 0·05% (w/v) sodium thioglycollate. On the completion of spore formation, the spores were harvested on the centrifuge, washed 6 times with water, and any remaining viable vegetative forms were killed by heating for 10–20 min. at a temperature sublethal to the spores (15–20° above the maximum growth temperature of the particular species).

Samples (0·2 ml.; containing about 10^7 spores) of the spore suspensions were dispensed in lightly plugged Pyrex tubes (9 × 88 mm.), freeze-dried, and the tubes sealed inside larger tubes (150 × 16 mm.) containing about 0·5 ml. of a salt solution or a saturated salt solution of known water activity a_w (Robinson & Stokes, 1955) or P_2O_5. In some experiments ampoules were prepared as described by Murrell & Scott (1957). The tubes were either sealed without evacuation or after evacuation to about 0·02 mm. Hg pressure. The tubes were stored in the dark at 25°. The effect of solutes was studied by adding to the spore suspension before drying, aqueous solutions to give the desired concentration in the undried suspension. Where necessary solutions were neutralized with NaOH.

At intervals during storage single tubes of each treatment were removed, opened, the spores rehydrated with 2 ml. 0·05 M-phosphate buffer (pH 7) and viable counts made. Viable counts of *Bacillus megaterium* and *B. stearothermophilus* were made on glucose Tryptone agar plates (in triplicate) at 30° and 50°, respectively. Viable counts of the clostridia were made at 30° in oval tubes containing pork infusion agar (Brewer, 1940) + 0·1 % (w/v) sodium mercaptoacetate (thioglycollate).

The heat resistance of the spores was determined by diluting the 2 ml. sample of rehydrated suspension from one ampoule to 20 ml. with phosphate buffer and heating 1·5 ml. samples of this in ampoules (9 × 75 mm.) sealed in air for suitable times at selected temperatures controlled to ± 0·05°. The survivors were determined by viable counts of appropriate dilutions of the heated suspensions, and survivor curves were plotted (see Fig. 5). The reproducibility of estimates of heat resistance of spore suspensions from replicate ampoules is shown in Table 4. The rate of decrease in viability was expressed as decimal reduction times (D values) both for the storage-viability and heat-resistance studies. The average D values were calculated from the regression coefficients for the linear regressions of log. survivors against time.

RESULTS

The moisture contents of the spores during storage at the various a_w values are given in Table 1. These values are the ranges for spores of six bacterial species obtained from water sorption isotherms at 25° (Marshall, in preparation).

Table 1. *The moisture content of spores at various a_w values at 25°*

The figures for the moisture content ranges are for the spores of six bacterial species (Marshall, in preparation).

a_w value	Moisture content (% dry wt.)	a_w value	Moisture content (% dry wt.)
0·00	0	0·60	9·5–16·0
0·10	4·8– 8·2	0·70	12·2–19·8
0·20	5·5–10·2	0·80	12·1–25·5
0·30	6·0–11·7	0·90	38·5–57·0
0·40	7·3–12·4	1·00	40 –88
0·50	8·5–12·6		

Viability of spores during 25° storage at various a_w values

The results with four species are given in Figs. 1–4; in Fig. 4 the results for two species mixed together are recorded.

Bacillus megaterium. During a storage period of more than 6 years at a_w values of 0·22, 0·43, 0·62 and 0·80, in air or in vacuum there was no significant decrease in viability (Fig. 1). However, at 0·00 a_w the rate of death was substantial both in air and in vacuum. During the first 40 weeks the viable counts decreased by about four log. units, subsequently the rates of destruction decreased appreciably. In other experiments *B. megaterium* spores were stored in vacuum at six a_w values between 0·75 and 1·00 for about 120 weeks, but at the high a_w values the spores were only slightly less stable than between 0·22 and 0·80 a_w. The viable counts of spores stored over pure water, however, decreased by a little over two log. units after two years in

vacuum. Of the spores stored at a_w values above 0·8 60–90 % usually stained with dilute crystal violet added after rehydration (see Bullock & Tallentire, 1952).

Bacillus stearothermophilus. The spores of this organism showed only small decreases in viability with any storage treatment, except in the treatments in vacuum at a_w 1·00, where the viable counts decreased rapidly (Fig. 2). At 0·00 a_w in air the viable counts decreased by over one log. unit in the first 40 weeks but thereafter remained constant; at 0·00 a_w in vacuum the initial decrease was less marked.

Clostridium bifermentans. Under conditions of extreme dryness (a_w 0·00) both in

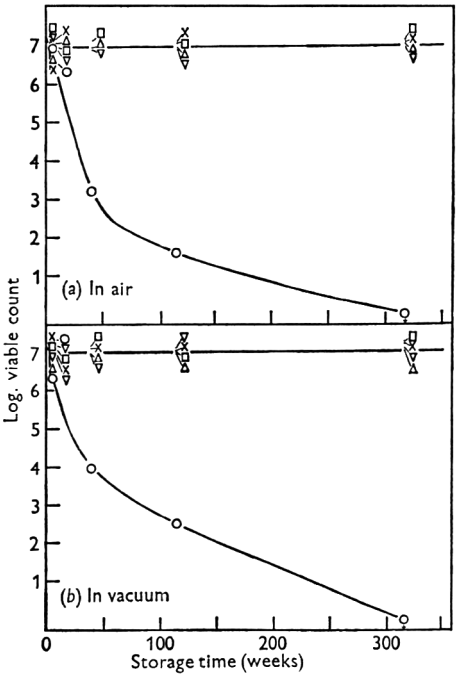

Fig. 1

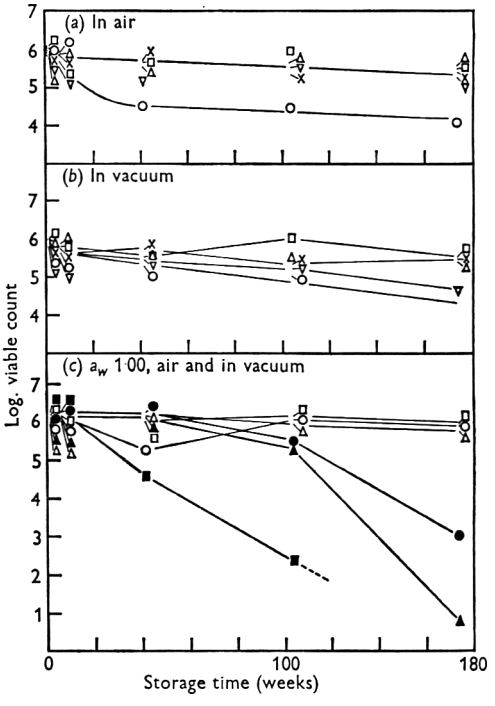

Fig. 2

Fig. 1. Survival of spores of *Bacillus megaterium* during storage in air and in vacuum at 25° at various a_w values. ○ 0·00 a_w; △, 0·22 a_w; □, 0·43 a_w; ×, 0·62 a_w; ▽, 0·80 a_w. The regressions for a_w values (excluding 0·00 a_w) were not significantly different, and the means for air and in vacuum were not significantly different from each other or from zero.

	Mean regression coefficient ($\times 10^3$)	S.E. ($\times 10^3$)	D.F.
Air	−0·021	0·096	18
In vacuum	−0·028	0·039	29

Fig. 2. Survival of spores of *Bacillus stearothermophilus* during storage at 25° at various a_w values. In (*a*) and (*b*), ○, 0·00 a_w; △, 0·22 a_w; □, 0·43 a_w; ×, 0·62 a_w; ▽, 0·80 a_w. In (*c*) open symbols, storage in air; closed symbols, storage in vacuum; □, 1·00 a_w, undried; △, 1·00 a_w, rehydrated in water; ○, 1·00 a_w, equilibrated through vapour phase. The regressions for air treatments (excluding 0·00 a_w) were not significantly different; their mean value was significant ($-0{\cdot}00034 \pm 0{\cdot}00016$), giving a D value of 2900 days with 95 % fiducial limits of 1500 and 50,000 days. The regressions for 0·22, 0·43, 0·62 and 0·80 a_w values in vacuum were not significantly different, and their mean value was not significantly different from zero.

air and in vacuum the decrease in viability was rapid (Fig. 3). Little killing occurred at the 0·22, 0·43, 0·62 and 0·80 a_w values in air or in vacuum although the viable counts were generally slightly less after storage in air than after storage in vacuum. The death rate in air was slightly greater than that in vacuum but not significantly so (see legend, Fig. 3). At a_w 1·00 the decrease in viability was more rapid in air than in vacuum, particularly when the spores were suspended in liquid water.

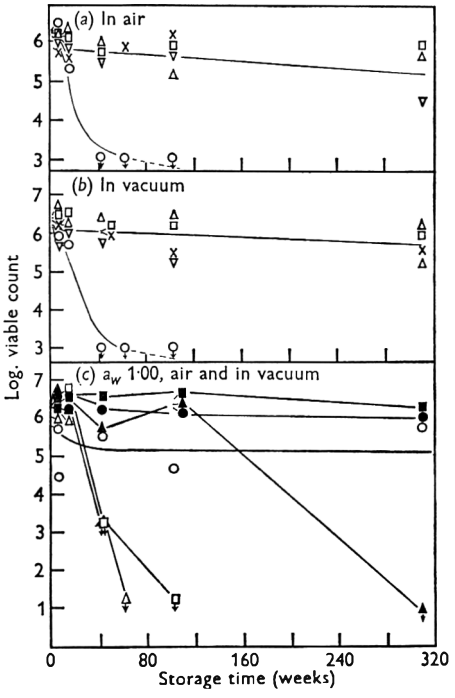

Fig. 3

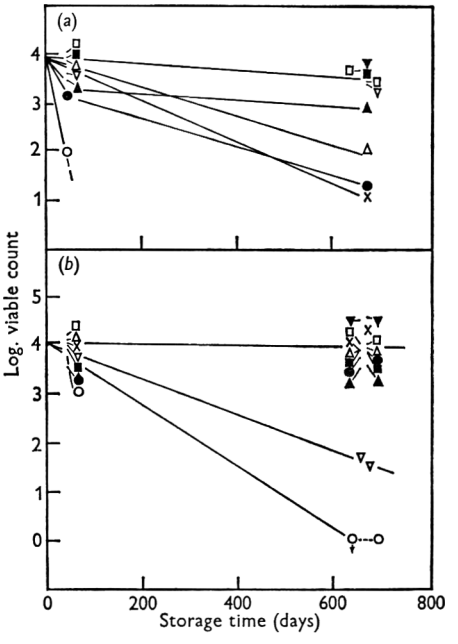

Fig. 4

Fig. 3. Survival of spores of *Clostridium bifermentans* at 25° at various a_w values. In (*a*) and (*b*), ○, 0·00 a_w; △, 0·22 a_w; □, 0·43 a_w; × ,0·62 a_w; ▽, 0·80 a_w. In (*c*) open symbols, in air; closed symbols, in vacuum; □, 1·00 a_w undried; △, 1·00 a_w rehydrated in water; ○, 1·00 a_w equilibrated through vapour phase. The regressions at the a_w values 0·22, 0·43, 0·62 and 0·80 in air or in vacuum were not significantly different and the mean value in air was not significantly different from that in vacuum.

	Mean regression coefficient ($\times 10^3$)	S.E. ($\times 10^3$)	D (days)	Fiducial limits (95 %)
In air	−0·310	0·097	3,226	1970 – 8850
In vacuum	−0·198	0·087	5,051	2670 – 47,600

Fig. 4. Survival of spores of (*a*) *Clostridium botulinum* type E and (*b*) *Bacillus stearothermophilus* dried together, at 25° in vacuum at various a_w values: ○, 0·00; ●, 0·05; △, 0·10; ▲, 0·20; □, 0·40; ■, 0·60; ×, 0·80; ▽, 0·90; ▼, 1·00 (liquid phase).

Clostridium botulinum Type E with *Bacillus stearothermophilus*. Spores of these two species were mixed together in suspension and, therefore, freeze-dried and equilibrated under identical conditions. A considerable decrease in viability of the *C. botulinum* type E spores occurred in 2 years, especially at the very low a_w values

(Fig. 4). On the other hand, the spores of *B. stearothermophilus* showed little decrease in viability except at a_w values 0·00 and 0·90.

Effect of storage at various a_w values on the heat resistance of spores. The effect of storage on the heat resistance of rehydrated spores was tested with three species (Tables 2–4). It is evident from Table 3 that storage for 6 years at the a_w values of 0·22, 0·43, 0·62 and 0·80 had very little adverse effect on the heat resistance of

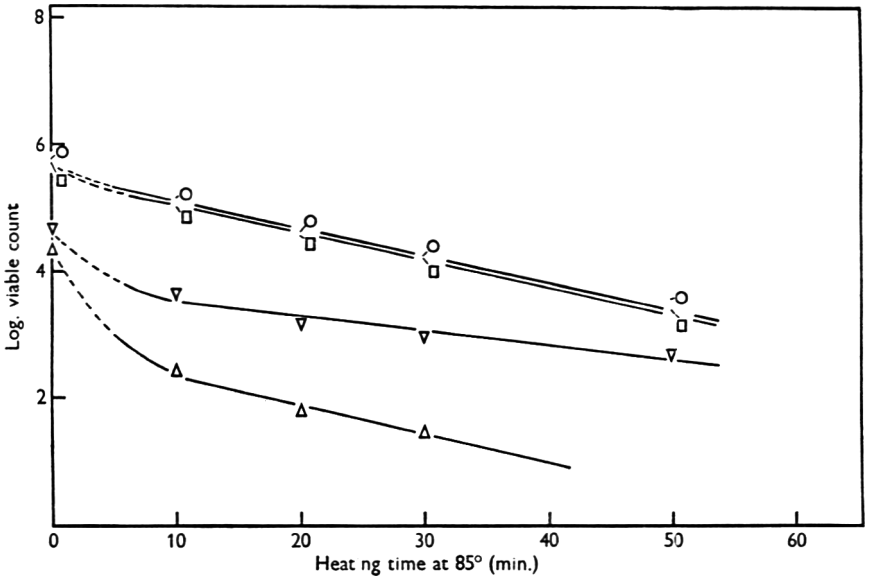

Fig. 5. Survival curves of rehydrated spores of *Bacillus megaterium*. Spores stored for 320 weeks at various a_w values before rehydration. Storage in vacuum at a_w values: ○, 0·22; □, 0·43; ▽, 0·62; in air: △, 0·62.

Table 2. *Heat resistance of rehydrated spores of* Clostridium bifermentans *after storage at various a_w values for* 310 *weeks*

Storage atmosphere	At a_w values of: 0·22	0·43	0·62	0·80	1·00		Original suspension
					Vapour phase	Undried	
	$D_{95°}$ values*						
Air	8·5 ± 0·8	9·9 ± 0·2	—	26·3 ± 7·6	2·9 ± 0·2	—	12·5 ± 0·5
Vacuum	14·4 ± 0·2	17·6 ± 0·7	20·8 ± 0·2	8·2 ± 0·2	9·8 ± 0·3	3·2	—

* Mean D values (decimal reduction time) in min. at temperature indicated of spores resuspended in 0·05 M-phosphate buffer (pH 7) sealed in air. Mean D values calculated from the regression coefficient ± S.E. for the regression of log. viable count on time.

Bacillus megaterium spores. Storage in vacuum, however, showed a slight but definite superiority over storage in air. The survival curves for *B. megaterium* (Fig. 5) showed an initial rapid decrease in several instances, which was due to the death of the spores in which germination had been initiated. This was confirmed by micro-

scopic observations of the presence of stained spores. The viable counts of unheated preparations, therefore, were excluded from the estimations of D values.

With *Clostridium bifermentans* spores also, storage in vacuum resulted in better retention of heat resistance (Table 2). In several instances with this species and with *Bacillus megaterium* (Table 3), an increase in heat resistance was observed, which was either a result of the drying process or of changes during storage (see Magoon, 1926). *B. stearothermophilus* spores in the mixed preparation with *C. botulinum* spores did not retain their heat resistance nearly as well as spores of the above two species (Table 4). This may, however, have been a consequence of their admixture with spores of *C. botulinum*. The viable counts of spores of *C. botulinum* type E had here decreased too much to enable satisfactory determinations of their heat resistance.

Table 3. *Heat resistance of rehydrated spores of* Bacillus megaterium *after storage at different a_w values for* 320 *weeks*

Storage atmosphere	Storage a_w values: 0·22	0·43	0·62	0·80	Original suspension
	$D_{85°}$ values				
Air	18·0 ± 2·7	24·8 ± 7·1	21·9 ± 3·8	9·10 ± 0·66	23·0 ± 2·4
Vacuum	22·9 ± 1·9	24·4 ± 1·0	47·3 ± 9·5	33·8 ± 12·1	—

Table 4. *Heat resistance of rehydrated spores of* Bacillus stearothermophilus *after storage of* 96 *weeks in vacuum at various a_w values*

	Storage a_w values: 0·05 (i)*	0·05 (ii)	0·10 (i)	0·10 (ii)	0·20 (i)	0·20 (ii)	0·40 (i)	0·40 (ii)	0·60 (i)	0·60 (ii)	0·80	0·90	1·00 (i)	1·00 (ii)	Original suspension†	
$D_{115°}$ value‡	11·5	7·0	8·7	9·7	13·6	12·2	16·8	12·4	9·7	6·7	7·7	8·9	11·3	11·0	22·2	(31·7)§
Limits (±)	0·9	2·2	1·0	1·0	1·0	1·9	2·1	0·4	0·9	1·2	—	0·8	1·2	0·5	0·6	(0·0)

* (i) and (ii) refer to suspensions derived from separate storage ampoules. † Suspension before storage. ‡ D value, mean, min. at 115°. § Heat resistance of the rehydrated freeze-dried spores before storage. —, Regression not significant.

Effect of solutes and storage temperatures on the viability of spores stored at 0·20 *and* 0·50 a_w. Various solutes have been shown to affect the viability of freeze-dried vegetative forms during storage and rehydration (Scott, 1958; Leach & Scott, 1959; Scott, 1960). It is not known what effect such substances may have on spore viability. Two experiments with two species were carried out to test the effect of some of these substances. In the first experiment, sucrose (0·25 M), glutamic acid (0·25 M), semicarbazide (0·05 M) and ribose (0·05 M) were added separately and in various mixtures before freeze-drying, to suspensions containing spores of both *Bacillus megaterium* and *B. stearothermophilus*. These solutes had no apparent effect on the viability of the spores during storage for 81 weeks at 10° and 30°.

In the second experiment the solutes DL-glyceraldehyde, ribose and diacetyl were tested (each at 0·05 M) again with both species of spore dried together. Glyceraldehyde decreased the viable count of both organisms by 6 log. units in 81 weeks at 30°

(Figs. 6, 7). The rate of decrease in viability was most rapid at the higher storage temperature and at the higher a_w value with both organisms. After the initial phase of rapid decrease in viability the rate of decrease in viability was less at the higher temperature. Diacetyl caused a rapid decrease in viability of up to 2 log. units within 10 days at the higher a_w value and temperature, with spores of both species; thereafter the viable count remained fairly constant (Figs. 6, 7). Ribose caused a definite decrease in viability, which was more pronounced at the higher temperature and with spores of *Bacillus megaterium* (Figs. 6, 7).

