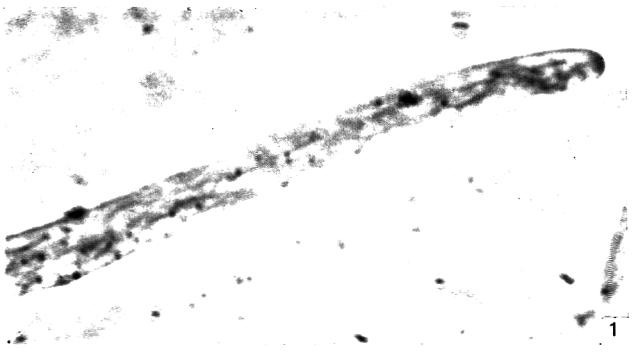
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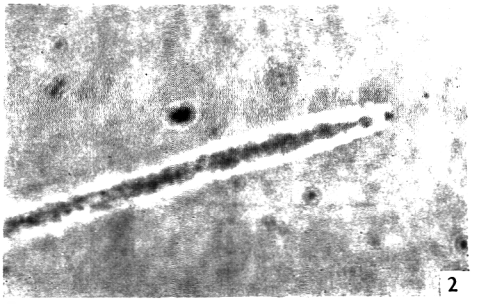
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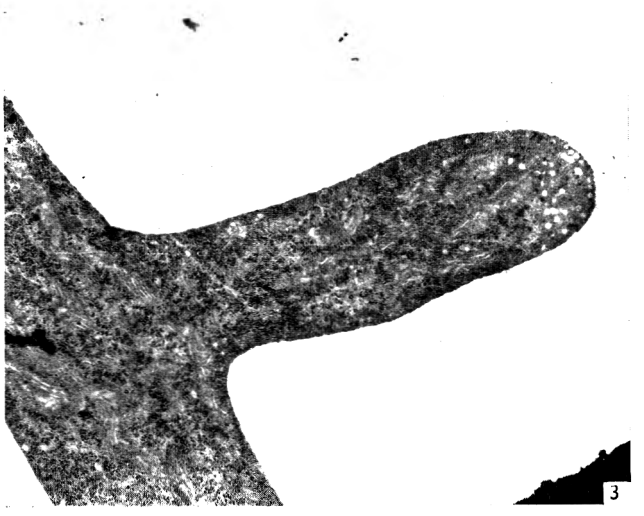
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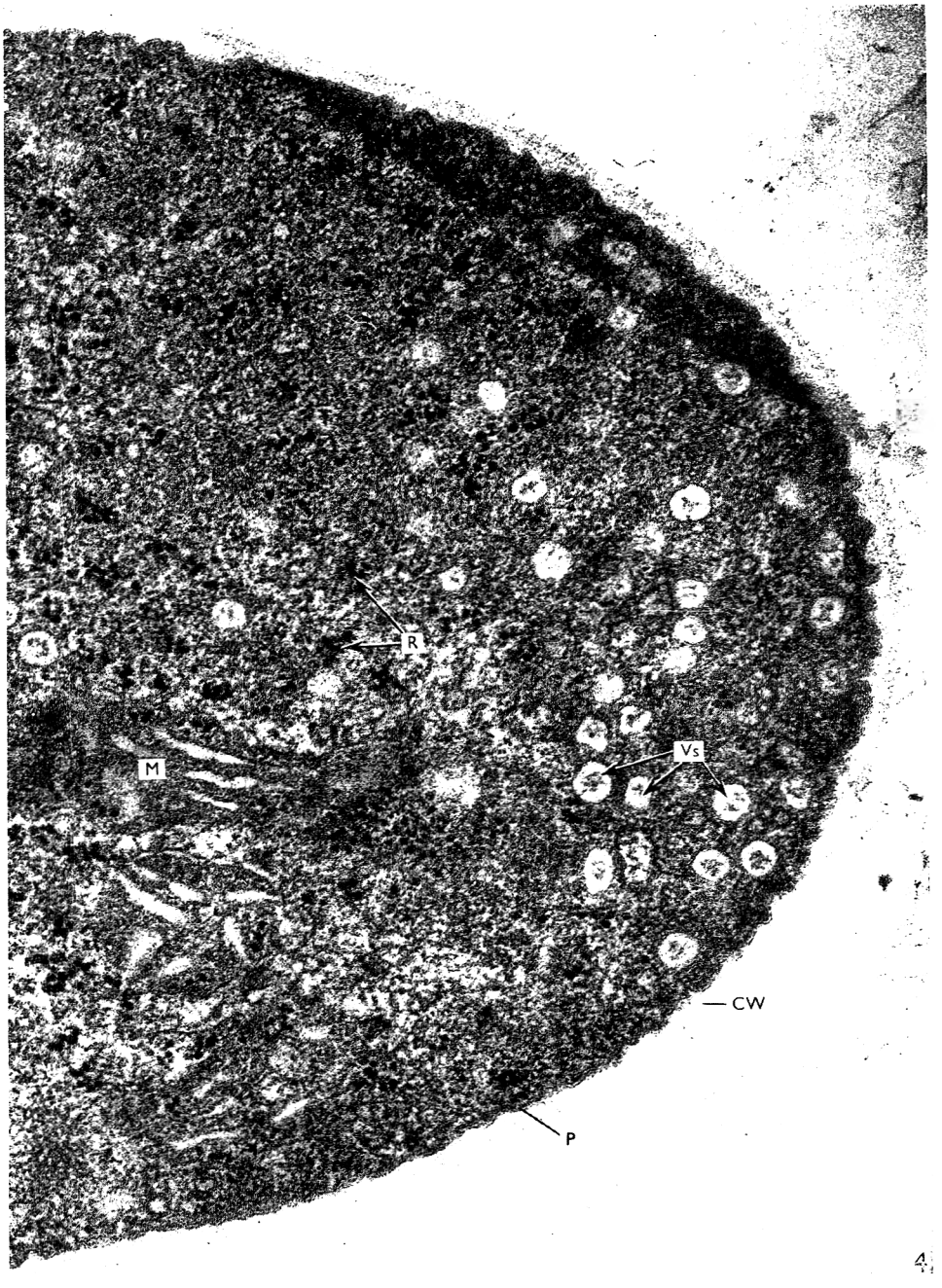


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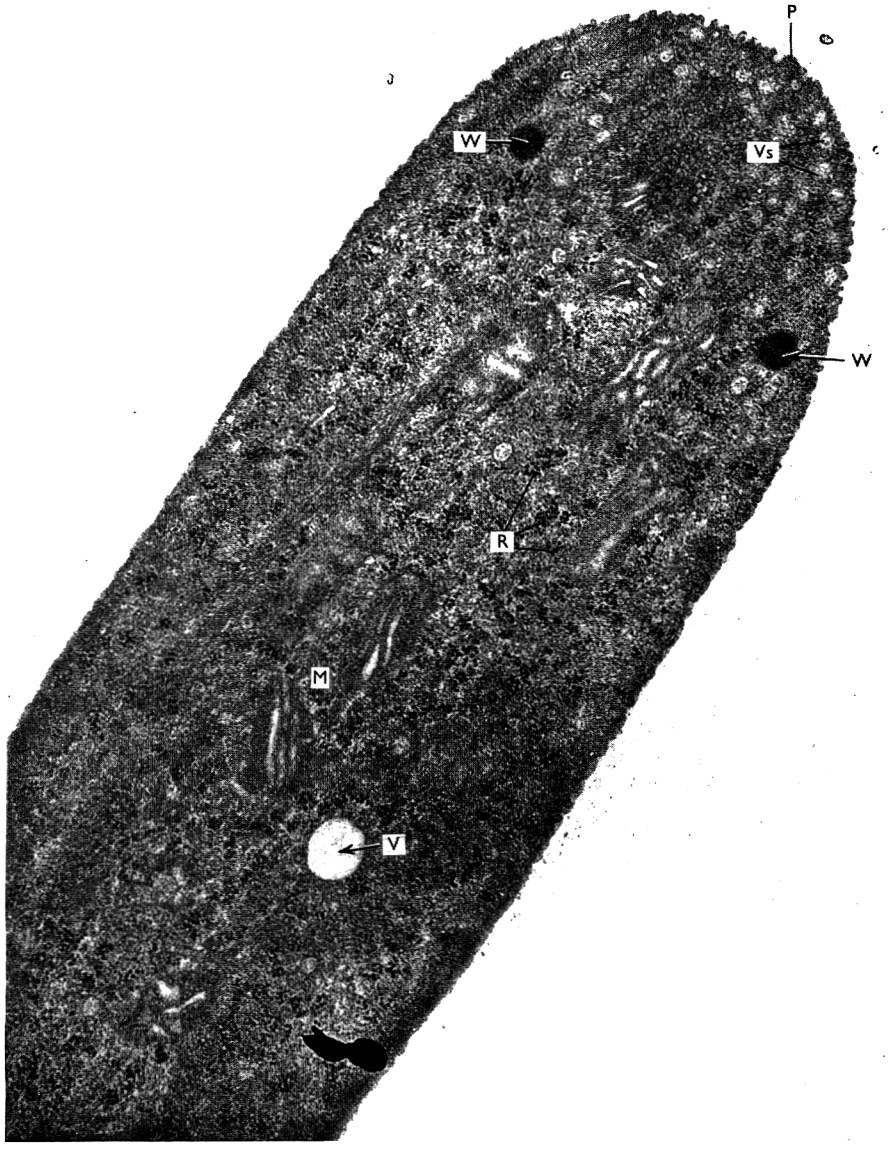


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# Acetate and Acetamide Mutants of *Pseudomonas aeruginosa* 8602

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SUMMARY

Mutants of *Pseudomonas aeruginosa* 8602 were isolated which were unable to grow with acetamide as sole carbon source. They were divided into five classes on the basis of growth studies and enzyme assays. The mutants able to grow on acetate but not acetamide ($Am^-$) lacked an inducible amidase. Mutants unable to utilize either acetamide or acetate ($At^-$) lacked one of the glyoxylate cycle enzymes (isocitrate lyase or malate synthase) or were deficient in citrate synthase or acetic thiokinase. Isocitrate lyase-negative mutants grew on propionate which is therefore not metabolized by the glyoxylate cycle. The amidase marker was not co-transduced with the isocitrate lyase, acetic thiokinase or citrate synthase markers.

## INTRODUCTION

*Pseudomonas aeruginosa* 8602 produces an inducible amidase (aliphatic acylamide amidohydrolase EC 3.5.1.4) in media containing acetamide and this enzyme accounts for the hydrolysis of acetamide to acetate and ammonia (Kelly & Clarke, 1962). Microbial growth on acetate is thought to involve the reactions of the glyoxylate cycle enzymes (Kornberg & Krebs, 1957) and the specific activities of isocitrate lyase and malate synthase are sufficiently high in acetate-grown pseudomonads to account for the biosynthesis of succinate and other compounds required for growth (Kornberg, Gotto & Lund, 1958; Kornberg & Lund, 1959). We have isolated several mutants of *P. aeruginosa* 8602 which are unable to grow on acetamide and acetate. These mutants have been examined by growth and enzyme studies to investigate the metabolism of acetate and related compounds. We have previously shown co-transduction of the amidase structural and regulator genes (Brammar, Clarke & Skinner, 1967). Similar transduction analysis was used to examine the genetic linkage of the acetate mutants. A brief report of part of this work has appeared previously (Skinner & Clarke, 1965).

## METHODS

*Organism.* The parent strain was *Pseudomonas aeruginosa* 8602. Amidase-negative mutants are referred to as Am 1, etc.; mutants unable to utilize acetate are referred to as At 1, etc. Cultures were maintained as previously described (Brammar *et al.* 1967).

*Media.* Liquid media were based on the minimal salt medium described by Brammar & Clarke (1964). Succinate, acetate, pyruvate and propionate (as sodium salts) were added to give a final concentration of 1% (w/v). Acetamide was sterilized by Seitz

filtration and added aseptically to sterile minimal salt medium to a final concentration of 1 % (w/v). Solid media contained 1·2 % (w/v) Oxoid no. 3 or Difco Noble agar. Succinate and acetate agar plates were prepared by adding the appropriate carbon source to minimal agar to a final concentration of 0·5 % (w/v). Acetamide agar plates were prepared by adding sterile acetamide to minimal agar to a final concentration of 0·5 % (w/v).

*Growth conditions.* Cultures were grown at 37° on a mechanical shaker in conical flasks of capacity 10 times that of the medium volume. Stationary phase cultures for enzyme assays were obtained after overnight growth (16 hr) from an inoculum of 0·1 ml./20 ml. medium. Bacteria were harvested at 0° in an MSE Angle 17 refrigerated centrifuge at 12,000*g* for 10 min.

*Preparation of cell-free extracts.* Bacteria were resuspended in cold 0·1 M-phosphate buffer (pH 7·2) and disrupted with an MSE 25 W ultrasonic oscillator. Samples (2·5 ml.) were subjected to an output of 1·3 A for 2 min. in a glass vessel designed for use with the $\frac{1}{4}$ in. probe. Bacterial debris was removed by centrifugation at 25,000*g* for 15 min.

*Estimation of protein.* The method of Lowry, Rosebrough, Farr & Randall (1951) was used to estimate protein, with bovine plasma albumin as a standard.

*Enzyme assays.* Amidase was measured by the hydroxamate method for transferase activity (Brammar & Clarke, 1964). For acetic thiokinase (EC 6.2.1.1.) the method of Jones & Lipmann (1955) was used to detect the formation of acetyl-coenzyme A from acetate in the presence of ATP, coenzyme A, glutathione and $Mg^{2+}$. Isocitrate lyase (EC 4.1.3.1.), malate synthase (EC 4.1.3.2) and citrate synthase (EC 4.1.3.7) were assayed according to the method of Dixon & Kornberg (1959) at room temperature by using a Carey Model 14 or a Unicam SP 800 recording spectrophotometer.

*Mutagenic treatment.* Ethylmethane sulphonate (EMS) and *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NMG) were used as described by Brammar *et al.* (1967).

*Replica plating.* Replica plating was done according to Lederberg & Lederberg (1952).

*Selection of mutants.* (*a*) Replica plating. Mutagen-treated cultures were plated on succinate agar and the colonies replicated to acetamide plates. Colonies which were present on succinate plates but absent from acetamide plates were picked off, resuspended in dilution buffer and streaked on succinate, acetate and acetamide plates. This differentiated mutants unable to utilize acetamide from those unable to grow on either acetate or acetamide. This method provided 6 strains unable to grow on acetate ($At^-$). (*b*) Limiting enrichment (Davis, 1949). Bacteria treated with a mutagen were spread on agar plates containing either 0·05 % (w/v) acetamide or 0·05 % (w/v) acetamide + 0·001 % (w/v) sodium succinate as carbon sources (Brammar *et al.* 1967). After 48 hr incubation, plates were examined for small or opaque colonies; these were picked off, resuspended in dilution buffer and streaked on succinate, acetate and acetamide plates.

*Transduction analysis.* The pseudomonad phage F 116 (Holloway, Egan & Monk, 1960) was used as described previously (Brammar *et al.* 1967).

*Reagents.* Acetamide was obtained from Hopkin and Williams Ltd. and recrystallized twice from ethanol for use in the amidase assay. *N*-acetylacetamide was synthesized from acetamide and acetic anhydride and twice recrystallized from methylethylketone. Glutathione and coenzyme A were obtained from C. F. Boehringer und

Soehne G.m.b.H. Isocitrate was prepared from DL-isocitric lactone obtained from L. Light and Co. Ltd. Acetyl-coenzyme A was prepared according to Stadtman (1957). Adenosine-5′-triphosphate.$3H_2O$ (ATP) was purchased as the disodium salt from the Sigma Chemical Company. Bovine plasma albumin was obtained from Armour Laboratories. Ethylmethane sulphonate was obtained from Kodak Ltd. *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine was obtained from the Aldrich Chemical Co.

## RESULTS

### *Mutants unable to grow on acetamide*

The mutant isolation procedures provided 32 strains of *Pseudomonas aeruginosa* unable to grow on acetamide as sole source of carbon and nitrogen; 22 of these strains were also unable to grow on acetate. The 32 strains were grown overnight in succinate

Table 1. *Growth of Pseudomonas aeruginosa 8602 wild-type and mutant strains*

| Strain | Carbon source for growth: Acetamide | Acetate | Succinate | Amidase* |
|---|---|---|---|---|
| Wild-type | + | + | + | Present |
| Am 1–Am 10 | − | + | + | Absent |
| At 1–At 22 | − | − | + | Present |

+, Growth after 24-hr incubation at 37°; −, no growth after 24 hr incubation at 37°.
* Cultures were grown overnight in 1% succinate medium with $10^{-2}$ M-*N*-acetylacetamide as inducer for amidase and assayed according to Brammar & Clarke (1964).

medium with *N*-acetylacetamide as a non-substrate inducer for amidase. The 10 strains which grew on acetate but not on acetamide all lacked amidase (Table 1); the specific activity was less than 1% of that of the fully induced wild-type strain. They were designated Am 1 to Am 10. The strains unable to utilize either acetamide or acetate had specific amidase activities which were not significantly different from those of the fully induced wild-type strain. These were classified as acetate-negative mutants and designated At 1 to At 22.

### *Growth of acetate-negative mutants*

Growth of pseudomonads on acetate as sole carbon source is thought to require the tricarboxylic acid and glyoxylate cycles (Kornberg & Krebs, 1957). As all the acetate-negative mutants were able to grow on succinate it seemed unlikely that they would have major defects in the tricarboxylic acid cycle enzymes. The mutants might be expected to be defective in either of the two specific enzymes of the glyoxylate cycle, isocitrate lyase or malate synthase, or in another enzyme required for acetate metabolism such as acetic thiokinase (Fig. 1).

The mutants were tested for growth in minimal medium containing acetate + glyoxylate. Unlike some other pseudomonads, *Pseudomonas aeruginosa* 8602 does not grow with glyoxylate as sole carbon source. Some mutants unable to grow on acetate might be expected to do so in the presence of added glyoxylate which could be condensed with acetyl-CoA to form malate. However, mutants lacking malate synthase would be unable to use glyoxylate in this way and would not grow on acetate + glyoxylate. The

addition of glyoxylate to an acetate medium would also have no effect on the growth of strains lacking acetate activating enzymes. *P. aeruginosa* 8602 grows on both propionate and propionamide and the acetate-negative mutants were tested to see whether they had also lost the ability to grow on propionate.

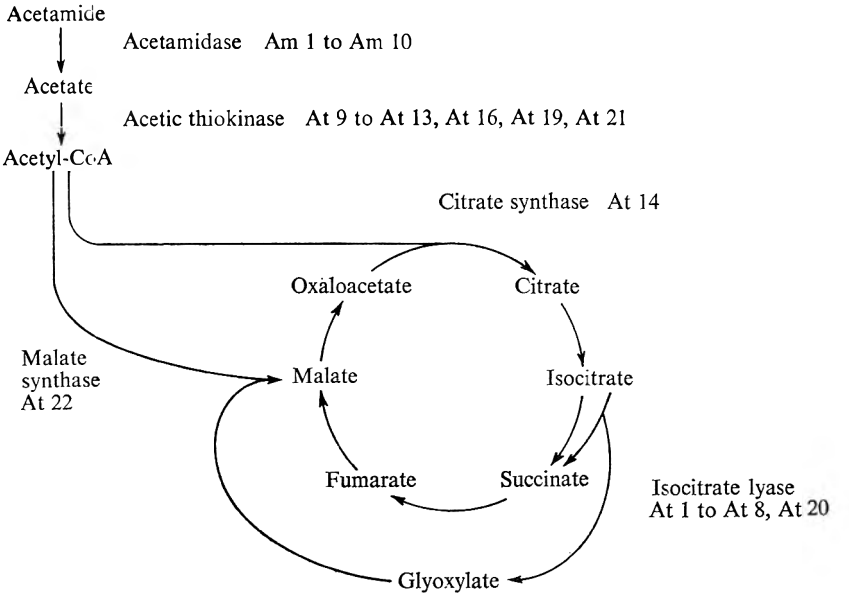


Fig. 1. *Pseudomonas aeruginosa* 8602; pathways of acetamide and acetate metabolism. Am 1–Am 10, amidase-negative mutants; At 1–At 22, mutants defective in enzymes required for acetate utilization.

Table 2. *Growth of Pseudomonas aeruginosa 8602 wild-type and acetate-negative mutants*

| Strain | Carbon source for growth: Acetate | Acetate + glyoxylate | Propionate | Possible enzyme defects |
|---|---|---|---|---|
| Wild-type | ++ | ++ | + | None |
| At 1, At 2, At 3, At 4, At 5, At 6, At 7, At 8, At 15, At 20 | (−) | ++ | + | Isocitrate lyase |
| At 14 | − | ++ | − | Citrate synthase, malate synthase, acetic thiokinase or other enzymes |
| At 9, At 16, At 21 | (−) | (−) | + | |
| At 10, At 11, At 12, At 13, At 19, At 22 | (−) | (−) | (−) | |

++, Growth (about $10^9$ bacteria/ml.) after 16–24 hr incubation at 37°; (−), no visible growth after 24 hr incubation at 37° (some cultures grew after a further 12 hr incubation); +, growth (about $10^9$ bacteria/ml.) after 24–36 hr incubation at 37°; −, no visible growth after 36 hr incubation at 37°.

Growth of cultures in acetate, acetate+glyoxylate or propionate media was examined visually at intervals during 36 hr. Table 2 shows that 11 mutants grew on acetate+glyoxylate and 10 of these also grew on propionate. These 10 were provisionally classified as isocitrate lyase-defective mutants. As they grew well on acetate +glyoxylate it seemed unlikely that the inability of the wild type to grow on glyoxylate alone was due to impermeability to this compound. Nine mutants were unable

to grow on acetate + glyoxylate and 6 of these were also unable to grow on propionate. The enzyme patterns of these 20 acetate-negative mutants were investigated. Two other mutants, At 17 and At 18, have not been examined in detail.

### *Isocitrate lyase*

The wild-type strain was grown on acetate, propionate and succinate and cell-free extracts were assayed for isocitrate lyase activity (Table 3). It is known that different growth substrates influence the specific activity of this enzyme in *Pseudomonas ovalis* Chester (Kornberg, Gotto & Lund, 1958; Kornberg & Lund, 1959) and in *Escherichia coli* (Kornberg, Phizackerley & Sadler, 1960; Vanderwinkel, Liard, Ramos & Wiame, 1963). A similar effect was found with *P. aeruginosa* 8602. Specific activities of isocitrate lyase in cell-free extracts from acetate-grown wild-type bacteria were approximately 30-fold greater than those from succinate-grown cultures (Table 3). Propionate-grown bacteria also produced isocitrate lyase, but the specific activity was only one quarter of that present in acetate-grown cultures.

Table 3. *Isocitrate lyase activities of cell-free extracts of wild-type and acetate-negative mutants of Pseudomonas aeruginosa 8602*

Assayed according to Dixon & Kornberg (1959). Cultures were harvested after 16 hr growth and broken by ultrasonic treatment. The supernatant fluid was used for isocitrate lyase determination.

| Strain | Carbon source for growth | Specific activity of isocitrate lyase* |
|---|---|---|
| Wild-type | Acetate | 14·3 |
| | Propionate | 3·5 |
| | Succinate | 0·45 |
| At 1 | Propionate | 0·01 |
| | Succinate | 0·01 |
| At 8 | Propionate | 0·04 |
| | Succinate | 0·02 |
| At 2 | Succinate | 0·06 |
| At 3 | | 0·06 |
| At 4 | | 0·07 |
| At 5 | | 0·04 |
| At 6 | | —† |
| At 7 | | 0·02 |
| At 20 | | —† |
| At 14 | Succinate | 0·06 |
| At 22 | | 0·10 |
| At 9 | Succinate | 0·38 |
| At 11 | | 0·57 |
| At 13 | | 0·44 |
| At 15 | | 0·60 |
| At 16 | | 0·58 |
| At 21 | | 0·46 |

* μmoles isocitrate utilized per mg. soluble protein per hr.
† Not determined quantitatively, but isocitrate lyase activity minimal in preliminary assays.

Enzyme assays of cell-free extracts from succinate-grown cultures of the 11 mutants which grew on acetate + glyoxylate showed that 9 strains (At 1 to At 8, At 20) were

deficient in isocitrate lyase (Table 3). It could be argued that the assays were performed on extracts of severely repressed (succinate-grown) cultures which would be expected to have low enzyme activity For this reason, two mutants (At 1, At 8) were grown in propionate medium and extracts assayed for isocitrate lyase activity. Under these partially derepressed conditions there was no significant increase in the specific activity. It was concluded that the 9 mutants were true isocitrate lyase-negative strains and since they all grew on propionate it appears that this glyoxylate cycle enzyme is not essential for propionate utilization by *Pseudomonas aeruginosa*.

Mutant At 15 also grew on acetate+glyoxylate and propionate but its isocitrate lyase activity did not differ significantly from that of the wild type. Mutant At 14 was the only mutant which grew on acetate+glyoxylate but not on propionate. It had only one third of the isocitrate lyase activity of the wild type strain under the same conditions (Table 3). However, as growth on propionate is not dependent on isocitrate lyase, the low activity of the enzyme in this strain was considered to be secondary to some other genetic defect.

Six of the 9 strains unable to grow on acetate+glyoxylate were also assayed for isocitrate lyase activity. In 5 of these strains (At 9, At 11, At 13, At 16, At 21) the enzyme activity was not significantly different from that of the wild-type strain. Mutant At 22, which was also unable to grow on propionate, had only a quarter of the isocitrate lyase activity of the wild-type strain. It was thought that like mutant At 14 a defective isocitrate lyase was not the primary genetic defect.

Table 4. *Malate synthase activities of cell-free extracts of wild-type and acetate-negative mutants of Pseudomonas aeruginosa 8602*

Assayed for malate synthase according to Dixon & Kornberg (1959); bacteria were harvested from succinate medium after 16 hr incubation and broken by ultrasonic treatment. The supernatant fluid was used for malate synthase determination.

| Strain | Genetic defect | Growth | | Specific activity of malate synthase* |
|---|---|---|---|---|
| | | Acetate+ glyoxylate | Propionate | |
| Wild-type | None | + | + | 1·6† |
| At 22 | Malate synthase | − | − | 0·1 |
| At 3 | Isocitrate lyase | + | + | 1·5 |
| At 15 | Not known | + | + | 1·3 |
| At 14 | Citrate synthase‡ | + | − | 1·7 |
| At 16, At 21 | Acetic thiokinase‡ | − | + | 1·5, 1·1 |
| At 11, At 12, At 13 | Acetic thiokinase‡ | − | − | 1·4, 1·3, 1·1 |

* μmoles acetyl-CoA utilized/mg. soluble protein/hr.
† The specific activity of an acetate-grown culture was 1·9.
‡ Identified in later experiments.

*Malate synthase*

The specific activity of malate synthase in cell-free extracts from the wild-type strain was similar for acetate-grown and succinate-grown cultures (Table 4). Mutant At 22, which was unable to grow on acetate+glyoxylate or on propionate, produced less than 10% of the malate synthase activity of the wild-type strain and it was concluded that

it was a malate synthase-deficient mutant. The low activity of isocitrate lyase in this mutant was thought to be a secondary effect due to metabolic repression. It is unlikely that a regulator mutation had resulted in co-ordinate repression of these two enzymes, since the activities of malate synthase, but not of isocitrate lyase, are similar in wild-type cultures grown on either acetate or succinate. Mutant At 14, which also had a low isocitrate lyase activity, had a normal malate synthase activity. Six other mutants, all of which were unable to grow on acetate + glyoxylate, also had normal malate synthase activities.

*Citrate synthase*

Citrate synthase activity was also similar in cell-free extracts from the wild-type strain grown on acetate or succinate (Table 5). The only mutant with a very low citrate synthase activity was At 14 which also produced less than the normal amount of

Table 5. *Citrate synthase activities of cell-free extracts of wild-type and acetate-negative mutants of Pseudomonas aeruginosa 8602*

Citrate synthase was assayed according to Dixon & Kornberg (1959). Bacteria were harvested after incubation for 16 hr in succinate medium and broken by ultrasonic treatment. The supernatant fluid was used for citrate synthase determination.

| Strain | Genetic defect | Growth | | Specific activity of citrate synthase* |
|---|---|---|---|---|
| | | Acetate + glyoxylate | Propionate | |
| Wild-type | None | + | + | 2·8† |
| At 14 | Citrate synthase | + | − | 0·2 |
| At 22 | Malate synthase | − | − | 2·3 |
| At 6 | Isocitrate lyase | + | + | 3·0 |
| At 8 | | | | 2·0 |
| At 15 | Not known | + | + | 1·6 |
| At 9 | Acetic thiokinase‡ | − | + | 2·4 |
| At 16 | | | | 1·6 |
| At 21 | | | | 2·2 |
| At 10 | Acetic thiokinase‡ | − | − | 1·8 |
| At 11 | | | | 1·7 |
| At 12 | | | | 2·0 |
| At 13 | | | | 1·1 |

* μmoles acetyl-CoA utilized/mg. soluble protein/hr.
† The specific activity of an acetate-grown culture was 3·3.
‡ Identified in later experiments.

isocitrate lyase on succinate medium (Table 2). A low citrate synthase activity could result in an increased intracellular concentration of oxaloacetate and consequently of phosphoenolpyruvate which is thought to be the specific co-repressor of isocitrate lyase synthesis in *Escherichia coli* (Kornberg, 1966). It is concluded that the acetate-negative character of mutant At 14 is due partly to a genetic deficiency of citrate synthase and partly to the consequent phenotypic deficiency of isocitrate lyase. The amount of citrate synthase produced by the mutant (7% of that of the wild type) is sufficient for growth on succinate but not on propionate.

The malate synthase mutant At 22 produced normal citrate synthase activity as did the two isocitrate lyase mutants tested (At 6, At 8). Cell-free extracts of mutants At 15

and 7 of the mutants unable to grow on acetate+glyoxylate also had high citrate synthase activities although in some cases these were less than that of the wild-type strain (Table 5).

*Acetic thiokinase*

The wild-type strain grown on succinate had only 30% of the acetic thiokinase activity of acetate-grown cultures (Table 6). Eight of the remaining unclassified mutants had significantly lower acetic thiokinase activities than the wild type in succinate medium. It was concluded that this defect could explain their inability to

Table 6. *Acetic thiokinase activities of cell-free extracts of wild-type and acetate-negative mutants of Pseudomonas aeruginosa 8602*

Acetic thiokinase was assayed according to Jones & Lipmann (1955). Cultures were harvested from succinate medium after 16 hr growth and broken by ultrasonic treatment. The supernatant fluid was used for acetic thiokinase determination.

| Strain | Growth on propionate | Specific activity of acetic thiokinase* |
|---|---|---|
| Wild-type | + | 1·3† |
| At 12 | − | 0·12 |
| At 13 | − | 0·40 |
| At 10 | − | < 0·04 |
| At 11 | − | < 0·04 |
| At 19 | − | < 0·04 |
| At 9 | + | 0·04 |
| At 16 | + | < 0·04 |
| At 21 | + | < 0·04 |

+, Growth (about $10^9$ bacteria/ml.) after 24–36 hr incubation at 37°; −, no visible growth after 36 hr incubation at 37°.

* μmoles acetyl-CoA formed/mg. soluble protein/hr.

† The specific activity of an acetate-grown culture was 4·5.

grow on acetate. A few acetate-negative mutants of all types grew slightly in liquid acetate medium after 36 hr incubation (Table 2) and mutant At 12 regularly did so. This could be due to the residual acetate activating activity (10% of wild type) although mutant At 13 which had a third of the wild-type activity seldom produced more than a trace of growth after 48 hr. Three of these mutants grew on propionate whereas the other 5 were unable to do so. The implications of this finding are discussed later.

Mutant At 15 was not tested for acetic thiokinase since it grew normally on acetate +glyoxylate and was presumed to activate acetate. It produced normal amounts of isocitrate lyase, malate synthase and citrate synthase and no enzyme defect has been detected.

*Transduction analysis*

The pseudomonad phage F 116 can be used to transduce genetic markers in *Pseudomonas aeruginosa* 8602. Phage lysates from the wild-type strain can be used to produce amidase-positive transductants from amidase-negative mutants (Skinner & Clarke, 1965; Brammar *et al.* 1967). No transductants were obtained when the amidase-negative mutants Am 4 or Am 5 were infected with a phage lysate propagated on mutant Am 9. This suggested that these mutations were closely linked. A detailed

analysis by the use of regulator mutants has shown that the amidase structural and regulator genes occur together on a small segment of the bacterial chromosome (Brammar *et al.* 1967).

When a lysate from mutant Am 9 was mixed with mutants lacking either isocitrate lyase At 3, or acetic thiokinase At 10, equal numbers of transductants were obtained on both acetate and acetamide plates. This suggested that the amidase gene is not closely linked to the isocitrate lyase or acetic thiokinase genes since co-transduction resulting from close linkage would have resulted in a decrease in the number of transductants on acetamide plates. This was confirmed by selecting $At^+$ transductants from an $Am^- \times At^-$ cross and replicating to acetamide plates. Using three different $At^-$ strains as recipients, no $At^+$ $Am^-$ colonies were found among the $At^+$ transductants tested (Table 7).