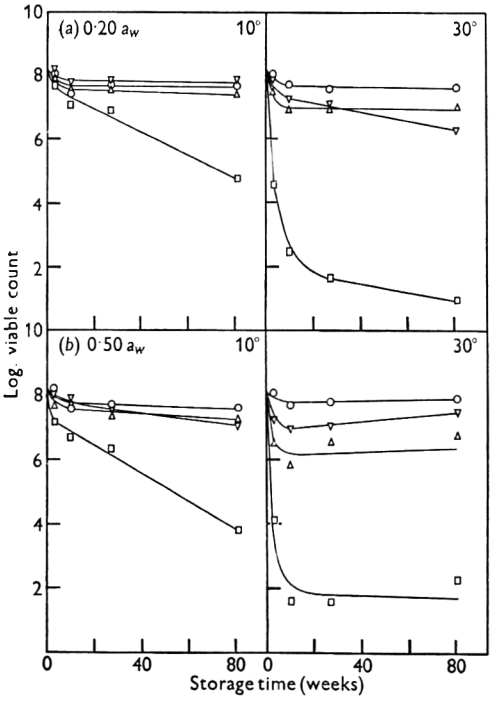

Fig. 6

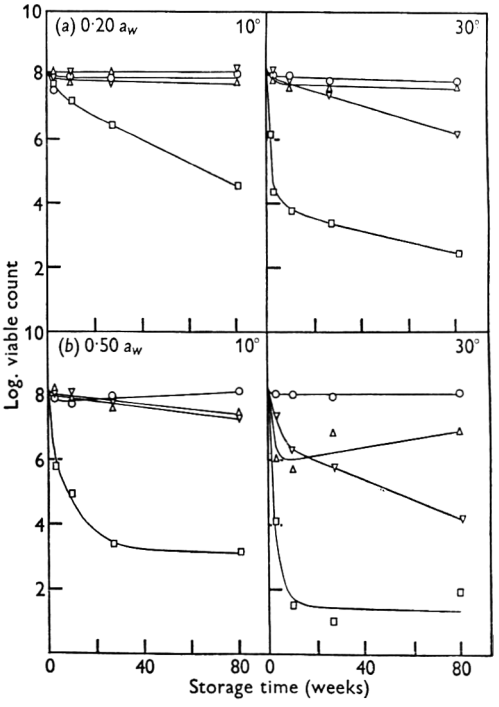

Fig. 7

Fig. 6. The effect of solutes, storage temperature and a_w value on the viability of dried spores of *Bacillus stearothermophilus* stored in vacuum. ○, water; □, DL-glyceraldehyde; △, diacetyl; ▽, D-ribose.

Fig. 7. The effect of solute, temperature and a_w value on the viability of dried spores of *Bacillus megaterium* stored in vacuum. ○, water; □, DL-glyceraldehyde; △, diacetyl; ▽, D-ribose.

DISCUSSION

Three points emerge from these experiments. First, spores of four bacterial species when stored under conditions of extreme dryness in air or in vacuum showed a marked decrease in viability. The cause of this instability has not been investigated, but it is interesting to note that the spores differed from vegetative organisms which at 0·00 a_w lost viability much more rapidly in air than in vacuum (Scott, 1958; Lion & Bergmann, 1961). Spores heated or irradiated under very dry conditions have also been shown to be less resistant to heat (Murrell & Scott, 1957) and to irradiation (Tallentire, 1958) than when similarly treated under moister conditions.

Secondly, the aerobic species showed instability in vacuum at a_w 1·00 and the anaerobic organisms instability in air at this a_w value (Figs. 2, 3). The understanding of this observation is complicated by the fact that under these moist conditions a high proportion of the spores showed initiation of germination in the rehydrated suspension. The possibility, therefore, exists that the aerobic spores which had begun germination lost viability in the absence of oxygen, and the spores of the anaerobes in which germination had been initiated became non-viable because of the presence of oxygen. An alternative but less likely interpretation is that the ungerminated aerobic spores under these moist conditions needed oxygen to respire and to retain viability. This interpretation may be more applicable to the facultative anaerobic thermophile (*Bacillus stearothermophilus*) which would be much less likely to begin germination at 25° at a_w 1·00 in vacuum.

Thirdly, it is evident that certain low molecular weight solutes were able to induce death of bacterial spores stored at water activities which were otherwise favourable for retention of viability and heat resistance. The active solutes were carbonyl compounds: this suggests that the Maillard reaction postulated by Scott (1960) may also be a mechanism which causes death of dry spores. In the case of spores the permeability of the spores to the solutes may be of considerable importance. If the solutes do not reach the spore protoplasm before freeze-drying, then they may have no effect on the retention of viability in the dry state. This may explain why ribose was less active than glyceraldehyde. The reaction involved in loss of viability in the presence of these solutes was accelerated at the higher temperature and a_w values.

It may be concluded from these studies that freeze-dried spores of some species do not survive well under extremely dry or very moist conditions, and that for maximum retention of viability and heat resistance storage in air or in vacuum at a_w values of 0·2–0·8 is recommended. In general at these a_w values storage in vacuum was superior to storage in air for retention of the original heat resistance of the spores.

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REFERENCES

ASHESHOV, I. N. (1941). Papain digest media and standardisation of media in general. *Canad. J. publ. Hlth*, **32**, 468.

BREWER, J. H. (1940). Clear liquid medium for the 'aerobic' cultivation of anaerobes. *J. Amer. med. Ass.* **115**, 598.

BULLOCK, K. & LIGHTBOWN, J. W. (1947). The inactivation of enzymes and micro-organisms in oils and powders. Part II. The effects of drying on the viability of bacteria, and their thermolability in powders. *Quart. J. Pharm.* **20**, 312.

BULLOCK, K. & TALLENTIRE, A. (1952). Bacterial survival in systems of low moisture content. Part IV. The effects of increasing moisture content on heat resistance, viability and growth of spores of *B. subtilis*. *J. Pharm., Lond.* **4**, 917.

DUBOVSKY, B. J. & MEYER, K. F. (1922). An experimental study of the methods available for the enrichment, demonstration, and isolation of *B. botulinus* in specimens of soil and its products, in suspected food, in clinical and necropsy material. *J. infect. Dis.* **31**, 501.

EVANS, F. R. & CURRAN, H. R. (1960). Influence of preheating, pH, and holding temperatures upon viability of bacterial spores stored for long periods in buffer substrates. *J. Bact.* **79**, 361.

HAWRYLEWICZ, E., GOWDY, B. & EHRLICH, R. (1962). Micro-organisms under a simulated Martian environment. *Nature, Lond.* **193**, 497.

LEACH, R. H. & SCOTT, W. J. (1959). The influence of rehydration on the viability of dried micro-organisms. *J. gen. Microbiol.* **21**, 295.

LION, M. B. & BERGMANN, E. D. (1961). The effects of oxygen on freeze-dried *Escherichia coli*. *J. gen. Microbiol.* **24**, 191.

MAGOON, C. A. (1926). Studies upon bacterial spores. I. Thermal resistance as affected by age and environment. *J. Bact.* **11**, 253.

MURRELL, W. G. & SCOTT, W. J. (1957). Heat resistance of bacterial spores at various water activities. *Nature, Lond.* **179**, 481.

OHYE, D. F. & MURRELL, W. G. (1962). Formation and structure of the spore of *Bacillus coagulans*. *J. Cell Biol.* **14**, 111.

ROBINOW, C. F. (1951). Observations on the structure of Bacillus spores. *J. gen. Microbiol.* **5**, 439.

ROBINSON, R. A. & STOKES, R. H. (1955). *Electrolyte Solutions*. Appendix 8.11, p. 490. London: Butterworths.

SCOTT, W. J. (1958). The effect of residual water on the survival of dried bacteria during storage. *J. gen. Microbiol.* **19**, 624.

SCOTT, W. J. (1960). A mechanism causing death during storage of dried micro-organisms. In *Recent Research in Freezing and Drying*, p. 188. Edited by A. S. Parkes & A. U. Smith. Oxford: Blackwell.

TALLENTIRE, A. (1958). An observed 'oxygen effect' during gamma-irradiation of dried bacterial spores. *Nature, Lond.* **182**, 1024.

WILLIAMS, O. B. (1929). Heat resistance of bacterial spores. *J. infect. Dis.* **44**, 421.

J. gen. Microbiol. (1963), **31**, 461–469
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Some Observations on the Nitrogen Metabolism of Rumen Proteolytic Bacteria

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SUMMARY

Isolates from the rumens of sheep of presumptively identified *Bacteroides amylophilus*, *B. ruminicola*, species of Bacteroides, Selenomonas, Butyrivibrio, Bacillus, Eubacterium, Clostridium and Gram-positive cocci were found to be proteolytic. Some of these strains also had exopeptidase and amidase activity, but significant deaminase activity was rare. Most of the strains preferentially utilized ammonia in synthesizing cellular constituents in media containing preformed amino acids. Few of the strains had urease activity.

INTRODUCTION

In a survey of the proteolytic bacteria from the sheep rumen (Blackburn & Hobson, 1962) it was found that proteolytic activity was possessed by strains of many kinds of bacteria. Proteolytic bacteria were selected on the basis of their capacity to produce some visible change in casein agar roll tubes, and overall proteolytic activity in liquid cultures was measured by determining the decrease in trichloroacetic acid-precipitated protein. The cultures isolated were not stored for more detailed investigation partly because of the difficulty of keeping many of the bacteria viable over prolonged periods. For the present work proteolytic strains of bacteria were re-isolated to provide further confirmation that the previously described bacteria are found in the rumen at all times, and the nitrogen metabolism was investigated in more detail. Previously it was found that a well-reduced medium was needed for good growth of proteolytic bacteria, and for most isolations media containing cysteine + dithionite were used, although media with cysteine alone were used initially. In the present work both types of media were used. As before, media containing a number of carbohydrates were used, to ensure that as large a number as possible of bacterial types grew. Since, as previously noted, proteolytic activity in liquid medium did not always coincide with a visible change in a solid casein medium, representatives of all colonial types which grew in the primary media were tested for proteolysis in liquid media.

METHODS

Sheep. Isolations were made from five sheep; sheep 6 was fed on a partially-defined casein diet, sheep 190, 74, 93 and 95 on hay-concentrate diets (Blackburn & Hobson, 1962). Sheep 95 by special experimental procedure contained no protozoa (Eadie & Hobson, 1962).

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Isolation and identification. The methods used were as previously described (Blackburn & Hobson, 1962), with the following modifications. Carbohydrates were added at 0·1 % concentration to discourage colonies from spreading. Isolation media 1D and 1C, 2D and 2C, 3D and 3C, 4D and 4C contained, respectively, no carbohydrate, carboxymethyl cellulose, glucose, and a mixture of xylose, maltose and cellobiose. Media designated D were reduced by cysteine + dithionite, media designated C were reduced by cysteine alone. Colony development was followed, any changes produced in the casein noted, and finally representatives of all colony types subcultured on agar slopes, a count being made of all similar colony types. The morphology of the organisms growing on the slopes was examined and the strains were inoculated into casein liquid medium to determine their proteolytic activity (Blackburn & Hobson, 1960*b*). Only proteolytic strains were examined further.

Nitrogen metabolism. This was studied in a basal medium to which various additions were made as indicated in Table 1. The media were incubated for 4 days before analysis.

Table 1. *Additions to the basal medium used in studying the nitrogen metabolism of proteolytic bacteria*

Additions and concentration	Media					
	C.Y. NH_3	T.Y. NH_3	T	T. NH_3	T.A. NH_3	T.Y.U.
Casein 0·5 %	+	−	−	−	−	−
Tryptose 0·5 %	−	+	+	+	+	+
Yeast extract 0·1 %	+	+	−	−	−	+
Urea 0·3 %	−	−	−	−	−	+
Asparagine 0·132 %	−	−	−	−	+	−
$(NH_4)_2SO_4$ 0·3 %	+	+	−	+	+	−

+ = The addition is present in the medium.
− = The addition is not present in the medium. Additions were at final concentrations as described above.
The basal medium contained per 100 ml.: salt solutions *a* and *b* (Blackburn & Hobson, 1962) minus ammonium sulphate, 15 ml. of each; centrifuged rumen fluid, 10 ml.; sodium bicarbonate 0·5 g.; L-cysteine-HCl. 0·05 g.; dithionite, 0·003 g.; pheno-safranine, 0·0001 g.; xylose, maltose and cellobiose, 0·1 g. of each.

Analytical methods. Uninoculated media were used as controls. A micro-Kjeldahl method (McKenzie & Wallace, 1954) was used to determine nitrogen in centrifuged bacterial deposits, and for trichloroacetic acid (TCA) soluble- and insoluble-nitrogen (Blackburn & Hobson, 1960*a*). Proteolytic activity was measured by decrease in TCA-insoluble nitrogen. Urease activity was measured by the production of ammonia from urea, amidase activity was measured by the production of ammonia from asparagine (Conway, 1957). Deaminative activity was measured by the production of ammonia in C.Y. NH_3, T.Y. NH_3 and T media (Table 1). A measure of the amino acid and peptide-nitrogen production in C.Y. NH_3 medium was obtained as follows. Samples (1·0 ml.) of the culture media were adjusted to pH 4·0 by adding citric acid (0·1 M) and diluted to 10 ml. by adding phosphate-citrate buffer (pH 4·0). After filtration through Whatman No. 42 paper, samples (0·1 ml.) of the filtrate were used for determining amino acid-nitrogen by the method of Lampson & Singher (1960) as modified by Abou Akkada & Howard (1962). Dipeptide nitrogen

was similarly determined on another sample of the culture adjusted to pH 5·5. Polypeptide-nitrogen was determined by mixing 1 ml. of the cell-free supernatant fluid of a centrifuged culture with 2 ml. 10 % (w/v) trichloroacetic acid, standing for 1 hr. and filtering through Whatman No. 42 paper. Samples (1·0 ml.) of the filtrate were mixed with 2N-NaOH, 1 ml., and biuret reagent, 8 ml., and the procedure of Layne (1957) followed. The qualitative and quantitative determinations of volatile fatty acids, except formic acid, were as previously described (Abou Akkada & Howard, 1960). Formic acid was detected by the ninhydrin method of Burness & King (1958). Quantitative determination of formic acid was carried out on a distillate of the culture medium by the colorimetric micro-method of Grant (1947). Succinic and lactic acids were identified by paper chromatography of the culture medium by a method similar to that of Stark, Goodban & Owens (1951).

RESULTS

One series of isolations was made from each sheep, 6, 190, 74, 93 and 95 and the runs were named B, C, D, E and F, respectively. The eight isolation media have been described (see page 462). The isolated bacteria were named according to the particular run, the medium and the dilution in which they appeared. Thus D1D6 would be a strain isolated in run D (sheep 74) from a medium containing no carbohydrate, reduced by cysteine + dithionite and occurring at 10^6 organisms/ml. rumen fluid. A further number, e.g. D1D63, would differentiate that strain from others isolated from the same dilution roll tube.

Media reduced by cysteine + dithionite were no more effective than those reduced by cysteine alone in producing a proteolytic flora. No particular carbohydrate source stimulated the growth of proteolytic bacteria but glucose appeared to have an inhibitory effect. Proteolytic colonies could easily be seen in the media containing no carbohydrate or carboxymethyl cellulose after incubation for 4 days. Probably no particular bacterial proteolytic type was associated exclusively with any sheep. Some colonies which produced no definite change or zone in the agar roll tubes produced marked hydrolysis of casein in liquid culture and vice versa. Proteolytic bacteria did not contribute to more than 10 % of the viable population on these media.

Representative proteolytic bacterial strains from each run were selected and these strains (32) were identified and their nitrogen nutrition examined. The properties of the bacteria were determined by using the same range of tests as did Blackburn & Hobson (1962) and they were classified in a similar manner. The products of fermentation which are helpful in classifying the bacteria are listed in Table 2.

Group 1. Bacteroides

(*a*) *Bacteroides amylophilus.* These seven strains (B3D71, B3D75, E4C6, E1D6, C1C6, C2C62, D1D61) were pleomorphic Gram-negative 1 μ coccoids, non-motile, and fermented dextrin, glycogen, starch and maltose.

(*b*) *Bacteroides ruminicola.* These three isolates (E2C7, E4D8, F2D7) were pleomorphic Gram-negative, non-motile rods 0·3 to 0·5 × 1·0 μ, and fermented a wide range of carbohydrates.

(*c*) *Bacteroides sp.* This strain (E4c6) was a Gram-negative small curved $0{\cdot}4 \times 1{\cdot}4\ \mu$ pointed-end rod; it produced butyric acid from glucose.

Group 2. Lachnospira. This single isolate, B2D8, was a Gram-negative, long ($0{\cdot}4 \times 2{\cdot}5\ \mu$), slightly curved, rod occurring in chains. It fermented only glycerol, glucose, mannose, maltose, sucrose, trehalose, carboxymethyl cellulose and cellobiose. The original colony produced no visible change in the casein of the roll tube.

Table 2. *Fermentation products in fluid medium after* 4 *days incubation at* 38° C

Isolate	Sugar fermented	Total† VFA m-equivalents/100 ml.	Succinic acid	Lactic acid	% of total VFA: Formic	Acetic	Propionic	Butyric	Final pH	Group
B3D71	Maltose	6·5	+	−	25	69	7	4	6·3	1
B3D75	Maltose	6·9	+	−	25	58	7	5	6·4	
E4C6	Maltose	3·2	+	−	22	69	3	4	5·1	
E1D6	Maltose	*	+	−	30	60	7	4	*	
C1C6	Maltose	2·9	+	−	43	44	9	4	7·1	
C2C62	Maltose	*	*	*	*	*	*	*	*	
D1D61	Maltose	3·7	+	−	30	60	7	4	5·3	
E2C7	Glucose	1·3	+	+	30	50	17	2	5·2	
E4D8	Glucose	1·8	+	+	0	84	11	2	5·8	
F2D7	Glucose	1·2	+	+	30	52	14	4	5·6	
E4C6	Glucose	5·3	+	−	30	21	4	49	5·4	
B2D8	Maltose	7·3	+	−	30	58	7	5	6·3	2
D1D4	Glucose	2·0	+	−	22	16	8	47	*	3
D4D6	Glucose	4·3	+	+	27	27	4	28	*	
F1D6	Glucose	2·5	+	+	32	28	6	46	5·6	
F2D6	Glucose	3·4	+	+	0	30	4	65	5·5	
F3D61	Glucose	3·1	+	+	20	18	6	52	5·3	
F4C6	Glucose	2·1	+	+	0	6	48	42	4·5	
E2C63	Glucose	1·8	−	+	10	57	9	25	4·6	4
E2C65	Glucose	1·6	−	+	8	53	10	28	4·4	
E2C62	Glucose	1·7	−	+	24	35	6	24	4·4	
F2C61	Glucose	1·4	−	+	0	78	20	5	5·0	
F4C6	Glucose	1·9	+	+	12	51	34	3	4·5	
F4D71	Glucose	2·1	−	+	*	46	27	9	4·2	
F3D6	Glucose	1·8	−	+	27	50	18	5	5·0	
C2C6	Maltose	6·3	−	−	20	57	8	22	6·1	
E2C64	Glucose	1·9	−	+	24	19	7	50	4·4	5
D2D6	Glucose	1·0	+	+	51	38	7	4	*	
E1C7	Glucose	1·5	−	+	8	53	20	3	5·8	
F4D7	Glucose	1·2	−	+	0	58	13	19	4·2	6
F3C6	Glucose	4·3	−	+	22	25	6	28	4·8	
F1D8	Glucose	1·3	−	+	30	49	13	9	4·7	

* Not determined. − No production. + Production.
† VFH = volatile fatty acids.

Group 3. Butyrivibrio. These six isolates (D1D4, D4D6, F1D6, F2D6, F3D61, F4C6) were Gram-negative curved rods 0·3 to $0{\cdot}5 \times 1$ to $2{\cdot}5\ \mu$, motile (except D1D4) by a single polar flagellum, and fermented a wide variety of carbohydrates.

Group 4. *Selenomonas ruminantium.* These eight isolates (E2C63, E2C65, E2C62, F2C61, F4C6, F4D71, F3D6, C2C6) were large Gram-negative crescents 0·4 to

1·2 × 2·0 to 4·0 μ, motile with flagellation similar to that described by Hobson & Mann (1961). Isolate F2C61 could be classified as *Selenomonas ruminantium* var. *lactyliticus*.

Group 5. Gram-positive or Gram-variable long rods

(*a*) Eubacterium. Isolate E2C64 was a non-motile, non-sporing, 0·6 × 2·3 μ rod in fairly long chains, and fermented arabinose, dextrin, fructose, glucose, galactose, glycogen, inulin, lactose, mannitol, mannose, maltose, sucrose and starch.

(*b*) Bacillus. Organism D2D6 was a Gram-positive, non-sporing 0·9 × 2 μ facultative anaerobic rod; it fermented a moderate range of carbohydrates.

(*c*) Clostridium. The isolate E1C7 was a Gram-variable non-motile rod 0·4 × 2·4 μ with a swollen medial spore, and fermented a moderate range of carbohydrates.

Group 6. Gram-positive cocci

The isolates F4D7, F3C6, F1D8, all 1·0 μ diameter cocci, were included in this group; F1D8 was facultatively anaerobic.

Peptidase activity

The end products of casein degradation by proteolytic bacteria were mainly amino acids and polypeptides (Table 3). No dipeptides were ever observed. Endopeptidase or proteinase activity was indicated by the percentage breakdown in casein after growth in casein liquid medium, exopeptidase activity by the ratio of amino acid–nitrogen to total amino acid + polypeptide–nitrogen (Table 3). Proteolytic activity varied within the presumptively identified groups as did exopeptidase activity and there was no correlation between the two except that the *Bacteroides amylophilus* strains had mostly high proteolytic activity and low exopeptidase activity while the *B. ruminicola* group had consistently high exopeptidase activity. No generalizations could be made regarding the Butyrivibrio and Selenomonas groups.

Ammonia production

The results of experiments to test ammonia production or utilization are tabulated (Table 4) in the form of increase or decrease in ammonia concentration over the control after incubation. The amount of bacterial growth was estimated only for the C.Y.NH_3 medium. In some cases this gave a very low value because of autolysis of the organisms, and sometimes the value was too high because denatured casein was sedimented with the centrifuge-deposit of organisms, this often being associated with amidase activity (Table 4). Probably the average bacterial nitrogen content should have been 1·0 to 2·0 μ equivalents nitrogen/ml. culture fluid. There was very little evidence of ammonia production from casein in the medium C.Y.NH_3; in general there was an uptake of ammonia corresponding roughly with the bacterial nitrogen. Deamination could result in an increase in ammonia in all the media. Media C.Y.NH_3, T.Y.NH_3 and T.A.NH_3 contained 5·0, 0·5 and 10·0 μ equivalents amide–nitrogen/ml., respectively, and thus amidase activity could also release ammonia in these media. Urease activity would be detected in T.Y.U. medium. The T.Y.NH_3 medium differed from the C.Y.NH_3 in containing a trypsin-hydrolysed casein, tryptose. In T.Y.NH_3 medium as in C.Y.NH_3 medium there was generally a net uptake of ammonia and there was no evidence that tryptose was a better

substrate for deaminases than was casein. A comparison between media T and T.NH_3 showed that in many cases the higher initial ammonia level resulted in decreased net production of ammonia. This was not true of the Butyrivibrio group 3. A comparison between T.NH_3 and T.A.NH_3 showed the amount of amidase activity. This property was found in all three strains of *Bacteroides ruminicola* group 1, and occurred

Table 3. *The products of casein hydrolysis after 4 days of incubation at* 39° C

Isolate	Casein hydrolysed (%)	Amino acid-nitrogen % total casein-nitrogen	Polypeptide-nitrogen as % total casein-nitrogen	Amino acid-nitrogen as % non-protein-nitrogen	Group
B3D71	86	15	61	20	
B3D75	86	14	65	18	
E4C6	30	5	26	15	
E1D6	35	11	26	34	
C1C6	90	16	68	20	
C2C62	94	31	54	37	1
D1D61	87	19	59	24	
E2C7	28	8	10	44	
E4D8	97	64	17	79	
F2D7	46	23	26	45	
E4C6	28	9	18	35	
B2D8	33	5	29	14	2
D1D4	55	23	29	44	
D4D6	16	5	13	28	
F1D6	81	19	58	25	3
F2D6	66	7	29	18	
F3D61	43	18	26	42	
F4C6	87	23	66	26	
E2C63	93	36	57	38	
E2C65	49	7	39	16	
E2C62	40	6	35	15	
F2C61	55	15	33	31	4
F4C6	12	2	8	16	
F4D71	96	17	71	20	
F3D6	21	4	14	20	
C2C6	34	8	27	23	
E2C64	47	6	42	13	
D2D6	26	6	17	26	5
E1C7	90	61	27	69	
F4D7	93	15	71	18	
F3C6	12	2	14	11	6
F1D8	79	10	64	14	

The initial casein concentration was 50 μ-equivalents nitrogen/ml. of medium C.Y. NH_3, the composition of which is in Table 1.

in Butyrivibrio group 3 (strains F2D6, F3D61, F4C6); in Selenomonas group 4 (strains E2C63, E2C65, F4C6, F4D71, F3D6) and in all strains in groups 5 and 6. The strains E4D8, F2D7 of *B. ruminicola*, Butyrivibrio F3D61, Selenomonas strains F3D6 and possibly F2C61, F4C6, F4D71, all strains in group 5, strain F4D7 in group 6 were considered to show some degree of deaminative activity. No significant urease activity was shown by any of these bacteria except possibly F4D7 and F2D6, and even there only a small amount of the substrate was attacked.

Table 4. *Production of ammonia from various substrates after 4 days of incubation at 39°*

The results are expressed in μ-equivalents of nitrogen per ml. of culture fluid. The initial concentrations of ammonia–nitrogen in μ-equivalents per ml. of uninoculated C.Y. NH_3, T.Y. NH_3, T, T.A. NH_3, T. NH_3 and T.Y.U. were 15·0, 17·0, 1·5, 15·3, 15·3 and 4·9 approximately, depending on the batch of medium. Media composition in Table 1.

Isolate	Bacterial nitrogen C.Y. NH_3	Increase in ammonia nitrogen C.Y. NH_3	T.Y. NH_3	T	T. NH_3	T.A. NH_3	T.Y.U.	Group
B3D71	0·00	−3·72	0·48	−0·54	*	*	*	
B3D75	0·00	−0·48	−0·46	−0·07	*	*	*	
E4C6	0·60	*	−2·86	−0·86	−1·64	0·71	1·21	
E1D6	0·90	*	−0·11	0·35	−1·19	0·71	−1·07	
C1C6	0·71	−1·18	−0·34	−0·15	*	*	*	
C2C62	*	−0·34	−1·43	0·33	−0·14	*	0·49	1
D1D61	0·00	−1·64	−2·86	−0·50	−2·00	−1·64	−3·00	
E2C7	0·63	−1·07	−1·43	0·63	−0·93	3·14	0·50	
E4D8	6·45	13·32	3·57	6·78	−3·00	15·71	3·58	
F2D7	7·85	4·28	−0·71	1·47	−0·71	3·57	4·64	
E4C6	4·67	*	−1·46	−0·64	−0·53	*	−1·26	
B2D8	1·93	−2·00	0·19	−0·59	*	*	*	2
D1D4	1·60	−6·72	−1·00	−1·36	−1·93	−0·87	−0·60	
D4D6	1·30	−0·38	−0·27	−0·28	0·43	−0·67	−0·20	
F1D6	2·00	−1·64	−3·66	−0·07	0·00	0·56	0·18	
F2D6	4·07	−1·93	−0·27	0·09	0·86	16·16	6·56	3
F3D61	2·71	2·86	3·36	0·09	0·93	15·85	2·71	
F4C6	5·00	0·24	−0·48	1·07	2·50	16·43	1·79	
E2C63	10·91	*	0·91	0·36	−1·53	8·71	0·50	
E2C65	9·14	*	−0·34	1·00	−1·33	9·30	0·15	
E2C62	7·14	*	0·48	0·50	−0·86	0·71	1·09	
F2C61	1·93	−1·93	−3·21	1·50	1·32	1·19	0·46	4
F4C6	2·50	−2·64	−3·24	1·14	3·31	15·30	0·51	
F4D71	1·93	−2·00	−4·96	1·29	1·50	13·83	1·79	
F3D6	0·23	1·14	0·00	5·07	0·71	5·79	1·50	
C2C6	3·07	−0·13	−1·00	−0·93	−0·26	0·81	−0·11	
E2C64	7·85	*	−0·26	4·14	−1·00	5·71	−2·43	
D2D6	8·14	1·50	2·07	0·90	0·19	3·36	−0·02	5
E1C7	4·72	13·36	4·36	13·14	2·71	19·57	3·14	
F4D7	2·50	0·00	−3·86	1·72	3·78	17·14	8·72	
F3C6	0·71	−1·29	−0·71	1·00	−2·00	4·50	2·07	6
F1D8	1·20	−0·68	0·33	1·14	−2·00	1·79	*	

* Not determined.

DISCUSSION

Species of Bacteroides, Selenomonas and Butyrivibrio have again been found associated with protein hydrolysis in the sheep rumen. This is in keeping with the observations of Blackburn & Hobson (1962), but none of the large Gram-negative butyric acid-forming rods (group 5, Blackburn & Hobson, 1962) was isolated. Proteolytic bacteria did not constitute a major portion of the strains which appeared on the isolation media, thus differing from the observations of Fulghum, King & Moore (1958) with cattle who found 30 % of their total count to be proteolytic. As reported previously (Blackburn & Hobson, 1962) no particular carbohydrate encouraged the

growth of all proteolytic bacteria but maltose was effective in culturing *Bacteroides amylophilus*, a particularly active protease producer. The total absence of carbohydrate or the presence of a less readily attacked carbohydrate such as carboxymethyl cellulose, resulted in the isolation of strains that might otherwise have been overgrown. Occasionally strains (e.g. c1c6, c2c62, f2c61) which had produced no change in the casein of the isolation medium, were found to be actively proteolytic in liquid culture. Generally, strains which produced a clear zone in the casein of the isolation medium subsequently produced hydrolysis of casein in liquid culture, but exceptions to this were noted, especially where the zone was not well defined.

A pure proteolytic enzyme such as crystalline trypsin will liberate little or no free amino nitrogen (Van Slyke, Dillon, MacFadyen & Hamilton, 1941). The present observations indicated that most of the strains had considerable exopeptidase activity, in addition to their proteolytic activity, which resulted in the liberation of free amino acids. This would be in accord with the results of Annison (1956) who found a rapid increase in amino acids and peptides in the rumen after feeding sheep a casein-containing diet. Considerable amidase activity was found in the present strains. The deamidation of casein by mixed rumen micro-organisms and by protozoa has been reported by Warner (1955) and Abou Akkada & Howard (1962). This amidase activity would explain the ability of propionamide to replace protein in ruminant diets (Hale, 1956).

Few of the isolates had any deaminase activity except in the *Bacteroides ruminicola* and Selenomonas groups; others such as the Clostridium and Gram-positive cocci are probably of less significance in the total rumen population under normal conditions. *B. ruminicola* and selenomonads isolated from the bovine rumen have been reported by Bladen, Bryant & Doetsch (1961) to have deaminase activity. Many of the species reported in the present paper and by Blackburn & Hobson (1962), which have been isolated for the first time from the sheep rumen, are similar to those reported in the rumen of cattle (Bryant, 1959) and their nitrogen metabolism is similar. In keeping with the observations of Bladen *et al.* (1961), many of the bacteria studied appeared to utilize ammonia as the main source of nitrogen even in the presence of pre-formed amino acids and although they were hydrolysing casein.

No significant urease activity was demonstrated among the present isolates. The possession of urease has not yet been associated with any of the more common rumen micro-organisms even though urea is rapidly hydrolysed in the rumen.

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REFERENCES

Abou Akkada, A. R. & Howard, B. H. (1960). The biochemistry of rumen protozoa. 3. The carbohydrate metabolism of Entodinium. *Biochem. J.* **76**, 445.

Abou Akkada, A. R. & Howard, B. H. (1962). The biochemistry of rumen protozoa. 5. The nitrogen metabolism of Entodinium. *Biochem. J.* **82**, 313.

Annison, E. F. (1956). Nitrogen metabolism in the sheep. Protein digestion in the rumen. *Biochem. J.* **64**, 705.

Blackburn, T. H. & Hobson, P. N. (1960*a*). Proteolysis in the sheep rumen by whole and fractionated rumen contents. *J. gen. Microbiol.* **22**, 272.

BLACKBURN, T. H. & HOBSON, P. N. (1960*b*). Isolation of proteolytic bacteria from the sheep rumen. *J. gen. Microbiol.* **22**, 282.

BLACKBURN, T. H. & HOBSON, P. N. (1962). Further studies on the isolation of proteolytic bacteria from the sheep rumen. *J. gen. Microbiol.* **29**, 69.

BLADEN, H. A., BRYANT, M. P. & DOETSCH, R. N. (1961). A study of bacterial species from the rumen which produce ammonia from protein hydrolyzate. *Appl. Microbiol.* **9**, 175.

BRYANT, M. P. (1959). Bacterial species of the rumen. *Bact. Rev.* **23**, 125.

BURNESS, A. T. & KING, H. K. (1958). Detection of fatty acids on paper chromatograms by means of ninhydrin. *Biochem. J.* **68**, 32P.

CONWAY, E. J. (1957). *Microdiffusion Analysis and Volumetric Error*, 4th ed. London: Crosby Lockwood and Son, Ltd.

EADIE, J. M. & HOBSON, P. N. (1962). Effect of the presence or absence of rumen ciliate protozoa on the total rumen bacterial count in lambs. *Nature, Lond.* **193**, 503.

FULGHUM, R. S., KING, K. W. & MOORE, W. E. C. (1958). Development and comparison of media for the isolation of proteolytic bacteria from the rumen. *Bact. Proc. 1958*, 22.

GRANT, W. M. (1947). Colorimetric micro-method for determination of formic acid. *Analyt. Chem.* **19**, 206.

HOBSON, P. N. & MANN, S. O. (1961). The isolation of glycerol-fermenting and lipolytic bacteria from the rumen of sheep. *J. gen. Microbiol.* **25**, 227.

HALE, W. H. (1956). Rumen metabolism of non-protein nitrogen. *J. Agric. Fd Chem.* **4**, 948.

LAMPSON, G. P. & SINGHER, H. O. (1960). Colorimetric determination of amino acids in presence of a peptide. *Proc. Soc. exp. Biol., N.Y.* **103**, 368.

LAYNE, E. (1957). Spectrophotometric and turbidimetric methods for measuring proteins. *Methods in Enzymology*, vol. 3, New York: Academic Press, Inc.

MCKENZIE, H. A. & WALLACE, H. S. (1954). The Kjeldahl determination of nitrogen. A critical study of digestion conditions—temperature, catalyst and oxidizing agent. *Aust. J. Chem.* **7**, 55.

STARK, J. B., GOODBAN, A. E. & OWENS, H. S. (1951). Paper chromatography of organic acids. *Analyt. Chem.* **23**, 413.

VAN SLYKE, D. D., DILLON, R. T., MACFADYEN, D. A. & HAMILTON, P. (1941). Gasometric determination of carboxyl groups in free amino acids. *J. biol. chem.* **141**, 627.

WARNER, A. C. I. (1955). Ph.D. Dissertation, Aberdeen University. Some aspects of the nitrogen metabolism of the micro-organisms of the rumen with special reference to proteolysis.

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Microbial Antagonism by *Staphylococcus aureus*

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SUMMARY

Seven strains antagonistic to indicator corynebacteria on solid media were found among 100 *Staphylococcus aureus* strains of human origin. These inhibitory strains all gave sharply defined inhibition zones, belonged to bacteriophage type 71, and were isolated from superficial infections. They were strongly active in direct antagonism tests against all the other 'aureus' strains, and against some coagulase-negative staphylococci, streptococci and other Gram-positive species; Gram-negative organisms were not susceptible. *S. aureus* strains producing hazy inhibition zones with corynebacteria showed similar though less extensive antibacterial activity. Inhibition by both kinds of staphylococci occurred under several different environmental conditions, but not in the presence of oleic acid. The antagonistic agents were relatively heat resistant, passed readily through cellophan, and showed considerable specificity in their action.

INTRODUCTION

It has long been known that some strains of staphylococci are capable of inhibiting the growth of other organisms, particularly corynebacteria, by the production of diffusible 'antibiotic' substances. The earlier literature was reviewed by Florey *et al.* (1949). Such strains were not thought to be associated with any particular kind of infection until Parker, Tomlinson & Williams (1955) showed that most staphylococci of bacteriophage 'type 71', but very few others, were able to prevent the growth of *Corynebacterium diphtheriae* on solid media, with the formation of sharply defined zones of inhibition. These strains of staphylococci were closely associated with impetigo contagiosa, and were rarely isolated from deeper infections (Barrow, 1955; Parker *et al.* 1955). A number of different staphylococci with antagonistic properties formed wider inhibition zones with 'hazy' edges, but had little else in common. The few remaining strains giving sharp zones of inhibition possessed other '71-like' characters, and were also associated with superficial skin infections (Parker, 1958). Later, Parker & Simmons (1959) found that type 71 staphylococci were weakly active in deferred antagonism tests against other strains of *Staphylococcus aureus*. The present paper concerns further observations on the range and significance of inhibitory activity by type 71 staphylococci on solid media.

METHODS

Organisms

Staphylococcus aureus. One hundred strains of coagulase-positive staphylococci, isolated from routine bacteriological specimens, were phage-typed at the Public Health Laboratory, Leeds, by the method of Anderson & Williams (1956). They

were derived from infections such as boils, abscesses and skin lesions, as well as from ear, nose and throat swabs, and were representative of all the phage groups. In addition, two strains of *Staphylococcus aureus* antagonistic to corynebacteria were obtained from the National Collection of Type Cultures: NCTC 6507—strain 'Inhibitor' isolated from an empyema and deposited by A. Fleming in 1943; and NCTC 8004—strain E 755, used by Gardner (1949). Each strain was examined for: (1) sensitivity to penicillin; (2) ability to inhibit the growth of corynebacteria on solid media; (3) production of opacity in horse serum agar medium, as described by Parker (1958); (4) production of opacity in egg-yolk broth (Oxoid), as described by Alder, Gillespie & Herdan (1953). These strains were also used in various inhibition tests. They were kept on Lemco nutrient agar slopes and subcultured infrequently.

Coagulase-negative staphylococci. Twenty-five strains were used, of which fifteen were isolated from routine bacteriological specimens and nine from normal skin. The remaining culture, *Staphylococcus saprophyticus* (NCTC 7291), was originally isolated from healthy skin. Each strain was examined for inhibitory activity against, and susceptibility to, other organisms.

Corynebacteria. A suitable 'indicator' strain, highly susceptible to inhibition by type 71 staphylococci, was selected from a collection of corynebacteria obtained from routine specimens. This strain, an unnamed diphtheroid organism referred to as 'Bradford' (BFD) was isolated from an ear swab, and was a short broad rod, forming flat whitish colonies with lobate edges and a dull granular surface. It fermented glucose and sucrose only, and showed some enhancement of growth around zones of inhibition. It was thus similar to the original 'Bradford' organism sent to, and used by, Parker & Simmons (1959). Later, a subculture of their nitrate-negative avirulent strain of *Corynebacterium diphtheriae mitis* (no. 51 in the collection of the Public Health Laboratory, Manchester) was also obtained and used in parallel with the BFD diphtheroid. This 'Manchester' organism (MC) showed typical 'mitis' morphology and cultural characters. Both indicator organisms were subcultured weekly on blood agar medium and kept on the bench. Stock cultures were also kept at 4°.

Haemolytic streptococci. Twenty representative streptococci comprising different serotypes of group A and members of other Lancefield groups, originally isolated from an outbreak of throat infections and impetigo in a school (Barrow, 1961), were used. In addition 38 different strains of *Streptococcus pyogenes*, 20 of which possessed M antigens, as well as 12 different strains from impetigo lesions, were obtained in the lyophilized state from the Streptococcus Reference Laboratory, Colindale. They were maintained by frequent subculture on blood agar medium, and were examined for inhibition of, and susceptibility to the action of, staphylococci.