Table 7. *Pseudomonas aeruginosa 8602: non-linkage of $At^-$ mutations to a mutation in the amidase structural gene*

Phage F 116 was propagated on strain Am 9 according to Brammar *et al.* (1967). Samples (1 ml.) of bacteria ($2–4 \times 10^9$ bacteria) were mixed with 1 ml. phage at a multiplicity 0·5–10. After 60 min. for phage adsorption, bacteria were centrifuged, resuspended in 2 ml dilution buffer and 0·2 ml. samples spread on acetate plates. Transductants appeared after 48 hr at 37° and were replicated to acetamide plates.

| Donor | Recipient | Altered enzyme | No. of $At^+$ transductants scored | $At^+$ transductants carrying $Am^+$ marker (%) |
|---|---|---|---|---|
| Am 9 | At 1 | Isocitrate lyase | 72 | 100 |
| Am 9 | At 10 | Acetic thiokinase | 100 | 100 |
| Am 9 | At 14 | Citrate synthase | 261 | 100 |

## DISCUSSION

It has been shown that an active amidase and an operative glyoxylate cycle are necessary for growth of *Pseudomonas aeruginosa* 8602 with acetamide as carbon source. A mutation leading to a deficiency in one of five enzymes results in an acetamide-negative phenotype. The metabolic pathway for acetamide and the location of the enzyme mutations identified in this study are given in Fig. 1. The 10 mutants able to grow on acetate but not on acetamide lacked amidase activity. Strains deficient in an amide permease would also be unable to grow on acetamide. Amide permease activity in this organism appears to be a constitutive character and no permease-negative mutants were isolated. The amidase-negative mutant Am 10 has been shown to take up 1-[$^{14}$C]acetamide at the same rate as the wild-type strain (Brammar, McFarlane & Clarke, 1966). Of the 20 acetate-negative mutants studied, 9 were considered to have defects in isocitrate lyase, one in malate synthase, one in citrate synthase and 8 in acetic thiokinase. It is not possible to say whether these were all mutations in structural genes or whether some may have been mutations in regulator genes. No enzyme defects were found for mutant At 15.

Mutants which lack isocitrate lyase have also been described for *Escherichia coli* (Ashworth & Kornberg, 1964; Kornberg & Smith, 1966). They are unable to grow with acetate but can grow with propionate, indicating that isocitrate lyase is not required for growth with propionate. We have shown that a similar situation exists in

*Pseudomonas aeruginosa*. The increased synthesis of isocitrate lyase on propionamide and propionate medium is therefore gratuitous. These results were confirmed and extended by Chapman & Duggleby (1967) with our strain of *P. aeruginosa*; they found that the isocitrate lyase-negative mutants At 1 and At 8 did not grow on the even-numbered monocarboxylic acids ($C_2$–$C_{10}$) which are degraded to acetyl-CoA by $\beta$-oxidation. Both strains grew on the odd-numbered mono-carboxylic acids ($C_3$–$C_9$) which produce propionyl-CoA as the terminal product of $\beta$-oxidation. Both mutants also grew on the even-numbered dicarboxylic acids ($C_3$–$C_8$) but not on the odd-numbered dicarboxylic acids ($C_3$–$C_9$) which are thought to produce acetyl-CoA by the decarboxylation of malonyl-CoA.

The metabolic pathway for propionate utilization by pseudomonads has not been studied in detail. Some experiments suggest that it may be converted to pyruvate via lactate (Vagelos, Earl & Stadtman, 1959; Sokatch, 1967). A preliminary report states that the enzymes of this pathway are present in *Escherichia coli* (Wegener, Reeves & Ajl, 1967). *Ochromas malhamensis* carboxylates propionate to form succinyl-CoA (Arnstein & White, 1962) and *Prototheca zopfii* converts propionate to acetyl-CoA via the malonic semialdehyde pathway (Callely & Lloyd, 1964; Lloyd & Callely, 1965). It is improbable that the last pathway is operative in *Pseudomonas aeruginosa* 8602 since it would require a functioning glyoxylate cycle and the presence of isocitrate lyase. If propionate were to be converted to succinyl-CoA via methylmalonyl-CoA, growth on succinate and propionate would be essentially similar. However, this is not the case, since mutant At 14 which has a low citrate synthase activity grows with succinate but not with propionate. The lactate-pyruvate pathway may therefore be the operative one for propionate utilization in *P. aeruginosa*.

The citrate synthase-deficient Pseudomonas mutant At 14 resembles *Escherichia coli* strain M 22–64 (Gilvarg & Davis, 1956). However, M 22–64, unlike At 14, is unable to grow on succinate unless this is supplemented with $\alpha$-oxoglutarate. The residual activity of citrate synthase in mutant At 14 (7%) may be sufficient to allow $\alpha$-oxoglutarate and related compounds to be synthesized at an adequate rate for growth. An alternative explanation is that other pathways operate for this purpose in pseudomonads. The lowered isocitrate lyase activity of this mutant is almost certainly due to an increase in the intracellular concentration of the metabolic repressor of the enzyme. This provides an interesting example of a secondary metabolic effect on the activity of an enzyme of a genetic lesion in a different enzyme. The variable citrate synthase activities of the acetic thiokinase mutants (Table 5) may reflect variation of intracellular concentrations of metabolic intermediates affecting the regulation of citrate synthase.

Eight Pseudomonas mutants were classified as defective in acetic thiokinase. This enzyme assay measures acetate activation in the presence of ATP and coenzyme A and the existence of other acetate activating enzymes in *Pseudomonas aeruginosa* 8602 cannot be ruled out. Kornberg & Madsen (1958) found acetic thiokinase in *P. fluorescens* but *Escherichia coli* is known to activate acetate by means of acetate kinase (EC 2.7.2.1) (Rose, Grunberg-Manago, Korey & Ochoa, 1954) and phosphotransacetylase (EC 2.3.1.8) (Gilvarg & Davis, 1956). The latter enzyme was reported in *P. aeruginosa* grown on valine (Sokatch, 1967).

Propionate and acetate are thought to be activated by the same thiokinase in *Rhodospirillum rubrum* (Eisenberg, 1955) and *Prototheca zopfii* (Callely & Lloyd, 1964).

Three of the 8 acetic thiokinase-deficient mutants of *Pseudomonas aeruginosa* 8602 were able to grow normally with propionate. A possible explanation for this finding is that the enzyme has been altered in such a way that it has retained sufficient activity to activate propionate but not acetate. Alternatively, acetate and propionate may be activated by different enzymes. This would seem unlikely since some of the acetate-activating enzyme mutants are also unable to grow on propionate.

It was not possible to locate any of these acetate genes on the bacterial chromosome. Transduction analysis is restricted to a small portion of the chromosome and could only reveal that the amidase gene did not appear to be linked to the isocitrate lyase, the acetic thiokinase or the citrate synthase genes.

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# Nutrition and Vitamin $B_{12}$ Metabolism of a Soil Bacterium

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SUMMARY

The growth requirements of a vitamin $B_{12}$-dependent soil bacterium (strain 544), provisionally classified as belonging to the genus *Arthrobacter*, were studied. The organism showed a high specificity for vitamin $B_{12}$; of the naturally occurring analogues tested, only α-(5-hydroxybenzimidazolyl)-cobamide cyanide and α-(2-methylmercaptoadenyl)cobamide cyanide possessed some growth-promoting activity (less than 10%). The anilide and the ethylamide of the monocarboxylic acid of vitamin $B_{12}$ inhibited growth and the inhibition was reversed by the vitamin. A medium and a procedure for vitamin $B_{12}$ assay using strain 544, is described. Comparative assays with *Ochromonas malhamensis* and *Escherichia coli* 7M are given and their differential usefulness for differentiating the various congeners of vitamin $B_{12}$ discussed.

Addition of vitamin $B_{12}$ to $B_{12}$-deficient cell suspensions of strain 544 stimulated the biosynthesis of methionine and the oxidation of propionate, valerate, isoleucine and valine. The endogenous respiration and oxidation of glucose, butyrate, leucine and lysine were only slightly enhanced by vitamin $B_{12}$.

## INTRODUCTION

Lochhead and co-workers have shown that the soil is an important habitat of vitamin $B_{12}$-requiring bacteria (Lochhead & Thexton, 1951, 1952; Lochhead & Burton, 1956, 1957). In a detailed study of vitamin $B_{12}$-requiring bacteria obtained from field soil by a 'non-selective' procedure, the majority of the isolates were considered to belong to the genus *Arthrobacter* (Lochhead & Burton, 1955). Ford & Hutner (1957), who studied the nature of the vitamin $B_{12}$ requirement of a number of these isolates, found that one strain responded to factor A, factor B and pseudovitamin $B_{12}$, while all the rest responded only to vitamin $B_{12}$ and vitamin $B_{12}$III and thus resembled *Ochromonas malhamensis* in their pattern of response to the various $B_{12}$ vitamins. The latter authors emphasized the promising features of several of these isolates as vitamin $B_{12}$ reagents. However, in a more detailed study of one of these organisms (Lochhead 38) Goldberg, Hutner & Ford (1957) found that although its response was limited to cyanocobalamin and vitamin $B_{12}$III, it was unsuitable for tube assays of the vitamin because of its granular and non-dispersible type of growth. Moreover Sanders & Seaman (1960) reported that 'Lochhead 38' also responded to α-(2-methyladenyl)cobamide cyanide, factor C and factor B.

This report describes the nutritional requirements and physiological characteristics of another of Lochhead's isolates, no. 544. The nature of the vitamin $B_{12}$ requirement of this organism was studied, and its specificity for the $B_{12}$ vitamins was compared to that of a $B_{12}$-requiring strain of *Escherichia coli* 7M and of *Ochromonas malhamensis*.

A growth medium and a procedure for a tube assay of vitamin $B_{12}$ using isolate 544 are described. Some metabolic effects of vitamin $B_{12}$ were studied using vitamin $B_{12}$-deficient suspensions of organisms.

## METHODS

*Micro-organisms used.* Isolate no. 544—a soil bacterium received from Dr A. G. Lochhead; *Escherichia coli*, 7M, a vitamin $B_{12}$ or methionine-requiring mutant of *E. coli* W; *Ochromonas malhamensis* ATCC 11352.

Cobinamide and the benziminazol and adenine analogues of vitamin $B_{12}$ were kindly supplied by Professor K. Bernhauer. The anilide and ethylamide of the monocarboxylic acids of vitamin $B_{12}$ were obtained through the courtesy of Dr E. Lester Smith. Factors $Z_2$, $Z_3$, which were isolated from digested sewage sludge, were kindly given by Dr Halina Neujahr. $\alpha$-(5,6-dimethylbenzimidazolyl)cobamide cyanide (vitamin $B_{12}$) was purchased from Nutritional Biochemicals Co., U.S.A. All compounds were dissolved in water and standardized spectrophotometrically at 361 m$\mu$ using vitamin $B_{12}$ as standard. Cobinamide and factors $Z_2$ and $Z_3$ were standardized at 355 m$\mu$.

*Growth. Escherichia coli* was grown in a glucose mineral medium (Davis & Mingioli, 1950). The quantitative assay of vitamin $B_{12}$ with this organism was done as described previously (Aronovitch & Grossowicz, 1958). *Ochromonas malhamensis* was grown in the dark according to the procedure and medium described by Ford (1953, 1958). The nutritional requirements of isolate 544 were studied by using the basal medium (Table 1), supplemented with vitamin $B_{12}$ (0·4 m$\mu$g./ml.). Utilization of various energy sources was tested in the basal medium with citrate and Tween 80 omitted. Cultures were grown in test-tubes (19 × 150 mm.) containing 5 ml. medium, or in Erlenmeyer flasks (50 ml.) containing 10 ml. medium. A 48 hr culture was diluted 1/10 with sterile medium and one drop of this culture was used to inoculate each assay tube.

Assays of vitamin $B_{12}$ and related compounds were done in the complete medium (Table 1). The inoculum was grown for 48 hr on the same medium, supplemented with vitamin $B_{12}$ 0·1 m$\mu$g./ml. One drop of this culture was used to inoculate each assay tube. The cultures were incubated at 28–30° on a reciprocal shaker (160–180 strokes/min.) for 48–72 hr. Growth was measured turbidimetrically in a Coleman nephocolorimeter at 590 m$\mu$. Cultures giving a scale reading higher than 0·50 were diluted 1/3 with water before reading.

*Manometric experiments.* The organisms were grown for 48 hr in the complete medium containing a low concentration of ammonium nitrate (0·02 %) and no Tween 80. The medium was supplemented with Na succinate (0·4 %) and a suboptimal concentration of vitamin $B_{12}$ (0·1 m$\mu$g./ml.). Organisms were collected by centrifugation, taken up in water and suspended in an equal volume of fresh medium without vitamin $B_{12}$, Casamino acids or energy source. The suspensions were aerated for 2 hr at 30° to decrease the endogenous reserves and then collected by centrifugation and washed once with water.

Oxygen consumption was measured in the conventional Warburg apparatus at 30°. Each vessel contained: suspension 1 ml., containing equiv. 4–6 mg. (dry wt organisms; phosphate buffer (pH 7·0), 10 $\mu$moles; tris-HCl buffer (pH 7·0), 50 $\mu$moles; substrate as indicated, 4 $\mu$moles; vitamin $B_{12}$, 40 m$\mu$g.; total volume, 2 ml. The centre well contained 0·1 ml. of 15 % KOH solution.

*Media.* The composition of the complete medium, vitamin $B_{12}$ omitted, is given in Table 1.

The basal medium was that described in Table 1 modified as follows: Casamino acids were omitted, and DL-methionine (20 $\mu$g./ml.) and trace elements added. Trace elements were supplied as sulphates to give final concentrations/l.: $Zn^{2+}$, 0·25 mg.; $Mn^{2+}$, 0·1 mg.; $Cu^{2+}$, 0·02 mg./l. Vitamin $B_{12}$, when included, 0·4 m$\mu$g./ml.

Table 1. *Complete liquid medium for vitamin $B_{12}$ assays with Arthrobacter isolate 544*

The medium was adjusted to pH 6·8–7·0, boiled, filtered and sterilized by autoclaving for 15 min at 115°. The medium was usually prepared as a five-fold concentrated solution and stored at −20° in polyethylene bottles. Glucose (solution) was added aseptically to the medium after autoclaving, to give 0·4% (w/v) glucose.

| | | | |
|---|---|---|---|
| Casamino acids (Difco) vitamin free | 2·0 g | $CaCO_3$ | 0·01 g |
| $K_2HPO_4$ | 1·0 g | Ferrous ammonium sulphate | 0·72 g |
| $KH_2PO_4$ | 0·4 g | Tween 80 | 1·0 g |
| $MgSO_4.7H_2O$ | 0·3 g | EDTA | 0·005 g |
| Potassium citrate | 0·5 g | Thiamine HCL | 0·002 g |
| $NH_4NO_3$ | 1·0 g | Distillled water to | 1 l |

## RESULTS

### *Morphology and physiology of isolate 544*

Young cultures of isolate 544 consisted of Gram-variable, non-sporeforming pleomorphic rods and some large coccoid forms. In older cultures, small coccoid forms, mostly Gram-negative, were predominant. Motility was not observed. Colonies, on suitable solid media, were small (1–2 mm in diameter), circular, convex, with smooth surface and of a soft consistency. On brain–heart infusion agar growth was abundant, with the production of a brownish water-soluble pigment. On nutrient agar there was no growth. In the complete solid medium, growth was colourless in young cultures and varied to pale brown in old ones. Acid was formed from glucose; starch was hydrolysed; nitrate was reduced to nitrite; indole was not formed; the organism was urease-negative and catalase-positive. Nitrate and ammonium were used as sole nitrogen sources. In the defined medium, vitamin $B_{12}$ and thiamine were required for growth. The organism was strongly aerobic. Good growth occurred between 20° and 30°; there was no growth at 37°. In accordance with these properties the strain was classified provisionally as belonging to the genus *Arthrobacter*.

*Nitrogen sources.* Utilization of various nitrogen sources for growth was tested by using the basal medium without methionine. Nitrogen compounds were added to a concentration of 0·1 % (w/v). Isolate 544 grew well on nitrate, ammonium salts (sulphate, chloride, acetate) and casein hydrolysate as sole sources of nitrogen. Casein hydrolysate served as an energy source as well.

*Energy sources.* Utilization of different energy sources was tested by using the basal medium from which methionine and Tween 80 were omitted. Glucose, sucrose, succinate and glycerol were found to be suitable energy sources, yielding comparable growth rates; starch, glycogen, inulin, cellobiose and rhamnose were utilized more slowly; there was no growth on acetate, propionate, tartrate or citrate. Addition of Tween 80 (up to 0·2 %) to the glucose-containing medium shortened the lag period and increased the total crop of organisms.

*Trace elements.* Growth on the basal medium was slow unless the medium was supplemented with trace amounts of $Zn^{2+}$, $Mn^{2+}$ and $Cu^{2+}$. The salt mixture supplying these cations could be replaced by 15–20% (v/v) boiled and filtered tap water. Addition of these trace elements proved unnecessary when casein hydrolysate was used as nitrogen source.

*Autolysis.* Cultures of isolate 544 grown in the complete medium without EDTA (Table 1) were prone to autolysis. Lysis usually began at the end of the exponential phase of growth, and became pronounced at suboptimal concentrations of vitamin $B_{12}$. The occurrence of autolysis interfered with the use of the isolate 544 for vitamin $B_{12}$ assays. However, addition of EDTA or spermine to the growth medium slowed down or even prevented autolysis, and allowed measurement of growth by extinction determinations (Table 2).

Table 2. *Prevention of autolysis of isolate 544 in complete medium by spermine and EDTA*

Isolate 544 was grown in the complete medium. When EDTA was used it was added to the medium before autoclaving it; spermine was added aseptically after autoclaving.

| Additions to growth medium (μg./ml.) | Incubation time (hr) | Vitamin $B_{12}$ (mμg./ml.) 0·025 | 0·050 | 0·100 |
|---|---|---|---|---|
| | | O.D. reading at 590 mμ | | |
| None | 42 | 0·16 | 0·32 | 0·57 |
| | 68 | 0·08 | 0·13 | 0·24 |
| Spermine (8·5) | 42 | 0·20 | 0·31 | 0·53 |
| | 68 | 0·20 | 0·45 | 0·80 |
| EDTA (5·c) | 42 | 0·17 | 0·35 | 0·55 |
| | 68 | 0·29 | 0·50 | 0·79 |

*Vitamin requirements.* Growth experiments showed that isolate 544 required vitamin $B_{12}$ and thiamine. The quantitative response to these vitamins is shown in Fig. 1*a*, *b*. Thiamine could not be replaced by the pyrimidine moiety of the vitamin (2-methyl-4-5-amino-methylpyrimidine) at concentrations up to 40 μg./ml.

*Functions of vitamin $B_{12}$.* The vitamin requirements of several Arthrobacter strains could not be satisfied by thymidine or methionine (Lochhead & Burton, 1955; Ford & Hutner, 1957). Furthermore, Goldberg *et al.* (1957) found no sparing of vitamin $B_{12}$ by methionine. Our studies with isolate 544 likewise showed that vitamin $B_{12}$ could not be replaced by methionine, thymidine or by acid and alkaline hydrolysates of DNA or RNA. However, we found that methionine (20–30 μg./ml.) had a sparing effect on the vitamin $B_{12}$ requirement in the basal medium (Fig. 2). This effect indicated the possibility of a vitamin $B_{12}$ function in the biosynthesis of methionine. The effect of vitamin $B_{12}$ on the methylation of homocysteine was therefore tested with vitamin $B_{12}$-deficient suspensions of isolate 544 (Table 3). As can be seen, addition of vitamin $B_{12}$ caused a three-fold increase in the amount of methionine formed from homocysteine and serine. Addition of vitamin $B_{12}$ to suspensions grown with optimal concentrations of vitamin $B_{12}$ had no effect on the synthesis of methionine.

*Effect of vitamin $B_{12}$ on respiration.* Although methionine spared vitamin $B_{12}$, it did not replace the vitamin for isolate 544. Additional functions of vitamin $B_{12}$ in this

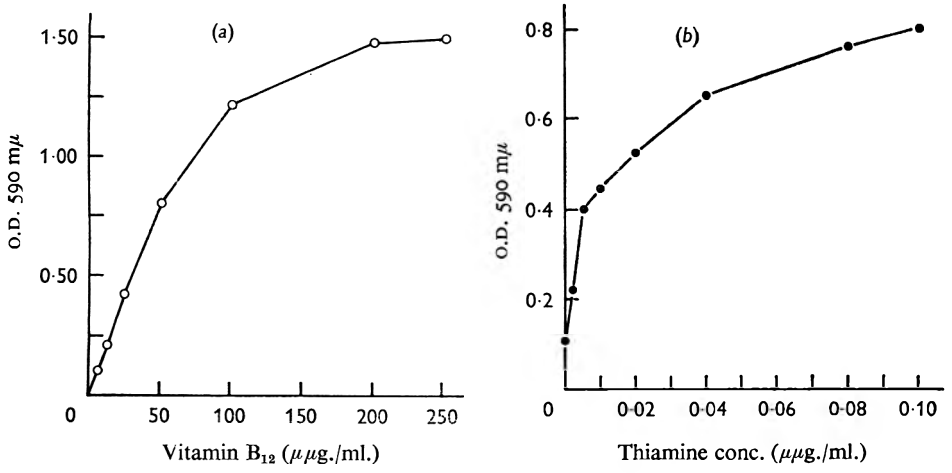


Fig. 1. Quantitative response of isolate 544 to thiamine and vitamin $B_{12}$. (*a*) Response to vitamin $B_{12}$, using the assay procedure described (see Methods); (*b*) response to graded amounts of thiamine in the thiamineless medium, supplemented with vitamin $B_{12}$ (0·5 mμg./ml.). The inoculum was taken from the same medium supplemented with thiamine (0·4 mμg./ml.) after incubation for 48 hr. Thiamine HCl was added aseptically after autoclaving the medium. Cultures were incubated for 48 hr at 30°.

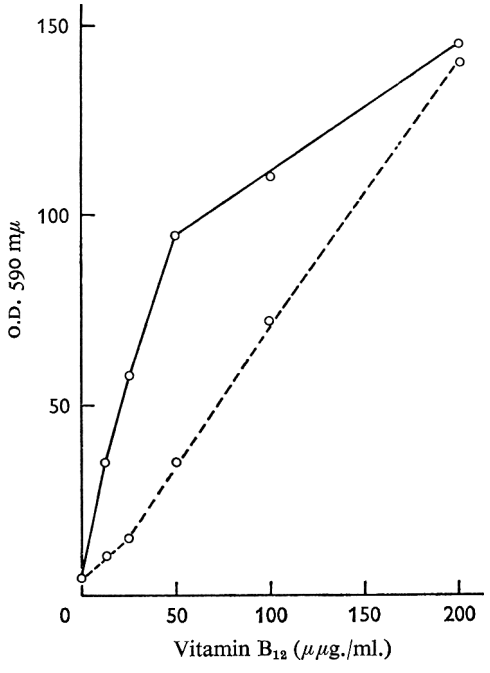


Fig. 2. Effect of DL-methionine on growth of isolate 544 in the presence of increasing concentrations of vitamin $B_{12}$. Broken line, basal medium (see Table 1); solid line, basal medium + DL-methionine 20 μg./ml. Incubation for 60 hr at 30°.

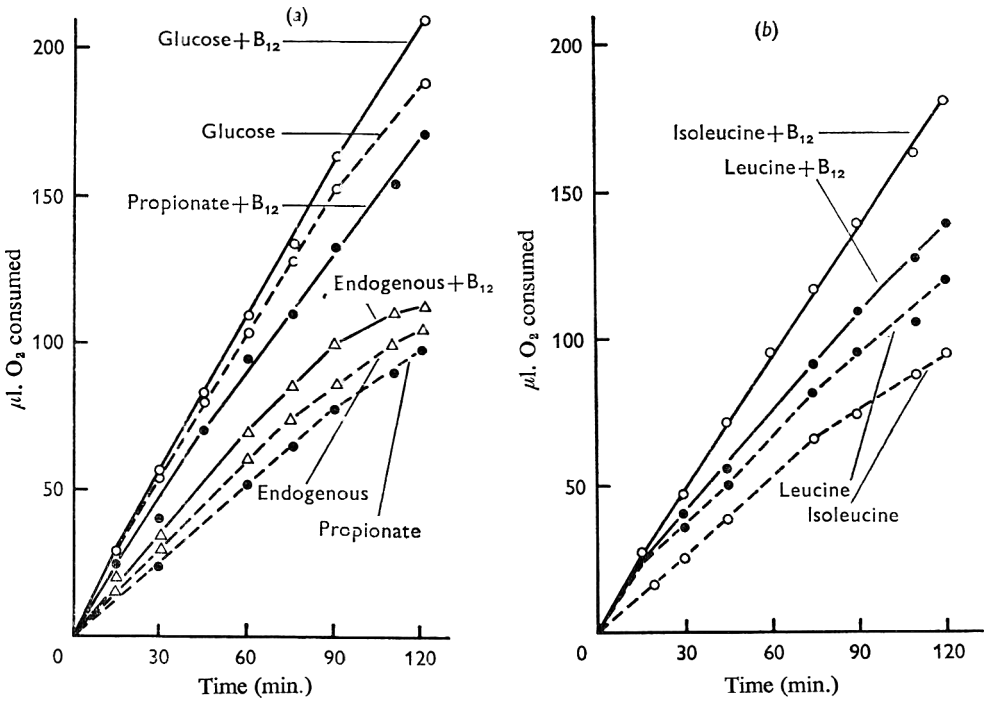


Fig. 3. Effect of vitamin $B_{12}$ on the endogenous respiration and the oxidation of various substrates by isolate 544. Solid lines, in presence of vitamin $B_{12}$; broken lines, without vitamin $B_{12}$.

Table 3. *Effect of vitamin $B_{12}$ on the methylation of homocysteine by suspensions of isolate 544*

Isolate 544 was incubated for 60 hr in basal medium (without methionine), supplemented with a suboptimal concentration of vitamin $B_{12}$ (0·075 m$\mu$g./ml.). Organisms were collected by centrifugation, washed twice with distilled water and incubated (equiv. 0·6 mg. dry wt organism/ml.) for 12 hr at 30° with shaking. Incubation mixtures contained mM: DL-homocysteine, 5; L-serine, 5; glucose, 25; vitamin $B_{12}$, 10 nM. Incubation was terminated by boiling the reaction mixtures for 5 min. and the organisms removed by centrifugation. Methionine in the supernatant fluids was assayed microbiologically with *Streptococcus equinus* (ATCC 7881) by using Difco methionine assay medium.

| Additions to reaction mixture | | | L-methionine formed ($\mu$moles/mg. dry wt organisms) |
|---|---|---|---|
| Homocysteine | Serine | Vitamin $B_{12}$ | |
| + | + | + | 0·65 |
| + | + | − | 0·20 |
| + | − | + | 0·38 |
| − | + | + | 0·10 |

organism have not been clearly defined. However, it was found that vitamin $B_{12}$-deficient isolate 544 organisms were unable to oxidize propionate unless supplied with vitamin $B_{12}$. The effect of vitamin $B_{12}$ on the respiration with glucose, various organic acids and amino acids was therefore tested. The endogenous respiration and the respiration with glucose, pyruvate, butyrate, leucine and lysine was only slightly

stimulated (5–20 %) by vitamin $B_{12}$. On the other hand, addition of the vitamin markedly increased (30–80 %) the oxygen uptake in presence of propionate, *n*-valerate, isovalerate, isoleucine and valine; the oxidation of Tween 80, acetate and succinate was stimulated to a lesser extent (25–45 %). The effect of vitamin $B_{12}$ on the respiration of isolate 544 in the presence of some of these compounds is shown in Fig. 3.

### *Growth response to vitamin $B_{12}$ analogues*

The growth response of isolate 544 to various vitamin $B_{12}$ derivatives was tested by using the tube assay. Table 4 compares the activities of some naturally occurring and some biosynthetically prepared analogues of vitamin $B_{12}$ for isolate 544, *Ochromonas malhamensis* and *Escherichia coli*; the activity of vitamin $B_{12}$ was taken as 100 %. The pattern of response of isolate 544 was similar to that of *O. malhamensis* (Bernhauer, 1956; Peterson, Hall & Bird, 1956; Coates & Kon, 1957). Isolate 544 responded neither to cobinamide (Factor B) nor to factors $Z_2$ and $Z_3$, which probably also lack the nucleotide component (Neujahr, 1956); α-(adenyl)cobamide cyanide (pseudovitamin $B_{12}$) and α-(2-methyladenyl) cobamide (Factor A) had little activity for both organisms (less than 1 %). Of the naturally occurring analogues, isolate 544 responded only to α-(5-hydroxybenzimidazolyl)cobamide cyanide (vitamin $B_{12}$ III and to α-(2-mer-

Table 4. *Activity for various micro-organisms of some naturally occurring and biosynthetically prepared analogues of vitamin $B_{12}$*

The activity of vitamin $B_{12}$ was taken as 100. Activities of the various $B_{12}$ analogues are means of 3–5 different assays. All compounds were tested over a wide range of concentrations (10–100-fold). Factors B, $Z_2$ and $Z_3$ were tested at concentrations up to 0·1 μg./ml.; the anilide and ethylamide of the monocarboxylic acid of vitamin $B_{12}$ were tested at concentrations up to 0·5 μg./ml. Considerable fluctuations in activities were noted with some of the $B_{12}$ analogues (benzimidazolylcobamide cyanide) even under carefully standardized conditions. Limit values given for the activities of the anilide and ethylamide analogues for isolate 544 were obtained after incubation times of 70 and 84 hr, respectively Values for *O. malhamensis* were obtained after incubation for 96 hr.

| Analogue | Test organism: Isolate 544 (*Arthrobacter* sp.) | *Ochromonas malhamensis* | *Escherichia coli* 7M |
|---|---|---|---|
| | Activity (%) | | |
| α-(5,6-Dimethylbenzimidazolyl)-cobamide cyanide (vitamin $B_{12}$) | 100 | 100 | 100 |
| α-(Benzimidazolyl)cobamide cyanide | 1·5–3 | 57–90 | 130–150 |
| α-(5-Methylbenzimidazolyl)cobamide cyanide | 9 | 72 | 100 |
| α-(5-Hydroxybenzimidazolyl)cobamide cyanide (vitamin $B_{12}$ III) | 8 | 32 | 30 |
| α-(Adenyl)cobamide cyanide (pseudovitamin $B_{12}$) | 0·05 | 0·02 | 60 |
| α-(2-Methyladenyl)cobamide cyanide (factor A) | 0·5 | 0·3 | 70 |
| α-(2-Methylmercaptoadenyl)cobamide cyanide (factor F) | 6 | 5 | 100 |
| Cobinamide (factor B) | 0·005 | 0·005 | 42 |
| Factor $Z_2$ | 0·4 | 0·1 | 60 |
| Factor $Z_3$ | 0·1 | 0·08 | 50 |
| Anilide analogue of vitamin $B_{12}$ | 0·04–0·1 | 0·025 | 0·025 |
| Ethylamide analogue of vitamin $B_{12}$ | 0·2–0·8 | 0·005 | 0·02 |

captoadenyl)cobamide cyanide (Factor F) with an activity of 8 % and 6 %, respectively. However, the response of isolate 544 to α-(5-hydroxybenzimidazolyl)cobamide cyanide was considerably weaker (8 %) compared to that of *O. malhamensis* (32 %).

The biosynthetically prepared benziminazole analogues tested (benzimidazolyl cobamide cyanide and 5-methylbenzimidazolyl cobamide cyanide) which are almost fully active for *Ochromonas malhamensis* had only a slight activity for isolate 544. The anilide and ethylamide of the monocarboxylic acids of vitamin $B_{12}$ are practically inactive as vitamin $B_{12}$ substitutes for *Escherichia coli* and *O. malhamensis*. Both analogues exhibited some growth-promoting activity for isolate 544; the activity of the ethylamide being about 8 times higher than that of the anilide. However, on prolonged incubation, *O. malhamensis* also showed some growth on the anilide.

Table 5. *Effect of substituted vitamin $B_{12}$ amides on the growth of isolate 544*

The cultures were incubated for 72 hr at 30°

| Analogue added (mμg./ml.) | Vitamin $B_{12}$ (mμg./ml.) | | | | |
|---|---|---|---|---|---|
| | 0 | 0·25 | 0·1 | 0·4 | 0·8 |
| | O.D. scale reading at 590 mμ | | | | |
| None | 0 | 0·21 | 0·90 | 1·42 | 1·50 |
| Anilide | | | | | |
| 10 | 0·15 | — | — | — | — |
| $1 \times 10^2$ | 0·65 | 0·97 | 1·11 | 1·30 | 1·35 |
| $1 \times 10^3$ | 0·76 | 0·86 | 0·90 | 1·06 | 1·28 |
| $1 \times 10^4$ | 0·03 | 0·05 | 0·05 | 0·06 | 0·06 |
| Ethylamide | | | | | |
| 10 | 0·76 | — | — | — | — |
| $1 \times 10^2$ | 1·24 | 1·10 | 1·28 | 1·44 | 1·50 |
| $1 \times 10^3$ | 1·18 | 1·12 | 1·16 | 1·20 | 1·49 |
| $1 \times 10^4$ | 1·04 | 1·09 | 1·06 | 1·04 | 1·08 |

Both the ethylamide and the anilide of vitamin $B_{12}$ are growth inhibitors for *Ochromonas malhamensis* and *Escherichia coli* (Ford, 1959; Baker *et al.* 1960; Smith, 1960). With isolate 544 it was found (Table 5), that the anilide ($1 \times 10^4$ mμg./ml.) completely inhibited growth in the presence of up to 0·8 mμg./ml. vitamin $B_{12}$. At this concentration of the analogue and increasing concentrations of vitamin $B_{12}$, the 50 % inhibition index was calculated to be 2500. On prolonged incubation (96 hr), the inhibition index for *O. malhamensis* was about 3000, and for *E. coli* 7M, which is more sensitive to cyanocobalamin, the inhibition index was about 10,000.