Other organisms. These were used in various inhibition tests and included miscellaneous strains of pneumococci, viridans streptococci, enterobacteria and aerobic and anaerobic spore-bearing organisms isolated in the laboratory. One strain of *Corynebacterium acnes* (NCTC 737) was also used.

Methods used in the study of inhibition on solid media

Blood agar medium was used because it allowed good growth of Corynebacterium MC and because the zones of inhibition were easily seen. The blood also provided a source of catalase, thus excluding the possibility of inhibition due to peroxide

formation. The nutrient base was Lemco infusion broth at pH 7·5, with the addition of 0·002 % (w/v) cystine hydrochloride and 1·6 % (w/v) New Zealand agar. Incubation was aerobic at 37° overnight, unless otherwise stated.

Direct antagonism

In these methods, the active or test strain was placed on solid medium shortly after spreading a sensitive indicator organism over the surface. The term 'direct' is used instead of 'simultaneous' antagonism (Gratia, 1946) because it avoids the suggestion that each organism is necessarily active against the other.

(1) *Stab inoculation.* Small loopfuls of broth cultures of the test strains were deposited on the surface of media previously sown with indicator organisms. Later, the test strains were stab-inoculated with a straight wire throughout the depth of the medium. On incubation, the indicator organisms grew as confluent lawns, with zones of inhibition round some of the 'spot' or stab inocula. Stab-inoculation was simple, rapid and gave consistent results with wider inhibition zones than surface inocula. It was also eminently suitable for examining single colonies. In accordance with Parker & Simmons (1959), inhibition of corynebacteria was considered positive (DI+) when the zones were 1·0 mm. or more in width; zones with sharply defined edges were designated DI+S, and those with hazy edges DI+H.

(2) *Overlapping drop method.* This was used to show competitive inhibition among staphylococci and was based on the cross-titration procedure of Rosebury, Gale & Taylor (1954). Pairs of overlapping drops from dilutions of two different broth cultures were placed on solid medium with a standard loop. The second drop of each pair was added when the first had dried. The plates were examined after incubation for inhibition near the area of overlap.

Deferred antagonism

In these methods, the active or test organisms were grown on solid media before inoculation of the passive indicator strains. They allowed growth of the test and indicator organisms under different conditions, as well as permitting the detection of inhibitory substances sometimes not revealed by direct antagonism.

(1) *Slide inoculation.* Test strains were grown as central spots or as streaks across plates. After incubation, passive organisms were inoculated at right angles to the primary growth by means of glass microscope slides held horizontally between thumb and fingers. The long edges of sterile slides were seeded from culture-soaked filter papers in Petri dishes. This method allowed inoculation close to the primary culture without touching it.

(2) *Colicine method.* The technique of Abbott & Shannon (1958) for typing *Shigella sonnei* by colicine production was used for staphylococci. After removal of the primary growth and exposure to chloroform, the passive organisms were quickly cross-inoculated by pressing glass slides alternately on culture-soaked filter papers as before and then on media. Later, a simple multiple-slide apparatus for the simultaneous and replicate inoculation of several different cross-streaks was developed for this and other purposes (Barrow & Ellis, 1962).

RESULTS

Inhibition of corynebacteria

The inhibitory activity of 100 strains of *Staphylococcus aureus* was first ascertained against the indicator corynebacteria by the 'spot' test. The results, with some properties of these staphylococci, are shown in Table 1. Seven strains were active and gave sharply defined zones of inhibition (DI+S) with both corynebacteria (Pl. 1, figs. 1, 2). These DI+S strains were isolated from superficial infections and comprised six type 71 cultures and one culture of phage pattern 55/71. They were re-tested at intervals, and the finding of Parker & Simmons (1959) that DI+S cultures lost their inhibitory activity on storage and showed a simultaneous increase in susceptibility to group II bacteriophages, was confirmed. When dilutions of cultures showing loss of activity were sown on plates with the corynebacteria, some inhibitory staphylococcal colonies were detected among the many inactive colonies. These active colonies were indistinguishable from freshly isolated DI+S cultures, and behaved in the same way on storage; active variants were never obtained from non-inhibitory colonies. The culture of phage pattern 55/71 also yielded on dilution a mixture of DI+S type 71 colonies and DI− colonies of pattern 55/71.

Table 1. *Phage groups and some properties of* 100 *strains of* Staphylococcus aureus *isolated from routine specimens*

		Number of strains of *S. aureus*				
S. aureus phage group		Penicillin resistant	Egg-yolk positive	Serum opacity positive	Active against Coryne-bacteria	Total no. of strains
I		27	38	1	0	41
II	Type 71	6	0	6	6	7
	Other patterns	4	7	1*	1*	8
III		12	15	3	0	19
Not typable		11	10	2	0	13
Not classifiable		8	12	0	0	12
Total		68	82	13	7	100

* This staphylococcal strain yielded inhibitory colonies of type 71 and non-inhibitory colonies of phage-pattern 55/71.

Because no inhibitory strains other than type 71 were identified, two 'aureus' strains active against corynebacteria were obtained from the National Collection of Type Cultures and used for comparison in some experiments. Strain NCTC 8004 (E 755), not susceptible to any of the typing phages in routine use, gave narrow inhibition zones with the BFD diphtheroid, and wide zones with hazy ill-defined margins (DI+H) with the MC diphtheria organism. Strain NCTC 6507 ('Inhibitor'), weakly susceptible to phage 29, also gave wide hazy zones with organism MC, but had little or no effect on the BFD organism (Pl. 1, figs. 1, 2). Neither of these aureus cultures showed much loss of inhibitory activity during storage.

Inhibition of staphylococci

(i) *Staphylococcus aureus*

(*a*) *Deferred antagonism.* The active (DI+) type 71 strains of *Staphylococcus aureus* were first tested by the colicine method for inhibitory power against a few non-inhibitory (DI−) staphylococci. The DI+S type 71 strains inhibited the DI− staphylococci. Next, these DI+S strains and the two DI+H NCTC strains were tested similarly against all the DI− staphylococci isolated from routine specimens, and then vice versa. In addition, 50 of the DI− strains were subsequently tested against each other. The combined results gave a chess board indicating mutual inhibitory activity. This showed that all the DI+S type 71 strains inhibited the growth of every DI− strain, and conversely, no DI− strain had any inhibitory activity against DI+S or DI+H staphylococci. Each of the two DI+H NCTC strains inhibited most, but not all, of the DI− staphylococci. Resistance to the

Table 2. *Inhibitory activity between representative strains of* Staphylococcus aureus *in deferred antagonism tests*

S. aureus phage group	Primary streak *S. aureus* strain no.	Passive cross-streaks *S. aureus*, strain no. 1	2	3	4	5	6	7	8	9	10	11	12
I	1	−	−	−	−	−	−	−	−	−	−	−	−
	2 (NCTC 6507)	++	−	+	++	++	+++	+	−	+	−	++	−
II Type 71	3	+++	+++	−	−	+++	+++	+++	+++	+++	+++	+++	+++
	4	+++	++	−	−	+++	+++	+++	+++	+++	+++	+++	+++
II Other patterns	5	−	−	−	−	−	−	−	−	−	−	−	−
	6	−	−	−	−	−	−	−	−	−	−	−	−
III	7	−	−	−	−	−	−	−	−	−	−	−	−
	8	−	−	−	−	−	−	−	−	−	−	−	−
Not typable	9	−	−	−	−	−	−	−	−	−	−	−	−
	10 (NCTC 8004)	++	−	±	+	++	++	++	+	−	−	++	+
Not classifiable	11	−	−	−	−	−	−	−	−	−	−	−	−
	12	−	−	−	−	−	−	−	−	−	−	−	−

+++ = Complete inhibition extending beyond area of overlap; ++ = complete inhibition over area of overlap; + = complete inhibition within area of overlap; ± = partial inhibition; − = no inhibition.

action of the DI+H strains was not, however, associated with any particular phage pattern. The inhibition zones produced by DI+ staphylococci were distinct, although resistant colonies sometimes occurred within them. Among the DI− staphylococci only weak doubtful activity against occasional strains was observed. The results of a typical experiment with representative DI+ and DI− organisms are shown in Table 2. When used as test organisms, active type 71 strains did not inhibit other DI+S strains, nor did the DI+H NCTC strains inhibit each other. Both DI+H strains, however, inhibited all the DI+S strains, and similarly each type 71 strain inhibited both DI+H NCTC strains. Loss of inhibitory power by type 71 strains against corynebacteria coincided with loss of activity against

staphylococci. Indeed DI− variants derived directly from DI+S type 71 strains were completely inhibited by the active parent cultures.

(*b*) *Direct antagonism.* The finding that DI+S type 71 staphylococci could inhibit the growth of all other strains of *Staphylococcus aureus* in deferred antagonism tests suggested that this ability might be of some advantage to them under natural conditions. It was important therefore to determine whether type 71 strains could suppress the growth of, or at least compete with, other staphylococci in direct antagonism experiments. With the spot test, inhibition was not generally observed when loopfuls of active type 71 broth cultures were deposited on lawns of DI− staphylococci. The effect of altering the ratio of organisms in the spot inocula and in the lawns was therefore investigated by the overlapping drop procedure. Undiluted DI+ broth cultures readily inhibited diluted broth cultures of DI− strains, but not vice versa. When examined in this way, a few representative staphylococci, including the two NCTC strains, gave results corresponding with those of the deferred antagonism tests.

Although direct antagonism between staphylococci was thus demonstrated, this method was not satisfactory for examining numerous organisms, and a more suitable technique was therefore sought.

(*c*) *Stab-inoculation method.* It was observed that active type 71 staphylococci stab-inoculated with a straight wire throughout the depth of solid media inhibited the corynebacteria to a greater extent than similar surface spot inocula (Pl. 1, figs. 1, 2). When tested with lawns of staphylococci prepared from broth cultures, good inhibition of DI− by DI+ strains was also observed, particularly when the straight wire was heavily charged with organisms from solid media. This technique was investigated and was found to provide a simple rapid method by which large inocula of one organism could easily be tested for direct antagonism against smaller inocula of other organisms.

It was thought that the smaller inhibition zones given by surface inocula were due to a decrease of the local antibiotic concentration by downward diffusion, and that antibiotic production throughout stab inocula gave higher surface gradients by diminishing this loss. This idea was supported by experiments in which antibiotic disks were placed on the surface, in the middle, and at the bottom of solid media, the surface of which had been seeded with a sensitive organism. After incubation, the surface disks gave narrower inhibition zones than the other disks. The importance of antibiotic loss by diffusion was emphasized by deferred antagonism experiments with media containing different concentrations of agar. Inhibition was weaker and the zones smaller in media containing 1 % or less agar compared with media containing higher concentrations of agar. Diffusion was therefore limited by using 2 % agar, by omitting the customary saline agar bases, and by making the depth of media slightly less than normal.

Using this technique, all the strains of *Staphylococcus aureus* were re-examined for direct antagonism against each other. The results were almost always the same as those obtained in deferred antagonism tests. All DI+ staphylococci inhibited most of the DI− strains, including non-inhibitory variants derived from active type 71 cultures. Conversely, none of the DI− strains showed any inhibitory activity against other staphylococci. Again, both DI+H NCTC strains were susceptible to the DI+S type 71 staphylococci; in turn these were inhibited, though to a lesser

extent, by the NCTC strains (Pl. 2, fig. 3). Neither NCTC organism inhibited the other, nor were any of the DI+S type 71 strains active against each other. By this method, type 71 strains were thus shown to be strongly and directly antagonistic to other 'aureus' strains.

(ii) *Staphylococcus albus*

Because of their remarkable inhibitory activity against other strains of *Staphylococcus aureus*, the interaction between type 71 staphylococci and 25 strains of coagulase-negative staphylococci was studied by the stab inoculation technique. Six of the *S. albus* strains were isolated from urines, four from urethral or vaginal discharges, and six from boils or other skin lesions. The remaining nine strains were from normal skin, one of which was *S. saprophyticus* NCTC 7291. It was found that 14 of the *S. albus* strains (including four from normal skin) were inhibited by type 71 stab inocula. On the other hand, only one of ten random DI – *S. aureus* strains was active in inhibiting three of the *S. albus* strains. Conversely, two coagulase-negative strains (both from normal skin) were active against all the type 71 strains, as well as against several DI – staphylococci. A few DI – *S. aureus* strains were also weakly susceptible to some (not necessarily the same) *S. albus* strains. When tested against the indicator corynebacteria, ten of the *S. albus* strains inhibited the BFD diphtheroid; two gave sharply defined zones of inhibition and eight gave hazy zones. Only three *S. albus* strains inhibited the MC organism, all producing sharply defined zones; these included the two *S. albus* strains active against type 71 staphylococci. There was thus no close association as compared with *S. aureus*, between ability to inhibit corynebacteria and ability to inhibit other *S. aureus* strains. Loss of power to inhibit corynebacteria by type 71 strains coincided with loss of activity against coagulase-negative staphylococci, and resulted in susceptibility to other active *S. albus* strains.

Inhibition of streptococci

Interaction between type 71 staphylococci and different types of haemolytic streptococci was studied to try to assess their relative importance in impetigo. Twenty representative streptococci, including strains other than group A, isolated from an outbreak of throat infections and impetigo in a school (Barrow, 1961) were examined for inhibition by type 71 stab inocula. Streptococci of group G were susceptible, but only doubtful activity was observed against occasional strains of *Streptococcus pyogenes*. Because M antigens, necessary for complete type identification of *Streptococcus pyogenes*, were not found in most of these strains, other cultures with known M and/or T antigens, obtained from the Streptococcus Reference Laboratory, were investigated. These included 20 stock M strains and 18 T strains of 'pyogenes', together representing 26 different serotypes. Also 12 different 'pyogenes' strains originally isolated from impetigo lesions and giving characteristic agglutination patterns, such as 3/13/B 3264, 8/IMP.19 and 5/11/27/44, were examined.

None of the 12 impetigo strains was inhibited by stab inocula of type 71 staphylococci on blood agar medium. Of the type strains of *Streptococcus pyogenes*, only the M and T cultures of type 23 were clearly and consistently inhibited by type 71 stab inocula. Most T strains were unaffected, although weak activity giving rise to hazy zones of minute colonies, was observed with several M strains. Examination

of selected single colonies from streptococcal cultures revealed variation in susceptibility to type 71 staphylococci, but inhibition could not be related directly to colonial morphology nor to the presence of particular antigens. DI – staphylococci used as controls had little or no effect on any of the streptococci. Blood agar media at different pH values and incubated under different conditions did not give more definite results. Inhibition of every streptococcal strain, however, was produced on nutrient agar without blood by many staphylococci, including DI – cultures, and it was therefore not comparable with type 71 activity. Loss of ability by type 71 strains to inhibit corynebacteria resulted in loss of activity against *S. pyogenes* type 23, but weak activity against other streptococci was not affected. None of the streptococcal strains was active in deferred antagonism tests against type 71 staphylococci.

Inhibition of other organisms

Gram-negative species. Active staphylococci were tested by the stab-inoculation method against strains of Haemophilus, Neisseria, and several different enterobacteria. In no instance was inhibition observed.

Gram-positive species. Several organisms, including strains of pneumococci, enterococci and viridans streptococci, as well as different species of Corynebacterium and Bacillus were inhibited by type 71 staphylococci and by both NCTC DI+H strains. Under anaerobic conditions, different species of Clostridium were also inhibited by type 71 stab inocula, but not by the NCTC strains. One strain of *Corynebacterium acnes* (NCTC 737) was susceptible to the action of type 71 staphylococci, but not to the DI+H NCTC strains. Conversely, the 'acnes' culture was not active in deferred antagonism tests against these staphylococci.

Properties of the inhibitory agents

Resistance to heat. Plates with central streaks of type 71 staphylococcal growth were prepared for cross-inoculation by the colicine method, and were then placed in an anaerobic container. This was partly immersed in boiling water for 3 hr. The maximum air temperature reached inside the container was 83°. After removal, the plates were cooled and inoculated with sensitive indicator organisms. Inhibition occurred on incubation and was similar to that produced on unheated control plates, indicating that the type 71 inhibitory substance withstood this amount of heat. The two DI+H NCTC organisms also behaved in this way.

Diffusion through cellophan. Small sections of cellophan (Visking) tubing were autoclaved and then carefully dried. These sterile cellophan cylinders were placed upright in solid media just before setting. Active organisms were inoculated on the medium inside the cylinders, and indicator organisms outside—either at the same time as, or after incubation of, the active cultures. When plates were prepared in this way, growth of active staphylococci produced good inhibition of the MC corynebacterium (Pl. 2, fig. 4), indicating that these inhibitory agents readily diffused through cellophan. This was also shown in direct and deferred antagonism experiments with sheets of commercial cellophan placed on the surface of solid media.

Specificity of action. All seven DI+S type 71 strains of staphylococci behaved in the same way and showed the same antibacterial range. With each, loss of activity against one organism always coincided with similar loss against other organisms,

suggesting that their antagonistic activity was due to one and the same substance. This was, however, different from the inhibitory agents produced by the DI+H NCTC organisms. Considerable specificity in their action was observed when DI+ strains were tested against colonies of staphylococci selected for their resistance to these inhibitory agents. Variants of DI− staphylococci resistant to one type 71 strain were equally resistant to the other type 71 strains, but they remained sensitive to the DI+H NCTC strains. Similarly DI− variants resistant to the DI+H NCTC organisms were not resistant to type 71 strains. From type 71 organisms which had lost their inhibitory power, variants were selected which, unlike the parent culture, were resistant to active type 71 cultures, but which were not active against corynebacteria. These variants nevertheless remained susceptible to the DI+H NCTC strains.

Influence of environment on inhibitory activity

A few experiments were made to determine whether type 71 staphylococci were active under some of the conditions which might be encountered in the skin. The factors considered were E_h, pH, temperature, salt concentration, the presence of unsaturated fatty acids, and the presence of blood, plasma or serum. Antibacterial activity by type 71 staphylococci was observed on solid media over a pH range from 5 to 9 after incubation at 37° under aerobic and under anaerobic conditions. It also occurred and was almost as effective under these conditions when the organisms were grown at 25–30°, even in the presence of 5% (w/v) NaCl. In contrast, the DI+H NCTC strains were not active when grown anaerobically. Inhibition by these staphylococci was detected by deferred antagonism methods after aerobic growth for as little as 5 hr.

Oleic acid was used to represent the unsaturated fatty acids present in sebum. Although not miscible with water, it was incorporated as fine globules in suspension by adding it to hot molten agar medium, mixing continuously until viscous, and then pouring rapidly on to plates. Some staphylococcal strains grew with difficulty on 0·5 or 1·0% oleic acid media, but type 71 strains, the DI+H NCTC organisms, and the BFD diphtheroid, grew well. Direct inhibition of corynebacteria or of DI− staphylococci was not observed on these plates. Inhibitory activity by DI+S type 71 staphylococci in solid media was not affected by the presence of heated serum from several different animal species, or by human blood or pooled human plasma or serum. Inhibitory activity was not prevented by standard commercial staphylococcal antitoxin, or by an immune rabbit serum prepared against DI+S type 71 organisms.

DISCUSSION

The present work confirms the finding of Parker (1958) that inhibition of corynebacteria by staphylococci, with the formation of sharply defined zones on solid media, is limited almost entirely to type 71 strains. These were all isolated from superficial infections, were penicillin resistant and produced opacity in horse serum agar but not in egg-yolk broth medium. An inverse relationship was found between the serum and egg-yolk opacity results, thus supporting Parker's conclusion that the serum opacity test provided a good indication of 'superficiality' among strains of *Staphylococcus aureus*. Gooder (1961) described a similar opacity reaction produced in broth by certain serotypes of *Streptococcus pyogenes*, including those

associated with skin infections. The present work has also confirmed the findings of Parker & Simmons (1959) that type 71 staphylococci lose their inhibitory activity on storage, and that this loss is accompanied by increased bacteriophage susceptibility. In contrast two staphylococcal strains which produced hazy zones of inhibition with corynebacteria remained stable.