### *Use of isolate 544 for assay of vitamin $B_{12}$*

Isolate 544 gives homogeneous growth in liquid media and grows well on suitable solid media. The strain is thus suitable for tube and plate assays of vitamin $B_{12}$.

*Procedure of tube assay.* The assay medium (Table 4), at 5/4 concentration was dispensed in 4 ml. amounts into 19 × 150 mm. test-tubes, and the volume made up to 5 ml. with either water or material to be tested.

*Standard curve.* A series of known concentrations of vitamin $B_{12}$ (standard curve) was included in each assay. One ml. samples of appropriate dilutions of a sterile stock solution of vitamin $B_{12}$ (1·5 mμg./ml.) were added to give final concentrations of

0·00625, 0·0125, 0·025, 0·050, 0·1, 0·15, 0·2 and 0·3 mμg./ml. Tubes without added vitamin $B_{12}$ were included as blanks.

*Inoculum.* The organism was maintained on solid assay medium supplemented with vitamin $B_{12}$ (1 mμg./ml.) and agar (1·5%). Growth from a 48 to 72 hr slope was removed with a wire loop and disposed in 10 ml. assay medium (in a 50 ml. Erlenmeyer flask) supplemented with vitamin $B_{12}$ (0·1 mμg./ml.) to obtain a barely turbid suspension. The culture was incubated with shaking for 48 hr at 30°; 0·1 ml. of this culture was used to inoculate each assay tube.

*Assay.* Vitamin $B_{12}$ was extracted from the material (adjusted to pH 4·5 with HCl) to be tested according to the procedure described previously (Aronovitch & Grossowicz, 1958) except for the acetate buffer and NaCN which were used at one-tenth of

Table 6. *Comparative assays by two organisms, isolate 544 and Escherichia coli 7M of vitamin $B_{12}$ in various materials*

Each sample was tested twice over a wide range of concentrations. Vitamin $B_{12}$ values of liver and soil extract are expressed as mμg./g., vitamin $B_{12}$ values of serum are given as mμg./ml.

| | Test organism | |
|---|---|---|
| | Isolate 544 | *E. coli* 7M |
| Material assayed | Vitamin $B_{12}$ conc. (mμg.) | |
| Human serum normal, pooled | 0·49 | 0·55 |
| Human myelogenous leukaemia serum, pooled | 2·20 | 2·10 |
| Rabbit serum | 10·90 | 12·80 |
| Human liver (myelogenous leukaemia) | 61·00 | 67·00 |
| Rat liver | 360·00 | 430·00 |
| Soil extract | 1·13 | 1·75 |

the previous concentration because of their adverse effect on the growth of isolate 544. One ml. samples of appropriate dilutions of the extracts to be tested were added to 4 ml. medium and sterilized by autoclaving for 15 min. at 115°. The assay tubes were incubated at 30° on a shaker for 48 hr. Growth was measured turbidimetrically. Figure 1 shows the response to graded amounts of vitamin $B_{12}$. The growth response was linear over a wide range of concentrations (0·005–0·1 mμg./ml.).

The assay medium when supplemented with an optimal concentration of vitamin $B_{12}$ was fully adequate for the growth of isolate 544; addition of defined growth factors and complex natural materials increased neither the growth rate nor the maximum yield of organisms; among the materials tested were various B vitamins, purine and pyrimidine mixtures, acid and alkaline hydrolysates of DNA and RNA and extracts of yeast, liver and soil.

Comparative assays of vitamin $B_{12}$ activity in various natural materials were made with isolate 544, *Ochromonas malhamensis* and *Escherichia coli* 7M. The vitamin $B_{12}$ values obtained with isolate 544 were similar to those obtained with *O. malhamensis*, and in general somewhat lower than those found with *E. coli*. Samples of human serum and liver gave similar values with all three bioassays. On the other hand, soil extracts showed significantly lower vitamin $B_{12}$ activity (65%) when assayed with isolate 544 than with *E. coli* 7M (Table 6).

## DISCUSSION

The morphological characteristics of isolate 544 fit the genus *Arthrobacter* as described by Conn & Dimmick (1947). However, biochemically, isolate 544 differs from the several species described in *Bergey's Manual* (1957). The vitamin requirements of isolate 544 are similar to those of *Arthrobacter duodecalis* (Lochhead, 1958), and to the organisms belonging to type II (Lochhead & Burton, 1955). However, isolate 544 differs from the latter in the production of acid from glucose, and is able to utilize nitrate and ammonium salts as sole nitrogen sources. Isolate 544 was therefore tentatively classified as *Arthrobacter* sp.

Although methionine was ineffective in replacing vitamin to $B_{12}$ for isolate 544, it spared the vitamin for growth. Addition of vitamin to $B_{12}$-deficient cell suspensions stimulated the methylation of homocysteine. A similar stimulatory effect was found with crude extracts prepared from these organisms. The sparing effect, as well as the enhancement of the biosynthesis of methionine by vitamin $B_{12}$, indicate that in this organism the vitamin is involved in the methylation of homocysteine. Isolate 544 differs in this respect from the Arthrobacter strains studied by Goldberg *et al.* (1957). A similar effect of vitamin $B_{12}$ on the biosynthesis of methionine has been shown in *Escherichia coli* (Gibson & Woods, 1960), and indicated in *Ochromonas malhamensis* (Johnson, Holdsworth, Porter & Kon, 1957; Dalal, Rege & Sreenivasan, 1961).

Other functions of vitamin $B_{12}$ in isolate 544 have not been well defined. The effect of the vitamin on the respiration of vitamin $B_{12}$-deficient organisms indicated that vitamin $B_{12}$ plays a role in the oxidation of odd-numbered and branched-chain organic acids. Vitamin $B_{12}$-deficient organisms of isolate 544 depended on the vitamin for oxidation of propionate. A similar effect of vitamin $B_{12}$ was found with vitamin $B_{12}$-deficient cells of *Ochromonas malhamensis* (Marchesi & Lajtha, 1961; Arnstein & White, 1962), and with two Flavobacterium strains (Ayers, 1962*a*, *b*). With isolate 544 and with one of the Flavobacterium strains, vitamin $B_{12}$ also stimulated the oxidation of some other odd-numbered organic acids as well as the oxidation of certain amino acids (valine, isoleucine), while the endogenous respiration and the oxidation of other amino acids (leucine, lysine) were only slightly affected. It seems likely therefore that all these substrates lead to the production of a common intermediate, as proposed by Ayers (1962*a*). The common intermediate, probably methylmalonyl-CoA, is isomerized to succinyl-CoA in a vitamin $B_{12}$-dependent reaction similar to that shown for other bacterial species (Stadtman, Overath, Eggerer & Lynen, 1960). Stimulation of the endogenous respiration by vitamin $B_{12}$ suggests that the endogenous substrate consists, at least in part, of compounds that yield methylmalonyl-CoA as an intermediate.

Of the naturally occurring $B_{12}$-analogues tested, isolate 544, like *Ochromonas malhamensis* and higher animals, utilizes only vitamin $B_{12}$III and factor F. The response of isolate 544 to vitamin $B_{12}$III is less than 10% of its response to cyanocobalamin, whereas the response of *O. malhamensis* is 30–70% (Kon & Pawelkiewicz, 1958). The sensitivity of isolate 544 to cyanocobalamin is similar to that of *O. malhamensis*. However, isolate 544 is faster growing and easier to maintain than *O. malhamensis* and unlike the latter grows well on solid media and may therefore be used in the convenient pad plate-assay. Only small amounts of vitamin $B_{12}$ are accumulated in isolate 544 organisms grown on optimal concentrations of the vitamin; large inocula may

therefore be used without washing. The medium used is suitable for the assay of vitamin $B_{12}$ in crude extracts; the response to the vitamin was not affected by the addition of defined growth factors or yeast extract. Isolate 544 has proved to be very stable; vitamin $B_{12}$-independent mutants have not been observed. Under proper aeration only minor variations in response were encountered in repeated assays. Isolate 544 seems therefore to be a suitable organism for use in the specific assay of cyanocobalamin in crude extracts.

The anilide and the ethylamide of the monocarboxylic acids of vitamin $B_{12}$ acted as vitamin $B_{12}$ antagonists for isolate 544. The growth inhibition caused by these analogues was annulled by vitamin $B_{12}$, indicating a competitive type of inhibition. Both analogues have vitamin $B_{12}$ activity at the concentrations used for inhibition studies, but growth on the analogues was slower than on vitamin $B_{12}$. The relative antagonistic activity was tested after a prolonged period of incubation (72 hr); under these conditions the anilide was a more active analogue than the ethylamide. A similar order of activities has been found for the growing chick (Coates, Davies & Harrison, 1960). The two amides have a reversed order of activities towards *O. malhamensis* and *E. coli* (Ford, 1959; Baker *et al.* 1960; Smith, 1960).

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# Study of a Small Amoeba from Mammalian Cell Cultures Infected with 'Ryan Virus'

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SUMMARY

A small amoeboid organism, found in mammalian tissue cultures inoculated with an infective agent formerly termed 'Ryan virus', is shown to have the morphological, cultural and behavioural characters of the free-living soil amoeba *Hartmannella castellanii* Douglas, 1930. Cytopathic changes occurred regularly in the infected monolayers; this was evidently due to action of the amoebae rather than the presence of any associated bacterial or viral agents. Strong circumstantial evidence suggests that the Ryan isolates of *H. castellanii* originated, either as trophozoites or cysts, from swabs of the human nasopharynx. Recovery of hartmannellid amoebae from this source is of interest in relation to some recently reported cases of pyogenic meningitis, apparently caused by free-living soil amoebae.

## INTRODUCTION

A transmissible cytopathic agent, provisonally called 'Ryan virus', was isolated in several laboratories by inoculation of tissue cultures with swabs of the upper respiratory tract from patients showing fever and respiratory symptoms (Pereira, Marsden Corbitt & Tobin, 1966). Some properties of the agent seemed to be virus-like, including cytopathic changes with intranuclear inclusions observed in all inoculated cultures. Subsequent cytological studies by light- and electron-microscopy indicated that the cytopathic effects were attributable, not to a virus, but to a motile cellular organism resembling the free-living soil amoeba *Hartmannella castellanii* (Armstrong & Pereira, 1967). In the present paper further details are given of the evidence leading to identification of the organism, including cultivation in the absence of mammalian cells, and morphological and behavioural characteristics distinguishing hartmannellid amoebae from somewhat similar free-living forms of the family Schizopyrenidae.

## MATERIALS AND METHODS

The agent described in this paper was the original isolate of the series reported by Pereira *et al.* (1966) and designated 'Ryan I'. HeLa cell cultures infected with Ryan I were kindly supplied by Dr M. S. Pereira (Virus Reference Laboratory, Central Public Health Laboratory, Colindale). HeLa cell monolayers were grown at 37° in stationary tubes containing coverslips, using a medium composed of Gey balanced salt solution containing 10% (v/v) human serum and 0·5% (w/v) lactalbumin hydrolysate with 100 units of penicillin and 100 $\mu$g. streptomycin/ml. For microscopy, monolayers were fixed in Bouin fluid, and stained with haematoxylin and eosin.

Two procedures were adopted for selective cultivation of amoebae. (1) Petri-dish

plates prepared from 1·5% (w/v) Ionagar (Oxoid: Oxo Ltd., Thames House, Queen St Place, London, E.C. 4) were spread with a suspension in saline of *Klebsiella aerogenes* (NCTC9667) which had been grown in broth and killed by heating at 56° for 2 hr. Surface water was dried from the plates, they were spread with drops of the supernatant fluid of cell cultures infected with Ryan I, and were then incubated at 36–37°. (2) For growth of pure (axenic) cultures, supernatant fluid of cell cultures infected with Ryan I, or amoebae grown on agar plates as shown, were inoculated into either Neff (1957) protease-peptone glucose medium in 5 ml. quantities in sloped McCartney bottles or into a modified 4% (w/v) mycological peptone (Adam, 1964) and incubated at 36·5°. The latter medium was prepared as follows: mycological peptone (Oxoid) was dissolved in M/15 phosphate buffer (pH 7). The solution was boiled to allow a precipitate to form, and then filtered. The filtrate was autoclaved in 5 ml. quantities in McCartney bottles for 20 min. at 121°. The bottles were sloped after inoculation, and subcultures were made at intervals of 4–7 days.

For studies of encystment and mitosis, permanent preparations were made by growing amoebae with living or killed *Klebsiella aerogenes* on microscope slides, coated with a thin layer of agar, in a moist atmosphere; the cultures were fixed *in situ* using Bouin or Carnoy fixative (Singh, 1950). Singh's technique was slightly modified by cutting small slits in the agar layer during inoculation of amoebae on to the slide. This enabled the amoebae to reach the glass surface more readily and to migrate under the agar. The agar layer was stripped off after fixation, and the amoebae adhering to the slide were washed in 70% (w/w) ethanol in water followed by distilled water, mordanted for 18 hr in 3% (w/v) ferric alum, washed in distilled water, and stained for 4–18 hr in 0·5% (w/v) haematoxylin. The stain was differentiated with 1% (w/v) ferric alum, and the preparations were counterstained, before final dehydration, in 2% (w/v) ethanolic aqueous eosin solution. Other preparations were made by allowing a drop of culture fluid containing many amoebae to lie on a slide for about 30 min. in a moist atmosphere, and then running on fixative to fix the amoebae which adhered to the slide. The Feulgen test was done according to Singh (1952).

In tests to determine whether the amoebae would transform into flagellates, the surface of an agar-plate culture of amoebae was flooded with distilled water, and observations were made at intervals of 1, 2, 4, 6, 9 and 24 hr by using an inverted microscope. In another experiment, axenic cultures were centrifuged and the 5 ml. of supernatant fluid discarded. The deposited amoebae were suspended in 10 ml. de-ionized water and samples were pipetted into Petri dishes for observation with an inverted microscope.

## RESULTS

On observation with the inverted microscope of living HeLa cell monolayers, infected with Ryan I, small amoebae were seen to move slowly across the cell sheet, sometimes remaining for a considerable time in one position close to a particular HeLa cell. Focal damage to the cell sheet was obvious to the naked eye 2 days after inoculation, and complete stripping of the monolayer often occurred by 4 days. Cytological features of the cytopathic effect due to Ryan isolates in tissue cultures were described by Armstrong & Pereira (1967), with particular reference to early changes involving the nuclei and nucleoli of the affected cells. Encystment of the amoebae with consequent detachment from the glass surface also occurred regularly, and cysts

were readily detected by microscopical inspection of the supernatant culture medium after 2–3 days of infection.

In agar-plate cultures, single amoebae could be detected after a few hours by using the inverted microscope. They left behind them tortuous trails in moving through the accumulated bacteria on the agar surface. At the end of each trial there could usually be found an indistinct amoeba with a clearly visible contractile vacuole emptying at intervals of about 2 min. When the agar surface had become drier, colonies of amoebae developed which were detectable by the naked eye as clear plaques on the confluent bacterial surface layer, similar to the plaques described for *Hartmannella castellanii* by Van Rooyen (1932*a*, *b*). Within about 4 days, or earlier in a dry atmosphere, most of the amoebae had encysted.

In 4% mycological peptone a heavy growth of amoebae (about $6 \times 10^4$/ml.) was produced after 4–7 days, which could be detected by viewing the culture with the naked eye in subdued daylight, when finely granular clouds of amoebae could be seen in the fluid as it was gently shaken. Examination of cultures by using the inverted microscope disclosed amoebae floating in the medium and adhering to the glass. Cysts were produced in this system after about 6 days, but the agar-plate method produced the maximum yields of cysts. The axenic cultures were subcultured by adding 0·1–0·5 ml. of a well-mixed 4- to 7-day-old culture to each fresh culture bottle. The inoculum could be stored for more than a month at 4° without appreciable loss of viability.

*Morphology of the trophozoites*

The cytoplasm consisted of an inner granular endoplasm, containing the cell organelles, and an outer hyaline ectoplasm. The pseudopodia were fine spike-like, or large lobose, extensions of the ectoplasm, which were often formed simultaneously. Movement of the organism was slow, but appeared to be slightly more rapid in the presence of tissue culture cells. Fifty living amoebae from an axenic culture in 4% mycological peptone at 36·5° were measured by using an eyepiece micrometer, and their mean diameter when rounded was 18·8 $\mu$ (range: 12·3–27·7 $\mu$). In fixed and stained preparations some shrinkage occurred, and the mean diameter was 14 $\mu$. Amoebae grown on agar surfaces with *Klebsiella aerogenes* (Pl. 1, figs. 1, 2) were larger than amoebae from HeLa cell cultures or from pure cultures. There was a single contractile vacuole, situated at the rear end during movement of the organism. Although amoebae were occasionally found which had 2 or more nuclei, there was generally only one nucleus, which was spherical and vesicular, with a delicate nuclear membrane. In life it was often 7 $\mu$ or more in diameter. The mean diameter in fixed and stained cultures with HeLa cells was 3·5 $\mu$ (range of 62 nuclei: 2·7–5·4 $\mu$). Amoebae grown on agar surfaces with *K. aerogenes* had a mean nuclear diameter, when fixed and stained, of 5·3 $\mu$ (range of 50 nuclei: 3·4–6·8 $\mu$). There was a strongly basophilic spherical karyosome or nucleolus, which often appeared hollow; it gave a negative Feulgen reaction. The nucleolar diameter was usually just over half that of the nucleus. The mean diameter of nucleoli from fixed and stained cultures with HeLa cells was 2·0 $\mu$ (range of 44 nucleoli: 1·4–2·7 $\mu$). In fixed and stained amoebae from cultures on agar surfaces, the mean nucleolar diameter was 3·2 $\mu$ (range of 50 nucleoli: 2·0–4·1 $\mu$). Slightly basophilic granules, just inside the nuclear membrane, gave a positive Feulgen staining reaction. These granules evidently represent the desoxyribonucleoprotein component of the nucleus.

*Cyst formation and morphology.* When the cysts were first formed they were rounded, with a thin wall, which became thicker and wrinkled when they ripened. The wall was then double-contoured and pierced by several circular ostioles (as many as 8), each sealed with an operculum. The ostioles were most easily visible in empty cysts (Pl. 2, figs. 7, 8, 9). The mean diameter of 50 living cysts from a pure culture was 18·6 $\mu$ (range 12·3–30·8 $\mu$). There was usually a single nucleus similar to that of the trophozoite. The peripheral part of the cytoplasm in the living cysts was occupied by a layer of dense granules. In excystment, which occurred in the presence of bacteria or HeLa cells, the amoeba apparently emerged through one of the ostioles, after dislodging the operculum (Pl. 2, figs. 8, 9). On agar plates smeared with *Klebsiella aerogenes*, most cysts had hatched in less than 6 hr at 36·5°. Cysts which had been kept under dry conditions at 20° for more than 3 months hatched readily when inoculated on to agar plates spread with *K. aerogenes*. Using viability as a test of survival, it was found that cysts were killed by exposure (for 30 min. at 20°) to methanol, 5% (v/v) Lysol, 70% (v/v) ethanol or 10% (v/v) formol saline. Under the same conditions, neither distilled water nor 0·3% (w/v) Pyroneg (a commercial glassware cleaner obtained from Diversey (U.K.) Ltd., 42–46 Weymouth Street, London, W. 1) killed the cysts.

*Mitotic division.* During mitosis the amoebae became round and ceased to move. In prophase the peripheral Feulgen-positive granules of the nucleus condensed to form chromosomes, whilst the nucleolus began to decrease in size (Pl. 1, fig. 3). When the nucleolus had completely disappeared, spindle fibres appeared, originating apparently from areas of the nuclear membrane which had broken down (Pl. 1, fig. 4). No centrioles were visible at any stage. The nuclear membrane then broke down, whilst the chromosomes became arranged on the equatorial plate and a typical metaphase figure developed (Pl. 1, fig. 5). When the daughter chromosomes moved apart in anaphase, they became more basophilic (Pl. 1, fig. 6). New nuclear membranes formed and two interphase nuclei were reconstituted before cytoplasmic division took place.

*Flagellate stage.* No flagellate stages were found when amoebae were suspended in distilled or de-ionized water for various lengths of time, although some amoebae disintegrated, some encysted, and some continued to move about for more than 2 days.

*Cytopathogenicity of amoebae grown with bacteria and in pure cultures.* Amoebae which had been subcultured 4 times on agar surfaces, with dead *Klebsiella aerogenes* as food, were suspended in saline and 3 serial 10-fold dilutions ($10^{-1}$, $10^{-2}$, $10^{-3}$) were inoculated into cultures of HeLa cells in stationary tubes with coverslips. Cytopathic effects beginning with nuclear changes typical of those reported for the Ryan isolates developed in all the inoculated cultures and led to destruction of the cell sheet. Parallel uninoculated cultures showed no such effects.

Amoebae grown axenically in Neff medium were similarly effective in producing the typical cytopathic effect in cultures of HeLa cells. These results, together with the absence of recognizable virus particles or of other regularly associated microbial agents in sections of the amoebae or infected tissue cultures examined by electron microscopy (Armstrong & Pereira, 1967) suggest that the hartmannellid amoebae were themselves responsible for the cytopathic effect produced by Ryan isolates in cultured mammalian cells. Moreover, the Ryan agent would not pass through filters which admitted viral agents. Further work on the nature and mechanism of the cytopathic effect is in progress.

## DISCUSSION

### *Taxonomy*

Amoebae of the '*Naegleria*' or '*Vahlkampfia*' type (family Schizopyrenicae, Singh, 1952) produce the elongated 'Limax' form readily, generally move rapidly in fresh cultures, and may produce flagellate forms when suspended in distilled water. Their mitosis in the trophozoite stage is distinctive, for the nucleolus is retained and divides to form 'polar masses' (Fig. 1 A). Hartmannellid amoebae (family Hartmannellidae, Singh, 1952) generally move more slowly, and filiform or spike-like pseudopodia are often present. They do not produce flagellate forms. Their mitotic cycle is similar to that found in higher animals and plants. The nucleolus is lost, and a spindle develops, with chromosomes arranged on the equatorial plate (Fig. 1 B). The mitotic cycle, the absence of a flagellate stage, the pseudopodia, the body form and the slow locomotion of the Ryan I amoeba are all characteristic of the family Hartmannellidae.

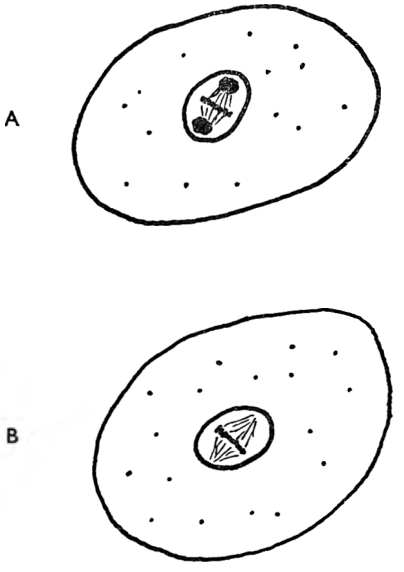


Fig. 1. Diagrammatic comparison of the metaphase figure of mitosis (as if stained with haematoxylin) in (A) a schizopyrenid amoeba and (B) a hartmannellid amoeba.

The structure of the nucleus, with a single large Feulgen-negative karyosome (nucleolus) and peripheral Feulgen-positive material, the absence of nuclear division in the cysts, the aerobic mode of growth and the size-range of the trophozoites separate the Ryan I amoeba from the genera in Hartmannellidae other than the genus *Hartmannella* Alexeieff 1912*a*, *b*, as described by Singh (1952) and Adam (1964). [Singh (1952) and Adam (1964) considered that Volkonsky (1931) split off '*Acarthamoeba*' from the genus *Hartmannella* on inadequate criteria; with this we concur.] The Ryan I amoeba accords well with the descriptions of *H. castellanii* Douglas (1930) given by Castellani (1930*a–d*), Douglas (1930), Volkonsky (1931) and Adam (1964), apart from our inability to find a centriole as described by Volkonsky. Three of the four species of free-living hartmannellid amoeba described by Singh (1952) have a different cyst morphology from the Ryan I amoeba; *H. rhysodes* Singh, 1952, is closely similar

to Ryan I, but we agree with the opinion of Adam (1964) that the name *H. rhysodes* is a synonym of *H. castellanii*. There is little doubt therefore that the Ryan I amoeba is a strain of *H. castellanii*. In the table we give a comparison of the diagnostic features of Ryan I and *H. castellannii* at the species level.

Table 1. *Comparison of the Ryan I amoebae with Hartmannella castellanii Douglas 1930*

| Body form | Nucleus | Cyst form | Cultivation |
|---|---|---|---|
| | *H. castellanii** | | |
| Diameter in life, 7·2–37·0 $\mu$ (mean 18·1 $\mu$). Very slow progression, 'Limax' form rare. Spike-like and lobose pseudopodia. Single contractile vacuole (period 56–137 sec.) | Diameter (fixed) 1·5–6·4 $\mu$. Single large nucleolus, 0·2–5·4 $\mu$ in diameter. Centriole, described by Volkonsky, in perinucleolar space | Diameter in life, 9–27 $\mu$ (mean 16·9$\mu$). Rounded at first, wrinkled and polygonal later. Ostioles closed with opercula at the angles of the cyst | On bacteria and yeasts, dead or alive, from 25° to 35°. Growth in peptone and defined media below 35° |
| | Ryan I amoeba | | |
| Diameter in life 12·3–27·7 $\mu$ (mean 18·8 $\mu$). Very slow progression. 'Limax' form not found. Spike-like and lobose pseudopodia. Single contractile vacuole (period 120 sec.) | Diameter (fixed) 2·7–6·8 $\mu$. Single large nucleolus, 1·4–4·1 $\mu$ in diameter. No centriole found in perinucleolar space | Diameter in life 12·3–30·8 $\mu$ (mean 18·6 $\mu$). Rounded at first, wrinkled and polygonal later. Ostioles closed with opercula at the angles of the cyst | On bacteria, dead or alive, at up to 37°. With tissue culture cells up to 37°. Growth in peptone media at 36·5° |

* Castellani (1930*a–d*), Douglas (1930), Volkonsky (1931), Adam (1964).

Our inability to demonstrate an intranuclear centriole in Ryan I amoebae may well be unimportant since the reported size of this organelle is at the limit of resolution of the light microscope, and its very existence in small amoebae is already disputed (Singh, 1952). With regard to the temperature requirements of different strains, Adam (1964) found that although the Castellani strain of *Hartmannella castellanii* would not grow in 4% mycological peptone at 35°, 11 of the 20 similar strains of hartmannellid amoebae she studied grew well at this temperature. She pointed out that many strains grow readily at (or near) mammalian body temperature.

The various observations reported in our paper refer directly to only one of the tissue culture isolates, namely Ryan I, of the series described by Pereira *et al.* (1966). Nevertheless, it has been confirmed that all isolates of Ryan agent contained similar free-living amoebae (Armstrong & Pereira, 1967), and the cytopathic picture in each case was identical. It is therefore highly probable that all of the Ryan isolates were strains of *Hartmannella castellanii*. Hartmannellid amoebae previously recovered by Wang & Feldman (1961) from cell cultures inoculated with material from human pharyngeal swabs, were considered by these authors to include *H. rhysodes*, *H. glebae* and *H. agricola*. They described the cyst of the form they considered to be *H. rhysodes*, and it is clearly comparable to that of *H. castellanii* and the Ryan I amoeba. The recent work of Chang, Hung Pan & Rosenau (1966) is relevant to the discussion of cytopathogenicity of small amoebae and their presence in tissue cultures. These authors consider the possibility that an infective agent known for several years as 'lipovirus', which was recovered in liver-cell cultures inoculated with human hepatitis

serum (Chang, 1961), is associated with a small amoeboid cell. Certainly the known properties of 'lipovirus' and 'Ryan virus' are strikingly similar, and it is even possible that they are one and the same agent.

*Significance of Hartmannella castellanii in tissue cultures*

Free-living hartmannellid amoebae have been positively identified in mammalian tissue cultures on several occasions, although their precise origin was often obscure (Jahnes, Fullmer & Li, 1957; Culbertson, Smith & Minner, 1958; Adam, 1964). When trophozoites were detected in supposedly normal monkey kidney cell cultures, it seemed reasonable to regard them as contaminants derived from infected renal tissue or from airborne cysts in the laboratory. Wang & Feldman (1961), using HeLa and Hep-2 cell cultures, recovered amoebae from 19 out of 10,000 swabs of the human pharynx, the positive subjects being mostly children. Here, as well as in the more recent Ryan isolates in HeLa cell cultures, inherent or accidental contamination seemed an unlikely explanation; in the Ryan series, for example, amoebae consistently failed to appear in the parallel uninoculated control cultures. There is strong circumstantial evidence that these amoebae were of human origin. The patients concerned were suffering from fever and influenza-like symptoms of varying severity; however, it has yet to be shown with what frequency *Hartmannella castellanii* is recoverable from the throats of symptomless people in different age-groups, and there is little reason at present for supposing the amoebae to be concerned directly in human respiratory disease.

An important and as yet unanswered question in relation to the pathogenic potentialities of *Hartmannella castellanii* is whether the organisms are capable of persisting in the human nasopharynx in the trophic form, or occur merely as occasional cysts either inhaled by chance from the atmosphere or taken in by mouth. Eldridge & Tobin (1967) by using a complement fixation test have found antibody (ranging in titre from 1 in 5 to 1 in 80) against Ryan isolates of Hartmannella in sera from 26 of 128 people in hospital. The sera from a small group from whose throats hartmannellid amoebae had previously been isolated all fixed complement with Hartmannella antigen, although the titres fell to low values during a period of 1–3 years. These findings may well be indicative of an infection with trophic forms of the amoebae and also suggest that it is by no means a rare occurrence. Culbertson and his co-workers (1958, 1959, 1961) have shown conclusively that at least some strains of Hartmannella are pathogenic for mice and for monkeys, following inoculation of trophozoites by the intracerebral or intranasal routes; their experimental studies provide factual support for speculations concerning the possible role of free-living soil amoebae in human disease. Recent reports possibly incriminating small free-living amoebae in more serious disease include the demonstration of abundant trophozoites in post mortem brain sections from 4 rapidly fatal cases of pyogenic meningitis in Australia (Fowler & Carter, 1965); furthermore, in 2 out of 3 similar cases in the United States (Butt 1966) the trophozoites were recognized, before death, in the cerebrospinal fluid.

We thank Dr Janet Niven, Dr F. Hawking and Dr C. G. Culbertson for encouragement and discussion. Drs M. S. and H. Pereira kindly supplied infected and uninfected HeLa cell cultures and also confirmed that amoebae grown axenically or with dead bacteria would produce the typical cytopathic effect. Mr J. B. Clark took the photomicrographs.