Although Parker & Simmons observed some activity by DI+S type 71 staphylococcal strains, but not by DI+H strains, in deferred antagonism tests against other staphylococci they did not observe any inhibition by direct antagonism. They concluded that inhibitory activity between different staphylococci was weak and probably of little significance. However, it has been shown in the present work that with suitable methods type 71 staphylococci can be strongly and directly active against all other strains of *Staphylococcus aureus*, as well as against many other Gram-positive species. Two DI+H NCTC staphylococcal cultures were similarly active against many, though not all, strains of *S. aureus*. Stab inoculation was useful for detecting such activity; it allowed inhibition which would otherwise not have been observed and directed attention to the importance of limiting diffusion in order to maintain high local antibiotic concentrations. This is important when examining organisms with similar growth rates, since a critical antibiotic concentration may be attained before growth of the sensitive population reaches a resistant level, as was found by Cooper, Linton & Sehgal (1958). These inhibitory agents were heat-resistant, able to pass through cellophan, and showed considerable specificity in action when tested against resistant staphylococcal variants. Similar substances, probably polypeptides, were detected among staphylococci by Fredericq (1946) and by Halbert, Swick & Sonn (1953). Many of these differed only in heat stability or in specificity of action, and were thus analogous to the colicines produced by enterobacteria (Fredericq, 1957). The similarity in behaviour of each type 71 strain, and the fact that loss of power against one organism coincided with similar loss against other organisms suggested that one particular substance was responsible for their activity. Type 71 staphylococci were active under most of the conditions likely to be encountered in the skin, and were also susceptible to the action of some commensal organisms. Nevertheless, as with other infections, there is still no proof that interaction between invading and resident organisms is actually concerned in the initiation of lesions.

Staphylococci isolated from human secretions and active against mucoid colonies of *Streptococcus pyogenes* were described by Murray & Loeb (1950). In the present work, little evidence was obtained with laboratory strains of 'pyogenes' to support the hypothesis that type 71 staphylococci suppress the growth of some streptococcal serotypes, thus giving an apparent association of other streptococci with impetigo. The streptococcal strains associated with superficial infections may, however, be better able to survive in the skin. Although microbial antagonism could not be related directly to infection, it is possible that, once established, type 71 staphylococci may prevent subsequent invasion of impetigo lesions by other organisms.

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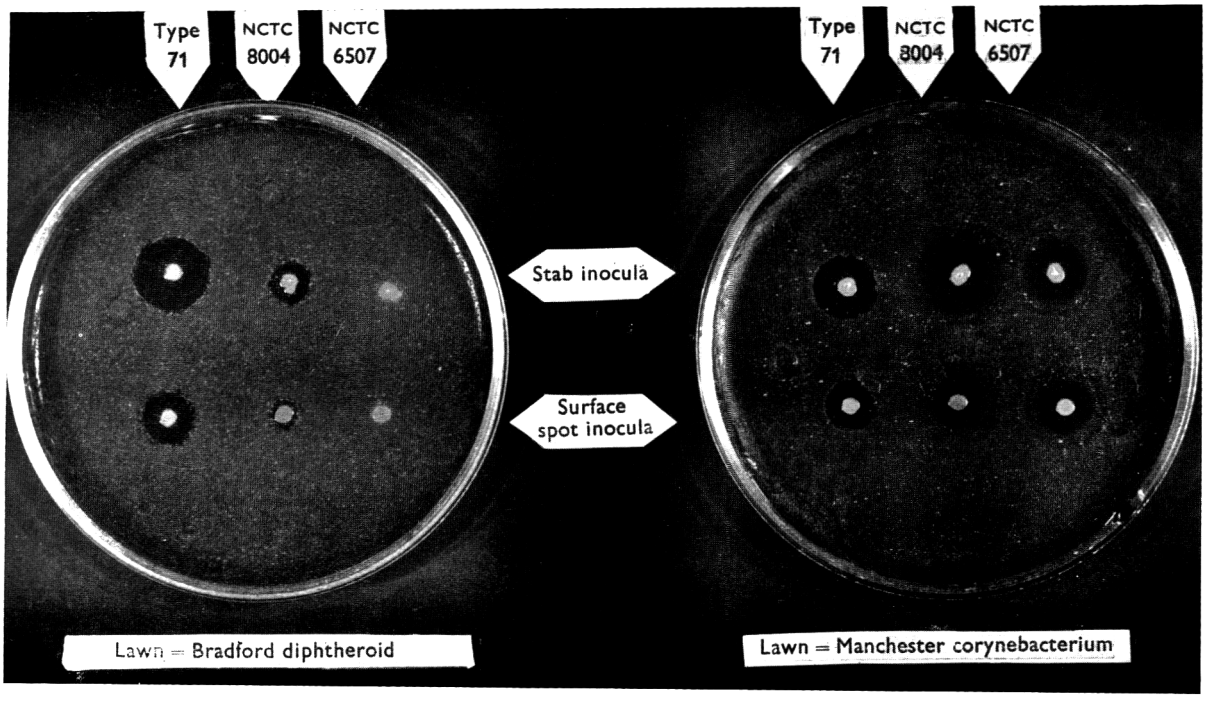
Type 71
NCTC 8004
NCTC 6507
Stab inocula
Surface spot inocula
Type 71
NCTC 8004
NCTC 6507
Lawn = Bradford diphtheroid
Lawn = Manchester corynebacterium

Fig. 1

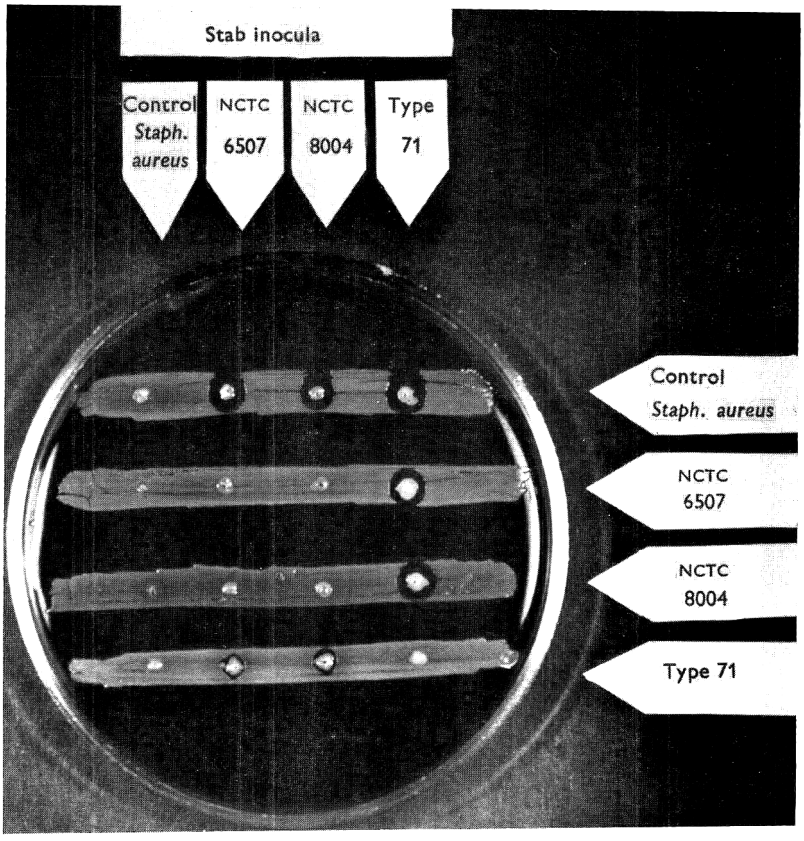
Stab inocula
Control Staph. aureus
NCTC 6507
NCTC 8004
Type 71
Control Staph. aureus
NCTC 6507
NCTC 8004
Type 71

Fig. 2

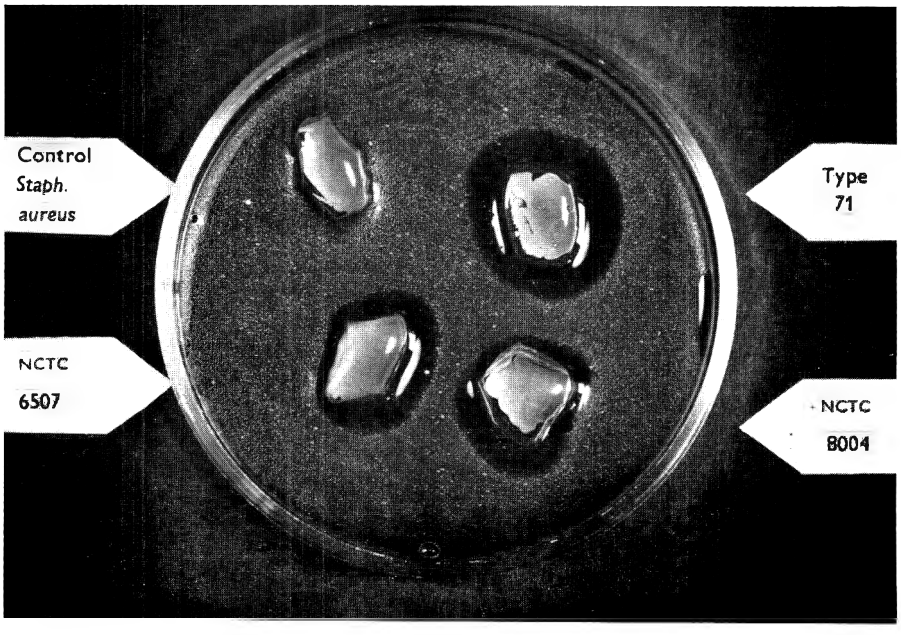
Control Staph. aureus
NCTC 6507
Type 71
NCTC 8004

Fig. 3

REFERENCES

ABBOTT, J. D. & SHANNON, R. (1958). A method for typing *Shigella sonnei* using colicine production as a marker. *J. clin. Path.* **11**, 71.

ALDER, V. G., GILLESPIE, W. A. & HERDAN, G. (1953). Production of opacity in egg-yolk broth by staphylococci from various sources. *J. Path. Bact.* **66**, 205.

ANDERSON, E. S. & WILLIAMS, R. E. O. (1956). Bacteriophage typing of enteric pathogens and staphylococci, and its use in epidemiology. A review. *J. clin. Path.* **9**, 94.

BARROW, G. I. (1955). Clinical and bacteriological aspects of impetigo contagiosa. *J. Hyg., Camb.* **53**, 495.

BARROW, G. I. (1961). An outbreak of streptococcal infection caused by different strains. *Mon. Bull. Minist. Hlth Lab. Serv.* **20**, 224.

BARROW, G. I. & ELLIS, C. (1962). Colicine typing of *Shigella sonnei* by replicate multiple-slide inoculation of indicator organisms. *Mon. Bull. Minist. Hlth Lab. Serv.* **21**, 141.

COOPER, K. E., LINTON, A. H. & SEHGAL, S. N. (1958). The effect of inoculum size on inhibition zones in agar media using staphylococci and streptomycin. *J. gen. Microbiol.* **18**, 670.

FLOREY, H. W., CHAIN, E., HEATLEY, N. G., JENNINGS, M. A., SANDERS, A. G., ABRAHAM, E. P. & FLOREY, M. E. (1949). *Antibiotics*, **1**, 493. London: Oxford University Press.

FRÉDÉRICQ, P. (1946). Sur la sensibilité et l'activité antibiotique des staphylocoques. *C.R. Soc. Biol., Paris*, **140**, 1167.

FRÉDÉRICQ, P. (1957). Colicins. *Annu. Rev. Microbiol.* **11**, 7.

GARDNER, J. F. (1949). An antibiotic produced by *Staphylococcus aureus*. *Brit. J. exp. Path.* **30**, 130.

GOODER, H. (1961). Association of a serum opacity reaction with serological type in *Streptococcus pyogenes*. *J. gen. Microbiol.* **25**, 347.

GRATIA, A. (1946). Techniques sélectives pour la recherche systématique des germes antibiotiques. *C.R. Soc. Biol. Paris*, **140**, 1053.

HALBERT, S. P., SWICK, L. & SONN, C. (1953). Characteristics of antibiotic-producing strains of the ocular bacterial flora. *J. Immunol.* **70**, 400.

MURRAY, R. G. E. & LOEB, L. J. (1950). Antibiotics produced by micrococci and streptococci that show selective inhibition within the genus *Streptococcus*. *Canad. J. Res.* (*E*), **28**, 177.

PARKER, M. T. (1958). Some cultural characteristics of *Staphylococcus aureus* strains from superficial skin infections. *J. Hyg., Camb.* **56**, 238.

PARKER, M. T. & SIMMONS, L. E. (1959). The inhibition of *Corynebacterium diphtheriae* and of other Gram-positive organisms by *Staphylococcus aureus*. *J. gen. Microbiol.* **21**, 457.

PARKER, M. T., TOMLINSON, A. J. H. & WILLIAMS R. E. O. (1955). Impetigo contagiosa. The association of certain types of *Staphylococcus aureus* and of *Streptococcus pyogenes* with superficial skin infections. *J. Hyg., Camb.* **53**, 458.

ROSEBURY, T., GALE, D. & TAYLOR, D. F. (1954). An approach to the study of interactive phenomena among micro-organisms indigenous to man. *J. Bact.* **67**, 135.

EXPLANATION OF PLATES

PLATE 1

Figs. 1, 2. Results given by three active strains of *Staphylococcus aureus* tested for direct antagonism against indicator corynebacteria. With the Manchester organism, type 71 strains gave sharply defined inhibition zones (DI+S), whereas the two NCTC strains gave zones with hazy ill-defined margins (DI+H). Stab inocula produced wider zones than similar surface spot inocula.

PLATE 2

Fig. 3. Results given by stab inocula of one control (DI−) and three active strains of *S. aureus* tested against each other for direct inhibition. The parallel streaks represent growth from single loopfuls of broth cultures. The stab inocula were from growth on solid media.

Fig. 4. Direct inhibition of the Manchester corynebacterium by diffusion of antibiotics produced by three strains of *S. aureus* through cellophan tubing cylinders placed in the medium before setting.

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Media for the Enumeration and Isolation of Heterotrophic Salt-Marsh Bacteria

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SUMMARY

An investigation was made into the relative merits of several media for counting salt-marsh bacteria. Although extracts of salt-marsh mud were superior to extracts of meadow soil, a nitrogenous medium, i.e. ZoBell's sea-water agar no. 2216, was apparently the least selective. For the salt-marsh muds studied, media with a salinity about the same as sea water gave the highest counts of bacteria, particularly for samples from the salicornietum. A possible correlation between this and the origin of the mud is discussed.

INTRODUCTION

Early studies of soil bacteria showed that media containing meat extract or other rich nitrogenous nutrients (e.g. sodium albuminate) were very selective for certain groups of soil bacteria. Indeed the work of Waksman (1922), Smith & Worden (1925), Lochhead & Chase (1943) and others led to the general use of soil-extract media for the isolation and enumeration of soil bacteria, and to the view that these were the least selective media available. Media used to isolate marine bacteria have usually been based on rich nitrogenous substances also, although fish extract was sometimes substituted for meat extract (Fischer, 1894). During recent years such media have been simplified and ZoBell (1941) proposed the use of a sea-water agar containing only 0·5 % peptone and 0·01 % ferric phosphate. Subsequently this medium was used widely, for example by Wood (1953) in Australia, Velankar (1955) in India, and Hudleston (1955) in Wales. Modifications of this medium have also been suggested (Oppenheimer & ZoBell, 1952) and have produced higher counts in some experiments (Carlucci & Pramer, 1957). Little use has been made of soil extracts for isolating marine bacteria, however, although Bayliss-Elliott (1930) and Turner & Pugh (1961) used mud- and soil-extract media to isolate salt-marsh fungi. Burkholder (1959) added mud extract to media in an attempt to promote the growth of bacteria from salt-marsh samples and James (1959) used soil-extract agar for counting bacteria in inland saline soils.

The salinity of the medium may be even more important than the concentration of nutrients, although the literature on this topic is contradictory. Berkeley (1919), in a study of the bacteria present in coastal waters, noted that the sea water was of low density, clearly indicating freshwater dilution. Nevertheless, none of his isolates grew in freshwater media at room temperature. Similarly, Lipman (1926) showed that maximum counts of bacteria were obtained on media containing un-

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diluted sea water. Pearse, Humm & Wharton (1942) and ZoBell (1959) reported essentially the same results for beach sand and marine mud. On the other hand, Lloyd (1930) found little or no difference between the counts obtained when water and mud from the Firth of Clyde were plated on saline or hardly saline media, whilst ZoBell (1941) obtained maximal counts of bacteria in mud and water from Mission Bay and San Diego Bay with media based on sea water diluted to 10–25% of the original concentration. ZoBell (1946) attributed these differing results to different degrees of terrigenous contamination. Results such as these give little guidance in the selection of a medium for the bacteriological examination of estuarine salt-marsh muds. Thus before studying the distribution of bacteria in a salt marsh at Gibraltar Point, Lincolnshire, it was decided to investigate the relative merits of various saline and non-saline media and to compare soil-extract and mud-extract media with the most widely used medium, namely ZoBell's no. 2216 sea-water agar.

METHODS

Sampling sites. The area being studied lay between a spit projecting in a south-westerly direction into the Wash and the River Steeping which flowed across the marsh some 500–600 yds. to the west. Samples of mud were collected from three sites in this area. These were: (1) the bank of the channel cut by the River Steeping through uncolonized mud flats, at a point which was covered by nearly all high tides; (2) the uncolonized mud at a point farther up the shore, covered by all but certain of the neap tides; (3) the salicornietum, which was covered only by the high spring tides. Samples of mud were also collected from the bank of the River Steeping at a point about 10 miles inland and well above the tidal region of the river.

Collection of samples. Samples of mud were obtained with a borer of diameter 5 cm. These samples were placed in polythene beakers and taken to the laboratory, keeping their temperature at about 2°. Subsamples of mud were removed from these cores with a sterile borer and small quantities from a depth of 1 cm. were placed in flasks containing 500 ml. sterile sea water (or river water for the non-saline mud) within 24 hr. of collection.

Methods of counting. The mud (about 0·5 g.) which had been transferred to the dilution flask was dispersed with the aid of a sterile glass rod. Serial tenfold dilutions were prepared and three replicate plates of each medium were made from each dilution. In certain cases the most probable number method of counting was used, in which case sets of four tubes containing 5 ml. of each medium were inoculated with each dilution of mud suspension. After incubation for 14 days at 18°, the colonies that had developed were counted; or by using the McCrady probability tables (McCrady, 1918) estimates of the original numbers of bacteria in the mud were made. The contents of the dilution flasks were evaporated to dryness to find the dry weight of the mud used.

Media. Freshly collected meadow soil (Keuper marl with a cover of *Dactylis glomerata*) and mud from the salicornietum were used to prepare soil-extracts and mud-extracts by the method of James (1958). These extracts were made with sea water or river water; they were incorporated in various media used to count the bacteria in mud from the different sites.