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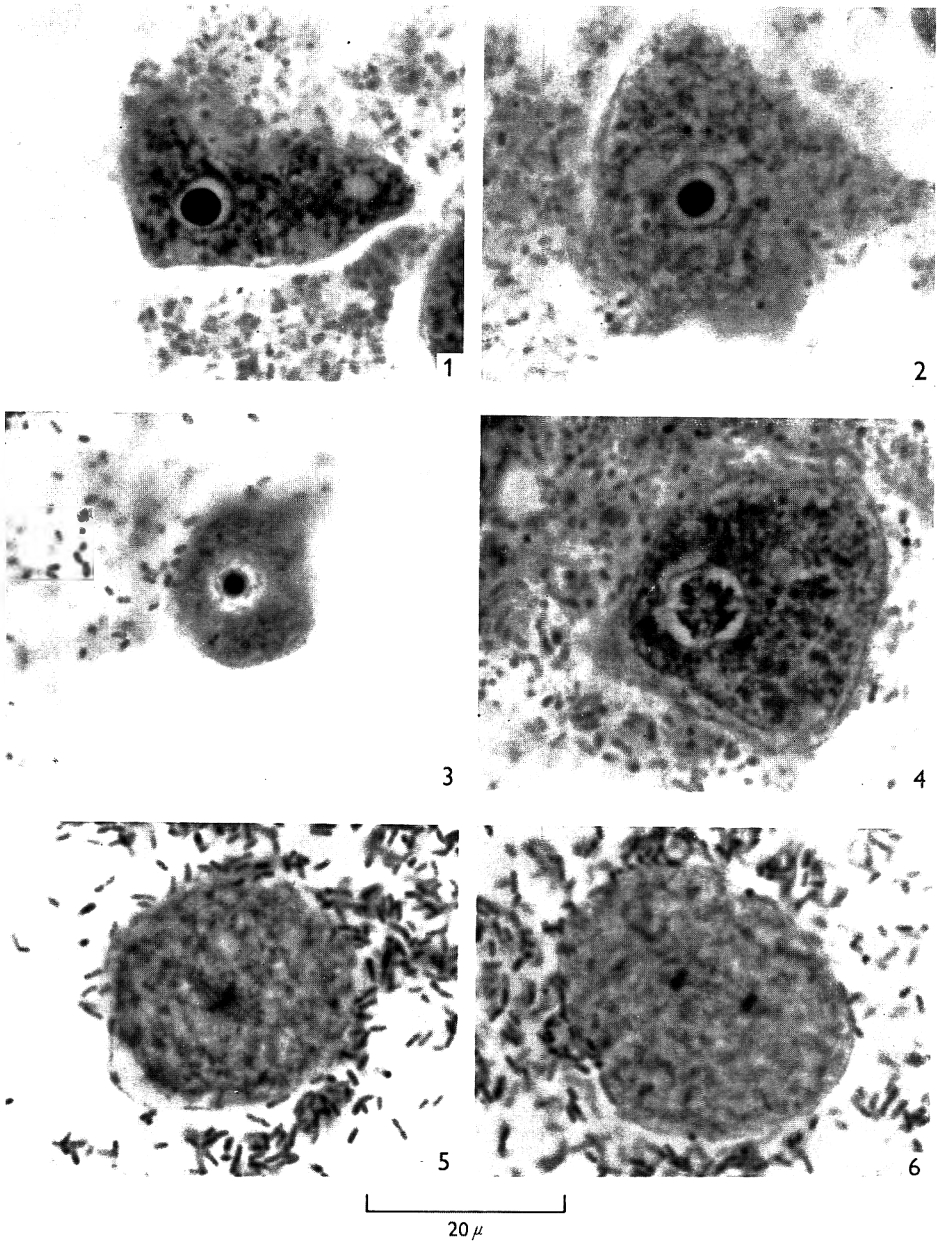


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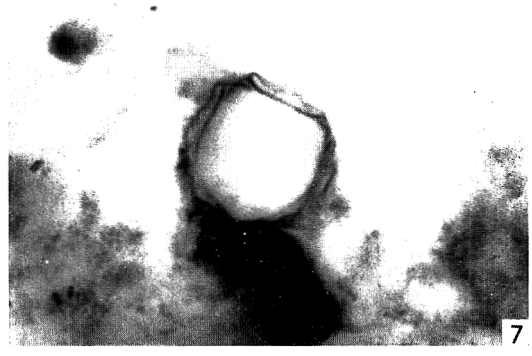

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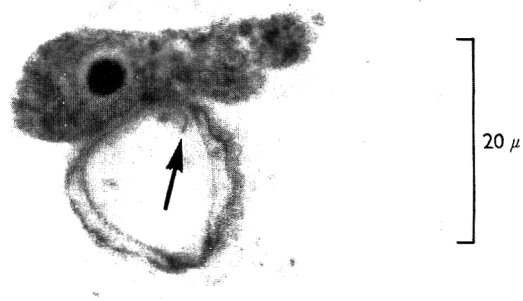


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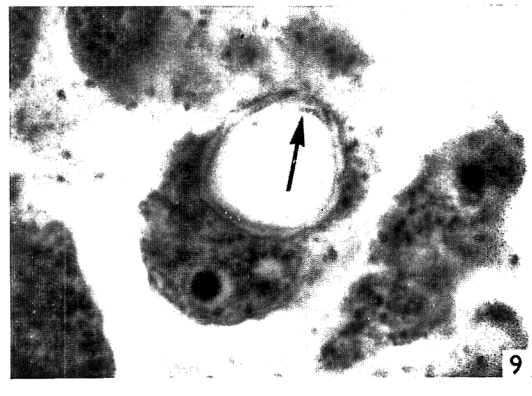

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## EXPLANATION OF PLATES

Photomicrographs of permanent preparations of the Ryan I amoeba, grown with *Klebsiella aerogenes* on agar-coated slides. The organisms were fixed with Bouin's fluid and stained in iron alum haematoxylin, with ethanolic eosin as a counterstain. The scale represents 20 $\mu$.

### PLATE 1

Morphology and mitosis of the trophozoites

Fig. 1. Interphase, showing the vesicular nucleus, with a densely staining single nucleolus, and the contractile vacuole.

Fig. 2. Interphase, showing the basophilic granules inside the nuclear membrane.

Fig. 3. Early prophase: the peripheral granules have condensed to form chromosomes, and the nucleolus is reduced in size.

Fig. 4. Late prophase: the nucleolus has disappeared whilst the spindle fibres and chromosomes are visible.

Fig. 5. Metaphase: chromosomes are arranged on the equatorial plate of the spindle. Note the absence of 'polar masses'.

Fig. 6. Anaphase: two groups of daughter chromosomes are moving apart. The spindle fibres are faintly visible.

### PLATE 2

Cyst-wall morphology in empty cysts

Fig. 7. Cyst showing the double-contoured wrinkled wall and 2 opercula *in situ*.

Fig. 8. Cyst showing dislodged operculum (arrow) and recently emerged (?) trophozoite.

Fig. 9. Cyst showing dislodged operculum (arrow) and recently emerged (?) trophozoite.

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# The Development of Flagella in Swarm Cells of the Myxomycete *Physarum flavicomum*

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SUMMARY

Myxamoebae of *Physarum flavicomum* and the stages of their development of flagella during transformation into biflagellate swarm cells were examined with the electron microscope. Fixation was with glutaraldehyde osmium. The flagellation process was similar to that reported in other systems, involving budding of the basal body into a primary flagellar vesicle which then forms the flagella sheath. Two basal bodies are present in the myxamoeba prior to flagellum development; other morphological features of myxamoebae and swarm cells, including systems of microtubules, are described. Mitochondria of both stages contain a dense core, flagella exhibit the typical '9 plus 2' arrangement, and the doublet fibrils of the axoneme become singlets near the rounded distal end of the flagellum.

## INTRODUCTION

Swarm cells of myxomycetes have been studied with the light microscope by several workers (Lister, 1911; Cadman, 1931; von Stosch, 1935; Elliott, 1949; Kerr, 1960) and appear uninucleate, elongated and in many cases biflagellate. An early study with the electron microscope (Cohen, 1959) yielded little new information, but a more recent investigation by Schuster (1965*a*) detailed subcellular structure of the myxamoebae, swarm cells, and stages in transformation of the former into the latter. Ultrastructural studies on other systems (Sotelo & Trujillo-Cenoz, 1958*a*, *b*; Sorokin, 1962; Schuster, 1963; Renaud & Swift, 1964; Dingle & Fulton, 1966; Stubblefield & Brinkley, 1966) have indicated at least two patterns of flagellum morphogenesis: (1) simple outgrowth of the axoneme at the cell surface (Dingle & Fulton, 1966); (2) development of a primary flagellar vesicle at the proximal end of the basal body, subsequent elongation of this vesicle with the growing axoneme, and ultimate fusion of this vesicle with the plasma membrane as the flagellum emerges (Sorokin, 1962; Renaud & Swift, 1964; Stubblefield & Brinkley, 1966). Other workers (Sotelo & Trujillo-Cenoz, 1958*a*, *b*; Schuster, 1963) have indicated sequences varying slightly from these basic patterns. The present study documents stages indicating that myxamoebae of *Physarum flavicomum* develop flagella as in pattern 2 above, contrasting with Schuster's report (Schuster, 1965*a*) that the process in *Didymium nigripes*, a related species, follows pattern 1 above.

I have recently shown (Aldrich, 1967) that the basal bodies of the myxamoebae are present in the spores after meiosis is complete, confirming earlier observations made by von Stosch (1935) with the light microscope. Study of details of the amoeba-

flagellate transformation in myxomycetes is considerably simplified by the fact that the transformation can be induced synchronously in a culture of amoebae by the addition of distilled water. Details of this system were described by Kerr (1960), who followed the transformation with the light microscope.

## METHODS

Single spores of the heterothallic myxomycete *Physarum flavicomum* were sown on half-strength cornmeal agar, and clones arising from such isolates were maintained with bacteria by successive subculture techniques. Flagellum development in such clones was induced by adding distilled water to agar cultures; within 2 hr essentially all cells were flagellated. Myxamoebae, various stages of cells developing flagella, and mature swarm cells were fixed in cold 1·5% or 2·5% glutaraldehyde, buffered with Sorenson phosphate to pH 7·5. After washing and staining with cold 1% osmium tetroxide for 1 hr, cells were washed and embedded in agar to facilitate handling, dehydrated, and embedded in epoxy resin mixture II of Mollenhauer (1964). Some samples received no glutaraldehyde pre-treatment, but were fixed in cold 1% osmium tetroxide with 0·002 M-calcium added. Blocks were sectioned with diamond knives on Sorvall models MT-1 and MT-2 and post-stained with 0·5% uranyl acetate followed by Reynolds lead citrate (Reynolds, 1963). Observations were made with RCA microscopes models EMU 3-D and 3-F and with a Siemens Elmiskop I.

## OBSERVATIONS

Myxamoebae beginning the transformation process (Pl. 1, fig. 1) contain typical organelles, including dictyosomes, endoplasmic reticulum, nucleus and mitochondria. The nucleus is beaked in the region near the basal bodies and dictyosomes, the association of the latter organelles having been noted by Schuster (1965*a*) and interpreted as the centrosphere of Bernhard & deHarven (1960). The nucleus contains a prominent nucleolus. Attached to the basal bodies is much of the accessory microtubular apparatus which will later be detailed in the swarm cell. Mitochondria contain the dense core reported by Schuster (1965*b*) to be DNA. Mitochondria at this stage assume various shapes, at times even including an electron-transparent vacuole. Food vacuoles contain bacteria being digested. Contractile vacuoles are also present, although not shown in Pl. 1, fig. 1. Several homogeneous globules probably represent a storage product.

After cultures are wet, basal bodies of transforming individuals develop a primary flagellar vesicle at their distal end (Pl. 2, fig. 2), as in systems investigated by Sorokin (1962), Renaud & Swift (1964) and Stubblefield & Brinkley (1966). This vesicle seems to form by the fusion of several smaller vesicles. Plate 2, figs. 3–5 show the flagellar bud elongating into this vesicle which adds membrane by fusion of secondary vesicles (Pl. 2, fig. 4), and which forms the flagellar sheath just as in the other systems mentioned. Evidently, just before emergence of the flagella, the two buds are within a common vesicle (Pl. 2, fig. 5); the significance of the electron dense globules in fig. 5 is unclear. Although they appear similar to familiar lead citrate artifacts, such dense bodies are only seen near developing flagella, which suggests that they may represent more than artifacts. Plate 2, fig. 2, 3 both show primary vesicles associated with the basal body of the longer flagellum, recognizable by its distinctive basal apparatus

represented by the row of dense granules along the side. Whether the developing second flagellum also has a primary vesicle is not known, but Pl. 2, fig. 5 indicates that the two flagellar buds emerge together.

Also noteworthy are the presence of satellites on budding basal bodies (Pl. 2, fig. 3) as noted by Sorokin (1962), and a discontinuity of the proximal end of the basal body (Pl. 2, fig. 3; Pl. 4, fig. 11), to which is attached an array of microtubules through a dense connexion. The significance of the satellites and of the discontinuity is unknown.

The mature swarm cell is elongated, the cone-shaped anterior region containing the basal bodies, Golgi apparatus, and the flat-topped nucleus. Posterior to the nucleus are mitochondria, regions of rough endoplasmic reticulum, and food vacuoles. Contractile vacuoles of a typical protozoan type occupy the posterior portion, which is amoeboid in behaviour, the anterior end of the cell being apparently held into a fairly rigid cone by microtubules attached to the longer flagellum.

These microtubules are in two concentric diverging arrays, the outer being attached to the basal body through dense bands partially encircling it. This outer array of approximately 40 microtubules, shown in grazing section in Pl. 4, fig. 12 and in cross-section in Pl. 4, fig. 13, runs posteriorly just under the plasma membrane and ends about the level of the posterior portion of the nucleus. An inner array attached to this same flagellum through a dense connexion (Pl. 4, figs. 11, 13) ends in the cytoplasm near the nuclear envelope.

The shorter flagellum, usually seen recurved close to the body of the swarm cell, has only a single parallel array of 6–8 microtubules attached, these running posteriorly just beneath the plasma membrane (Pl. 4, figs. 11, 13). The microtubules just described appear typical of those found in the cytoplasm of a variety of plant and animal cells, with an osmiophilic outer cylinder and an electron-transparent core. The model of the anterior end of the swarm cell, shown in Pl. 3, fig. 9, illustrates the arrangement of these tubules.

The flagella themselves show the typical 9 plus 2 arrangement of fibrils when sectioned transversely (Pl. 3, fig. 8), and appear blunt in longitudinal section (Pl. 4, fig. 10). Near the tip, the outer doublets become singlets (Pl. 3, fig. 7), suggesting that the terminal structure is similar to that of cilia of *Diplodinium ecaudatum* (Roth & Shigenaka, 1964) and gill cilia of mussels (Satir, 1965).

## DISCUSSION

The type of flagellum development described here is similar to that first outlined by Sorokin (1962) in bird and mammal fibroblasts and smooth muscle cells, subsequently in Allomyces by Renaud & Swift (1964), and now most recently in Chinese hamster fibroblasts by Stubblefield & Brinkley (1966). Earlier workers (Sotelo & Trujillo-Cenoz, 1958*a*, *b*) reported that the basal body first contacts the plasma membrane, causing it to invaginate to form the ciliary sheath; however, no such invaginations or necessity for contact of basal body with plasma membrane were apparent in the present study.

Schuster (1965*a*) reported that flagella of two other species of myxomycetes develop simply by the outgrowth of the axoneme at the surface of the cell, much as shown by Dingle & Fulton in Naegleria (1966). My own experience indicates that early developmental stages in the proper orientation are infrequently encountered, making reconstruction of the sequence difficult. I feel, however, that the sequence presently reported

is the usual one for *Physarum flavicomum*. Kerr's (1960) observation that flagella of *Didymium nigripes* appear quite suddenly fits with a sequence involving initial axoneme development inside the cell in association with a primary vesicle, followed by emergence by fusion of this vesicle with the plasma membrane, as described herein. However, this does not preclude the possibility that some species might develop differently. This possibility gains credibility when considered in view of the facts that meiosis in myxomycetes may vary in position among different species (Aldrich, 1967), and that mitosis in haploid stages, according to Schuster (1965*a*), is of an astral type (terminology of Roth, 1964), while the mitotic spindle in diploid stages is intranuclear (Schuster, 1964; Koevenig & Jackson, 1966; Aldrich, 1967). Further details of these mechanisms will be dealt with in a later paper, but these anomalies serve to illustrate the non-uniformity of myxomycetes in several respects, and it may well be that flagellum development follows different patterns in different species.

The morphology of the myxamoebae and swarm cells as reported here agrees well with that reported earlier for *Didymium nigripes* (Schuster, 1965*a*). All mature swarm cells examined were biflagellate, with the second flagellum shorter and recurved near the body of the swarm cell. The shorter flagellum is inactive in locomotion of living cells viewed with phase contrast, which may explain difficulties of light microscopists in finding the second flagellum. (For review of controversy concerning number of flagella in myxomycetes see Alexopoulos, 1963.) The coincidence of inactivity of this shorter flagellum with its small complement of associated microtubules, compared with the extensive arrays associated with the longer, active flagellum, suggests that the microtubules anchor the flagellum into the cytoplasm and contribute to the observed inflexibility exhibited by the anterior end of the swarm cell in the living condition.

The results just discussed indicate that myxomycetes share ultrastructural characters with diverse groups of organisms, including the universal flagellum morphology and the mode of flagellum development typical of fungi and higher animals. Certain unique features of the group, including synchronous mitosis in the plasmodial state, different mechanisms of karyokinesis in haploid and diploid stages, and spectacular reversible streaming of the plasmodium, make myxomycetes potentially extremely useful subjects for future research.

Portions of this work were included in a dissertation submitted to the Graduate School, University of Texas, in partial fulfilment of the requirements for the Ph.D. degree. Support is gratefully acknowledged from the following sources: NIH Training Grant in Cell Biology 5TI-GM-789 to the University of Texas, NSF Grant GB-2738 to C. J. Alexopoulos, an American Cancer Society Institutional Grant to the University of Florida, and the Graduate School, University of Florida.

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## EXPLANATION OF PLATES

### SYMBOLS USED

| | | | |
|---|---|---|---|
| 1 | Basal body of long flagellum | N | Nucleus |
| 2 | Basal body of short flagellum | O | Outer microtubular array |
| C | Contractile vacuole | P | Parallel microtubular array |
| D | Dense body | PM | Plasma membrane |
| G | Golgi apparatus | PV | Primary flagellar vesicle |
| I | Inner microtubular array | S | Satellite |
| M | Mitochondrion | V | Food vacuole |

### PLATE 1

*Physarum flavicomum*

Fig. 1. Myxamoeba before transformation into swarm cell. Three food vacuoles contain phagocytized bacteria. Nucleus is pear-shaped. Mitochondria show characteristic dense core. Centrosphere region at upper right contains Golgi apparatus and basal bodies. × 24,000.

### PLATE 2

Fig. 2. Primary flagellar vesicle is evidently forming at arrow by fusion of several smaller vesicles. × 90,000.

Fig. 3. Flagellar bud is elongating. Note discontinuity of basal body at arrow. × 90,000.

Fig. 4. Secondary vesicle fusing with primary flagellar vesicle (arrow). × 86,000.

Fig. 5. Both flagella at time of emergence. × 70,000.

### PLATE 3

Fig. 6. Longitudinal section of whole swarm cell. × 12,000.

Fig. 7. Cross-section of flagellum near tip. Nine outer fibres are single. Diameter is reduced (compare with fig. 8, printed at same magnification). × 197,000.

Fig. 8. Cross-section of flagellum. Arms are present on doublet fibres. × 197,000.

Fig. 9. Model of anterior portion of swarm cell.

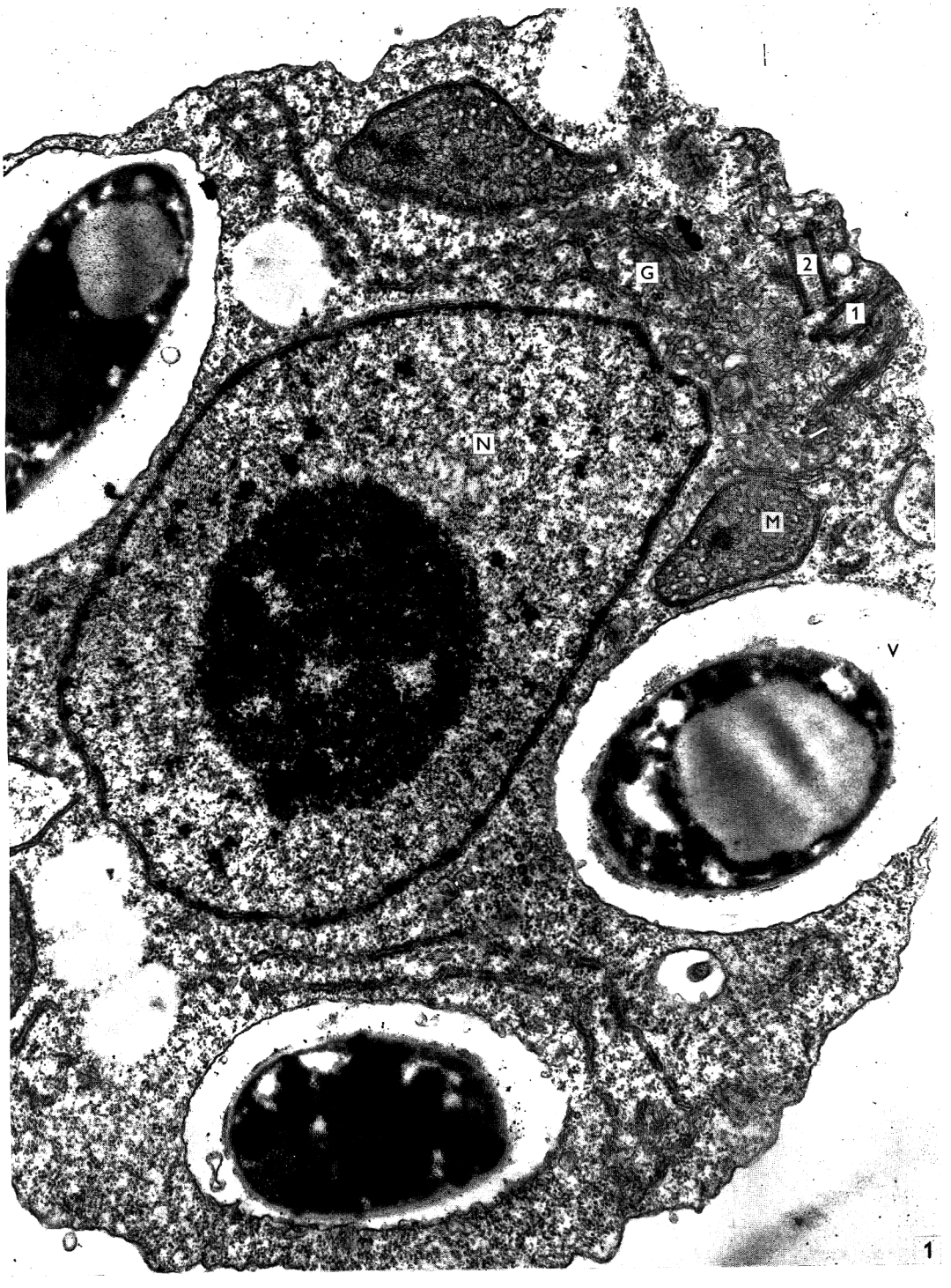
### PLATE 4

Fig. 10. Longitudinal section of tip of longer flagellum. × 66,000.

Fig. 11. Anterior of swarm cell, long section. Note dense body attached to basal body and discontinuity of latter. Line marks plane of section in fig. 13. × 55,000.

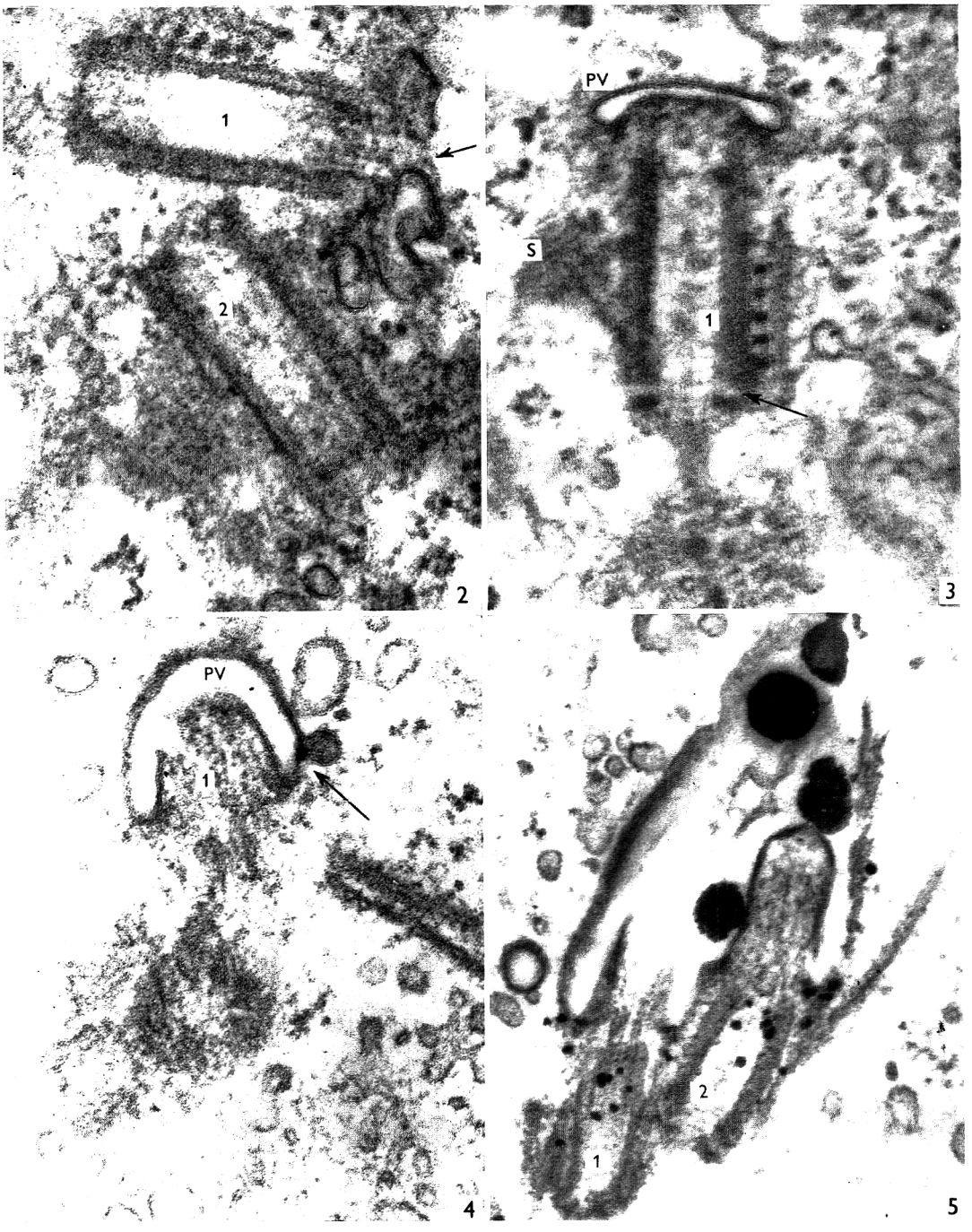
Fig. 12. Grazing section of anterior of swarm cell, showing outer diverging microtubular array attached to longer flagellum. × 53,000.

Fig. 13. Anterior of swarm cell, cross section, showing all three arrays of microtubules.




H. C. ALDRICH

(*Facing p.* 222)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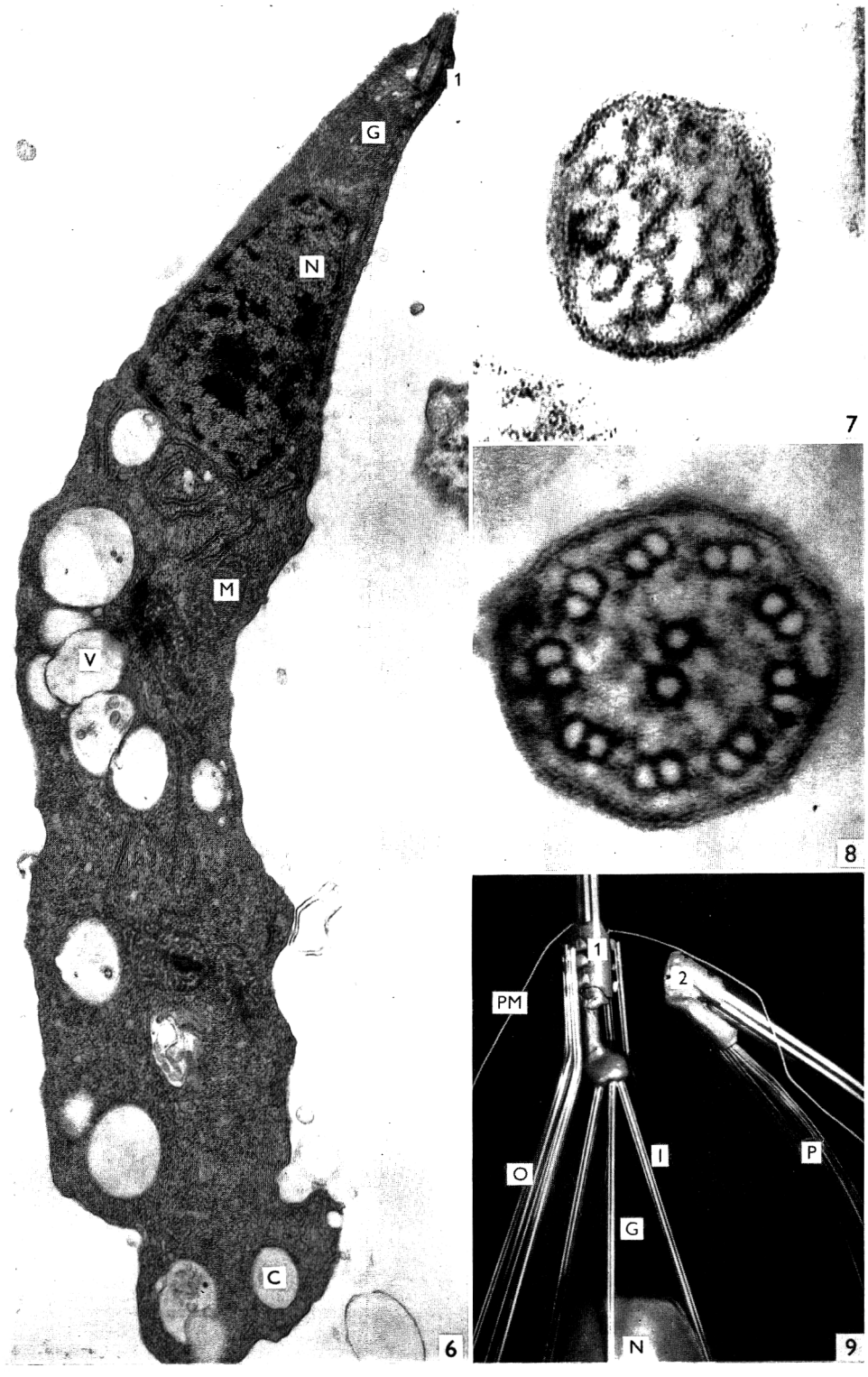
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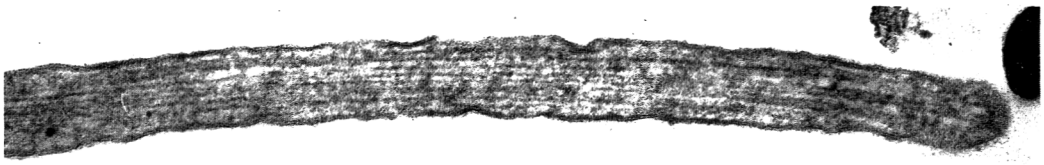

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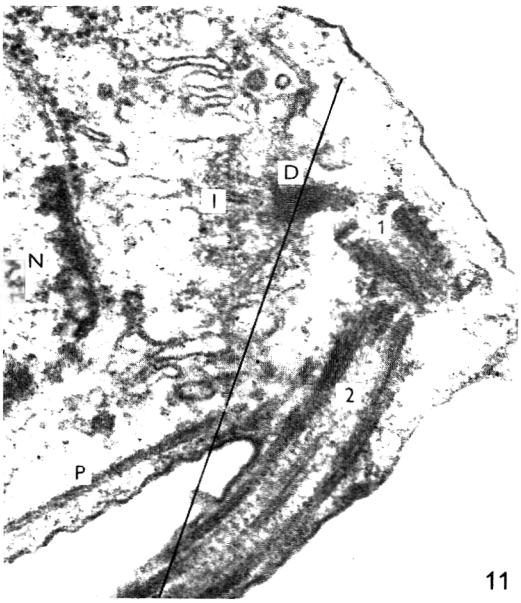


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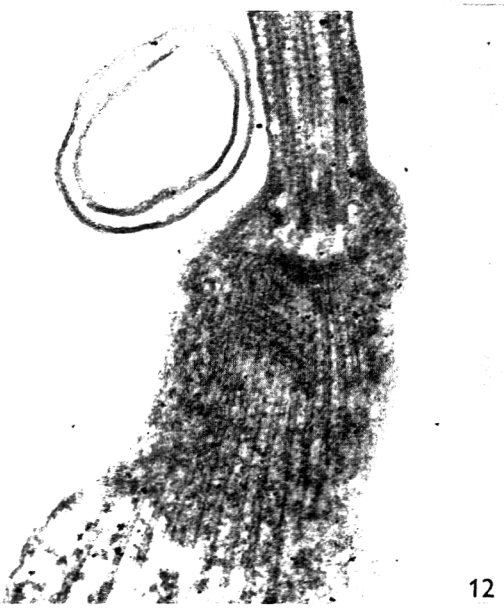

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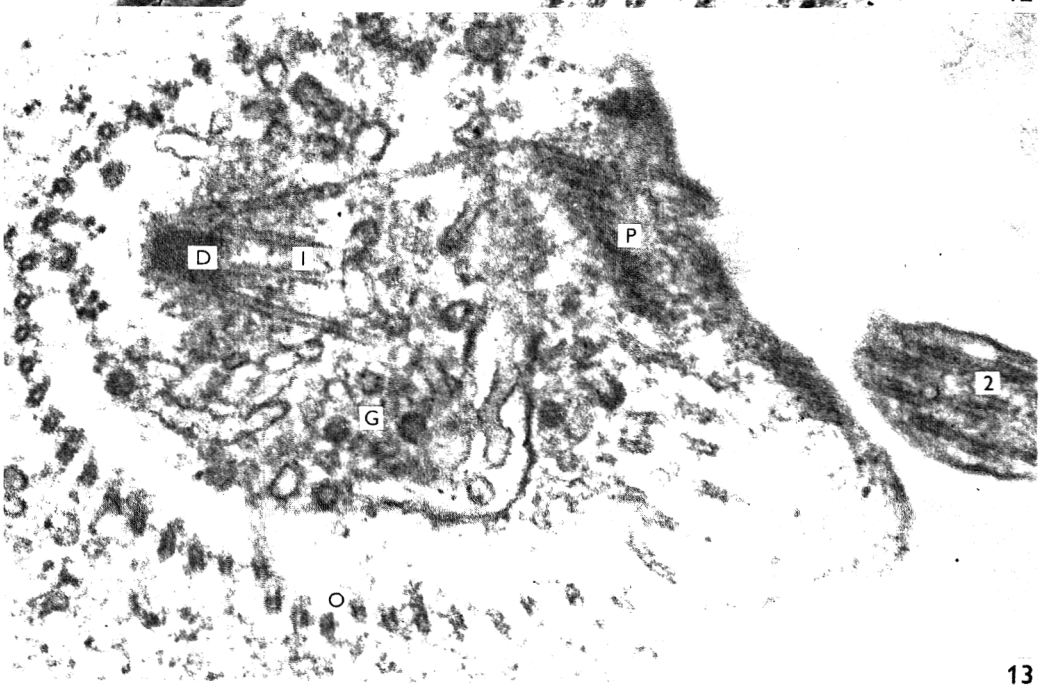


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# A Piecemeal Description of *Streptomyces griseus* (Krainsky) Waksman and Henrici

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SUMMARY

Forty-six of the 48 strains named *Streptomyces griseus* in our collection have in common a distinctive pattern of properties and are believed to represent a recognizable taxonomic group, or aggregate, of this polytypic species. Seventy-seven additional strains bearing 51 different species names were found to possess the same pattern of characteristics. The distinguishing properties of this aggregate are described and compared with those of *S. albus*, *S. fradiae* and *S. somaliensis*.

## INTRODUCTION

Early in this study of the streptomycetes wide variation was observed among cultures of individual strains not only in their pigmentation but also in their formation of aerial hyphae, sporulation and the fragmentation of their substrate hyphae (Gordon & Mihm, 1962). Certain physiological properties, on the other hand, were shared by the parent strains and their soft fragmenting variants. Because the basic taxonomic unit (designated here by the old-fashioned term 'species') can be recognized in different ways, a search was begun for a group of relatively stable characters by which each species might be described and differentiated from the other species of streptomycetes and nocardias in our collection.

According to the criteria investigated, certain species, typified by *Streptomyces fradiae*, were comparatively homogeneous and distinct; either all the strains, or nearly all the strains, gave the same reaction to each test applied (Gordon, 1967). A distinguishing pattern of characters for recognizing *S. fradiae* was thus easily found. Other species were more heterogeneous, or polytypic, and were, apparently, composed of aggregates of strains with intermediate strains connecting the aggregates. *S. griseus* (Krainsky, 1914) Waksman & Henrici (1948) is an example of a polytypic species, and its composition, as represented in our collection, confirmed Pridham's (1964) description of *S. griseus* as a 'species comprising many sub-species'.

On the basis of physiological properties, 46 of the 48 strains named *Streptomyces griseus* in the Institute of Microbiology, Rutgers, The State University, New Jersey (IMRU), collection were relatively similar and are representative of a single aggregate joined to other aggregates by intermediate strains. These 46 strains have been kept as separate strains, although some are duplicates maintained for several years in other laboratories and some are variants developed for specific purposes. The pattern of reactions by which these 46 strains can be recognized, and a list of other strains in this IMRU collection with the same pattern of characters are presented.

## METHODS

The 123 strains listed in Tables 1 and 2 and the strains examined by Gordon (1966, 1967) were compared by means of observations and tests described in the same reports. In addition, the following tests were used.

*Hydrolysis of hippurate.* Cultures were prepared in hippurate broth (tryptone, 10 g.; beef extract, 3 g.; yeast extract, 1 g.; glucose, 1 g.; $Na_2HPO_4$, 5 g.; Na hippurate, 10 g.; distilled water, 1000 ml.). After 6 weeks of incubation at 28°, 1 ml. of each culture, as free from clumps of growth as possible, was mixed with 1·5 ml. of 50% (v/v) sulphuric acid. The appearance of finely divided crystals in the acid mixture after 4 hr at room temperature indicated the presence of benzoic acid (Baird-Parker, 1963). When the identity of the crystals was in doubt, their melting point was compared with that of benzoic acid.

*Oxidation of glucose.* The oxidative or fermentative utilization of glucose was determined by the Hugh & Leifson (1953) test. The basal medium (peptone, 2 g.; NaCl, 5 g.; $K_2HPO_4$, 0·3 g.; agar 3 g.; 0·04% (w/v) aqueous solution of bromcresol purple, 15 ml.; distilled water, 1000 ml.; pH 7·1) was tubed (7·5 ml./16 mm. diam. tube), autoclaved and quickly cooled. Before the agar solidified, 0·5 ml. of 15% (w/v) aqueous solution of glucose, also sterilized by autoclaving, was added to each tube. As soon as the agar hardened, duplicate tubes were inoculated by stabbing from a 10- to 14-day culture in glucose broth; one tube was then sealed with a mixture of petroleum jelly and paraffin (paraffin, 60% (w/w); petroleum jelly, 40% (w/w)). During the first 24 hr of incubation, some cultures had to be resealed by heating the glass around the jelly in a small flame. The cultures were incubated at 28° and observed at 7 and 28 days. A culture that grew both in the aerobic and the anaerobic tubes, but formed the acid colour of the indicator only in the aerobic tube was recorded as oxidizing glucose. A culture that grew and formed the acid colour of the indicator both in the aerobic and the anaerobic tubes was recorded as fermenting glucose. A culture that grew in both tubes but did not change the colour of the indicator was recorded as not attacking glucose in the medium of Hugh & Leifson.

*Resistance to salicylate.* A tube of glucose broth (peptone, 5 g.; beef extract, 5 g.; yeast extract, 5 g.; glucose, 5 g.; distilled water, 1000 ml.) and a tube of glucose broth containing 0·2% (w/v) Na salicylate were inoculated from a 10- to 14-day culture in glucose broth with a loop 2 mm. outside diameter. The tubes were examined immediately and those containing inoculum that might be mistaken for growth were replaced. Growth of the cultures was observed after 7 and 28 days of incubation at 28°. This test was suggested by the method of Tsukamura (1962).

*Utilization of benzoate, lactate and oxalate.* Modifications of Koser's (1924) citrate agar were made by adding 2 g. Na benzoate, Na lactate or Na oxalate to: 1 g. NaCl, 0·2 g. $MgSO_4.7H_2O$, 1 g. $(NH_4)_2HPO_4$, 0·5 g. $KH_2PO_4$, 15 g. agar, 1000 ml. distilled water, 20 ml. 0·4% (w/v) phenol red solution. The pH value of each medium was adjusted to ensure a slightly acid colour of the indicator after autoclaving. Cultures on slopes of each medium were observed for the alkaline colour of the indicator after 7 and 28 days of incubation at 28°.

*Determination of diaminopimelic acid (DAP).* The method of Becker, Lechevalier, Gordon & Lechevalier (1964) was used to establish the form of DAP in hydrolysates of the whole organisms.

## RESULTS

After 5 days of incubation, cultures of the strains (Tables 1, 2) grown on glycerol agar and stained by a modified Ziehl-Neelsen method were composed of short or long slender branching filaments that did not retain the carbol fuchsin. In some cultures the filaments were vacuolated, in others they were broken into uneven fragments or bacillary forms. Spores, when formed on the glycerol agar, appeared as spherical or oval bodies.

Table 1. *Reference strains of Streptomyces griseus (Krainsky) Waksman & Henrici*

| Laboratory no. | Source, strain name or number |
|---|---|
| 3326 | N.J. Agr. Exp. Station (Waksman & Curtis 360) |
| 3326a | Centraalbureau voor Schimmelcultures (CBS) (Waksman & Curtis 360) |
| 3326b | A. Kelner, Biological Lab., Cold Spring Harbor, N.Y. (mutant 1 of Waksman & Curtis 360) |
| 3378 | F. A. Wolff, Duke Univ. (Yucatan) |
| 3463, 3464 | N.J. Agr. Exp. Station (18-16, soil; D-1, chicken's throat) |
| 3464a | N.J. Agr. Exp. Station (sporulating variant of D-1) |
| 3475 | H. W. Anderson, Univ. of Ill. (variant 42.1 of 4) |
| 3478, 3481 | N.J. Agr. Exp. Station (25-G, peat; 22, soil) |
| 3480 | Schenley Labs., Inc., Lawrenceberg, Ind. (SL-842) |
| 3492–3494, 3498 | N.J. Agr. Exp. Station (variants 1, 9, 19 and $C_1$ of 18-16) |
| 3496, 3495 | N.J. Agr. Exp. Station (4 and rhodomycetin producing variant of 4) |
| 3499 | C. H. Reilly, N.J. Agr. Exp. Station (C3, a streptomycin producing variant of D-1) |
| 3510 | W. Garson, N.J. Agr. Exp. Station (HF, cow manure) |
| 3511 | Parke, Davis and Co., Detroit (04506) |
| 3512 | N.J. Agr. Exp. Station (2 PR, a phage-resistant variant of 3475) |
| 3523, 3524 | Merck and Co., Rahway, N.J. (7R2557 and unnumbered); CBS 3496 and 3493) |
| 3527 | N.J. Agr. Exp. Station (Mexican soil) |
| 3533 | A. B. Kupferberg, N.J. Agr. Exp. Station (31) |
| 3536–3539 | H. Umezawa, Nat. Inst. of Health, Tokyo (S-1, S-2, S-3 and S-4) |
| 3546 | F. Bustinza, Univ. of Madrid (Z) |
| 3563 | W. Gold, N.J. Agr. Exp. Station; Univ. of Wis. |
| 3567a, 3567b | N.J. Agr. Exp. Station (intestine of earthworm) |
| 3570 | H. A. Lechevalier, N.J. Agr. Exp. Station (C 135, produces candicidin) |
| 3586–3589 | R. S. Weiser, Univ. of Wash. (214, 305, 1260 and 2017) |
| 3616 | P. P. DeSomer, Rega Inst., Louvain (R 33, a variant of 3464) |
| 3620, 3621 | H. E. Bailey, Wayne Univ. (103 and 105) |
| 3646 | R. G. Benedict, U.S. Dept. Agr., Peoria, Ill. (NRRL B-683) |
| 3734 | J. Ehrlich, Parke, Davis and Co., Detroit (A 4903-PD 04797-NRRL 2426) |
| 3754 | H. B. Woodruff, Merck and Co., Rahway, N.J. (L-118 MA-45) |
| 3903 | H. A. Lechevalier, Rutgers Univ. (LP-16, produces geosmin) |
| A10137 | American Type Culture Collection (ATCC) (10137); S.A. Waksman (4) |

On soil extract and Bennett agars, the colonies were densely or loosely filamentous. The substrate hyphae were extensive, fine, wavy, branching and intertwined with the substrate hyphae of adjacent colonies. The aerial hyphae varied from sparse to abundant, straight to loosely coiled, and short to long, intertwining sometimes with the aerial hyphae of adjacent colonies. The aerial hyphae of some cultures branched in clusters or whorls. During the 14-day incubation chains of bead-like spores were formed.

Macroscopically the cultures on yeast dextrose agar at 7 days were abundant and spreading and finely or coarsely wrinkled or flat. The growth was cream-

Table 2. *Strains identified as Streptomyces griseus (Krainsky, 1914) Waksman & Henrici (1948)*

| Laboratory no. | Name when received, source, and strain name or number |
|---|---|
| 3845 | *Actinomyces cyaneofuscatus* Kudrina (1957); G. F. Gauze, Inst. of Antibiotics, Moscow (92/54) |
| 413 | *A. keratolytica*; J. M. Coffey, N.Y. State Dept. of Health (5229) |
| 3765 | *A. longissimus* Krassil'nikov (1941); H. J. Kutzner, Inst. f. Biochemie des Bodens, Braunschweig-Volkenrode, Germany (116); M. S Todorović |
| 3347 | * *A. roseus* Krainsky (1914); N.J. Agr. Exp. Station |
| 3007 | *A. sulphureus*; N.J. Agr. Exp. Station |
| 506 | *Nocardia alba*; T. Thibault, Inst. Pasteur, Paris |
| N 3258, N 4520 | *N. asteroides*; NCTC (3258, 4520) |
| 3301 | *N. farcinica*; N.J. Agr. Exp. Station; CBS |
| 676 | *N. haemolytica*; E. N. Azarowicz, Univ. of Calif. (13) |
| 3856 | *N. italica* Spalla, Amici & Bianchi (1961); A DiMarco, Farmitalia, Milan (soil) |
| A 6855 | *N. leishmani*; ATCC (6855); BCTC (658) |
| A 6245 | *N. madurae*; ATCC (6245); C. W. Emmons (9902) |
| 505 | *N. odorifera* (Rullmann, 1895) Castellani & Chalmers (1913); P. Thibault |
| A 4919 | *N. sylvodorifera*; ATCC (4919); A. Castellani |
| 511 | *N. violacea* (Rossi-Doria, 1891) Chalmers & Christopherson (1916); P Thibault |
| 638, 712, 713 | *Nocardia* spp ; D. B. Tilden, Chicago Zoological Park (lung of a kangaroo, lung lesion of a sitatunga, lung of a black-tailed deer) |
| 699 | *Nocardia* sp.; C. W. Emmons, Nat. Inst. of Health, Bethesda, Md. (9940); A. L. Carrion (mycetoma) |
| 3049 | *Streptomyces acidoresistans*; N.J. Agr. Exp. Station; Král Collection |
| 3861 | *S. albidoflavus* (Rossi-Doria, 1891) Waksman & Henrici (1948); P M. Arnow, Rutgers Univ.; H. Okada |
| N 7807 | *S. albus*; NCTC (7807); W. R. Maxted |
| 3005, 3006 | *S. albus*; N.J. Agr. Exp. Station; CBS; H. W. Wollenweber (potatoes) |
| 3019 | *S. albus*; N.J. Agr. Exp. Station; CBS |
| 3361 | *S. albus*; N.J. Agr. Exp. Station |
| 3448 | *S. albus*; M. Welsch, Univ. de Liège |
| 3462 | *S. albus*; C. H. Meredith, Glenleigh Lab., Highgate, Jamaica, B.W.I. (11, antagonistic to *Fusarium oxysporum cubense*) |
| 3307 | *S. annulatus*; N.J. Agr. Exp. Station; CBS |
| 3309A | *S. aureus* (Waksman & Curtis, 1916) Waksman & Henrici (1948); N.J. Agr. Exp. Station |
| 3811 | *S. baarnensis* Pridham, Hesseltine & Benedict (1958); Lederle Labs., Pearl River, N.Y.; CBS |
| 3312 | *S. californicus* (Waksman & Curtis, 1916) Waksman & Henrici (1948); N.J. Agr. Exp. Station |
| 3593 | *S. californicus*; H.B. Woodruff, Merck and Co., Inc. Rahway, N.J. (51 R 3639) |
| A 4879 | *S. candidus*; ATCC (4879); S. A. Scudder (23314) |
| 3416 | *S. candidus*; N.J. Agr. Exp. Station; ATCC (4878) |
| 3758 | *S cavourensis* Waksman (1961); R. Craveri, S. A. Montecatini, Milan (829. Linate) |
| 3313A, 3313B | *S. cellulosae* (Krainsky, 1914) Waksman & Henrici (1948); N.J. Agr. Exp. Station (variants of 3313) |
| 3657 | *S. chrysomallus* Lindenbein (1952); K. B. Raper, U.S. Dept. Agr., Peoria, Ill. (NRRL 2250); Schenley Labs., Inc. |
| 3036 | *S. erytheus* (Waksman, 1923) Waksman & Henrici (1948); CBS |
| 3316 | *S. exfoliatus* (Waksman & Curtis, 1916) Waksman & Henrici (1948); CBS (Waksman & Curtis) |
| 3671 | *S. flavochromogenes* (Krainsky, 1914) Waksman & Henrici (1948); Heyden Chemical Corp., Princeton, N.J. (8) |
| 3590 | *S. gougerotii* (Duché, 1934) Waksman & Henrici (1948); CBS |
| 3068 | *S. griseobrunneus* Waksman (1961); N. J. Agr. Exp. Station (218) |
| 3325 | *S. griseolus* (Waksman, 1923) Waksman & Henrici (1948); N.J. Agr. Exp. Station. |
| 3328 | *S. halstedii* (Waksman & Curtis, 1916) Waksman & Henrici (1948); N.J. Agr. Exp. Station |
| 3329 | *S. intermedius*; H. W. Wollenweber, Forschungs Inst. f. Kartoffelbau, Berlin |

Table 2. (*contd.*)

| Laboratory no. | Name when received, source and strain name or number |
|---|---|
| 3330 | *S. lavendulae*; N.J. Agr. Exp. Station |
| 3331 | *S. lipmannii* (Waksman & Curtis, 1916) Waksman & Henrici (1948); N.J. Agr. Exp. Station |
| 3332 | *S. microflavus* (Krainsky, 1914) Waksman & Henrici (1948); CBS; A. Krainsky |
| 3334 | *S. odorifer* (Rullmann, 1895) Waksman (1953); N.J. Agr. Exp. Station; Král Collection |
| 3677 | *S. parvullus* Waksman & Gregory (1954); F. J. Gregory, N.J. Agr. Exp. Station (G-375) |
| 3686 | *S. pravus* (Krainsky, 1914) Waksman & Henrici (1948); R. G. Benedict, U.S. Dept. Agr., Peoria, Ill. (NRRL B-1455) |
| 3374 | *S. praecox* (Millard & Burr, 1926) Waksman (1953); W. A. Millard, Univ. of Leeds |
| 3344 | *S. reticuli* (Waksman & Curtis, 1916) Waksman & Henrici (1948); N.J. Agr. Exp. Station |
| 3689 | * *S. roseochromogenus* (Jensen, 1931) Waksman & Henrici (1948); R. W. Thoma, E. R. Squibb and Sons, New Brunswick, N.J. (SC 1624) |
| 3350 | *S. rutgersensis* (Waksman & Curtis, 1916) Waksman & Henrici (1948); N.J. Agr. Exp. Station |
| 3371 | *S. sampsonii* (Millard & Burr, 1926) Waksman (1953); W. A. Millard |
| 3351 | *S. saprophyticus*; N.J. Agr. Exp. Station |
| 3029 | *S. scabies* (Thaxter, 1892) Waksman & Henrici (1948); N.J. Agr. Exp. Station; M. Shapovalov (Morse 17) |
| 3352 | *S. scabies*; CBS |
| A 10246 | *S. scabies*; ATCC (10246); W. H. Burkholder (N 54); R. W. Goss |
| 3375 | *S. setonii* (Millard & Burr, 1926) Waksman (1953); W. A. Millard |
| N 3236 | *S. somaliensis*; NCTC (3236); Wellcome Tropical Research Labs. (mycetoma) |
| 3418 | *S. sylvodoriferus*; N.J. Agr. Exp. Station; ATCC (4919) |
| 3367 | *S. tetanusemus*; N.J. Agr. Exp. Station: Pribram Collection |
| A 11861 | * *S. vinaceus* Waksman (1953); ATCC (11861); NRRL (B-2285) |
| 3372 | *S. viridis* (Millard & Burr, 1926) Waksman (1953); W. A. Millard |
| 3690 | *S. viridis*; R. W. Thoma (SC 1633) |
| A 6867 | *S. willmorei* (Erikson, 1935) Waksman & Henrici (1948); ATCC (6867); NCTC (1856); J. G. Willmore |
| 581 | *Streptomyces* sp.; C. C. Campbell, Army Med. Service Graduate School. Washington, D.C. (698) |
| 985 | *Streptomyces* sp.; T. G. Pridham, U.S. Dept. of Agr., Peoria, Ill. (D) |
| 3069 | *Streptomyces* sp.; N.J. Agr. Exp. Station (142) |
| 3482 | *Streptomyces* sp.; R. L. Mayer, Ciba Pharmaceutical Co., Summit, N.J. |
| N 4523 | *Streptothrix buccalis* Goadby (1903); NCTC (4523); ATCC (3311); Pribram Collection |
| 3311 | *Streptothrix buccalis*; N.J. Agr. Exp. Station; C.-E. A. Winslow |

* Illegitimate specific epithet.

coloured, yellow, greenish yellow, olive drab or brown. Some cultures did not form aerial hyphae visible to the unaided eye; others showed patches or a thin coating of whitish aerial hyphae; still others were abundantly covered with greyish white or grey aerial hyphae. Soluble pigments were greenish yellow, greenish brown, reddish brown or red (Gordon & Horan, 1968, pl. 2).

Properties (Table 3) shared by all 46 reference strains of *Streptomyces griseus*, or those shared by a high percentage of the strains were: decomposition of adenine, casein, hypoxanthine, tyrosine, urea and xanthine; lack of growth at 45°; growth at 10°; survival after 8 hr at 50°; use of citrate, lactate, malate and succinate as sole carbon source; failure to utilize benzoate, mucate or oxalate; sensitivity to lysozyme and

Table 3. *Pattern of reactions of Streptomyces griseus*

| Property | 46 reference strains (% positive strains) | 77 similar strains (% positive strains) |
|---|---|---|
| Decomposition of | | |
| Adenine | 96 | 100 |
| Casein | 100 | 100 |
| Hypoxanthine | 100 | 100 |
| Tyrosine | 100 | 100 |
| Urea | 96 | 97 |
| Xanthine | 100 | 100 |
| Growth at | | |
| 45° | 0 | 0 |
| 40° | 13 | 45 |
| 10° | 100 | 100 |
| Survival at 50° for 8 hr | 100 | 99 |
| Nitrite from nitrate | 83 | 68 |
| Utilization of | | |
| Benzoate | 0 | 0 |
| Citrate | 100 | 99 |
| Lactate | 93 | 88 |
| Malate | 100 | 100 |
| Mucate | 0 | 0 |
| Oxalate | 0 | 0 |
| Succinate | 100 | 100 |
| Resistance to | | |
| Lysozyme | 2 | 0 |
| Salicylate | 0 | 3 |
| Oxidation of glucose | 100 | 97 |
| Fermentation of glucose | 0 | 0 |
| Hydrolysis of | | |
| Hippurate | 2 | 6 |
| Starch | 100 | 100 |
| Acid from | | |
| Adonitol | 57 | 31 |
| Arabinose | 41 | 75 |
| Dulcitol | 0 | 0 |
| Erythritol | 0 | 1 |
| Galactose | 100 | 99 |
| Glucose | 100 | 100 |
| Inositol | 20 | 13 |
| Lactose | 100 | 100 |
| Maltose | 100 | 100 |
| Mannitol | 98 | 96 |
| Mannose | 100 | 100 |
| Melibiose | 0 | 0 |
| α-Methyl-D-glucoside | 100 | 97 |
| Raffinose | 2 | 3 |
| Rhamnose | 24 | 34 |
| Sorbitol | 0 | 0 |
| Trehalose | 100 | 99 |
| Xylose | 100 | 100 |

salicylate; oxidation of glucose; inability to form benzoic acid from hippurate; hydrolysis of starch; formation of acid from galactose, glucose, lactose, maltose, mannitol, mannose, α-methyl-D-glucoside, trehalose and xylose; failure to attack dulcitol, erythritol, melibiose, raffinose or sorbitol. Variable characteristics of the 46

Table 4. *Comparison of properties of four species of streptomyces* (% *positive strains*)

| Property | *S. griseus* (123 strains) | *S. albus* (23 strains) | *S. fradiae* (23 strains) | *S. somaliensis* (22 strains) |
|---|---|---|---|---|
| Decomposition of | | | | |
| Adenine | 98 | 4 | 100 | 0 |
| Casein | 100 | 100 | 100 | 100 |
| Hypoxanthine | 100 | 100 | 0 | 0 |
| Tyrosine | 100 | 100 | 100 | 100 |
| Urea | 97 | 100 | 0 | 0 |
| Xanthine | 100 | 83 | 0 | 0 |
| Growth at | | | | |
| 50° | 0 | 100 | 0 | 0 |
| 45° | 0 | 100 | 91 | 55 |
| 40° | 33 | 100 | 100 | 91 |
| 10° | 100 | 0 | 5 | 9 |
| Survival at 50° for 8 hr | 99 | 100 | 91 | 50 |
| Nitrite from nitrate | 73 | 60 | 100 | c |
| Utilization of | | | | |
| Citrate | 99 | 100 | 0 | 18 |
| Lactate | 90 | 100 | 26 | c |
| Malate | 100 | 100 | 95 | 37 |
| Mucate | 0 | 0 | 0 | c |
| Oxalate | 0 | 57 | 0 | c |
| Succinate | 100 | 100 | 100 | 45 |
| Resistance to | | | | |
| Lysozyme | 1 | 21 | 0 | 0 |
| Salicylate | 2 | 100 | 0 | 0 |
| Oxidation of glucose | 98 | 96 | 100 | 0 |
| Fermentation of glucose | 0 | 4* | 0 | 0 |
| Hydrolysis of | | | | |
| Hippurate | 5 | 30 | 0 | 5 |
| Starch | 100 | 0 | 100 | 36 |
| Acid from | | | | |
| Adonitol | 43 | 100 | 0 | 0 |
| Arabinose | 63 | 17 | 91 | 0 |
| Dulcitol | 0 | 0 | 0 | 0 |
| Erythritol | 1 | 100 | 0 | 0 |
| Galactose | 99 | 100 | 100 | 0 |
| Glucose | 100 | 100 | 100 | 50 |
| Inositol | 15 | 65 | 0 | 0 |
| Lactose | 100 | 100 | 63 | 0 |
| Maltose | 100 | 100 | 100 | 64 |
| Mannitol | 97 | 100 | 0 | 0 |
| Mannose | 100 | 100 | 100 | 5 |
| Melibiose | 0 | 13 | 0 | 0 |
| α-Methyl-D-glucoside | 98 | 100 | 0 | 0 |
| Raffinose | 2 | 0 | 0 | 0 |
| Rhamnose | 31 | 0 | 0 | 0 |
| Sorbitol | 0 | 4 | 0 | 0 |
| Trehalose | 99 | 91 | 100 | 0 |
| Xylose | 100 | 100 | 100 | 0 |

reference strains were: growth at 40°; reduction of nitrate or nitrite; acid production from adonitol, arabinose, inositol, and rhamnose.

The following 21 strains (Tables 1, 2) were found to contain *LL*-diaminopimelic acid: 3029, 3068, 3475, 3523, 3527, 3567a, 3567b, 3856, N3236, N3258, N4520, N4523,

N7808, 413, 505, 506, 511, 638, 676, 712 and 713. The 101 remaining strains of *Streptomyces griseus* were not tested for the form of diaminopimelic acid.

The reactions of the 77 strains listed in Table 2 are given in Table 3, and in Table 4 these reactions are combined with those of the 46 reference strains to represent one aggregate of *Streptomyces griseus*. The pattern of characters of these strains is compared (Table 4) with those of the strains in our collection of *S. albus* (Rossi-Doria) Waksman & Henrici, *S. fradiae* (Waksman & Curtis) Waksman & Henrici and *S. somaliensis* (Brumpt) Waksman & Henrici. As a group the following criteria served to differentiate this aggregate of *S. griseus* from the three other species of streptomycetes: decomposition of adenine, hypoxanthine, urea, and xanthine; growth at 10° but not at 45°; use of citrate; sensitivity to salicylate; oxidation of glucose; hydrolysis of starch; acid production from galactose, lactose, mannitol, mannose, α-methyl-D-glucoside, trehalose and xylose; and lack of acid formation from erythritol.

## DISCUSSION

The usual practice of author citation of the name under which each strain was received was difficult to follow. After reading the available original descriptions of the species named in Table 2, we attempted to decide whether or not each strain was representative of its original strain. Because many of the descriptions of these strains relied on variable properties, and because of human fallibility in the preparation of descriptions and in the maintenance of cultures, the task proved to be impossible. We accepted, therefore, all strains whose authenticity was not definitely challenged either by the first description or by the recognition of very different strains as representatives of the particular species. For example, a strain deposited in this collection as *Nocardia madurae* and subsequently found to be *Streptomyces griseus* was obviously not typical of *N. madurae*, because other very different strains are accepted as representing *N. madurae*.

The names of the strains in Table 2 that are followed immediately and without intervening semicolon by the name of the author(s) are regarded as synonomous with *Streptomyces griseus*. Some of these strains came from the author(s) of the species. Other strains whose histories cannot be traced to the describer of the species may, however, be typical of the original strains, since they were found to possess characters that do not appear to be at variance with the original species descriptions.

Strain 3347 was accepted as conforming to Krainsky's (1914) description of *Actinomyces roseus*, although previously Namyslowski (1912) had given the name to another strain. In 1916, Chalmers & Christopherson assigned Krainsky's *A. roseus* to the genus *Nocardia*. *N. roseus* has priority therefore over *A. roseochromogenus*, a name assigned to Krainsky's *A. roseus* by Jensen (1931). The legitimate name for Krainsky's species is *Streptomyces roseus* (Chalmers & Christopherson) Pridham, Hesseltine & Benedict (1958). Mayer *et al.* (1951) named ATCC 11861 *S. vinaceus* but did not describe it; the necessary description came from Waksman (1953). Although ATCC 11861 is considered as authentic, the name *S. vinaceus* is considered an illegitimate one for the species because Jones (1951) gave the name *S. vinaceus* to another strain.

Beijerinck (1912), cited by Waksman (1953), used the name *Actinomyces* (*Streptothrix*) *annulatus*, but since he did not describe the strain, his name has no nomenclatural

status. Although Wollenweber (1920) ascribed the name to Krainsky, he gave no reference to the Krainsky work, and we have been unable to find it. Wollenweber (1920) also described and named some isolates of his own *A. annulatus*; in our opinion he was correctly cited by Hütter (1963) as the original author of the species name.

We are unable to give the authority for the name borne by ATCC 4879, because this strain and strain 4878 were deposited in the ATCC in 1933 by S. A. Scudder and appear in the ATCC catalogue as *Actinomyces candidus* Petruschky (1903). Waksman (1953) assigned *A. candidus* Krassil'nikov (1941), which he did not regard the same as *Streptothrix candida* Petruschky, to the genus *Streptomyces* and in 1961 designated ATCC 4878 (IMRU 3416) as the type strain of *S. candidus* (Krassil'nikov) Waksman.

Waksman (1953) cited Krüger (1809) as the author of *Oospora intermedia*, but Krüger did not mention this name in his report. Wollenweber (1920) named and described some isolations of his own as '*Actinomyces intermedius* (Krüg.) Wr.' but did not identify Krüger's paper. Kudrina's (1957) and Pridham's (1964) listing of Wollenweber as the authority for the specific epithet is therefore in accord with the rules of nomenclature.

*Actinomyces saprophyticus* was named by Gasperini (1892); later Chalmers & Christopherson (1916) assigned the same specific epithet to a species in the genus *Nocardia*. An authority for the transfer of either species to the genus *Streptomyces* was not found. A search of the literature did not reveal the author of the name and description of *N. hemolytica*, *S. acidoresistans* or *S. tetanusemus*. Strains labelled *N. sylvodorifera* and *S. sylvodorifera* could be traced to Castellani, but Castellani was unable to furnish the reference (personal communication), and the original characterization and naming of this species was not discovered.

Among the strains listed in Table 2, those labelled *Actinomyces sulphureus*, *Nocardia asteroides*, *N. farcinica*, *N. madurae*, *Streptomyces albus*, *S. lavendulae* and *S. somaliensis* were unlike recognized strains of these species and were therefore regarded as mis-named. Strain 413 was not accepted as representing *A. keratolytica* because Acton & McGuire (1931) characterized their species as forming limpet-shaped colonies, black or red in colour, with deep roots penetrating the media, and as resembling, in certain respects, cultures of *A. bovis* and *A. madurae*. ATCC 6855 was unacceptable as *N. leishmani* Chalmers & Christopherson (1916), a species originally described as acid-fast by Birt & Leishman (1902).

A number of the strains listed in Table 2 were previously recognized as strains of *Streptomyces griseus* by Baldacci & Comaschi (1956), Benedict *et al.* (1955), Burkholder, Sun, Anderson & Ehrlich (1955), Ettlinger, Corbaz & Hütter (1958), Hütter (1963) Krassil'nikov (1949), Pridham (1964), and others. Our findings, based mainly on different observations, support their conclusions.

The pattern of properties presented is for the identification of only one aggregate of strains of the heterogeneous species *Streptomyces griseus* and is not intended as a delination of the species. Strains labelled *S. coelicolor*, for example, which were recognized as *S. griseus* by Hütter (1963), Pridham (1964) and others, have, with three exceptions, the same pattern of characters. The three characters of strains of *S. coelicolor* not shared by the strains of this aggregate of *S. griseus* do not, however, offer a definite separation of the two groups, because other strains possess one or two of the three properties and are clearly intermediate. Other aggregates of strains of *S. griseus* are also represented in our collection and are bound together by inter-

mediate strains. With the exception of the taxonomic group described here, the representation in our collection of the aggregates of *S. griseus* is inadequate, and we are unable to offer at this time a pattern of properties for the identification of the entire species.

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# *Nocardia dassonvillei*, a Macroscopic Replica of *Streptomyces griseus*

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SUMMARY

Twenty-six strains of *Nocardia dassonvillei* (Brocq-Rousseau) Liégard & Landrieu from the soil and from infections of man and animals are described, and a pattern of characteristics for their identification and differentiation from *N. asteroides*, *N. brasiliensis*, *N. caviae*, *N. madurae* and *N. pelletieri* is presented. Strains of *N. dassonvillei* resembled strains of *Streptomyces griseus* (Krainsky) Waksman & Henrici in the macroscopic appearance of their growth and in many of the physiological properties examined.

## INTRODUCTION

The strains of nocardias and streptomycetes examined by Becker, Lechevalier, Gordon & Lechevalier (1964) for the form of diaminopimelic acid (DAP) in hydrolysates of whole organisms included a small group isolated from pulmonary infections of animals in the Chicago Zoological Park. The strains of this group macroscopically resembled strains of *Streptomyces griseus*, and two strains had the same pattern of physiological characteristics as *S. griseus*. Only two properties, at that time, separated the others from *S. griseus*. Later Becker *et al.* (1964) reported that the two strains had the *LL*-form of DAP and the other strains the *meso*-form. Determination of the form of DAP was therefore added to our observations of those nocardias and streptomycetes in our collection of whose specific identity we were uncertain.