Three liquid media, based on the formulae of Lochhead & Chase (1943) and

Taylor (1951) were used for the most probable number counts. These were as follows. *Basal medium* (*medium B*): glucose, 1·0 g.; potassium nitrate, 0·5 g.; ferric phosphate, 0·1 g., dissolved in 1 l. artificial sea water (NaCl, 24·0 g.; Na_2SO_4, 4·0 g.; $MgCl_2.6H_2O$, 11·0 g.; $CaCl_2.6H_20$, 2·0 g.; KCl, 0·7 g.; $NaHCO_3$, 0·2 g.; KBr, 0·1 g.; $SrCl_2.6H_2O$, 0·04 g.; H_3BO_3, 0·03 g.; $Na_2SiO_3.9H_2O$, 0·005 g.; NaF, 0·003 g.; demineralized water, 1 l. *Medium CG* consisted of 1 l. basal medium B + vitamin-free casein hydrolysate (Difco), 4·0 g.; inositol, 0·05 g.; thiamin, 100 μg.; biotin, 0·1 μg.; pyridoxin, 200·0 μg.; pantothenic acid, 100·0 μg.; nicotinic acid, 100·0 μg. *Medium SY* consisted of 750 ml. basal medium B + 250 ml. meadow-soil extract made with artificial sea water + 0·1 g. yeast extract (Difco).

Six solid media, four of which were soil-extract or mud-extract media were used for a series of dilution plate counts. Sea-water and river-water extracts of meadow soil (media SS and SR) and of salt marsh mud (media MS and MR) were supplemented with 0·1 g. ferric phosphate and 15·0 g. agar (Oxoid Kobe no. 1). These were compared with ZoBell's medium no. 2216 (peptone, 5·0 g.; ferric phosphate, 0·1 g.; agar, 15·0 g.; sea water, 1 l.) and a variant of this medium made with river water (media ZS and ZR, respectively).

The effect of salinity of the medium was examined by means of further dilution counts on media ZS and ZR and on media of the same formula but made with artificial sea water or with solutions of sodium chloride in glass-distilled water at concentrations of 3·5 %, 10·0 % and 20·0 % (w/v) (media ZAS and ZNaCl). All these media were adjusted to pH 7·8 with sodium hydroxide immediately before use. The agar media were dispensed directly into Petri dishes by means of a sterile agar-medium dispenser (Gray, 1961).

Chloride determination. The chloride contents of the various mud samples were determined by titration of the soil solution with silver nitrate, with potassium chromate as an indicator (Piper, 1947).

RESULTS

The use of soil-extract and mud-extract media

The numbers of bacteria from three salt-marsh muds which developed in media B, CG and SY were determined by using the most probable number method. The results of five such experiments are given in Table 1. The numbers of bacteria which developed in media B and SY are expressed as a percentage of the number which developed in medium CG. The percentage of bacteria which grew in medium B was low. There was little difference between the three sampling sites, the numbers of bacteria varying haphazardly between 0·1 and 3·2 % of the numbers that grew in medium CG. Medium SY gave much larger counts, but except for the salicornietum bacteria, gave smaller counts than did medium CG. It was therefore considered that soil-extract media were probably of little advantage for counting salt marsh bacteria, at least as compared with nitrogenous media of known composition.

It is a commonly held view that the origin of soil for preparation of soil extract is of little consequence (Smith & Worden, 1925; James, 1958). Nevertheless, in the present work it was thought that an extract of salt marsh mud might prove superior to an extract of meadow soil. To investigate this, dilution plates were poured with six media (ZS, ZR, SS, SR, MS, MR) to count the numbers of bacteria in the

salicornietum mud. To test whether the non-saline mud would support growth, bacteria in mud from the freshwater site on the bank of the River Steeping were also counted. The results are given in Table 2. The numbers of bacteria capable of developing on the various media are all expressed as a percentage of those which developed on medium MS. The greatest number of colonies from the salicornietum mud developed on medium ZS and was three to four times as great as on medium MS. There were still fewer colonies on medium SS and very few on the non-saline media (ZR, MR, SR). In contrast, the highest counts of bacteria from the non-saline mud samples were on the non-saline media; but again the peptone medium gave higher counts than the soil-extract and mud-extract media. Apparently mud-extract media were less selective than soil-extract media.

Table 1. *The relative numbers of bacteria developing in medium B and medium SY when compared with medium CG*

Medium	Site	Numbers of bacteria in media B and SY as percentage of those in medium CG (five replicate experiments)				
B	River Steeping (saline)	0·1	1·5	3·2	1·8	1·4
	uncolonized mud	0·4	1·0	1·4	0·3	0·2
	salicornietum	0·4	0·2	1·0	0·4	1·4
SY	River Steeping (saline)	70·0	43·0	32·0	55·0	80·0
	uncolonized mud	43·0	25·0	46·0	100·0	45·0
	salicornietum	100·0	100·0	140·0	17·0	1000·0
CG	All three sites	100·0	100·0	100·0	100·0	100·0

Table 2. *The numbers of bacteria/g. of oven-dry mud as determined on several media, expressed as percentage of the number on medium MS*

	Salicornietum mud			Non-saline mud	
	Sample 1	Sample 2	Sample 3	Sample 2	Sample 3
Medium	Percentage of bacteria developing on various media compared with medium MS				
ZR	—	50	69	360	1,950
ZS	390	330	330	115	160
SR	45	4	22	230	507
SS	92	61	28	39	55
MR	36	36	106	290	1,000
MS	100	100	100	100	100

The salinity of the medium

The use of saline and non-saline counting media was studied in greater detail. Table 3 summarizes the results of a series of dilution plate counts on media ZS and ZR. Mud from four sites was used: the salicornietum; uncolonized mud; uncolonized mud near the River Steeping; the freshwater mud referred to previously. The numbers of colonies developing on the non-saline medium are expressed as a percentage of those developing on the saline medium. The saline media again gave much higher counts for mud from the saline sites, whilst the non-saline medium gave higher counts for mud from the freshwater site. In the salicornietum, where

high chloride concentrations were found (see Table 3) because of longer periods of tidal exposure, the percentage of bacteria which developed on the river water medium was particularly low, except in August, 1959.

Some attempts were made to subculture the salt-marsh bacteria which grew on the river-water medium on further river-water medium; growth was poor, however, and very poor when the isolates were reconstituted from freeze-dried cultures.

Table 3. *The number of colonies on medium ZR expressed as percentage of the colonies on medium ZS in four sampling sites*

	Number of colonies on medium ZR expressed as percentage of the colonies on medium ZS			
	Salicornietum	Uncolonized mud	River Steeping (saline)	Non-saline mud
June 1959	3·5	3·0	14·0	192·0
	—	—	14·0	—
July 1959	3·0	—	3·0	225·0
	1·1	7·7	13·0	290·0
August 1959	3·0	5·0	8·0	—
	17·0	—	—	—
September 1959	4·0	8·0	—	—
October 1959	5·0	—	—	—
December 1959	4·5	—	—	320·0
Chloride content (g./kg. oven-dried mud).	15·0–43·7	14·9–28·1	9·8–24·6	O–a trace

Table 4. *The number of bacteria from the salicornietum developing on media of different salinities expressed as percentage of those developing on medium ZS*

	Number of bacteria developing on different media as percentage of those on medium ZS		
Medium	Expt. 1	Expt. 2	Expt. 3
ZS	100	100	100
ZAS	105	94	90
ZR	13	9	4
ZNaCl (3·5 %)	107	80	85
ZNaCl (10·0 %)	33	48	45
ZNaCl (20·0 %)	18	16	30

A second series of counts was made to compare medium ZR with a series of saline media, namely ZS, ZAS and the three variants of ZNaCl. The numbers of colonies which developed on the different media are recorded in Table 4, expressed as a percentage of the number which developed on medium ZS. Again, media of salinity about that of sea water gave the highest counts. There were comparatively small numerical differences between the counts on media ZS, ZAS and ZNaCl (3·5 %). However, the non-saline medium and the two media with high salt concentrations gave marked decreases in the counts obtained.

The appearance of the colonies on the different media also varied. Media ZS and

ZAS supported a mixture of pigmented (yellow, red, orange) and non-pigmented forms, whilst on medium ZR most of the colonies were pigmented. Media made with sodium chloride supported very few pigmented colonies at all.

DISCUSSION

Previous workers have used soil extracts and mud extracts to isolate micro-organisms from saline areas, with varying degrees of success. As far as fungi are concerned, Bayliss-Elliott (1930) found that other media were superior for isolation purposes. Similarly, Burkholder (1959) noted that the growth of marine bacteria was greatly stimulated by nitrogenous substances and Thalassia extracts, stimulated to some extent by dextrose, but not stimulated by the additions of mud-extract, or phosphate and ammonium nitrate. The results in the present work appear to support these results, and to show that soil-extract and mud-extract agars are not suitable for the enumeration of salt marsh bacteria. The only other report of the use of soil extracts to count bacteria from saline sites is that of James (1959) who investigated an inland saline area; he found that soil-extract media were superior to nitrogenous media. The reasons for this difference are obscure. For isolating organisms from salt marshes, as opposed to counting, mud extract may still prove useful as it may support a wider range of bacterial types than the peptone media used. Clearly the response of bacteria from saline sites to soil extracts and mud extracts needs further investigation.

The evidence also suggests that, contrary to the usual view (James, 1959) the origin of the soil used in preparing soil extract is of some importance, since saline-mud extracts gave substantially larger counts than meadow-soil extracts. Starr (1956) showed that vitamin B_{12} was produced more rapidly than it was destroyed in salt marshes, so that it is possible that mud extracts may contain a greater quantity or variety of growth factors than soil extracts.

The most satisfactory peptone media used in the present work were those based on sea water or artificial sea water; these gave slightly higher counts than a medium based on 3·5% sodium chloride. This is in agreement with the findings of Tyler, Bielling & Pratt (1960) who showed that for marine bacteria, cations other than sodium were important for growth. River-water media, which apparently contained sufficient nutrients and growth factors to permit maximum counts of river-mud bacteria to be obtained, were unsuitable for salt-marsh bacteria, even for those isolated from close to the River Steeping. This reaction of salt-marsh bacteria has been confirmed by experiments on the effect of salinity on the growth of individual isolates (Gray, 1962). This evidence, together with the inability of the salt-marsh bacteria isolated on river-water media to grow well on subcultivation, indicates the possible marine origin of these bacteria. Macleod & Onofrey (1956) reported that sea-water bacteria subcultured onto media of low salinity showed poor growth and often failed to survive more than one subculture. The possibility that terrestrial bacteria may become adapted to higher salinities cannot be ruled out however.

The origin of the mud in the marsh is of importance. Thus the present findings may not apply to all salt marshes. It was noted by Barnes & King (1961) that at Gibraltar Point muddy sand was predominant, partly blown, but mainly washed over the spit or through a depression in it. In fact the main areas of accretion in the

marsh that they examined were opposite this depression in the spit which separates the marsh from the North Sea. Hardy (1959) pointed out that there was an inflow of water from the North Sea into the Wash basin. Presumably, the predominant bacteria in the marsh will be derived from this mud and water. If large numbers of terrestrial bacteria are also swept into the area they must either be killed and replaced by marine forms or they must rapidly adapt themselves to saline conditions. It is possible that in other estuaries where it is known that the mud is largely of terrestrial origin, sea water media may prove to be less satisfactory for counting. Preliminary samples from the Humber estuary suggest that these muds contain rather more bacteria that would grow on non-saline media.

On the basis of the work described in this paper, an investigation into the distribution of bacteria in the Gibraltar Point salt marsh has been made. For this work, peptone media, namely ZoBell's medium no. 2216 and variants of it, were mostly used. The work is to be published later.

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REFERENCES

BARNES, F. A. & KING, C. A. M. (1961). Salt marsh development at Gibraltar Point, Lincolnshire. *East Midl. Geographer*, **15**, 20.

BAYLISS-ELLIOTT, J. S. (1930). The soil fungi of the Dovey salt marshes. *Ann. appl. Biol.* **17**, 284.

BERKELEY, C. (1919). A study of marine bacteria. Straits of Georgia, B.C. *Trans. Roy. Soc. Canad.* (Ser. 3) **13**, 15.

BURKHOLDER, F. R. (1959). Some microbiological aspects of marine productivity in shallow waters. *Proc. Salt-marsh Conf. Sapelo Island*, 1958, p. 70.

CARLUCCI, A. F. & PRAMER, D. (1957). Factors influencing the plate method for determining the abundance of bacteria in sea water. *Proc. Soc. exp. Biol., N.Y.* **96**, 392.

FISCHER, B. (1894). Die Bakterien des Meeres bei den Untersuchungen der Plankton-Expedition unter gleichzeitiger Berücksichtigung einiger älterer und neuerer Untersuchungen. *Ergebn. Plankton-Expedition der Humboldt-Stiftung*, **4**, 1.

GRAY, T. R. G. (1961). A sterile agar-medium dispenser. *J. appl. Bact.* **24**, 1.

GRAY, T. R. G. (1962). Effect of salinity on the growth of certain salt-marsh bacteria. *Nature, Lond.* **194**, 560.

HARDY, A. C. (1959). *The Open Sea. II. Fish and Fisheries.* London: Collins.

HUDLESTON, M. (1955). Marine bacteria of Cardigan Bay. II. Bacteria in rock pools. *J. appl. Bact.* **18**, 29.

JAMES, N. (1958). Soil extract in soil microbiology. *Canad. J. Microbiol.* **4**, 363.

JAMES, N. (1959). Plate counts of bacteria and fungi in a saline soil. *Canad. J. Microbiol.* **5**, 431.

LIPMAN, C. B. (1926). The concentration of sea water as affecting its bacterial population. *J. Bact.* **12**, 311.

LLOYD, B. (1930). Bacteria of the Clyde sea area. A quantitative investigation. *J. mar. biol. Ass.* **16**, 879.

LOCHHEAD, A. G. & CHASE, F. E. (1943). Qualitative studies of soil micro-organisms. V. Nutritional requirements of the predominant bacterial flora. *Soil Sci.* **55**, 185.

MACLEOD, R. A. & ONOFREY, E. (1956). Nutrition and metabolism of marine bacteria. II. Observations on the relation of sea water to the growth of marine bacteria. *J. Bact.* **71**, 661.

MCCRADY, M. H. (1918). Tables for the rapid interpretation of fermentation tube results. *Canad. Publ. Hlth J.* **9**, 201.

OPPENHEIMER, C. H. & ZOBELL, C. E. (1952). The growth and viability of sixty three species of marine bacteria as influenced by hydrostatic pressure. *J. mar. Res.* **11**, 10.

PEARSE, A. S., HUMM, A. J & WHARTON, C. W. (1942). Ecology of sand beaches at Beaufort, North Carolina. *Ecol. Monogr.* **12**, 135.

PIPER, C. S. (1947). *Soil and Plant Analysis.* Adelaide: University Press.

SMITH, N. R. & WORDEN, S. (1925). Plate counts of soil micro-organisms. *J. agric. Res.* **31**, 501.

STARR, T. J. (1956). Relative levels of vitamin B_{12} in detritus from oceanic and estuarine environments. *Ecology*, **37**, 658.

TAYLOR, C. B. (1951). Nutritional requirements of the predominant bacterial flora of soil. *Proc. Soc. appl. Bact.* **14**, 101.

TURNER, M. & PUGH, G. J. F. (1961). Species of Mortierella from a salt-marsh. *Trans. Brit. mycol. Soc.* **44**, 243.

TYLER, M. E., BIELLING, M. C. & PRATT, D. B. (1960). Mineral requirements and other characters of selected marine bacteria. *J. gen. Microbiol.* **23**, 153.

VELANKAR, N. K. (1955). Bacteria in the inshore environment at Mandapam. *Indian J. Fish.* **2**, 96.

WAKSMAN, S. A. (1922). Microbiological analysis of soil as an index of soil fertility. II. Methods of study of the numbers of micro-organisms in soil. *Soil Sci.* **14**, 283.

WOOD, E. J. F. (1953). Heterotrophic bacteria in marine environments of Eastern Australia. *Aust. J. fresh. mar. Res.* **4**, 160.

ZOBELL, C. E. (1941). Studies on marine bacteria. I. The cultural requirements of heterotrophic aerobes. *J. mar. Res.* **4**, 42.

ZOBELL, C. E. (1946). *Marine Microbiology* Waltham, Mass., U.S.A.: Chronica Botanica Co.

ZOBELL, C. E. (1959). Introduction to marine microbiology. *N.Z. Oceanogr. Inst. Mem.* 3, *Contrib. marine Microbiol.* 7.

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Fine Structure of the Young Vegetative Hyphae of *Pythium debaryanum*

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SUMMARY

The fine structure of *Pythium debaryanum* Hesse differs from that of Rhizopus species and some other fungi in the abundant regularly distributed endoplasmic reticulum, the presence of typical Golgi-bodies and the irregularly tubular structure of the cristae mitochondriae. Some of these characters resemble those of some algae and the liverwort Anthoceros and the significance of this is discussed. Typical lomasomes are present.

METHODS

Young hyphae of *Pythium debaryanum* Hesse grown on 3% malt agar for 40 hr. at 20° were fixed with Luft's permanganate (Luft, 1956), embedded in Araldite, sectioned by the methods previously described (Hawker & Abbot, 1963) and examined and photographed with a Phillips E.M. 200 electron microscope.

RESULTS

The cell wall shows no structure and is represented only by a narrow electron transparent zone (Pl. 3, fig. 5).

The plasmalemma or ectoplast is represented by a wavy line bounding the endoplasm (Pl. 1, figs. 1 and 2; Pl. 2, fig. 4) and situated just within the wall except where lomasomes (*L*) (Pl. 2, fig. 4; Pl. 3, fig. 6) are present. These contain amorphous matter and are similar to those shown by Moore & McAlear (1961) for several fungi.

The endoplasm enclosed within the plasmalemma is of complex nature and shows a regularly distributed reticulum appearing as two outer dark layers and a wider light inner layer (*ER* in the photographs). Portions of this reticulum may widen to form irregular cisternae (*C*) or globules (*G*). Occasionally these cisternae are stacked together to give typical Golgi-bodies or dictyosomes (*D*, Pl. 1 and 2; Pl. 4, fig. 9) towards the periphery of the endoplasm. The matrix of the endoplasm is homogeneous or slightly granular.

The mitochondria are ovoid with a regular outline. They are surrounded by a membrane appearing as two dark layers enclosing a wider light one. The inner dark layer projects into the interior to form more or less tubular, irregularly shaped cristae (*MC*). Occasionally the outer membrane of a mitochondrion appears to be continuous with the endoplasmic reticulum (Pl. 3, fig. 6, z_3) and apparently gives rise to small vesicles (Pl. 2, fig. 4), the nature and further development of which could not be determined. The central matrix of the mitochondria resembles that of the endoplasm.

The nuclei are of irregular shape and size. The nuclear membrane is of three layers, an inner light layer enclosed by two thin dark ones, and is interrupted by

numerous pores (Pl. 3, fig. 6; Pl. 4, fig. 8) and occasionally by larger gaps (Pl. 3, fig. 6). The nuclear membrane shows connexions with the endoplasmic reticulum (Pl. 2, fig. 4; Pl. 3, fig. 6) as has been shown by other workers for a variety of organisms (e.g. Weier & Thomson, 1962, for leaf mesophyll; Manton, 1961, for the liverwort Anthoceros; Lindergren, 1962, for yeasts). Serial sections suggest that contact occurs only over a limited area of the nuclear membrane.

Vacuoles were not present in young hyphae, but typical stellate vacuoles occurred in older material, finally coalescing to give a large central vacuole in mature hyphae. The endoplasmic reticulum is progressively less regularly arranged as the hyphae age. The mitochondria (Fig. 1) and nuclei remain distinct even in the oldest material examined.