Strains from other sources were encountered with the three correlating properties that distinguished them from *Streptomyces griseus*, and only one more property was found to widen the separation between the two taxa. Because the strain bearing the oldest name among those with the *meso*-form of DAP was *Nocardia dassonvillei* (Brocq-Rousseu, 1907) Liégard & Landrieu (1911), we assigned this name to the group. A pattern of characteristics for the recognition of strains of *N. dassonvillei* is given here and contrasted with the properties of *S. griseus* and those of five other species of *Nocardia*.

## METHODS

*Organisms.* The strains presented in Table 1 were compared with those listed by Gordon & Horan (1968) and by Gordon (1966).

*Tests.* The strains were examined according to the procedures described by Gordon (1966, 1967), and for the the form of diaminopimelic acid (Becker *et al.* 1964).

## RESULTS

Smears of the 26 cultures of *Nocardia dassonvillei*, grown on glycerol agar for 5 days and stained by a modified Ziehl-Neelsen method, showed long filaments. Approximately half the smears also contained rods of varying lengths and a few coccoid bodies. The cultures were not acid-fast.

Table 1. *Strains identified as Nocardia dassonvillei*

| Strain no. | Name when received, source, strain name or number |
|---|---|
| 509 | * *Nocardia dassonvillei* (Brocq-Rousseu) Liégard & Landrieu; P. Thibault, Inst. Pasteur, Paris |
| N3255 | *Nocardia madurae*; (NCTC 3255) |
| 575 | *Nocardia* sp.; C. W. Emmons, Nat. Inst. of Health, Bethesda, Md. (A 826); G. Altmann (1344, ulcerating lesion on leg of patient with elephantiasis) |
| 639, 711, 714–718 | *Nocardia* sp.; E. B. Tilden, Chicago Zoological Park, Brookfield, Ill. (lung lesion of a sitatunga; P 2, lung of a penguin; 6, 9, 10, 11, 12, lung of a kangaroo) |
| 1288 | *Nocardia* sp.; C. W. Emmons (10,006, nasal washings) |
| 1289 | *Nocardia* sp.; N. F. Conant, Duke Univ.; H. A. Campana (Malloch, chronic ulcer following bite of a dog) |
| 1322–1330 | *Nocardia* spp.; M. P. Lechevalier, Rutgers Univ. (G 1, G 2, H 1, J 2, J 3, L 10, L 13, 11–36, soil; Cross Fil., bovine streptothricosis) |
| N434 | *Streptomyces listeri* (Erikson) Waksman & Henrici; NCTC; (434, human) |
| 1236 | *Streptomyces* sp.; C. W. Emmons (B-2031, pulmonary lymph node) |
| 1250 | † *Streptomyces* sp.; D. Taplin, Univ. of Miami (Babcock, case of erosive plantar disease) |
| 794 | Unidentified isolation; H. Cohen, Kaiser Foundation Hosp., Los Angeles, Calif. (Robinson, pleural biopsy) |
| 1213 | Unidentified isolation; G. Ryvarden, Kaiserin Elizabeth Hospital, Vienna (venous blood of patient with malignant lymphoma) |

* ATCC 23218; NCTC 10488.
† ATCC 23219; NCTC 10489.

Under the conditions of the examination, the 26 strains formed loosely or densely filamentous colonies; colonies with smooth margins were not observed. Aerial hyphae were not produced by four of the cultures and in the remaining cultures varied from sparse to abundant. These hyphae were short and straight, or long and branching, straight or coiled (Pl. 1). Sporulation was abundant. The colonies of a few strains displayed many purple-coloured crystals identified by Gerber (1966) as crystals of iodinin.

Cultures grown on 'yeast dextrose (glucose) agar' were macroscopically abudant, coarsely wrinkled or flat. The growth was yellowish, greenish yellow or reddish brown, and produced a yellowish, greenish yellow or brown soluble pigment. The aerial hyphae formed by some cultures were not visible to the unaided eye but on other cultures showed as a sparse coating or a few whitish patches; still other cultures were thickly covered with greyish aerial hyphae. The resemblance of cultures of *Nocardia dassonvillei* to cultures of *Streptomyces griseus* is illustrated in Pl. 2.

The 26 strains of *Nocardia dassonvillei* in our collection dissolved adenine, casein, hypoxanthine, tyrosine, and xanthine; did not grow at 45° and 10°; utilized lactate, malate and succinate, but not mucate or oxalate; were sensitive to lysozyme and salicylate; did not ferment glucose; hydrolysed hippurate and starch; formed acid from glucose, maltose, mannitol, and mannose; did not produce acid from adonitol, dulcitol,

Table 2. *Comparison of some reactions of Nocardia dassonvillei and of Streptomyces griseus*

| Property | *Nocardia dassonvillei* (26 strains) | *Streptomyces griseus* (123 strains) |
|---|---|---|
| | Positive strains (%) | |
| Decomposition of | | |
| Adenine | 100 | 98 |
| Casein | 100 | 100 |
| Hypoxanthine | 100 | 100 |
| Tyrosine | 100 | 100 |
| Urea | 35 | 97 |
| Xanthine | 100 | 100 |
| Growth at | | |
| 45° | 0 | 0 |
| 40° | 80 | 33 |
| 10° | 0 | 100 |
| Survival at 50° for 8 hr | 92 | 99 |
| Nitrite from nitrate | 92 | 73 |
| Utilization of | | |
| Citrate | 96 | 99 |
| Lactate | 100 | 90 |
| Malate | 100 | 100 |
| Mucate | 0 | 0 |
| Oxalate | 0 | 0 |
| Succinate | 100 | 100 |
| Resistance to | | |
| Lysozyme | 0 | 1 |
| Salicylate | 0 | 2 |
| Oxidation of glucose | 85 | 98 |
| Fermentation of glucose | 0 | 0 |
| Hydrolysis of | | |
| Hippurate | 100 | 5 |
| Starch | 100 | 100 |
| Acid from | | |
| Adonitol | 0 | 43 |
| Arabinose | 60 | 63 |
| Dulcitol | 0 | 0 |
| Erythritol | 0 | 1 |
| Galactose | 62 | 99 |
| Glucose | 100 | 100 |
| Inositol | 0 | 15 |
| Lactose | 0 | 100 |
| Maltose | 100 | 100 |
| Mannitol | 100 | 97 |
| Mannose | 100 | 100 |
| Melibiose | 31 | 0 |
| α-Methyl-D-glucoside | 23 | 98 |
| Raffinose | 0 | 2 |
| Rhamnose | 65 | 31 |
| Sorbitol | 0 | 0 |
| Trehalose | 65 | 99 |
| Xylose | 81 | 100 |
| *Meso*-diaminopimelic acid | 100 | 0* |
| *LL*-diaminopimelic acid | 0 | 100* |

* Twenty-one strains analysed.

erythritol, inositol, lactose, raffinose or sorbitol; had the *meso*-form of DAP in hydrolysates of whole organisms (Table 2). Survival at 50° for 8 hr, reduction of nitrate to nitrite, and utilization of citrate were considered to be fairly reliable properties, although not shared by all the strains. Because of their variability, the remaining characteristics listed in Table 2 were not included in the pattern of reactions of the species. A comparison (Table 2) of the properties of *N. dassonvillei* with those of *Streptomyces griseus* (Gordon & Horan, 1967) discloses that the two taxa are differentiated only by growth at 10°, hydrolysis of hippurate, acid formation from lactose and the form of their DAP.

Table 3. *Some criteria for identifying six species of Nocardia*

| Property | *N. asteroides* (137 strains) | *N. caviae* (24 strains) | *N. brasiliensis* (62 strains) | *N. madurae* (44 strains) | *N. pelletieri* (15 strains) | *N. dassonvillei* (26 strains) |
|---|---|---|---|---|---|---|
| | Positive strains (%) | | | | | |
| Acid-fastness | 55 | 66 | 80 | 0 | 0 | 0 |
| Decomposition of | | | | | | |
| Adenine | 0 | 4 | 3 | 0 | 0 | 100 |
| Casein | 0 | 0 | 98 | 100 | 100 | 100 |
| Hypoxanthine | 4 | 100 | 94 | 100 | 100 | 100 |
| Tyrosine | 1 | 0 | 100 | 86 | 100 | 100 |
| Urea | 96 | 92 | 100 | 0 | 0 | 35 |
| Xanthine | 0 | 100 | 0 | 0 | 0 | 100 |
| Growth at | | | | | | |
| 45° | 41 | 50 | 2 | 36 | 13 | 0 |
| 10° | 15 | 13 | 37 | 0 | 67 | 0 |
| Survival at 50° for 8 hr | 94 | 88 | 0 | 100 | 100 | 92 |
| Nitrite from nitrate | 86 | 100 | 90 | 100 | 100 | 92 |
| Utilization of | | | | | | |
| Citrate | 38 | 29 | 98 | 80 | 7 | 96 |
| Malate | 97 | 100 | 100 | 84 | 53 | 100 |
| Succinate | 92 | 100 | 100 | 80 | 0 | 100 |
| Resistance to lysozyme | 100 | 100 | 100 | 5 | 0 | 0 |
| Hydrolysis of | | | | | | |
| Aesculin | 100* | 100 | 100* | 100 | 0 | 12 |
| Hippurate | — | — | — | 2 | 0 | 100 |
| Starch | 67 | 54 | 55 | 100 | 13 | 100 |
| Acid from | | | | | | |
| Adonitol | 0 | 0 | 0 | 91 | 0 | 0 |
| Arabinose | 0 | 4 | 0 | 100 | 0 | 60 |
| Dulcitol | 0 | 0 | 0 | 0 | 0 | 0 |
| Erythritol | 7 | 0 | 2 | 0 | 0 | 0 |
| Galactose | 27 | 0 | 94 | 81 | 7 | 62 |
| Glucose | 98 | 100 | 97 | 100 | 100 | 100 |
| Inositol | 3 | 100 | 100 | 61 | 0 | 0 |
| Lactose | 0 | 0 | 0 | 55 | 0 | 0 |
| Maltose | 6 | 18 | 4 | 32 | 0 | 100 |
| Mannitol | 1 | 90 | 94 | 100 | 7 | 100 |
| Mannose | 17 | 36 | 68 | 61 | 0 | 100 |
| Melibiose | — | 0 | — | 0 | 0 | 31 |
| α-Methyl-D-glucoside | 0 | 0 | 0 | 0 | 0 | 23 |
| Raffinose | 0 | 0 | 0 | 0 | 0 | 0 |
| Rhamnose | 32 | 5 | 0 | 100 | 0 | 65 |
| Sorbitol | 0 | 0 | 0 | 0 | 0 | 0 |
| Xylose | 0 | 5 | 0 | 100 | 0 | 81 |

* Fifty strains examined.

The pattern of reactions that seems distinctive for *Nocardia dassonvillei* and separates the species from the other species represented in our collection was compared (Table 3) with the same reactions of five other species of *Nocardia* (Gordon, 1966). The following combination of characteristics sets the strains of *N. dassonvillei* apart from the strains of *N. asteroides*, *N. caviae*, *N. brasiliensis*, *N. madurae* and *N. pelletieri*: lack of acid-fastness; decomposition of adenine, casein, hypoxanthine, tyrosine, and xanthine; survival at 50° for 8 hr; utilization of citrate and succinate; hydrolysis of hippurate; sensitivity to lysozyme; acid production from maltose, mannitol, and mannose; no acid from adonitol or inositol. Inability to hydrolyse aesculin was a somewhat less dependable property.

## DISCUSSION

The original strains of *Nocardia dassonvillei*, named *Streptothrix dassonvillei* by Brocq-Rousseu (1907), were isolated from mildewed grain and fodder. Two years later, Liégard & Landrieu (1911) isolated a strain from a case of ocular conjunctivitis. They concluded that this strain was the same as Brocq-Rousseu's *S. dassonvillei* and suggested its assignment to the genus *Nocardia*. Accounts by Brumpt (1913) Forbes (1924) and Langeron (1922) are the only reports we have found in the literature of other strains named *N. dassonvillei*. *Actinomyces listeri*, described by Erikson (1935) and transferred to the genus *Streptomyces* by Waksman & Henrici (1948), is considered in the synonymy of *N. dassonvillei*.

The distribution of the first strains of *Nocardia dassonvillei* is repeated in the histories of the strains listed in Table 1. Nine of the 26 strains came from soil, and 15 were described as isolations from human or animal infections. Although pathogenicity is outside the scope of our studies, we are tempted to speculate on the discarding in medical laboratories of cultures of *N. dassonvillei* as contaminants because of their macroscopic resemblance to cultures of *Streptomyces griseus*.

With allowance for microbial variation, the combination of 32 properties given here is believed to be a reliable basis for differentiating strains of *Nocardia dassonvillei* from the other strains of aerial hyphae-forming nocardias and streptomycetes in our collection. We cannot, however, suggest a combination of a few properties as a presumptive test for the recognition of *N. dassonvillei*. Among the properties of the six species of nocardias in Table 3, the decomposition of adenine is useful for separating *N. dassonvillei* from the other five species and, if the nocardias were limited to six species, could be proposed as a presumptive test for *N. dassonvillei*. The genus *Nocardia*, however, is not so obligingly simple.

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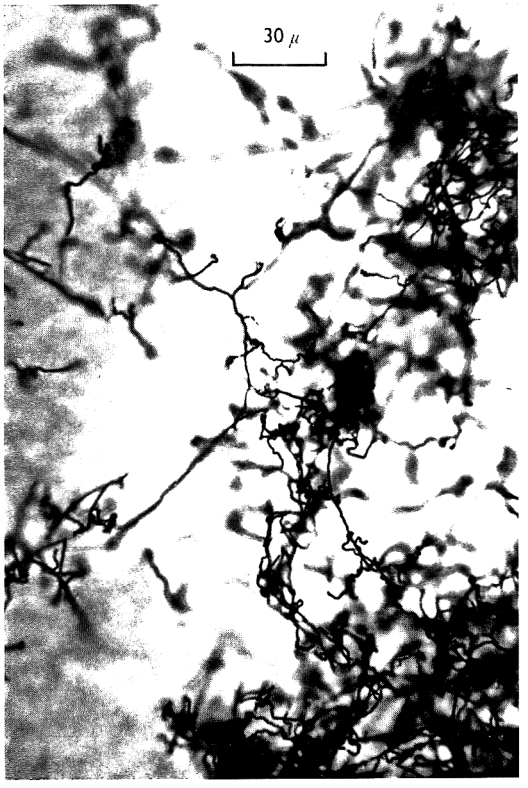
## EXPLANATION OF PLATES

### PLATE 1

Aerial hyphae of *Nocardia dassonvillei*, strain 794, on yeast dextrose agar after 10 days of incubation at 28°.

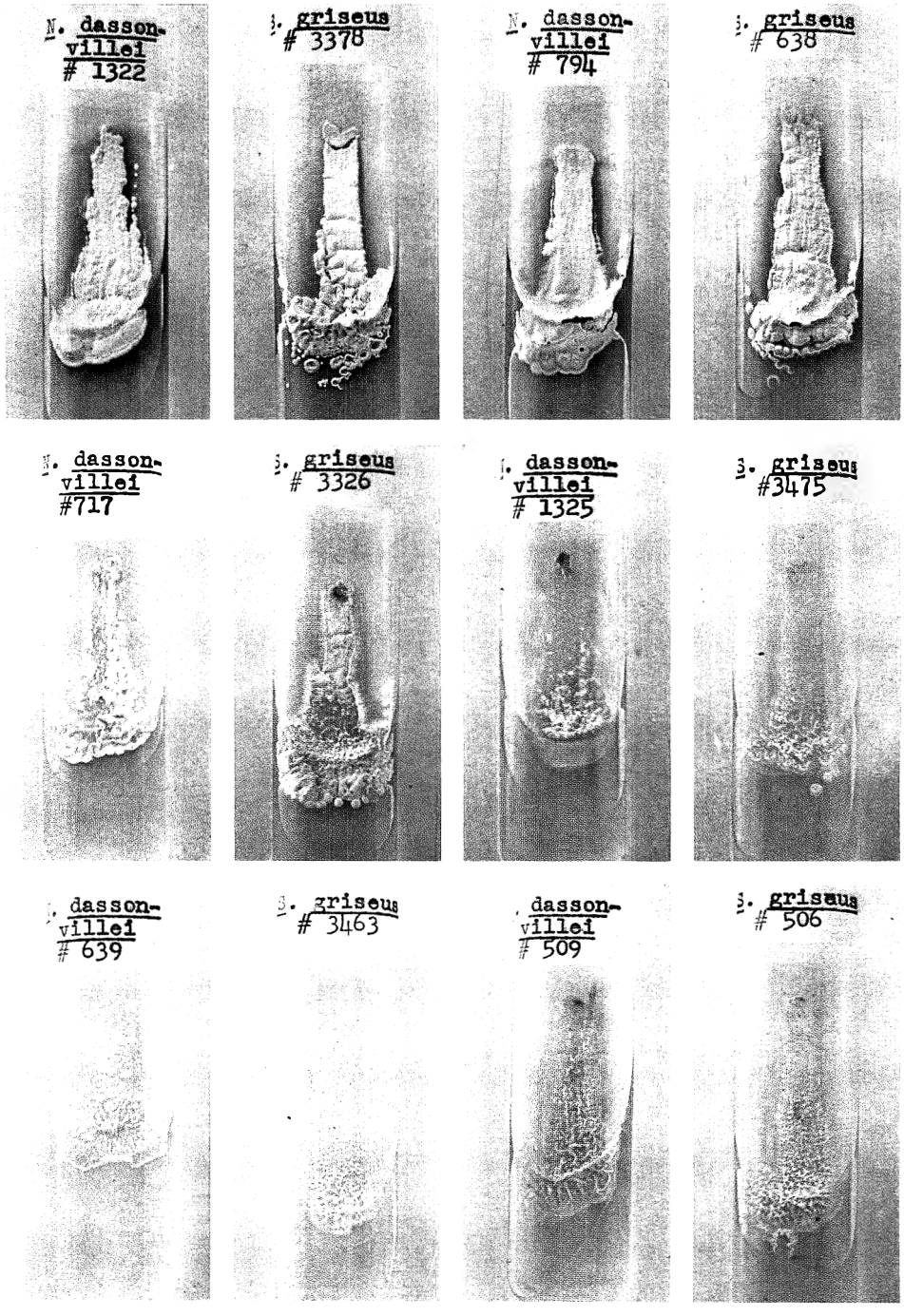
### PLATE 2

Growth of cultures of *Nocardia dassonvillei* and *Streptomyces griseus* on slants of yeast dextrose agar after 14 days of incubation at 28°.






(*Facing p.* 240)


N. dasson-
villei
# 1322
S. griseus
# 3378
N. dasson-
villei
# 794
S. griseus
# 638
N. dasson-
villei
#717
S. griseus
# 3326
N. dasson-
villei
# 1325
S. griseus
#3475
N. dasson-
villei
# 639
S. griseus
# 3463
N. dasson-
villei
# 509
S. griseus
# 506

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# On the Nature of the Recipient Ability of *Salmonella typhimurium* for Foreign Deoxyribonucleic Acids

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SUMMARY

A fertile mutant (*fer*) isolated from *Salmonella typhimurium* LT-7*mut* with a mutator gene *mut* was found to have a high recipient ability when used as a recipient of the *Escherichia coli* chromosome and F′ and R factors, whereas LT-7*mut*$^+$ and *S. typhimurium* LT-2*mut*$^+$, both of which lack the *mut* gene, were poor recipients of these foreign deoxyribonucleic acids. LT-7*mut* exhibited an intermediate recipient ability. These episomal elements, however, were transferred from LT-7*mut* and LT-7*mut*$^+$ to the *mut*, *mut*$^+$ and *fer* substrains at frequencies comparable to an *E. coli* recipient. In contrast, the frequencies of transfer of these episomes from LT-7*fer* to the other substrains of *S. typhimurium* were considerably lower than those to LT-7*fer*. The efficiencies of plating (*e.o.p.*) of phage P-22 grown on LT-7*fer* were likewise lower on LT-7*mut*, LT-7*mut*$^+$ and LT-2*mut*$^+$ than on LT-7*fer*. The *e.o.p.* of phage P-22 on LT-7*mut*, LT-7*mut*$^+$ and LT-7*mut*$^+$ were increased to the value on LT-7*fer* by its growth on LT-7*mut*$^+$ or LT-2*mut*$^+$. The frequencies of transduction of an R factor to these substrains of *S. typhimurium* with phage P-22 grown on various substrains were parallel with the *e.o.p.* of this phage on these strains. It was further shown that the deoxyribonucleic acid of phage P-22, which had previously been propagated on LT-7*fer*, injected into LT-7*mut*$^+$ and LT-2*mut*$^+$ is rapidly degraded, whereas appreciable breakdown did not occur in LT-7*fer*. These results with P-22 indicate that LT-7*fer* is a mutant which is impaired in its capacity of restriction and modification of this phage. The growth of P-22 on LT-7*mut* resulted in partial increase of its *e.o.p.* on LT-7*mut*$^+$ and LT-2*mut*$^+$, indicating that the restriction and modification capacities of LT-7*mut* are partially affected. The above results, that LT-7*fer* acts as a good recipient in the conjugal transfer of *E. coli* chromosome and non-viral episomes and that the frequencies of transfer of the non-viral episomes among the substrains of *S. typhimurium* are comparable to those to LT-7*fer* and *E. coli*, are interpreted as due to the restriction- and modification-less nature of LT-7*fer*.

## INTRODUCTION

Sexual recombination between *Escherichia coli* and Salmonella strains was first discovered by Baron, Carey & Spilman (1958) and intensively studied by several groups of investigators (Baron, Carey & Spilman, 1959; Baron, Spilman & Carey, 1959; Zinder, 1960; Miyake, 1962). A characteristic feature of the mating of *E. coli*

with salmonellas was the low recipient ability of the Salmonella strains. Baron, Carey & Spilman (1959) succeeded by chance in isolating a good genetic recipient in a salmonella, which is capable of producing recombinants at a much higher frequency than the original strain. They were led by this finding to propose that the low frequency of recombination initially observed represented the selection of rare, high-frequency recipients from an otherwise sterile culture (Baron, Spilman & Carey, 1959). Miyake (1962) reported that in the crosses of *E. coli* Hfr strains with *Salmonella typhimurium* LT-7*mut*, which carries a mutator gene *mut* (Miyake, 1959; Miyake, 1960), recombinants for a range of donor markers can be produced but only at very low frequencies. Fertile recipients with a high recipient ability (LT-7*fer*) were isolated from LT-7*mut* by indirect selection, without previous participation in recombination. Thus populations of LT-7*mut* were considered heterogeneous with respect to the ability to mate with *E. coli* Hfr donors (Miyake, 1962). Strains of *S. typhimurium* are usually poor recipients for the transfer of R factors (see Watanabe, 1963) and the sex factor F from *E. coli* (Watanabe, 1962). We have found that the frequencies of transfer of an R factor and an F′ factor (Jacob & Adelberg, 1959; Hirota & Sneath, 1961) from *E. coli* K-12 to LT-7*fer* are considerably higher than those to LT-7*mut* and LT-7*mut*$^+$, which does not carry the mutator gene *mut*, and to *S. typhimurium* LT-2, that is regarded as *mut*$^+$ (Miyake, 1962). We have also found that the conjugal transfer of these episomal elements from LT-7*mut*, LT-7*mut*$^+$ and LT-2*mut*$^+$ to these strains occurs at frequencies almost equal to those to *E. coli* K-12 and LT-7*fer*. These results will be reported in the present paper and the nature of fertility of the Salmonella recipients will be discussed.

## METHODS

*Bacterial strains. Salmonella typhimurium* LT-7*mut* carrying the mutator gene *mut* and a stable mutant derived therefrom, LT-7*mut*$^+$ without the *mut* gene, were employed. *S. typhimurium* LT-2 (originally colicin I-resistant) and its cysteine-requiring mutant cysD-36 were also used. Strain cysD-36 was supplied by H. Ozeki. Cysteine-requiring mutants isolated in our laboratory from LT-7*mut*, LT-7*mut*$^+$ and LT-7*fer*, respectively, were used as well. An Hfr strain of *E. coli* K-12 used was W2252/*col* I (methionine requiring and colicin I-resistant, it injects its chromosome from lactose genes in a counter-clockwise direction). CSH-2/*col* I (methionine-requiring and colicin I-resistant carrying an *F′-lac*$^+$ ($F_{13}$ of Hirota & Sneath, 1961), W677/PTS (requiring threonine, leucine, proline, thiamine; not fermenting mannitol, xylose, maltose, galactose, lactose; resistant to phage T6 and high concentrations of streptomycin, $F^-$), and W3102/*col* I (non-fermenting galactose and resistant to colicin I, $F^-$), all of which are substrains of *E. coli* K-12, were also used.

*Phage strains.* Phage P-22 (identical to PLT-22) wild type and its clear plaque mutant were employed.

*Culture media and conditions of cultivation.* Liquid cultures were prepared in Penassay broth (Difco) with gentle shaking. Plating media were nutrient agar (Difco), a minimal medium agar described by Davis & Mingioli (1950) and this minimal medium agar without glucose and sodium citrate. When the utilization of lactose was the selected marker, this sugar was added in 0·2 % to the minimal medium without glucose and sodium citrate (minimal lactose agar medium). Semi-solid nutrient agar medium for phage titrations contained 0·7 % agar. Incubation was at 37°.

*Drugs.* The drugs used were sulfathiazole (Takeda), dihydro-streptomycin sulfate (Takeda), chloromycetin powder (Parke, Davis & Co.) and tetracycline hydrochloride (Lederle) and are abbreviated as Su, Sm, Cm and Tc, respectively. The concentrations of these drugs incorporated in the media were the same as those used by Watanabe & Fukasawa (1961*a*); 500 $\mu$g./ml. for Su and 25 $\mu$g./ml. for Sm, Cm and Tc.

*Method for proving the presence of the mut gene.* Whether or not the bacterial strains carry the mutator gene *mut* was studied by the procedure described by Treffers, Spinelli & Belser (1954) and Miyake (1960); each colony was suspended in a small amount of saline and streaked on nutrient agar containing Sm 100 $\mu$g./ml. After overnight incubation of the plates, the streaks of the *mut* clones gave rise to numerous Sm-resistant colonies, whereas those of the *mut*$^+$ strains produced very few or no such colonies.

*Procedure for isolating fer mutants of Salmonella typhimurium* LT-7. Fertile mutants *fer* of *S. typhimurium* LT-7 were isolated by Miyake's printing method (1962); approximately 100 bacteria of LT-7 *mut* per plate were plated on nutrient agar. After overnight incubation the developed colonies were replica-plated on minimal lactose agar medium which had previously been inoculated with 0·1 ml. of an overnight broth culture of W2252/*col* I. After incubation of the replica plates for 48 hr some of the replica-plated colonies gave heavy growth due to the lactose-fermenting (*lac*$^+$) recombinants produced. Corresponding colonies on master plates were picked and retested for their high recipient ability by using the standard procedure for conjugation, described below.

*Method for transduction of an R factor.* The method for transduction of an R factor 222 with phage P-22 in *Salmonella typhimurium* was the same as that described by Watanabe & Fukasawa (1961*b*).

*Procedure for conjugation. Escherichia coli* K-12 strain W2252/*col* I was used as a donor for sexual recombination. The donor and recipient strains were separately grown in Penassay broth to about $3 \times 10^8$ bacteria/ml. A 1 ml. portion of the donor culture was mixed with 9 ml. of a recipient culture in a 200 ml. Erlenmeyer flask and the flask incubated in a water bath for 2 hr without agitation. Dilutions in physiological saline of this mixed culture were plated on properly enriched minimal medium to select for the recombinants. When the recipients were the substrains of *Salmonella typhimurium*, the donor and recipient strains were mixed in a 1:1 or 1:9 ratio, and 0·1 ml. of the dilutions of the mixture was immediately plated on the selective medium (Zinder, 1960). In parallel with this procedure, the filter membrane method devised by Sanderson & Demerec (1965) was used in some experiments. The mixture of about $3 \times 10^8$ donor bacteria and about $3 \times 10^9$ recipient bacteria was placed on a Millipore filter (45 mm. diameter; HA type) by filtering the mixture of the Penassay broth cultures through the filter, and the filter with the bacteria on it was placed on the surface of a semisolid nutrient agar plate, which was then incubated for 5 min. to permit the formation of effective cell contacts. The Millipore filter was then transferred to 10 ml. Penassay broth in a 200 ml. Erlenmeyer flask and the bacteria were gently resuspended in the broth, and the mating was allowed to proceed for 90 min; Varying dilutions in physiological saline of this mixed culture were plated on minimal lactose agar medium. The resultant *lac*$^+$ recombinants were scored after incubation for 48 hr.

*Procedure for transfer of an R factor and an F'-lac*$^+$ *factor.* CSH-2/*col* I (222) was used as a donor for the transfer of the R factor 222 and W3747/*col* I (*F'-lac*$^+$) as

a donor for the transfer of the *F'-lac*$^+$. The recipients were prototrophs of *Salmonella typhimurium*. The donor and recipient strains were separately grown in Penassay broth to about $3 \times 10^8$ bacteria/ml. A 5 ml. portion of the donor culture was mixed with 5 ml. of recipient culture in a 200 ml. Erlenmeyer flask, and the flask was incubated in a water bath without aeration for 16 hr (R) or 3 hr (*F'-lac*$^+$). Varying dilutions in physiological saline of the mixed culture with the R$^+$ donor were plated on minimal agar containing Cm 25 $\mu$g./ml. In the case of the transfer of the F'-*lac*$^+$, varying dilutions in physiological saline of the mixed culture were plated on minimal lactose agar. After incubation for 48 hr the colonies of the clones which received the R factor or F'-*lac*$^+$ were scored.

*Method for detection of spontaneous segregants of an R factor.* Spontaneous segregation of an R factor 222 and its drug resistance determinants in various substrains of *Salmonella typhimurium* was studied by the method described by Watanabe & Lyang (1962): a Penassay broth culture of each substrain of *S. typhimurium* carrying 222 was diluted and plated on nutrient agars containing Sm, Cm, Tc 25 $\mu$g./ml. + Su 500 $\mu$g./ml. Two of the well-isolated colonies of each strain, which had thus been confirmed resistant to the four drugs, were used as starting materials. Five ml. of Penassay broth in a test-tube was inoculated with each colony and incubated for 24 hr without aeration. Each culture was then diluted with fresh Penassay broth by a factor of $10^3$ and 0·1 ml. of this dilution transferred to 5 ml. fresh Penassay broth; subcultures were daily prepared thereafter in a similar fashion. After 10 subcultures, the cultures were diluted to $10^{-5}$ and $10^{-6}$, and 0·1 ml. each of these dilutions was plated on drug-free nutrient agar. The patterns of drug resistance of the developed colonies were studied with a replica plating technique (Lederberg & Lederberg, 1952).

*Method for detection of colicin I production.* The production of colicin I by various substrains of *Salmonella tyhphimurium* was studied with the method described by Fredericq (1957).

*General phage techniques.* The general phage techniques followed were those described by Adams (1950).

*Method for study of the breakdown of the injected phage nucleic acid.* $^{32}$P-labelled phage P-22 was prepared and purified by the procedure described by Watanabe *et al.* (1966). The method used to study the breakdown of the injected phage nucleic acid was also similar to that described by Watanabe *et al.* (1966).

## RESULTS

### *Production of colicin I by various substrains of Salmonella typhimurium*

Wild-type strain of LT-7 is known to produce colicin I (Ozeki, Stocker & Smith, 1962). We have shown that LT-7*mut*$^+$, LT-7*mut* and LT-7*fer* all produce the same colicin. This was shown by the finding that *Escherichia coli* CSH-2 but not CSH-2/*col* I is killed by the colicin produced by these strains. LT-2*mut*$^+$ did not produce any detectable colicin. The production of colicin I by LT-7 necessitated the use of colicin I-resistant mutants of *E. coli* K-12 as recipients in the crosses with LT-7 substrains.

### *Sexual recombination between an Hfr strain W2252/col I of Escherichia coli and substrains of Salmonella typhimurium*

As shown in Table 1, the frequencies of recombinants were highest in an *Escherichia*

*coli* recipient and second highest in LT-7*fer*. No recombinants were found with LT-7*mut*$^+$ and LT-2*mut*$^+$ as recipients. LT-7*mut* gave intermediate frequencies of recombinants. W2252/*col* I injects its chromosome with the lactose genes as the leading region, and the proline genes are so closely linked to the lactose genes that they are transferred to W677/PTS at almost equal frequencies.

Table 1. *Frequencies of recombinants in the crosses of Escherichia coli W2252/col I* with various substrains of Salmonella typhimurium*

The procedure for conjugation was that of Sanderson & Demerec (1965) which is described in the text.

| | Recipient | | | | |
|---|---|---|---|---|---|
| Recombinants | W677/PTS† | LT-7*fer* | LT-7*mut* | LT-7*mut*$^+$ | LT-2*mut*$^+$ |
| Selected marker | *pro*$^+$‡ | *lac*$^+$‡ | *lac*$^+$ | *lac*$^+$ | *lac*$^+$ |
| Frequency per donor cell | $2{\cdot}0\times10^{-2}$ | $3{\cdot}3\times10^{-3}$ | $1{\cdot}4\times10^{-5}$ | $< 10^{-8}$ | $< 10^{-8}$ |

* W2252/*col* I is a methionine-requiring, colicin I-resistant Hfr strain of *E. coli* K-12.

† W677/PTS is an F$^-$ substrain of *E. coli* K-12, requiring threonine, leucine, proline and thiamin, non-fermenting mannitol, xylose, maltose, galactose and lactose, and resistant to phage T6 and high concentrations of streptomycin.

‡ Abbreviations: *pro*$^+$ non-requirement for proline; *lac*$^+$ fermentability of lactose.

### *Transfer of an R factor 222 to substrains of Salmonella typhimurium*

The mixtures of the donor and recipient cultures in Penassay broth were incubated overnight without aeration. The frequencies of transfer of an R factor 222 from CSH-2/*col* I (222) to LT-7*mut*, LT-7*mut*$^+$ and LT-2*mut*$^+$ were very low even with overnight incubation and much lower than those to *Escherichia coli* W3102/*col* I and LT-7*fer* (Table 2). The filter membrane method (Sanderson & Demerec, 1965) did not improve the frequencies of R transfer. The frequencies of R transfer between the substrains of *Salmonella typhimurium* were rather high except with LT-7*fer* (222) as a donor and almost comparable to those between the substrains of *E. coli* K-12. Thus, the poor recipient ability of these strains was specific for the transfer of the R factor from *E. coli* K-12. LT-7*fer* (222) transferred its R factor at a high frequency to *E. coli* K-12, at a lower frequency to T-7*fer* and LT-2*mut*$^+$ and at even lower frequencies to LT-7*mut* and LT-7*mut*$^+$. When LT-7*fer* was a recipient, the frequencies of transfer of the R factor were always high regardless of whether the donor was a substrain of *E. coli* K-12 or a substrain of *S. typhimurium*. Similarly, when W3102/*col* I was a recipient, the frequencies of transfer of the R factor were always high regardless of whether the donor was a substrain of K-12 or a substrain of *S. typhimurium*. The frequencies of R transfer from LT-2*mut*$^+$ (222) to LT-7*mut* and LT-7*mut*$^+$ were slightly lower than those between the other strains of *S. typhimurium*. The frequencies shown in Table 2 cannot be taken to reflect the frequencies of transfer very accurately, because the frequencies varied to some extent from experiment to experiment.

### *Spontaneous segregation of an R factor in various substrains of Salmonella typhimurium*

The Su, Sm, and Cm resistance markers of the R factor 222 segregated at high frequencies in all of the substrains of *Salmonella typhimurium*. In contrast, the

segregation of the Tc resistance marker occurred at much lower frequencies in all of these strains. These results are analogous to those reported previously by Watanabe & Lyang (1962) with *S. typhimurium* LT-2 and there seems to be no specificity in the frequencies and patterns of segregation of the drug resistance markers of the R factor 222 in these substrains of *S. typhimurium*.

Table 2. *Frequencies of transfer of an R factor 222* among various substrains of Escherichia coli K-12 and Salmonella typhimurium*

The procedure for the transfer of the R factor is described in the text. The duration of mixed cultivation was 16 hr. The values in this table represent the frequencies of R transfer in 1 ml. of the mixed cultures

| | Frequency of transfer of an R factor 222 by the following donor | | | | |
|---|---|---|---|---|---|
| | CSH-2/*col* I† (222) | LT-7 *cys*⁻‡ *fer* (222) | LT-7 *cys*⁻‡ *mut* (222) | LT-7 *cys*⁻‡ *mut*⁺ (222) | LT-2 *cys*⁻‡ *mut*⁺ (222) |
| W3102/*col* I§ | $2{\cdot}6\times10^{6}$ | $1{\cdot}0\times10^{7}$ | $4{\cdot}0\times10^{6}$ | $3{\cdot}0\times10^{6}$ | $5{\cdot}0\times10^{6}$ |
| LT-7 *fer* | $9{\cdot}2\times10^{4}$ | $2{\cdot}4\times10^{5}$ | $3{\cdot}3\times10^{6}$ | $4{\cdot}5\times10^{5}$ | $4{\cdot}4\times10^{5}$ |
| LT-7 *mut* | $2{\cdot}0\times10^{1}$ | $1{\cdot}9\times10^{4}$ | $8{\cdot}5\times10^{5}$ | $5{\cdot}6\times10^{5}$ | $2{\cdot}1\times10^{3}$ |
| LT-7 *mut*⁺ | $4{\cdot}0\times10^{2}$ | $9{\cdot}5\times10^{3}$ | $1{\cdot}2\times10^{6}$ | $1{\cdot}3\times10^{6}$ | $3{\cdot}2\times10^{3}$ |
| LT-2 *mut*⁺ | $<10^{1}$ | $1{\cdot}2\times10^{5}$ | $5{\cdot}6\times10^{5}$ | $3{\cdot}8\times10^{6}$ | $3{\cdot}5\times10^{4}$ |

* R factor 222 carries the markers of resistance to sulphonamides, streptomycin, chloramphenicol and tetracycline.

† CSH-2/*col* I is an F⁻, methionine-requiring, colicin I-resistant substrain of *E. coli* K-12.

‡ *cys*⁻ stands for cysteine-requirement. LT-2 *cys*⁻ *mut*⁺ is identical to cysD-36.

§ W3102/*col* I is an F⁻, galactose-non-fermenting, colicin I-resistant substrain of *E. coli* K-12.

Table 3. *Frequencies of transduction of an R factor 222* to various substrains of Salmonella typhimurium with phage P-22*

The procedure for transduction of the R factor is the same as that described by Watanabe & Fukasawa (1961*b*)

| Recipient | Frequency of chloramphenicol-resistant transductants per donor phage with the following donor | | | |
|---|---|---|---|---|
| | LT-7 *fer* (222) | LT-7 *mut* (222) | LT-7 *mut*⁺ (222) | LT-2 *mut*⁺ (222) |
| LT-7 *fer* | $1{\cdot}7\times10^{-8}$ | $2{\cdot}2\times10^{-8}$ | $9{\cdot}0\times10^{-8}$ | $1{\cdot}2\times10^{-7}$ |
| LT-7 *mut* | $<4{\cdot}8\times10^{-10}$ | $8{\cdot}0\times10^{-10}$ | $1{\cdot}6\times10^{-8}$ | $7{\cdot}1\times10^{-8}$ |
| LT-7 *mut*⁺ | $<4{\cdot}8\times10^{-10}$ | $8{\cdot}0\times10^{-10}$ | $1{\cdot}7\times10^{-7}$ | $1{\cdot}8\times10^{-7}$ |
| LT-2 *mut*⁺ | $<4{\cdot}8\times10^{-10}$ | $4{\cdot}0\times10^{-10}$ | $4{\cdot}0\times10^{-7}$ | $2{\cdot}5\times10^{-7}$ |

* R factor 222 carries the markers of resistance to sulphonamides, streptomycin, chloramphenicol and tetracycline.

### *Transduction with phage P-22 of an R factor 222 in various substrains of Salmonella typhimurium*

The frequencies of transduction of an R factor 222 with phage P-22 grown on LT-7 *mut*⁺ or LT-2 *mut*⁺ carrying the R factor 222 to various substrains of *Salmonella typhimurium* were about equal as seen in Table 3. In contrast, the frequencies of transduction of the R factor 222 with P-22 grown on LT-7 *fer* (222) were about $10^{-8}$ per infective phage in LT-7 *fer* but much lower in LT-7 *mut*, LT-7 *mut*⁻ and LT-2 *mut*⁺ (less than $4{\cdot}8\times10^{-10}$; Table 3). The phage grown on LT-7 *mut* (222) gave a similar but less pronounced tendency of transduction to the phage grown on LT-7 *fer*.

### *Frequencies of transfer of an F'-lac⁺ to various substrains of Salmonella typhimurium*

The mixtures of the donor and recipient cultures in Penassay broth were incubated for 3 hr without aeration. The frequencies of transfer of the F'-*lac*⁺ to various substrains of *Salmonella typhimurium* from W 3747/*col* I (F'–*lac*⁺) and various substrains of *S. typhimurium* carrying the F'-*lac*⁺ are shown in Table 4. The frequencies of *lac*⁻ bacteria were scored on each donor culture for the transfer experiments, because the frequencies of spontaneous segregation of the F'-*lac*⁺ were rather high in Salmonella strains. As seen in Table 4, the frequencies of transfer of the F'-*lac*⁺ to LT-7*fer* were higher than those to the other substrains of *S. typhimurium* when the donor was a substrain of K-12. The same tendency was observed also with LT-7*fer* (F'-*lac*⁺) as a donor. In contrast, the frequencies of transfer of the *F'-lac*⁺ from LT-7*mut*, LT-7*mut*⁺ and LT-2*mut*⁺ did not show marked differences among the various recipients (Table 4). These data gave some fluctuations in several independent experiments.

Table 4. *Frequencies of transfer of an F'-lac⁺* among Escherichia coli K-12 and various substrains of Salmonella typhimurium*

The procedure for studying the frequencies of transfer of the *F'-lac*⁺ is described in the text. The duration of mixed cultivation was 3 hr in this experiment. The spontaneous segregants without *F'-lac*⁺ were determined on each donor in the transfer experiments and were found to range between 0 and 60 %; LT-7*cys*⁻*fer* 0 %, LT-7*cys*⁻*mut* 50 %, LT-7*cys*⁻*mut*⁺ 60 %, and LT-2*cys*⁻*mut*⁺ 50 %. The values in this table show the frequencies of F' transfer in 1 ml. of the mixed cultures of the donor and the recipient.

| | Frequency of transfer of an *F'-lac*⁺ by the following donor | | | | |
|---|---|---|---|---|---|
| Recipient | W 3747/*col* I† (*F'-lac*⁺) | LT-7*cys*⁻*fer* (*F'-lac*⁺) | LT-7*cys*⁻*mut* (*F'-lac*⁺) | LT-7*cys*⁻*mut*⁺ (*F'-lac*⁺) | LT-2*cys*⁻*mut*⁺‡ (*F'-lac*⁺) |
| LT-7*fer* | $6{\cdot}0 \times 10^6$ | $2{\cdot}0 \times 10^6$ | $2{\cdot}2 \times 10^4$ | $4{\cdot}0 \times 10^4$ | $2{\cdot}0 \times 10^5$ |
| LT-7*mut* | $4{\cdot}7 \times 10^4$ | $2{\cdot}8 \times 10^4$ | $4{\cdot}4 \times 10^3$ | $7{\cdot}8 \times 10^3$ | $1{\cdot}2 \times 10^5$ |
| LT-7*mut*⁺ | $3{\cdot}7 \times 10^4$ | $9{\cdot}5 \times 10^3$ | $2{\cdot}1 \times 10^3$ | $5{\cdot}0 \times 10^3$ | $1{\cdot}5 \times 10^5$ |
| LT-2*mut*⁺ | $2{\cdot}1 \times 10^2$ | $1{\cdot}8 \times 10^5$ | $3{\cdot}5 \times 10^3$ | $3{\cdot}5 \times 10^3$ | $2{\cdot}1 \times 10^5$ |

* *F'-lac*⁺ is $F_{13}$ of Hirota & Sneath (1961).
† W 3747/*col* I is an F⁻, methionine-requiring, colicin I-resistant mutant of *E. coli* K-12.
‡ LT-2*cys*⁻*mut*⁺ is identical to cysD-36.

### *Efficiencies of plating (e.o.p.) of phage P-22 on various substrains of Salmonella typhimurium*

It was previously noted by Miyake (1962) that phage grown on LT-7*fer* gave a high *e.o.p.* on LT-7*fer*, a lower *e.o.p.* on LT-7*mut* and an even lower *e.o.p.* on LT-7*mut*⁺. The adsorption of this phage took place equally well to these strains. We have shown that a clear mutant of this phage gives an *e.o.p.* on LT-2*mut*⁺ as low as on LT-7*mut*⁺ (Table 5). The *e.o.p.* of this mutant phage on LT-7*mut*, LT-7*mut*⁺ and LT-2*mut*⁺ were found to be increased by its growth on any of these bacterial strains. The *e.o.p.* on LT-7*mut*, LT-7*mut*⁺ and LT-2*mut*⁺ of the phage grown on LT-7*mut*⁺ or LT-2*mut*⁺ were equal to those on LT-7*fer*, whereas the phage grown on LT-7*mut* gave slightly lower *e.o.p.* on LT-7*mut*, LT-7*mut*⁺ and LT-2*mut*⁺ than on LT-7*fer*.

We made single-plaque isolations from the plaques developed on LT-2*mut*⁺ plated

with phage P-22 which had previously grown on LT-7*fer*. A broth suspension of each purified plaque was prepared and LT-2*mut*$^+$ was infected with it. All the lysates thus obtained gave equal *e.o.p.* on every substrain of *Salmonella typhimurium*. Conversely, the propagation on LT-7*fer* of phage P-22 which had previously grown on LT-2*mut*$^+$ resulted in the reduction of *e.o.p.* on LT-7*mut*, LT-7*mut*$^+$ and LT-2*mut*$^+$.

Table 5. *Efficiencies of plating of a clear mutant of phage P-22 on various substrains of Salmonella typhimurium*

| Indicator | Efficiency of plating of phage P-22 grown on: LT-7*fer* | LT-7*mut* | LT-7*mut*$^+$ | LT-2*mut*$^+$ |
|---|---|---|---|---|
| LT-7*fer* | 1·0 | 1·0 | 1·0 | 1·0 |
| LT-7*mut* | $1{\cdot}0\times10^{-4}$ | $4{\cdot}0\times10^{-1}$ | $8{\cdot}6\times10^{-1}$ | 2·1 |
| LT-7*mut*$^+$ | $3{\cdot}0\times10^{-5}$ | $1{\cdot}3\times10^{-2}$ | $8{\cdot}3\times10^{-1}$ | 1·4 |
| LT-2*mut*$^+$ | $6{\cdot}0\times10^{-5}$ | $2{\cdot}0\times10^{-2}$ | 1·2 | 1·4 |

Since LT-7*mut* cultures are heterogeneous, containing fertile and infertile cells as was pointed out above, the results of the *e.o.p.* of the phage propagated on LT-7*mut* on the other indicators might be due to the heterogeneous population of the phage, some phage particles being propagated on the fertile cells and other phage particles on the infertile cells. The following single burst experiments were carried out to test this possibility.

### *Single burst experiments with a clear mutant of phage P-22 in Salmonella typhimurium LT-7mut*

A culture of LT-7*mut* containing $8{\cdot}4\times10^7$ bacteria/ml. was infected with a clear mutant of phage P-22 propagated on LT-7*mut* at a multiplicity of input of 4. The infected culture was diluted with Penassay broth so that 1 ml. contained 0·3 infected bacterium and distributed in 50 small test-tubes in a volume of 1 ml. in each tube. The content of each tube, after incubation for 2 hr, was divided into two equal portions and was separately plated for phage titrations with LT-7*fer* and LT-7*mut*$^+$, respectively, as indicators. All of the 13 samples which gave bursts yielded consistently higher titres on LT-7*fer* than on LT-7*mut*$^+$, the ratio between them being about 3:1. Similar results were obtained in several other independent experiments. These results suggest that the low *e.o.p.* on LT-7*mut*$^+$ of the phage grown on LT-7*mut* is probably not due to its growth on the heterogeneous population of LT-7*mut*.

### *Isotope studies on the fate of the injected phage nucleic acid*

It was found that 30–40% of the deoxyribonucleic acid (DNA) of a clear-plaque mutant of phage P-22, which had previously been propagated on LT-7*fer*, was degraded to acid soluble fractions in 10 min. in LT-7*mut*$^+$ and LT-2*mut*$^+$, whereas less than 10% of the $^{32}P$ label of the phage DNA became acid-soluble in LT-7*fer* in 10 min. In contrast, less than 10% of the DNA of the clear mutant of P-22, which had previously grown on LT-2*mut*$^+$, was degraded to acid-soluble fractions in 10 min. in any of the strains LT-7*fer*, LT-7*mut*$^+$ and LT-2*mut*$^+$.

## DISCUSSION

As reported in the present paper, the conjugal transfer of F′ and R factors occurred most frequently to LT-7*fer* among various substrains of *Salmonella typhimurium*. We have also confirmed with an Hfr strain a previous finding of Miyake (1962) that LT-7*fer* has a high recipient ability in the crosses with different Hfr strains of *Escherichia coli* K-12. Thus LT-7*fer* acts as an unusually good recipient among the substrains of *S. typhimurium* in the conjugal transfer of bacterial chromosomes and episomal elements.

We have shown that phage P-22 grown on LT-7*fer* gives a very much reduced *e.o.p.* on any other derivatives of *Salmonella tyhphimurium*, namely LT-7*mut*, LT-7*mut*$^+$ and LT-2*mut*$^+$. Phage P-22 grown on LT-7*mut*$^+$ or LT-2*mut*$^+$, however, gave almost equal *e.o.p.* on any of the substrains of *S. typhimurium*. In contrast, the phage propagated on LT-7*mut* gave slightly lower *e.o.p.* on LT-7*mut*, LT-7*mut*$^+$ and LT-2*mut*$^+$ than on LT-7*fer*. We have further shown that the DNA of a clear-plaque mutant of phage P-22, which had previously been propagated on LT-7*fer*, is rapidly degraded after its injection into LT-7*mut*$^+$ and LT-2*mut*$^+$, whereas appreciable breakdown did not occur in LT-7*fer*. On the contrary, the DNA of the clear mutant of P-22, which had previously grown on LT-2*mut*$^+$, was not degraded appreciably in any of the substrains of *S. typhimurium*. These results indicate that the observed phenomena are due to host-controlled restriction and modification as defined by Arber & Dussoix (1962) and Arber (1965). They may be interpreted in the following two alternative ways: (1) that LT-7*fer* is a mutant which lacks the capacity of host-controlled restriction and modification; (2) that LT-7*mut*$^+$ and LT-2*mut*$^+$ lack the capacity of modification, whereas LT-7*fer* and LT-7*mut* are possessed of this capacity. In other words, it can be said that P-22 is modified by growth on LT-7*fer* or LT-7*mut* so that it will be restricted in LT-7*mut*$^+$ and LT-2*mut*$^+$. Thus phage P-22 acquires new host specificity (Arber & Dussoix, 1962) by growth on LT-7*fer* and will be restricted in LT-7*mut*, LT-7*mut*$^+$ and LT-2*mut*$^+$. The fact that P-22 grown on LT-7*fer* is still restricted on LT-7*mut* indicates that modifications of P-22 by LT-7*fer* and LT-7*mut* may be different. It should be pointed out that LT-7*fer* is regarded as restriction-less in either interpretation. The first interpretation seems more reasonable, since it is known that restriction and modification are usually impaired together (Arber & Morse, 1965; Boyer, 1964; Wood, 1966).

The results of transduction with phage P-22 of host chromosomal markers reported previously by Miyake (1962) and of an R factor reported in the present paper with various substrains of *Salmonella tyhpimurium* as recipients are also compatible with these interpretations, since the frequencies of transduction with P-22 grown on LT-7*fer* or LT-7*mut* were considerably higher to LT-7*fer* than to the other strains. In contrast, the phage grown on LT-7*mut*$^+$ or LT-2*mut*$^+$ transduced the R factor to various substrains at almost equal frequencies. Spontaneous segregation of an R factor or its drug-resistance markers occurred equally frequently in various substrains of *S. typhimurium*, indicating that the R factor replicates equally well in the various substrains, once it has started replication.

It has been found in several systems of host-controlled restriction and modification that a wide variety of foreign DNA are restricted and modified in common, including phage genomes, non-viral episomes and bacterial chromosomes (Arber, 1964; Arber,

1965; Arber & Morse, 1965; Boice & Luria, 1963; Pittard, 1964; Watanabe *et al.* 1964; Watanabe *et al.*, 1966). Our finding that LT-7*fer* acts as a good recipient for bacterial chromosomes and F′ and R factors can be easily understood, if the restriction and modification are exerted in common upon these foreign DNA. In fact, as we will report in a following paper, we have been able to show that a number of mutants of LT-2 with high recipient abilities selected by the transfer of an R factor from *Escherichia coli* CSH-2 (222) behaved, after elimination of the R factor with acridine dyes, as good recipients of K-12 chromosomes. Furthermore, phage P-22 grown on any substrains of LT-2 and LT-7 gave highest *e.o.p.* on these mutants. These results seem to suggest that the aforementioned pleiotropic phenomena are caused by single genetic events.

If we adopt the first interpretation as to the modification of phage P-22, its modification is complete on LT-7*mut*$^+$ and LT-2*mut*$^+$ in view of the *e.o.p.* on the substrains of *Salmonella typhimurium* of the phage propagated on these strains. The modification of F′ and R factors, however, did not give clear-cut results. As the results in Table 4 indicate, the frequencies of transfer of an F′ from LT-7*mut*$^+$ (*F′-lac*$^+$) to LT-7*mut*, LT-7*mut*$^+$ and LT-2*mut*$^+$ were considerably lower than those to LT-7*fer*, whereas the frequencies of transfer of the F′ from LT-2*mut*$^+$ (F′-*lac*$^+$) to those strains were almost equal. On the other hand, the frequencies of transfer of the R factor 222 from LT-2*mut*$^+$ (222) to LT-7*mut*, LT-7*mut*$^+$ were much lower than that to LT-7*fer*, whereas the frequencies of R transfer from LT-7*mut*$^+$ (222) to various substrains of *S. typhimurium* were almost equal (Table 2). These results are not completely parallel with the results of the *e.o.p.* of phage P-22 on the substrains of *S. typhimurium*. This discrepancy may be at least partly due to the experimental conditions we used: we incubated the mixtures of the donor and recipient cultures overnight or for 3 hr, because the frequencies of transfer of R and F′ factors were too low in some recipients to detect in the ordinary 1 hr incubation. The incubation for long durations may have caused some distortion of the frequencies of transfer of these episomes. Some other unknown factors might also be involved.

Eisenstark (1965) succeeded in isolating fertile mutants from LT-2*mut*$^+$ through treatment with a mutagen, nitrosoguanidine; Miyake (1962) had presented some evidence that such mutations may occur in LT-2*mut*$^+$ spontaneously. These results are compatible with our results and hypothesis that fertility is given by the restrictionless mutation. The fact that fertile mutants are easily isolated in LT-7*mut* is considered to be merely because this strain is a mutable one due to the presence of the mutator gene.

The hybrids between *Escherichia coli* and Salmonella exhibit a high recipient ability when used as recipients in the crosses with *E. coli* donors (Baron, Carey & Spilman, 1959; Miyake, 1962). This finding was interpreted as due to the selection of high-frequency recipients and this fits well our data and hypothesis. Johnson, Falkow & Baron (1964), however, later demonstrated that the increased recipient ability of the hybrids of *E. coli* and *S. typhi* is due to the presence of integrated *E. coli* genetic material. They further showed that the recipient ability of these hybrids was increased only when their substituted *E. coli* genetic section matched the leading region of the Hfr chromosome. They also mentioned that the Salmonella population is probably homogeneous with respect to its initial ability to mate with *E. coli* on the basis of the finding that the transfer of an *F′-lac*$^+$ element occurred only

slightly less efficiently from an *E. coli* F′ donor to Salmonella than it did to an *E. coli* F⁻ recipient. In contrast, in our results, the transfer of an F′ from *E. coli* to the substrains of *S. typhimurium* except to LT-7*fer* occurred at much lower frequencies than to *E. coli* recipients. The discrepancies between the results of Johnson *et al.* (1964) and ours may probably be due to the difference in the strains employed; Johnson *et al.* (1964) employed *S. typhi*, whereas we used *S. typhimurium*.

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# The Effect of the Pressure Shock Wave and Some Electrical Quantities in the Microbicidal Effect of Transient Electric Arcs in Aqueous Systems

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SUMMARY

Micro-organisms in aqueous suspensions were killed when voltages above a certain threshold value were discharged through electrodes immersed in the suspensions. The relation of the peak pressure, peak current and the arcing time to the killing effect were studied, but no clear-cut relationship was ascertained. At the discharges shock waves with durations of 20–105 $\mu$sec. and pressure amplitudes of 40–250 bar were generated. Although bigger and more solid objects were considerably damaged, the micro-organisms were inconspicuously affected by the pressure shock wave alone.

## INTRODUCTION

When micro-organisms suspended in water are subjected to submerged high-voltage electrical discharges, a large proportion of them are killed (Brandt *et al.* 1962; Allen & Soike, 1966). The spectrum of sensitive organisms is wide and the efficiency high, which might make this method useful for practical disinfection purposes. Several physical and chemical activities are initiated by the electrical discharge. From the plasma channel emanate a pressure shock wave, photon radiation and more or less unstable chemical compounds. Information about the relation of the microbicidal effect to the different non-biological effects of the discharge might elucidate the mechanism of action and lead to a high microbicidal efficiency of the process being obtained.

## METHODS

### *The electric circuit used and its physical and electrical quantities*

The electric circuit used is shown in Fig. 1. The step-up transformer (3) is supplied with a variable voltage from a variac (2) connected to the a.c. mains (1). The high voltage capacitor (6) is charged through a resistor (4) and a rectifier (5). The capacitor voltage will reach a ceiling value, $U$, determined by the spark gap (8) operating in air. The breakdown of gap (8) causes a current to flow between the submerged eletrodes (9). The capacitor will discharge through the circuit elements (7–8–9) of Fig. 1. The capacitance, $C$, is measured in microfarad ($\mu$F.) and the inductance, $L$, in microhenry ($\mu$H.). The electrodes (9) were submerged in water.

To initiate a breakdown of the water between the submerged electrodes and to bridge the electrodes with an arc the capacitor must be initially charged with a voltage $U \geqslant U_b$. The breakdown voltage ($U_b$) is a function of the electrode separation ($s$), the water conductivity and the capacitance ($C$). The current through the submerged gap is a damped sinusoidal current (e.g. Fig. 5 of Brandt *et al.* 1962). The arc is extinguished after one or more half-cycles of current.

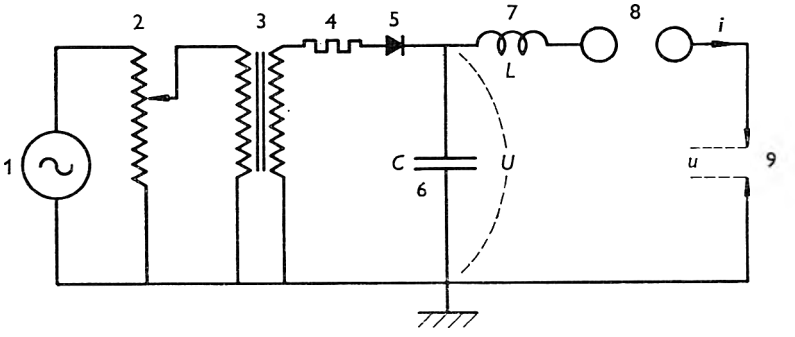


Fig. 1. The electric circuit. 1, Mains; 2, variac; 3, step-up transformer; 4, resistor; 5, rectifier; 6, capacitor; 7, choke; 8, spark gap; 9, submerged electrodes. $U$, initial capacitor voltage; $u$, voltage between submerged electrodes; $i$, discharge current; $C$, capacitance; $L$, inductance.

The pressure shock wave originates from the plasma arc between the submerged electrodes. It travels through the liquid with the speed of sound. The peak pressure develops during the rise of current during the first current cycle. The variation of the peak pressure with voltage ($U$), choke inductance ($L$) and electrode separation ($s$), was studied with the electrodes submerged in tap water in a steel tank of about 500 l. capacity (fig. 2 of Brandt *et al.* 1962). The pressure was recorded by a piezoelectric tourmalin crystal connected with a cathode-ray oscilloscope. The crystal diameter (0·25 in.) limited the measuring accuracy of the pressure rise time. The pressure was also recorded behind membranes of variable thickness.

Standard deviation, $\sigma = \sqrt{[(x-\bar{x})^2/n-1]}$, where $n$ is the number of observations, $x$ the measured value and $\bar{x}$ the arithmetic mean.

### *Microbiological methods*

*Escherichia coli* B was cultivated in 200 ml. nutrient broth (Difco) on a shaker table at 37°. After incubation for 18 hr the culture was cooled to 0°, centrifuged, the deposited organisms washed once in 0·01 M-sodium phosphate (pH 7·0) and then suspended in distilled water. The total count was estimated after dilution and counting in a Buerker Chamber with 0·01 mm. depth. Colony counts were made by spreading 0·1 ml. of tenfold dilutions on the surface of nutrient agar plates. A standard diluent containing Difco nutrient broth, 1 g.; NaCl, 5 g.; distilled water, 1000 ml., was used throughout. The plates were incubated at 37° for 1 day, and those with 20–400 colonies were counted. Each value given is the arithmetic mean of three individual determinations. Phage suspensions were prepared according to Frick (1961) and titrated by the plaque technique (Adams, 1959).

The experiments on the microbicidal effect of the discharges were made in a vertical stainless-steel tube vessel (Fig. 2). Immediately after the discharge 0·5 ml. of treated microbial suspension was transferred to 4·5 ml. diluent and kept in an ice-water bath until further dilution and plating. This storage did not appreciably affect the colony count. When no dilution was to be done, 0·1 ml. was directly pipetted on to nutrient agar plates.

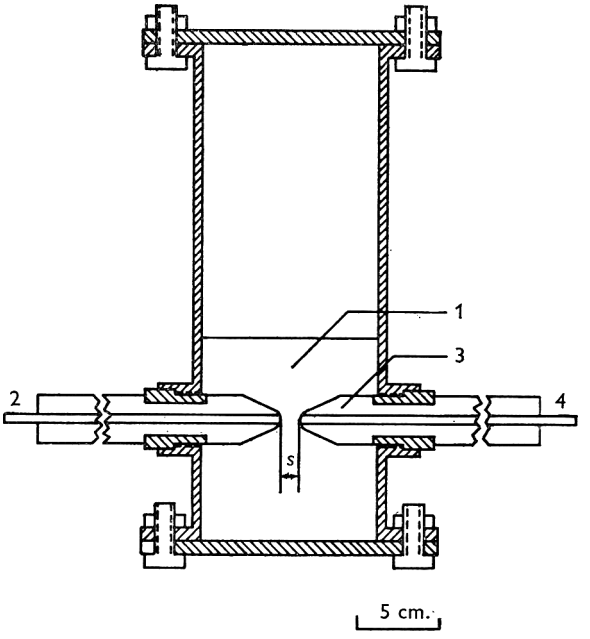


Fig. 2. Vertical stainless steel tube discharge vessel. 1, Water; 2, high-voltage electrode; 3, plastic insulation; 4, earthed electrode.

In some experiments the suspension of micro-organisms was isolated from all other recognized effects of the submerged discharges except the pressure by putting the suspension in a metal box placed on the bottom of the discharge vessel (Fig. 2). The box-wall facing the electric arc consisted of a steel membrane of different thicknesses. In other experiments the suspension was contained in a cellophan bag (tube diameter 6·35 mm.) and placed on the bottom of the discharge vessel. Samples of these suspensions were taken with syringe and needle.

## RESULTS

Separate discharges performed in the same kind of liquid with the same electrical set-up produced different results with respect to the peak value and duration of the pressure and the current, and to the killing effect (Table 1). Also the sound of the bang accompanying the discharge varied markedly. A loud bang was generally accompanied by a higher bactericidal effect than a weak one. This variation is supposed to be inherent in the nature of the discharge, which is rapid and far from equilibrium. Several experiments were therefore made to determine the results of a certain type of discharge.

At voltages $U < U_b$ no plasma channel was formed in the water. At such voltages the noise accompanying the discharge was much weaker or inconspicuous, and no killing effect or pressure shock wave were recorded. At higher voltages ($U > U_b$) the peak pressure and the peak current increased with voltage. At these voltages an oscillating current was produced (e.g. fig. 5 of Brandt *et al.* 1962). As seen from Fig. 3,

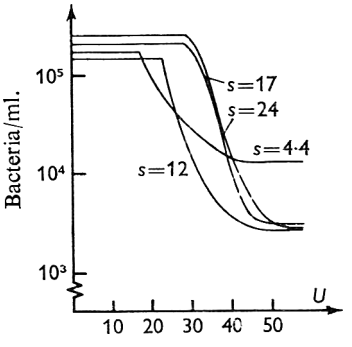


Fig. 3

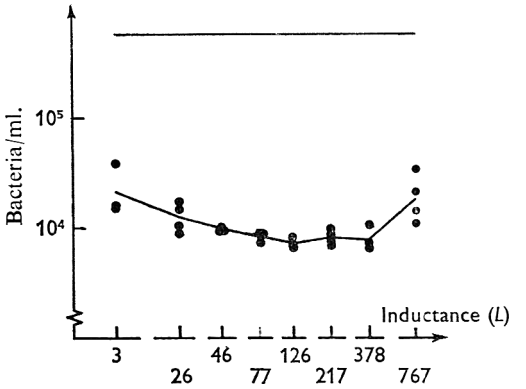


Fig. 4

Fig. 3. Effect of voltage and electrode separation ($s$) on the killing of *Escherichia coli* B17 suspended in tap water (1200 ml.). $C = 0{\cdot}6\ \mu$F., $L = 46\ \mu$H.

Fig. 4. Effect of inductance ($L$) on the killing of *Escherichia coli* B15 suspended in tap water. $C = 0{\cdot}6\ \mu$F., $U = 45{\cdot}5$ kV., $s = 32{\cdot}5$ mm. Points = colony counts of individual experiments 1 min. after the discharge; the line joins their geometric means. Upper horizontal line = no discharge.