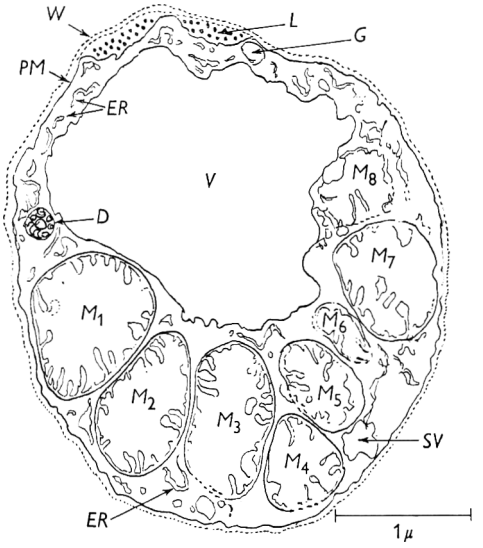

Fig. 1. Tracing of an electron micrograph of a mature hypha of *Pythium debaryanum* (? permanganate fixed) showing mitochondria (*M*), possible remains of a Golgi-body (*D*), unidentified spherical globule (*G*), endoplasmic reticulum (*ER*) which is less abundant and less regularly distributed than in the younger hyphae shown in Pls. 1–4, plasmalemma (*PM*), large vacuole (*V*) surrounded by tonoplast, small stellate vacuole (*SV*). Mitochondria 1–3 are still more or less intact but 4–7 show signs of breakdown. M_8 is in an advanced state of breakdown. *L* = lomasome (shown diagrammatically). *W* = cell wall.

DISCUSSION

As has already been pointed out (Hawker, 1963) the fine structure of *Pythium debaryanum* shows many points of resemblance to that of some algae and green plants and differs in some respects from that of species of Rhizopus and other filamentous fungi and yeasts. The absence of visible structure in the cell wall is in marked contrast to material from Rhizopus species fixed in a similar way (Hawker & Abbott, 1963). This is not surprising in view of the known difference in the chemical nature of the largely cellulose wall of Pythium and the chitinous one of Rhizopus.

The mitochondrial cristae are much less regular in shape and arrangement than those of Rhizopus (Hawker & Abbott, 1963) or of certain yeasts (Thyagarajan, Conti & Naylor, 1961). They resemble those of many algae and protozoa and also the irregularly distended ones of the liverwort Anthoceros illustrated by Manton

(1961, fig. 18). Manton, however, considers that this appearance may be due to osmic distortion and that the cristae of most green algae and plants are normally flat plates. In her figure 17, representing material fixed with potassium permanganate, the cristae are not distended and are more or less plate-like, but even here they are much less regular than those of Rhizopus (Hawker & Abbott, 1963) which were also fixed with permanganate. The permanganate fixed material of Pythium is, however, very different from similarly fixed material of Rhizopus, the cristae being almost certainly irregularly tubular in contrast to the regular flat plates of Rhizopus. In general the mitochondria of Pythium show at least a superficial resemblance to those of many algae and some green plants and are unlike those shown in published figures of other fungi.

The most striking difference between Pythium and other fungi, e.g. Rhizopus and yeasts, is the regularly distributed endoplasmic reticulum of the former and, in particular, the presence of typical Golgi-bodies resembling those of the Chrysophycean flagellate *Paraphysomonas vestita* (Manton & Leedale, 1961) and the liverwort Anthoceros (Manton, 1961). No such highly organized Golgi-bodies were seen in Rhizopus. Moore & McAlear (1962) observed such structures in *Neobulgaria pura* only, in a survey of over 50 genera. Thus the fine structure of the fungus *Pythium debaryanum* resembles that of certain lower green plants in a number of ways, the most striking being the nature of the endoplasmic reticulum. This is of interest taxonomically, since many mycologists consider that the Oomycetes may be closely related to certain green algae. On the other hand the presence of typical lomasomes is a fungal characteristic, since these are thought by Moore & McAlear (1961) to be found only in fungi.

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REFERENCES

HAWKER, L. E. (1963). Fine structure of *Pythium debaryanum* Hesse. *Nature, Lond.* **197**, 618.

HAWKER, L. E. & ABBOTT, P. McV. (1963). Fine structure of vegetative hyphae of *Rhizopus*. *J. gen. Microbiol.* **30**, 401.

LINDEGREN, C. C. (1962). Origin of endoplasmic reticulum. *Nature, Lond.* **195**, 1225.

LUFT, J. H. (1956). Permanganate—a new fixative for electron microscopy. *J. biophys. biochem. Cytol.* **2**, 799.

MANTON, I. (1961). Plant cell structure; in *Contemporary botanical thought*, edited by A. M. McLeod & L. S. Cobley. Edinburgh: Oliver and Boyd, p. 171.

MANTON, I. & LEEDALE, G. F. (1961). Observations on the fine structure of *Paraphysomonas vestita* with special reference to Golgi bodies and the internal origin of scales. *Phycologia*, **1**, 35.

MOORE, R. T. & MCALEAR, J. H. (1961). Fine structure of Mycota 5. Lomasomes—previously uncharacterized hyphal structures. *Mycologia*, **53**, 194.

MOORE, R. T. & MCALEAR, J. H. (1962). Characterization of the Golgi dictyosome of the fungus *Neobulgaria pura* in *Rept. Vth Int. Congr. Electron Microscopy*. New York: Academic Press.

THYAGARAJAN, T. R., CONTI, S. F. & NAYLOR, H. B. (1961). Electron microscopy of yeast mitochondria. *Exp. Cell Res.* **25**, 216.

WEIER, T. E. & THOMSON, W. W. (1962). Membranes of mesophyll cells of *Nicotiana rustica* and *Phaseolus vulgaris* with particular reference to the chloroplast. *Amer. J. Bot.* **49**, 807.

EXPLANATION OF PLATES

Pythium debaryanum—electron micrographs

W = cell wall, *N* = nucleus, *NM* = nuclear membrane, *NP* = pores in nuclear membrane, *M* = mitochondria, *MC* = cristae, *ER* = endoplasmic reticulum, *C* = cisternae, *G* = globules, *D* = Golgi-body or dictyosome, *PM* = plasmalemma or ectoplast, *L* = lomasome.

All material fixed Luft's permanganate

PLATES 1 AND 2

Figs. 1 and 2. Slightly oblique serial sections through a young hypha.

Fig. 3. Part of Fig. 1 enlarged. Matrix of nuclei shows faint reticulate structure.

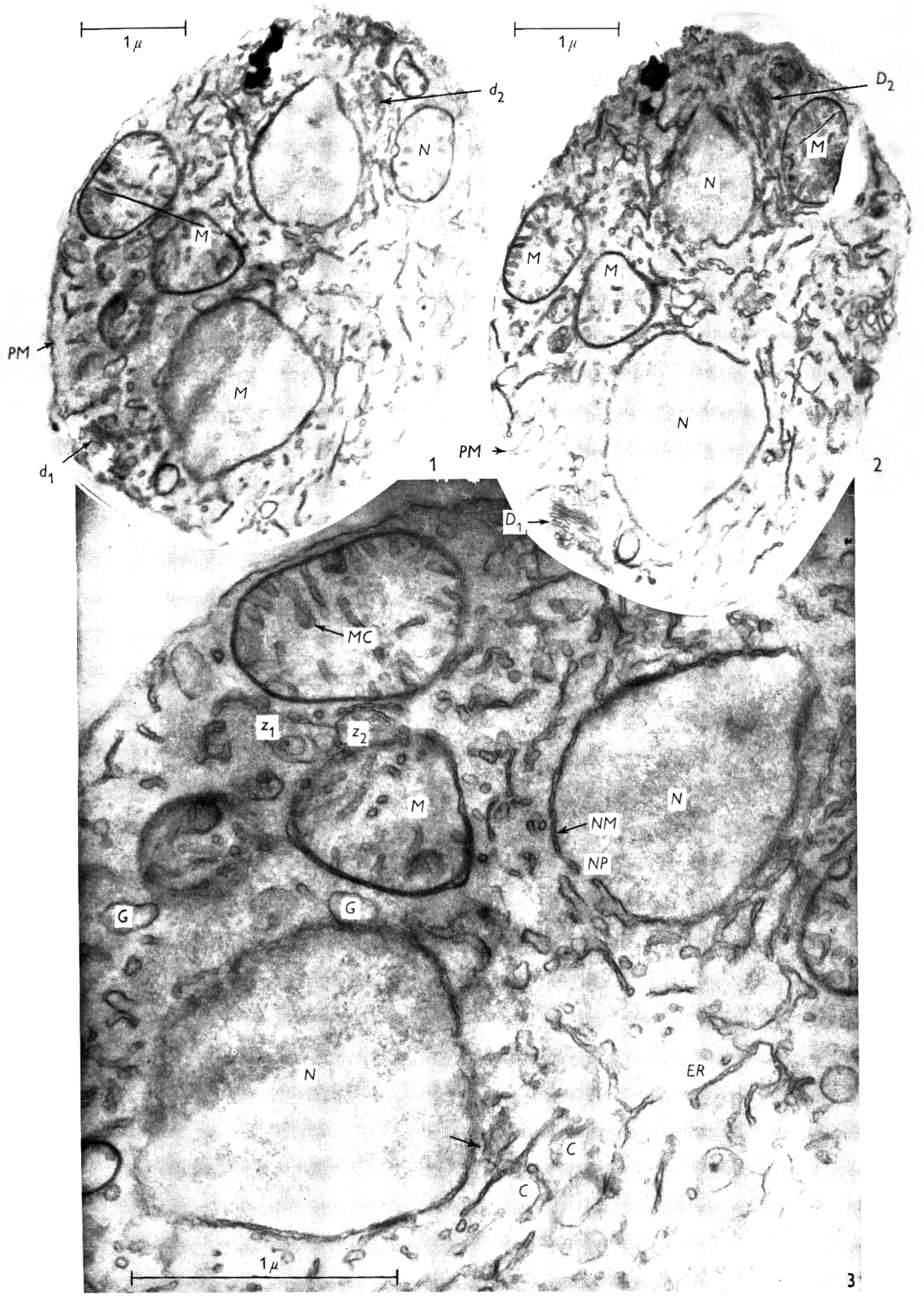
Fig. 4. Same hypha as in Fig. 2 enlarged. Note typical Golgi-bodies. Compare with serial section shown in Fig. 3. Note contact between nuclear membranes and endoplasmic reticulum at point *X* and compare with same point *x* in Fig. 3; distinct Golgi-body D_1 and less distinct one D_2 and compare with same points (marked d_1 and d_2) in Fig. 1; small globose bodies adjacent to mitochondrion at points Z_1, Z_2 and compare with similar detached body at point z_1 (Fig. 3) and blister-like body apparently being formed by the mitochondrion at z_2 in Fig. 3; changes in form of various globules and cisternae in the two sections; faint reticulate structure of nuclear matrix as in Fig. 3; irregular tubular structure of cristae mitochondriae (compare with same mitochondria in Fig. 3).

PLATES 3 AND 4

Figs. 5 and 7. Oblique serial sections through another hypha of similar age to that shown in Pls. 1 and 2 and adjacent to it on the grid.

Figs. 6 and 8. Parts of same sections enlarged. Note continuity between nuclear membrane and endoplasmic reticulum at points R_1, R_2, R_3 and R_4 in Fig. 6 and compare with similar points r_1, r_2, r_3 and r_4 in Fig. 8; large gap in nuclear membrane at *S* in Fig. 6 and more or less complete membrane at similar position *s* in Fig. 8.

Fig. 9. More or less transverse section of part of a third hypha showing a Golgi-body in which some tubules are at right-angles to others.



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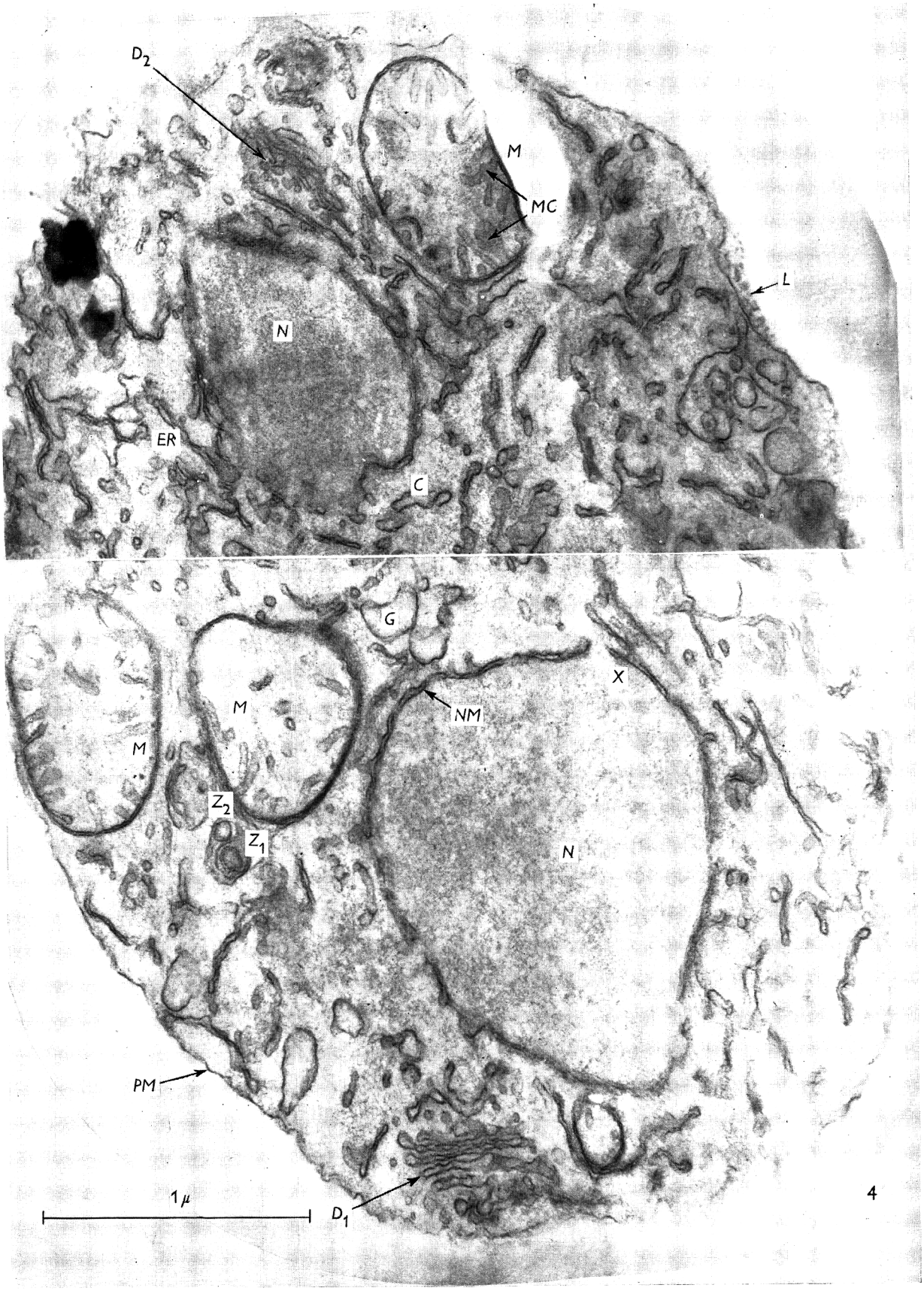
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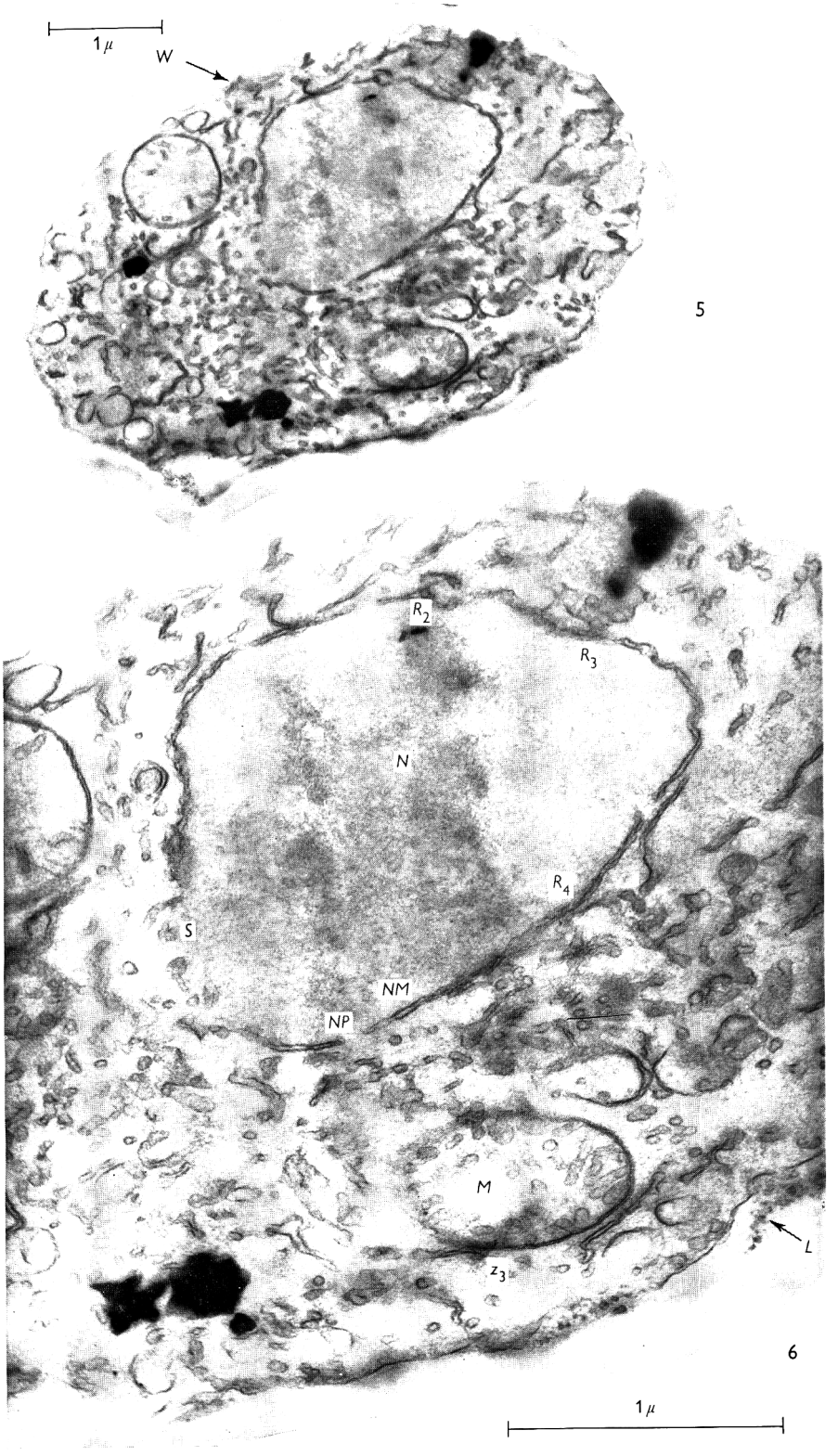

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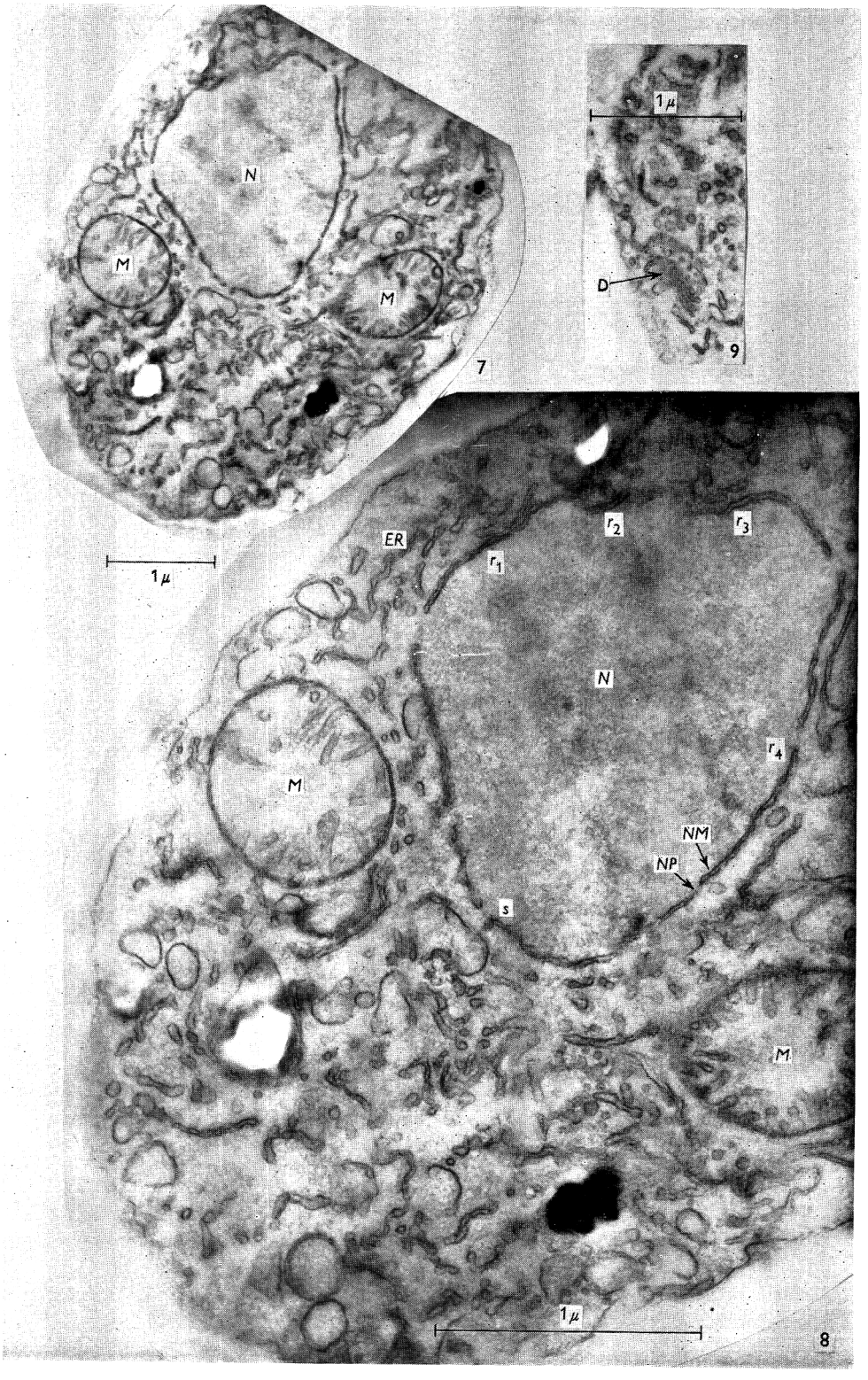
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Printed in Great Britain

Electron Microscopic Observation of the Sporangial Structure of an Actinomycete, *Microellobosporia flavea*

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(*Received* 20 *November* 1962)

SUMMARY

Ultrathin sections of sporangia of the actinomycete *Microellobosporia flavea* were examined with an electron microscope. The sporangial wall is a thin, wrinkled membrane which seems to be an extension of the outer layer of the cell wall of the sporangiophore. A substance, probably a liquid, is located between the sporangial wall and the sporangiospores. The spores are ovoid and consist of a laminated wall in which two layers can be differentiated. The wall is 30–40 mμ thick but thickens to 90–120 mμ at the point of contact between two spores. Inside the spores one can differentiate a finely granular nucleus, large vacuoles which are probably filled with a fatty substance, and a coarsely granular cytoplasm. The cytology of the sporangiospores of *M. flavea* is similar to that of the conidia of *Waksmania rosea*.

INTRODUCTION

More than a decade ago the isolation of actinomycetes which formed motile sporangiospores was reported (Couch, 1949). Later Couch proposed to call such organisms by the generic name *Actinoplanes* (Couch, 1950) and he described, under the name *Streptosporangium* (Couch, 1955), actinomycetes which formed non-motile sporangiospores. He also suggested that the family Actinoplanaceae be created to group all sporangia-bearing actinomycetes (Couch 1955*a*). Strains of both Actinoplanes and Streptosporangium form globose to spherical sporangia containing numerous spores which are randomly distributed at maturity. Recently, organisms which form non-motile sporangiospores arranged in a short single row have been described under the new generic name, *Microellobosporia* (Cross, Lechevalier & Lechevalier, 1963). Two species of this new genus of *Actinoplanaceae*, differing from each other mainly in pigmentation, were described: *M. cinerea*, the type species; and *M. flavea*. Cross and his co-workers showed the presence of the sporangial wall by light microscopy and by electron microscopy, and they followed with light microscopy the formation of the spores within the sporangia. They noted that even though all the spores within a sporangium were of the same age, they were not necessarily of the same size. In the present paper electron microscopy is used to probe the structure of sporangia and sporangiospores of *M. flavea*. Sporangia are borne both on the substrate and on the aerial mycelium, but only the structure of the more accessible aerially-borne sporangia was investigated. These are club-shaped structures borne on the swollen tips of sporangiophores and usually contain from 2 to 5 spores. They range in length from 2 to 9μ and in diam. from 1·5 to 3·6μ.

METHODS

Organism. Microellobosporia flavea, No. 3858 (Culture collection of the Institute of Microbiology of Rutgers).

Growth and preliminary treatment of the organism. Potato + carrot agar medium (Segretain, Drouhet & Mariat, 1958) was poured into Petri dishes. The surface of the solid medium was covered with a film of cellophan (Chabbert & Patte, 1960) and inoculated with a cell suspension which was spread over the surface of the cellophan. The plates were incubated at 28° until mature sporangia were abundant (about 10 days). The cellophan membranes were removed from the medium and the growth that they carried was scraped off with an inoculating needle. The material thus collected was suspended in acetate-veronal (sodium barbital) buffer (Kellenberger, Ryter & Séchaud, 1958) and was sedimented by centrifugation.

Fixation. The procedure of Kellenberger *et al.* (1958) was followed with minor modifications. The fixative was a 1 % (w/v) solution of osmium tetroxide in acetate-veronal buffer (pH 5·9) to which traces of calcium chloride were added just before use. The previously sedimented organisms were suspended in this fixative and left there overnight. After proper centrifugation and washing, the fixed organisms were embedded in 2 % (w/v) agar which was cut in small (1–2 $mm.^3$) blocks. These were suspended in 0·5 % (w/v) aqueous uranium acetate for 1 hr.

Embedding. After fixation, the specimens were dehydrated with ethanol. After having been passed twice through propylene oxide, they were embedded in Epon 812 (Shell Chemical Co., San Francisco) according to the method of Luft (1961) by using 3 volumes of his mixture A for 7 volumes of his mixture B.

Thin sectioning. After proper paring, the resin blocks were sectioned either with a Porter-Blum microtome (Ivan Sorwall, Inc., Norwalk, Connecticut, U.S.A.) equipped with glass knives or with an LKB Ultratome microtome (L.K.B. Instruments, Inc., 4800 Rugby Avenue, Washington 14, D.C., U.S.A.) equipped with a diamond knife. The sections were floated on 10 % (v/v) acetone in water and were picked up on collodion coated grids.

Electron microscopy. The sections were examined with an RCA-3D electron microscope (aperture, 25 μ) operated at 50 Kv., and photographed on Eastman Kodak Lantern Slides at an instrumental magnification of 8000.

RESULTS AND DISCUSSION

Not a single section was obtained which passed through the whole sporangium and sporangiophore. This is not surprising when one considers the large size of this whole structure. At best, some sections were obtained which passed through the apex of a sporangiophore to which two spores were still attached (Pl. 1, fig. 1), or sections were obtained through three spores but not the sporophore (Pl. 1, fig. 2).

The sporangial wall appears as a thin, loosely-wrinkled membrane which looks like an extension of the outer covering of the cell wall of the sporangiophore (Pl. 1, figs. 1 and 3). Inside the sporangium, between the spores and the sporangial wall, is located an electron light, reticulated to granular substance. It is difficult to measure accurately the thickness of the sporangial wall, since one is never sure that the wall has not been cut obliquely, but this thickness was estimated at 5–6 mμ.

Sectionsof spores, cut perpendicularly to the long axis of the sporangium, are round. Cutting the spores through the long axis of the sporangium revealed an ovoid shape. The walls of the spores are laminated and two main layers of different density can be seen in most sections, the outer layer being the darker one. The spore walls are 30–40 mμ at the equator and thicken into pads at the point of contact between two spores. These pads (90–120 mμ) are mainly due to the thickening of the outer layer. The spore walls exhibit another anatomical feature which is always located in the area where the thickening of the interspore pads starts. It is reminiscent of a plasmodesm and might represent a point of cytoplasmic contact between two adjacent immature spores before they are separated by the obesity of maturity, or it might indicate a point of contact, at some stage of maturation, between the cytoplasm of the spores and the sporangial membrane. Such structures can be seen on Pl. 1, figs. 1 and 2; in fig. 1 two arrows point to a pair of such structures located on adjacent spores. In addition, the interspore pads and more strikingly the spore-wall pad which rests on the sporangiophore, exhibit two electron light areas which look like an extension of the inner spore wall layer. On Pl. 1, figs. 1 and 3, these areas appear as two oblique lines converging toward the inside of the spore.

Within the spores three main cytological structures can be noted. (1) A large ovoid, more or less centrally located mass of a very finely granular substance; by its very location, one would be tempted to call it a nucleus. Its structure is different from that previously observed in 'nuclei' of spores of Streptomyces which had been fixed, stained and embedded by the same method used here for *Microellobosporia flavea*. (2) Numerous electron-light vacuoles. (3) A coarsely granular material, darker than the 'nucleus', which fills up whatever space is left, and which is probably the cytoplasm. Since the spores of *M. flavea* were easily stained with Sudan Black it is a fair assumption that the large vacuoles are formed by the deposition of a fatty storage material. An attempt to identify this compound as poly-β-hydroxybutyric acid failed (Slepecky & Law, 1960).

Macroscopically, the growth of strains of Microellobosporia resembles that of Streptomyces; microscopically, at least at first glance, it is reminiscent of Waksmania (Lechevalier & Lechevalier, 1957). It is interesting that sections of spores of *M. flavea* are very similar to sections of spores of *W. rosea* (Pl. 1, fig. 4) and somewhat different from what we would consider a typical section of a spore of a Streptomyces (Pl. 1, fig. 5). By comparing these pictures of organisms which have all been treated in the same manner one will note the greater abundance of vacuoles in Microellobosporia and Waksmania than in Streptomyces, and the rather light unevenly-dense nuclear material of the Streptomyces which contrasts with the evenly-granular structures of the other two organisms. Study of additional strains of these and related organisms may permit some generalizations with phylogenic implications. Along this line of thought, we are tempted to compare the sporangial wall in Microellobosporia with the outer layer of the sporogenic hyphae of Streptomyces, which breaks up into spore-sized segments, and which finally enrobes each mature spore (Glauert & Hopwood, 1961). Microellobosporia could then be considered as short chained Streptomyces in which the outer layer of the sporogenic hyphae does not break up and does not cover each spore. Rather, this outer layer remains continuous around the whole chain of spores, and some fluid is probably

present between the sporangial wall and the spores. Waksmania may well be a link between Microellobosporia and Streptomyces since its spores are non-sporangial like the Streptomyces, and its cytology is strikingly similar to Microellobosporia.

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REFERENCES

CHABBERT, Y. A. & PATTE, J. C. (1960). Cellophan transfer. *Appl. Microbiol.* **8**, 193.

COUCH, J. N. (1949). A new group of organisms related to actinomyces. *J. Elisha Mitchell sci. Soc.* **65**, 315.

COUCH, J. N. (1950). Actinoplanes, a new genus of the actinomycetales. *J. Elisha Mitchell sci. Soc.* **66**, 87.

COUCH, J. N. (1955). A new genus and family of the actinomycetales, with a revision of the genus *Actinoplanes*. *J. Elisha Mitchell sci. Soc.* **71**, 148.

COUCH, J. N. (1955*a*). Actinosporangiaceae should be actinoplanaceae. *J. Elisha Mitchell sci. Soc.* **71**, 269.

CROSS, T., LECHEVALIER, M. P. & LECHEVALIER, H. A. (1963). A new genus of the Actinomycetales: *Microellobosporia* gen.nov. *J. gen. Microbiol.* **31**, 421.

GLAUERT, A. M. & HOPWOOD, D. A. (1961). The fine structure of *Streptomyces violaceoruber* (*S. coelicolor*). III. The walls of the mycelium and spores. *J. biophys. biochem. Cytol.* **10**, 505.

KELLENBERGER, E., RYTEF, A. & SÉCHAUD, J. (1958). Electron microscope study of DNA-containing plasms. *J. biophys. biochem. Cytol.* **4**, 671.

LECHEVALIER, M. P. & LECHEVALIER, H. A. (1957). A new genus of the Actinomycetales: *Waksmania* gen.nov. *J. gen. Microbiol.* **17**, 104.

LUFT, J. H. (1961). Improvements in epoxy resin embedding methods. *J. biophys. biochem. Cytol.* **9**, 409.

SEGRETAIN, G., DROUHET, E. & MARIAT, F. (1958). *Diagnostic de Laboratoire en Mycologie Médicale*. St. Mandé, France: Editions de la Tourelle.

SLEPECKY, R. A. & LAW, J. H. (1960). A rapid spectrophotometric assay of α,β-unsaturated acids and β-hydroxy acids. *Analyt. Chem.* **32**, 1697.

EXPLANATION OF PLATE 1

Electron microphotographs of thin sections of spores of Actinomycetes.

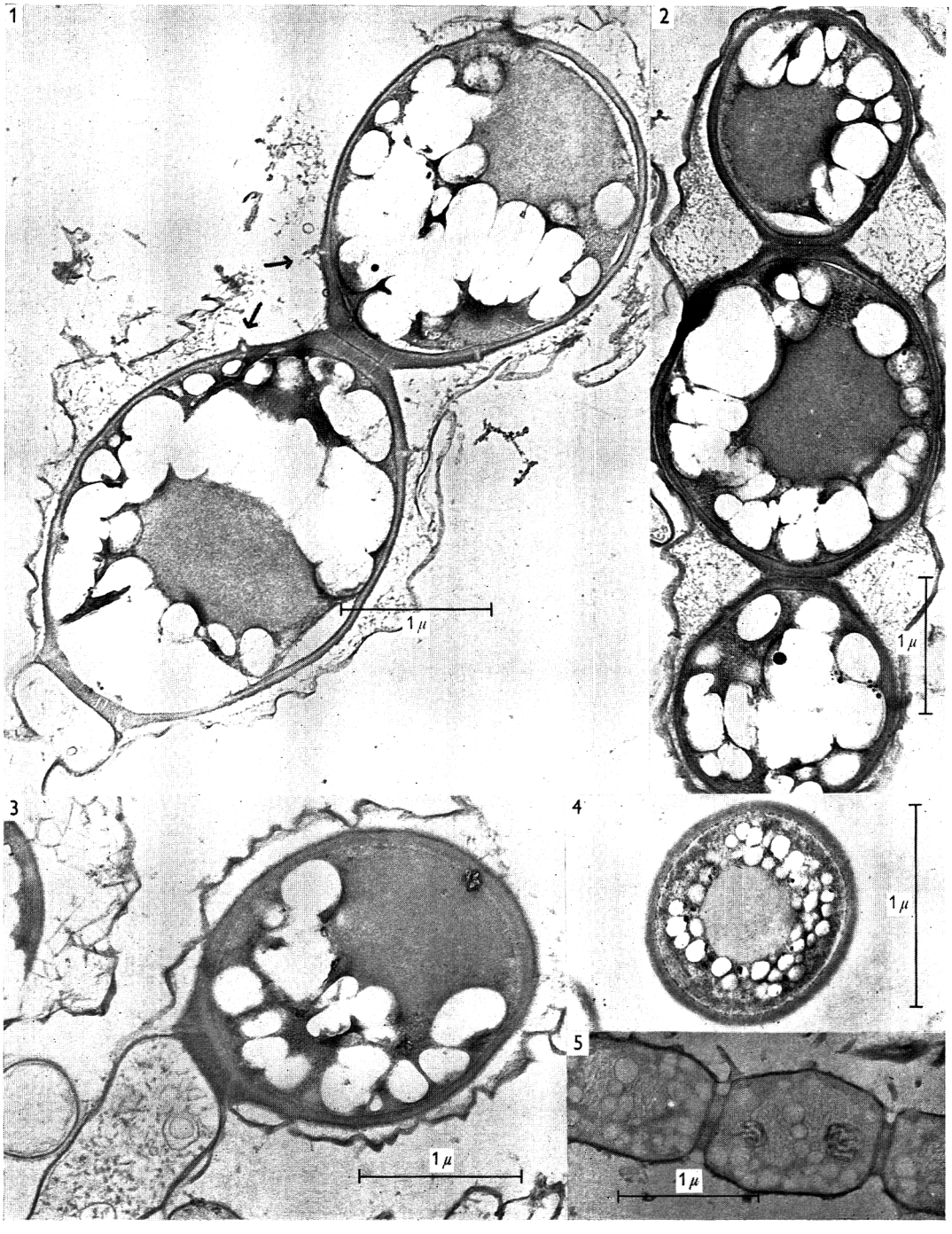
Fig. 1. Section of sporangiophore and sporangium of *Microellobosporia flavea* 3858. × 22,200.

Fig. 2. Section of sporangium of *M. flavea* 3858. × 20,000.

Fig. 3. Section of sporangiophore and sporangium of *M. flavea* 3858. × 24,000.

Fig. 4. Section of spore of *Waksmania rosea* 3748. × 29,600.

Fig. 5. Section of spores of *Streptomyces violaceus* 829. × 21,000.



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Books Received

Die Bedeutung physikalisch-chemischer Faktoren (pH und rH 2) für die Lebenstätigkeit der Mikroorganismen. By I. L. RABOTNOWA. Published by VEB Gustav Fischer Verlag Jena, Villengang 2, Jena, Germany. 226 pp. Price 58.20 DM.

The First Book of Microbes. By L. Z. LEWIS. Published by Edmund Ward (Publishers) Ltd., 194–200 Bishopsgate, London, E.C. 2. 62 pp. Price 10*s.* 6*d.*

The Handling of Chromosomes. By C. D. DARLINGTON and L. F. LA COUR. Published by George Allen and Unwin Ltd., 40 Museum Street, London, W.C. 1. 247 pp. Price 30*s.*

Modern Microbiology. By W. W. UMBREIT. Published by W. H. Freeman and Co. Ltd., Hyde House, West Central Street, London, W.C. 1. 507 pp. Price 48*s.*

Nomina und Synonyma. By O. MRÁZ. Published by VEB Gustav Fischer Verlag Jena, Villengang 2, Jena, Germany. 488 pp. Price 49.70 DM.

Peripheral Circulation in Man. British Medical Bulletin, **19**, no. 2. May 1963. Published by the Medical Department, The British Council, 65 Davies Street, London, W.1. Price 30*s.*

Respiratory Physiology. Ed. by P. HUGH-JONES and E. J. M. CAMPBELL. *British Medical Bulletin*, **19**, no. 1. January 1963. Published by the Medical Department, The British Council, 65 Davies Street, London, W.1. Price 30*s.*

Some Methods of Water Analysis for Limnologists. By F. J. H. MACKERETH. Scientific Publication no. 21. 1963. Published by Freshwater Biological Association. 70 pp. 5*s.* 6*d.*

Terrestrial and Freshwater Radioecology: A Selected Bibliography. By A. W. KLEMENT and V. SCHULTZ. Published by the United States Atomic Energy Commission, Division of Technical Information, Washington, 25. D.C., U.S.A. 79 pp. Price $1.00.

Revista Venezolana de Sanidad y Asistencia Social. Vol. XXVII, nos. 1 and 2. Published by Ministerio de Sanidad y Asistencia Social, Caracas, Venezuela, S. America.

Revista Venezolana de Sanidad y Asistencia Social. Suplemento II, nos. 3 and 4. Published by the Ministerio de Sanidad y Asistencia Social, Caracas, Venezuela, S. America.

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