Table 1. *Escherichia coli B17 suspended in tap water and subjected to one discharge*

Ten experiments were run at each inductance value. Initial colony count $1{\cdot}1 \times 10^5$ bacteria/ml. $C = 0{\cdot}63\ \mu$F., $U = 29{\cdot}2$ kV., $s = 11$ mm. The statistical calculations were performed on the logarithms of the colony counts.

| Inductance ($\mu$H.) | Viable count (bact./ml.) | | | Standard deviation, $\sigma$ (%) |
|---|---|---|---|---|
| | Maximum | Minimum | Mean ($\bar{x}$) | |
| 3 | $7{\cdot}0 \times 10^4$ | $2{\cdot}6 \times 10^4$ | $4{\cdot}9 \times 10^4$ | 23 |
| 126 | $1{\cdot}7 \times 10^4$ | $7{\cdot}8 \times 10^3$ | $1{\cdot}2 \times 10^4$ | 20 |
| 767 | $3{\cdot}3 \times 10^4$ | $1{\cdot}2 \times 10^4$ | $2{\cdot}2 \times 10^4$ | 38 |

the threshold voltage for killing effect was higher at greater electrode separation ($s$) and the killing effect increased with voltage until a plateau was reached. This maximum killing effect was smaller and was attained at lower voltages at shorter distances between the electrodes.

When the circuit inductance was increased, the peak pressure and the peak current decreased and the arcing time increased (L. Edebo & I. Selin, unpublished). The bactericidal effect was affected by changes in the circuit inductance as shown in Table 1. For several different micro-organisms, voltages and electrode separations, the maximum bactericidal effect was achieved at an intermediate inductance value in the range of 40–400 $\mu$H. (Fig. 4).

The pressure behind steel membranes of different thicknesses was oscillographically

recorded and compared with recordings without membrane (L. Edebo & I. Selin, unpublished). Within each group there was considerable difference between individual discharges. The average peak pressure behind a membrane, however, deviated less than $\pm 11\%$ from the pressure without a membrane. This deviation is smaller than the standard deviation of individual discharges from the same experimental set-up. Consequently, the insertion of a thin metallic membrane between the arc and a suspension of micro-organisms should not cause any appreciable change in the pressure effect on the micro-organisms.

Table 2. *Effect on Escherichia coli* B *in a steel box with different membrane thicknesses and in a cellophan bag*

Two series of experiments were made. Discharge liquid = 1200 ml. tap water. $C = 0{\cdot}6\ \mu$F., $L = 214\ \mu$H., $U = 29$ kV., $s = 8{\cdot}5$ mm.

| Container | No. of discharges | Colony count (bacteria/ml.) Series 1 | Series 2 |
|---|---|---|---|
| No treatment | 0 | $7{\cdot}8 \times 10^6$ | $6{\cdot}9 \times 10^6$ |
| Box, membrane 0·06 mm. | 40 | $6{\cdot}5 \times 10^6$ | $6{\cdot}1 \times 10^6$ |
| Box, membrane 0·093 mm. | 40 | $6{\cdot}6 \times 10^6$ | $6{\cdot}5 \times 10^3$ |
| Box, membrane 0·291 mm. | 40 | $6{\cdot}2 \times 10^6$ | $6{\cdot}8 \times 10^3$ |
| Box, lid 3·83 mm. | 40 | $6{\cdot}7 \times 10^6$ | $7{\cdot}7 \times 10^3$ |
| Cellophan bag | 4 | $2{\cdot}4 \times 10^5$ | $3{\cdot}3 \times 10^5$ |

Table 3. *Effect on coliphage T 5 in different containers*

Discharge liquid = 1200 ml. 0·0013 M-NaCl. $C = 0{\cdot}63\ \mu$F., $L = 175\ \mu$H., $U = 36$ kV., $s = 11$ mm.

| Container | No. of discharges | Plaques/ml. |
|---|---|---|
| No treatment | 0 | $5{\cdot}7 \times 10^5$ |
| Box, membrane 0·06 mm. | 20 | $7{\cdot}1 \times 10^4$ |
| Cellophan bag kept in the box above | 20 | $5{\cdot}3 \times 10^5$ |
| Cellophan bag on the bottom of the discharge vessel | 3 | 5 |

A steel box with a steel membrane cover and containing a suspension of *Escherichia coli* was submerged in water in which electrical discharges were initiated. No significant change in the number of colonies was observed (Table 2). When very dilute suspensions of micro-organisms were used, a minor killing effect was noticed, but such an effect was produced even without discharges. When, instead of enclosing the bacteria in a metal box, the bacteria were kept in a cellophan bag, a considerable killing effect was achieved with discharges. Whereas 40 discharges did not produce any significant decrease in colony count of a suspension kept in the metal box, 4 discharges decreased the colony count 20–40-fold in the cellophan bag. No killing effect by discharges was observed in experiments when the bag was placed in the metal box, or placed below a piece of U-shaped iron plate standing on its legs, or when it was wrapped into a piece of black plastic sheet.

Experiments similar to those described for *Escherichia coli* B were also made with bacteriophages. Suspensions of coliphage T5 enclosed in the steel box and subjected to discharges showed little or no inactivation of the phages. When, however, phage enclosed in a cellophan bag was subjected to a much smaller number of discharges, there was almost complete inactivation (Table 3).

## DISCUSSION

The experiments described in this paper were planned to investigate whether the killing effect might be correlated to any one of the main physical or electrical activities of the discharge. By increasing the circuit inductance, the peak pressure, the pressure gradient and the peak current were decreased without affecting the energy content of the discharge. Since a moderate increase of the circuit inductance increased the microbicidal effect, none of the above-mentioned activities can be held solely responsible for the killing effect.

It was possible to separate the pressure shock wave from other activities by screening off the discharge gap with a thin steel membrane. Although pressure measurements showed no appreciable change of the shock wave when passing through the membrane, the killing effect was completely extinguished. We conclude that the pressure alone had no microbicidal effect.

It might seem surprising that pressure shock waves which were capable of buckling 0·5 mm. thick iron plate did not kill the micro-organisms. However, when the physical nature of the shock wave in relation to the micro-organism was considered, the results seemed less strange. Assume a pressure wave with a peak pressure of 50 bar and a rise time of 10 $\mu$sec. The velocity of a pressure shock wave in water is about 1·5 mm./$\mu$sec. Hence the corresponding pressure front is about 15 mm. and the pressure gradient 50/15 bar/mm. An *Escherichia coli* bacterium is generally shorter than 3 $\mu$ along its long axis This means that the pressure difference along the length of the bacterium is about $0{\cdot}003 \times (50/15)$ bar, i.e. 0·01 bar. This is quite small compared with the intracellular pressure of *E. coli* which has been estimated as 4 bar (Mitchell & Moyle, 1956). Consequently, the mechanical shearing stress on a bacterium by the pressure shock wave is negligible. Electron photomicrographs of bacteria subjected to 40 discharges, which left a surviving fraction less than $10^{-5}$, did not show any morphological changes in the bacteria. Staphylococci and streptococci, which are smaller and have considerably higher intracellular pressure than *E. coli*, were affected to approximately the same extent by the electrical discharges (Brandt *et al.* 1962). All these data support the view that the killing effect of the discharges depended little on the pressure gradient.

Besides the shearing stress, the pressure gradient can also be considered as a sudden squeezing of the micro-organisms. Hydrostatic pressures of the magnitude measured in our experiments have no known killing action on micro-organisms (Larson, Hartzell & Diehl, 1918; Johnson & Lewin, 1946; Rutberg, 1964; Hedén, 1964). For *Escherichia coli* B a pressure of 1058 atmospheres for 5 min. is required to cause an almost 90% decrease of the colony count (Rutberg, 1964). The sudden application and removal of the moderate pressures formed by discharges did not seem to affect their innocuousness for micro-organisms.

Bacteria kept in a cellophan bag were killed by the discharges, in contrast to bacteria within a steel box. This might indicate that, instead of pressure, the photon radiation was important for the killing effect. This supposition was further supported by the fact that bacteria suspended in water but not in broth or milk were effectively killed by discharges.

Below a certain threshold voltage no killing effect could be demonstrated. At higher voltages an increase in voltage enhanced the killing effect until a plateau was reached.

Since the energy consumption of the discharge increases with the square of the voltage, a voltage increase after the plateau has been reached would lead to waste of energy under these conditions. Studies on the maximum efficiency will be reported later.

Recently a method for sterilization by electrohydraulic treatment has been described (Allen & Soike, 1966). In those experiments the microbicidal effect of multiple discharges of 14 kV. and 5–95 $\mu$F. has been investigated. Although the voltage was lower and the capacitance higher than those used by us, their results indicate that the activities responsible for the killing effect are similar.

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# The Effect of the Photon Radiation in the Microbicidal Effect of Transient Electric Arcs in Aqueous Systems

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SUMMARY

The importance of photon radiation for the microbicidal effect of electrical discharges in water has appeared from a series of experiments. (*a*) An ultraviolet (u.v.)-sensitive strain of *Escherichia coli* was more susceptible to discharges than a u.v.-resistant mutant derived from it. (*b*) The kinetics of the inactivation by discharges were under certain conditions similar to those of continuous u.v. irradiation. (*c*) Addition of u.v.-absorbing substances to the discharge liquid decreased the bactericidal efficiency of the discharges to an extent which paralleled the u.v. absorbance. (*d*) The bactericidal effect decreased as the distance from the spark increased. (*e*) Bacteria enclosed in a cellophan bag were killed by discharges outside the bag, even when the bag was kept in the air above the discharge liquid. (*f*) Also discharges in air were active. (*g*) Bacteria inactivated by discharges were accessible to photoreactivation, but the magnitude of the reactivation was generally less than that obtained with bacteria inactivated by continuous u.v.-irradiation. In addition to direct radiation effects other kinds of microbicidal activities were produced by the electric discharges.

## INTRODUCTION

Submerged electrical discharges in water have a potent microbicidal effect for a wide range of micro-organisms (Brandt *et al.* 1962; Allen & Sioke, 1966). The physicochemical effect of the discharges consists of, amongst others, ionization, heat, a pressure shock wave, radiation, and chemical effects. Previous experiments (Edebo & Selin, 1968) showed that the microbicidal effect of the pressure shock wave was insignificant, nor could any of the main electrical quantities be correlated with the killing effect. However, a microbial suspension enclosed in a dialysis bag was inactivated by discharges, unless it was shielded from the electric arc (Edebo & Selin, 1968). These results indicated that radiation from the arc might be essential for the microbicidal effect.

## METHODS

The *Escherichia coli* B strains B15 (u.v.-sensitive) and B17 (u.v.-resistant) were obtained from Dr G. Bertani, Department of Microbial Genetics, Karolinska Institutet, Stockholm.

The electrical equipment, cultivation of micro-organisms and colony counts were made as described previously (Edebo & Selin, 1968). When the killing effect in free suspensions in the discharge vessel (fig. 2 of Edebo & Selin, 1968) was investigated, each value was calculated as the arithmetic mean of three individual determinations.

The difference between each one did not usually exceed 30%. In some experiments suspensions of bacteria were enclosed in four separate cellophan bags, fastened to a metal frame and placed in the discharge vessel. Discharges were made and then samples taken from the cellophan bags by means of a syringe and needle. The spread of colony count values was greater in these cases, sometimes, particularly at high killing effects, the largest value being nearly 9 times the smallest. Therefore each such experiment was calculated as the geometric mean of the four individual determinations.

After treatment with discharges or continuous u.v. irradiation 3 ml. samples of the bacterial suspensions in test-tubes were subjected to visible light (Philips Attralux, 24 V., 150 W, > 3800 A.) for photoreactivation. The samples were immersed in a water bath (24–28°) to prevent heating, and light was admitted through 45 × 62 mm. windows in the wall of the container. The distance from the wall of the container to the centre of the test-tube was 90 mm., the illumination time 30 min.

Standard electrical arrangement (SEA): capacitance ($C$) = 0·6 $\mu$F., inductance ($L$) = 43 $\mu$H., discharge voltage = 45 kV.; electrode separation = 11 mm.; electrode tips consisted of copper + tungsten alloy; liquid volume in the discharge vessel = 1200 ml.

Symbols in the Figures unless otherwise stated: solid circles and continuous lines represent samples diluted 1/10 in the diluent (nutrient broth 1 g., NaCl 5 g./l.) immediately after the discharges; open circles and broken lines those after overnight incubation in the refrigerator. Each circle is the result of one experiment from which three different samples were taken.

The characteristics of the tap water were: colour < 5 mg. Pt/l., permanganate 4–6 mg. $KMnO_4$/l., conductivity 354–413 $\mu$mho./cm., pH 7·1–7·7, total hardness calculated as Ca 101–108 mg./l.; ammonium < 0·1, manganese < 0·05, bicarbonate 262–286, chloride 18–21, sulphate 26–34, nitrate 9–36, nitrite < 0·01, phosphate < 0·01 mg./l. Bacteria: nutrient gelatine at 20° for 48 h < 1 colony/ml.; lactose broth 37° < 1/100 ml.

## RESULTS

### *Microbicidal effect of discharges with relatively high energy content (620 J.)*

The microbicidal effect on various concentrations of bacteria was investigated for *Escherichia coli* strains B15 and B17 in 0·001 M-KCl and in tap water (Figs. 1–4). Potassium chloride was added to distilled water, because a certain conductivity was needed to produce this type of discharge (Edebo, Holme & Selin, unpublished results). When suspensions of *E. coli* B15 in 0·001 M-KCl were subjected to discharges of SEA (see methods), a large proportion of the bacteria were killed. When the logarithm of the colony count was plotted against the number of discharges, it approached a straight line (Fig. 1). The killing effect might therefore be approximately described by $S = e^{-NK}$, where $S$ is the fraction of surviving colony-forming units, $N$ is the number of discharges, and $K$ is a constant characteristic for the electrical arrangement the suspending fluid and the micro-organism investigated. For practical reasons the base 10 was chosen instead of $e$, $S = 10^{-Nx}$, and the microbicidal effect of the individual discharges ($-x$) expressed as log per discharge (l.p.d.). For suspensions of *E. coli* B15 whose initial colony counts were between $2 \times 10^6$ and $2{\cdot}5 \times 10^7$ bacteria/ml. the microbicidal effect was 3·0–2·8 l.p.d. For more concentrated suspensions it became less, successively diminishing with higher bacterial concentrations. When the bacterial

suspensions which had been subjected to discharges were left overnight in the refrigerator before dilution and plating, the number of colonies recovered was further decreased, while the count of untreated bacteria remained almost constant. Suspensions with initial colony counts of $10^7$ bacteria/ml. and less did not show any growth (i.e. < 10 bacteria/ml.), when subjected to one discharge and kept overnight at 4°. For *E. coli* B 15 in tap water the microbicidal effects at the initial concentrations $2{\cdot}2 \times 10^6$, $2{\cdot}2 \times 10^7$ and $2{\cdot}2 \times 10^8$ bacteria/ml. were 2·4, 1·9 and 0·4 l.p.d. respectively (Fig. 2). No protracted killing effect was observed when these suspensions were kept overnight in the refrigerator.

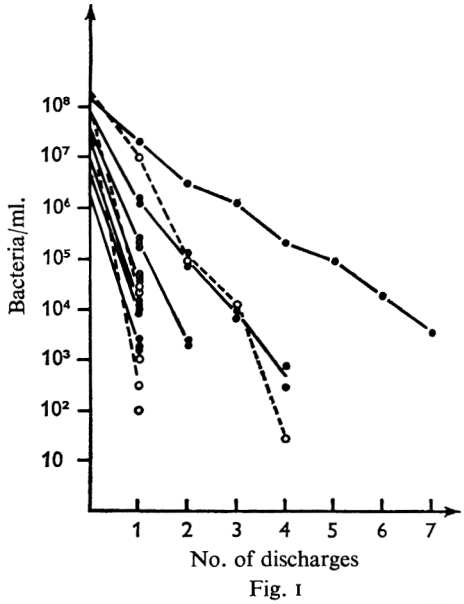


Fig. 1

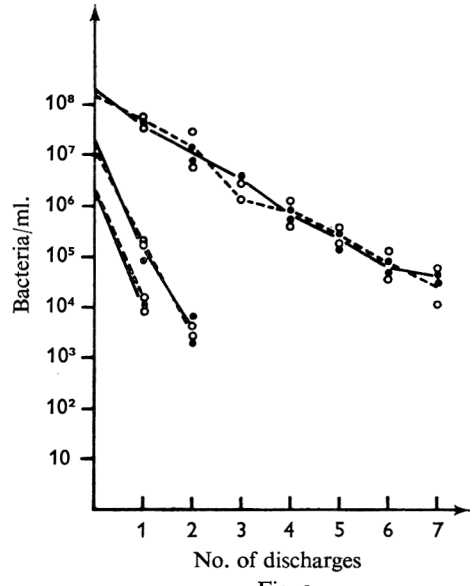


Fig. 2

Fig. 1. *Escherichia coli* B 15 suspended in 0·001 M-KCl and subjected to discharges (SEA and symbols, see Methods).

Fig. 2. *Escherichia coli* B 15 suspended in tap water and subjected to discharges (SEA).

The microbicidal effect on *Escherichia coli* B 17 suspended in 0·001 M-KCl at concentrations between $9{\cdot}6 \times 10^5$ and $8{\cdot}3 \times 10^6$ bacteria/ml. was 2·0 l.p.d. (Fig. 3). At moderately higher concentrations the inactivation was less but fairly constant in consecutive discharges. At an initial concentration higher than $10^8$ bacteria/ml. the killing curve deviated considerably from a straight line. The fourth and the fifth discharge had the greatest killing effect. The protracted killing effect achieved by keeping overnight in the refrigerator was, however, considerable also at high concentrations; only for initial concentrations above $10^7$ bacteria/ml. were the colony counts sufficiently high to allow an approximate estimation. In tap water at concentrations of $2{\cdot}7 \times 10^6$ and $2{\cdot}9 \times 10^7$ bacteria/ml. the bactericidal effect varied between 1·6 and 1·2 l.p.d. (Fig. 4). At a concentration of $2{\cdot}5 \times 10^8$ bacteria/ml. it was decreased to about 0·2 l.p.d. The protracted effect was generally negligible.

In these experiments the u.v.-sensitive B 15 strain of *Escherichia coli* was more strongly inactivated by discharges than the u.v.-resistant strain B 17. However, the

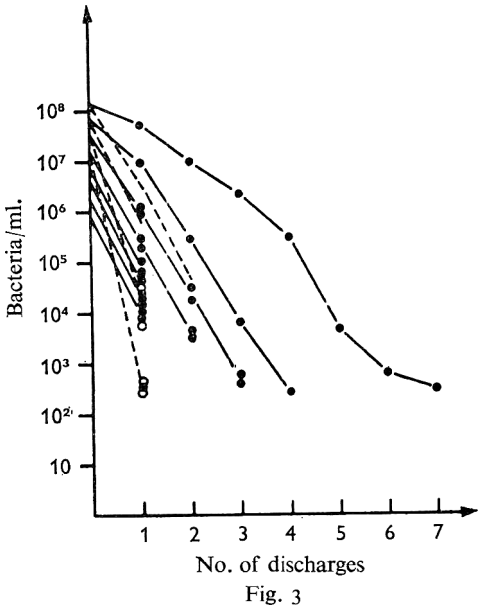


Fig. 3

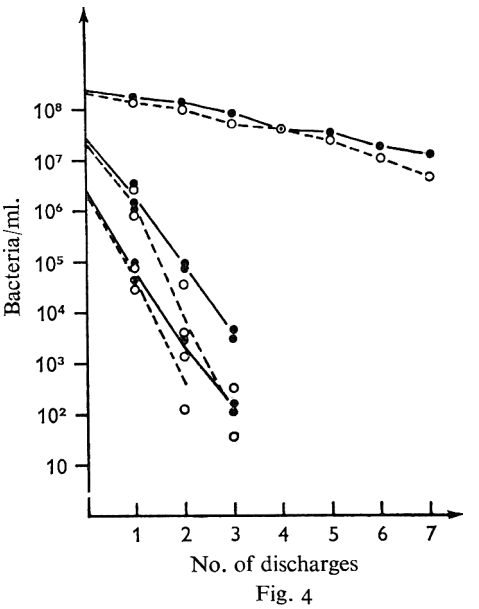


Fig. 4

Fig. 3. *Escherichia coli* B 17 suspended in 0·001 M-KCl and subjected to discharges (SEA).

Fig. 4. *Escherichia coli* B 17 suspended in tap water and subjected to discharges (SEA).

kinetics of the inactivation of strain B 17 deviated considerably from that of continuous u.v. radiation.

*The influence of albumin on the microbicidal effect*

The presence of albumin at 100 mg./l. ($A_{260m\mu}$ = 0·125) in a liquid containing *Escherichia coli* B 15 decreased the immediate killing effect of discharges slightly

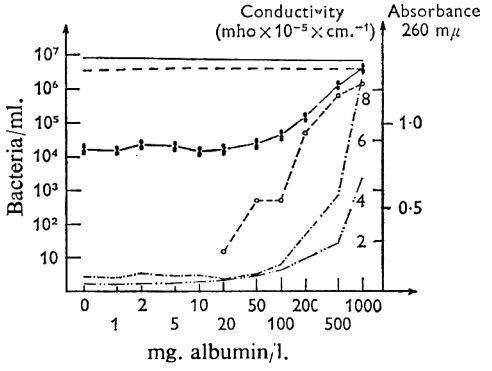


Fig. 5

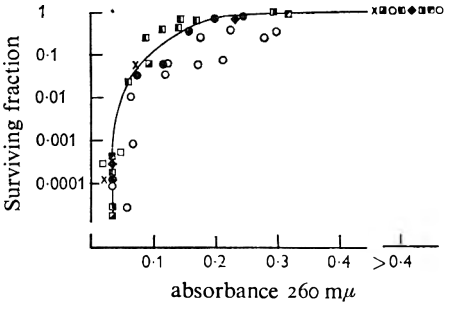


Fig. 6

Fig. 5. *Escherichia coli* B 15 suspended in different concentrations of albumin and subjected to one discharge (SEA). Upper horizontal lines represent viable counts of samples not subjected to discharges. —·—, Conductivity; —··—, absorbance.

Fig. 6. The relation of the u.v.-absorbing capacity of the discharge liquid to the bactericidal effect on *Escherichia coli* B 17 enclosed in cellophan bags and immersed in the discharge liquids. SEA, two discharges. ●, DNA; ◆, RNA; ×, albumin; □, dextran; ◩, blue dextran; ◪, green dextran; ◧, yellow dextran; ◨, red dextran; ○, yeast suspension.

(Fig. 5); at higher concentrations the effect was gradually decreased. In suspensions containing albumin, 1 g./l., no bactericidal effect was observed. The protracted bactericidal effect was still more sensitive to albumin. No protracted effect could be demonstrated at concentrations $\geqslant$ 200 mg./l.; at 50–100 mg./l., 98–99 % of the bacteria surviving immediately after the discharge were killed later, and at 20 mg./l. and below, < 10 to 40 bacteria/ml. were recovered after keeping overnight in the refrigerator. These experiments showed that albumin decreased the immediate and the protracted killing effect. They did not show, however, whether this was due to protective action on the bacteria themselves or to a general neutralization of some physical or chemical activities of the discharges.

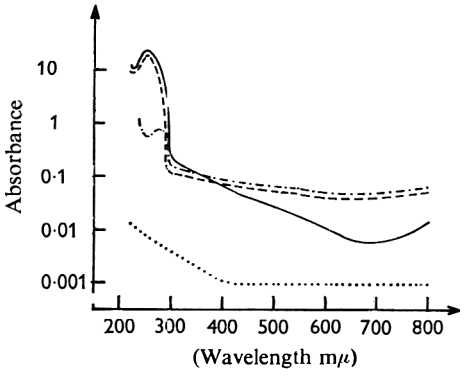


Fig. 7

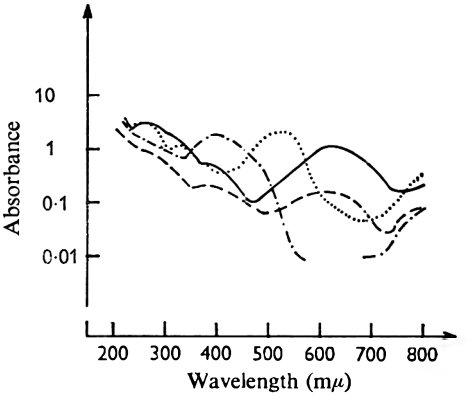


Fig. 8

Fig. 7. Absorbance of 1 g./l. solution of DNA (——), RNA (– – –), albumin (–·–·), and dextran (· · · ·).

Fig. 8. Absorbance of g./l. solution of variously coloured dextrans: (——), blue; (– – –), green; (–·–·), yellow; (· · · ·), red.

*The microbicidal effect on Escherichia coli suspensions enclosed in cellophan tubing immersed in discharge liquids containing light-absorbing substances*

When cellophan tubing containing *Escherichia coli* B 17 was placed on the bottom of the discharge vessel and two discharges made, the microbicidal effect was strongly influenced by the nature of the surrounding liquid (Fig. 6). Substances with high u.v. absorbing capacity, e.g. DNA, RNA, albumin (Fig. 7), decreased the microbicidal effect even at low concentrations; the nucleic acids were particularly active. Dextran at 1 g./l. influenced the bactericidal effect little (this solution had low u.v. absorbance); however, with a dextran concentration at 10 g./l. the bactericidal effect was almost completely extinguished. This dextran solution (10 g./l) was viscous and the sound accompanying a discharge was weak, which might imply that the discharge itself was affected. Before the discharges all the dextran was not dissolved; after the discharges, however, no undissolved material remained. When solutions with chromophores chemically bound to the dextran molecules were used (substances provided by AB Pharmacia, Uppsala), the decrease of the killing effect was considerably greater. These substances showed a fairly strong absorption in the u.v. region (Fig. 8) which paralleled their decrease of the killing effect. No correlation with other parts of the spectrum was observed.

*The microbicidal effect on suspensions enclosed in cellophan tubing and placed at different distances from the spark*

When a suspension of *Escherichia coli strain* B was enclosed in a cellophan tube and placed in a large discharge vessel (fig. 2 of Brandt *et al.* 1962) at different distances from the spark gap, the bactericidal effect became less at greater distances from the spark gap. At a distance of 8 cm. one 45 kV. discharge decreased the colony count more than 300-fold; at distances more than 20 cm. the killing effect was hardly measurable.

*The microbicidal effect of discharges in liquid and in air*

When different volumes of an *Escherichia coli* strain B 15 suspension were subjected to one discharge, the bactericidal effect was dependent on the volume (Fig. 9). The surviving fraction had a maximum at 600 ml., i.e. when the surface of the liquid was just below the electrodes so that the discharge took place in the air. In these experiments it should be observed that at the same spark gap (fig. 1:8, Edebo & Selin, 1968) the discharge voltage with electrodes in the air was higher than that with submerged electrodes.

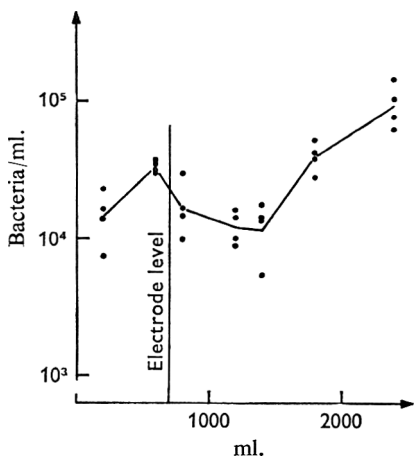


Fig. 9. The effect on different volumes of a suspension of *Escherichia coli* B 15 in tap water of discharges in the suspension (> 700 ml). and in the air above the suspension (< 700 ml.). One discharge. SEA except spark gap = 12 mm. (i.e. 38 kV. with electrodes in water) and $s$ = 12·9 mm. No discharge = $1{\cdot}6 \times 10^5$ bacteria/ml.

Cellophan tubing containing *Escherichia coli* B 15 was placed on the bottom of the discharge vessel and at the same distance above the electrodes, and discharges were made with different volumes of tap water in the vessel, similar to the experiments described in Fig. 9. With no liquid and 600 ml. in the discharge vessel the discharge took place in the air, with 800 and 1200 ml. it occurred in the water. The cellophan tubes placed above the electrodes were surrounded by air. Except when no tap water was added the tubes below the electrodes were immersed in water. The colony counts of the samples kept in either medium were greatly decreased by discharges in water and in air. The greatest killing effect on the average was obtained with 600 ml. water in the discharge vessel. With regard to the original colony count the differences between the

results with the different volumes were small. Therefore the efficiency of discharges in water and in air could not be adequately compared, since also reflexion, refraction and absorption of the radiation varied between different experiments, and at the same spark-gap a higher voltage was needed for discharges in air. Experiments where air was injected through the lower of two vertical electrodes while discharges were performed gave low bactericidal effects.

*The microbicidal effect of submerged discharges with relatively low energy content (91 J.) compared with discharges in air and continuous u.v. radiation*

The bactericidal effect of 620 J. discharges did not show the typical u.v. inactivation curves on *Escherichia coli* B 17 (Figs. 3, 4). Therefore discharges with less energy

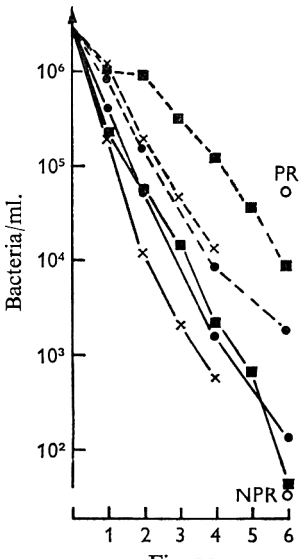


Fig. 10

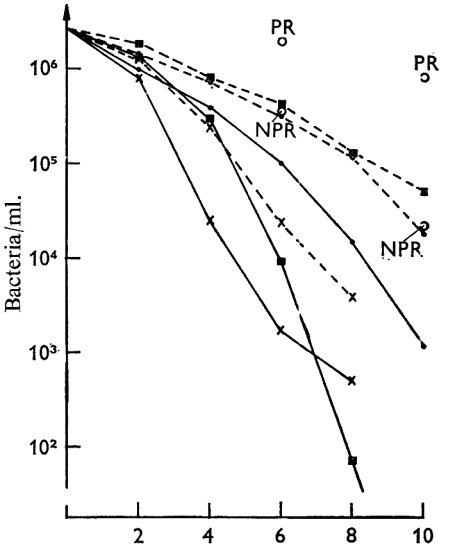


Fig. 11

Figs. 10, 11. *Escherichia coli* after treatment with submerged (1200 ml., ●) and air (200 ml., ×) discharges, and with continuous u.v. irradiation (■). The units along the abscissa signify no. of discharges or min. u.v. treatment. Solid lines and NPR = without photoreactivation (PR), broken lines and PR = with PR. ○, bacteria enclosed in cellophan tubing. Fig. 10 = *E. coli* strain B 15 (u.v.-sensitive), Fig. 11 = *E. coli strain* B 17 (u.v.-resistant).

($C$ = 0·6 $\mu$F., $L$ = 43 $\mu$H., spark gap 5 mm., i.e. 17.4 kV with electrodes in water, $s$ = 4·5 mm., iron electrodes, 91 J. per discharge) were used to compare the bactericidal effect of discharges in water and in air with that of continuous irradiation from an ordinary u.v. lamp (Philips TUV, 6 W., 2537 Å., distance to sample 15 cm.). The effect of submerged discharges was tested with 1200 ml. water and that of air discharges with 200 ml. water in the discharge vessel. In Figs. 10 and 11 the units along the abscissa signify number of discharges or minutes of u.v. treatment. Due to few colonies viable counts < 100 bacteria/ml. were not reliable. All the inactivation curves of *E. coli* strain B 15 ran fairly close (Fig. 10); the general tendency was that they were slightly concave upwards. With the units chosen the inactivation by air discharges was somewhat greater; the energy content of the air discharges was greater and the volume treated smaller than that of submerged discharges. The curves for *E. coli* strain B 17

(Fig. 11) were more convex upwards and did not follow each other so closely. The B 17 organisms were particularly sensitive to high doses of continuous u.v. radiation. The upward bend of the air-spark curve at low colony counts was presumably an artifact due to contamination from the part of the wall of the discharge vessel which had been located in the shadow.

*Photoreactivation*

*Escherichia coli* strains B 15 and B 17 inactivated by submerged discharges were reactivated by illumination with visible light (Figs. 10, 11). At comparable inactivation rates the magnitude of the reactivation of bacteria inactivated by discharges in the suspension was smaller than that of continuous u.v. irradiation and discharges in air. In contrast, bacteria inactivated by submerged discharges while enclosed in cellophan tubing were very liable to photoreactivation.

## DISCUSSION

As a consequence of earlier results (Edebo & Selin, 1968) experiments were designed to test the hypothesis that the electric arc used produced bactericidal quantities of u.v. radiation. Two different strains of *Escherichia coli* B were used: strain B 15 was u.v.-sensitive and strain B 17 was u.v.-resistant. When the bacteria were suspended in 0·001 M-KCl (Figs. 1, 3) or tap water (Figs. 2, 4) at concentrations less than $2{\cdot}5 \times 10^7$ bacteria/ml. the fraction of bacteria killed by one discharge was fairly constant. Strain B 15 was more susceptible than was strain B 17. The bactericidal effect per discharge was decreased at higher concentrations of bacteria and when albumin was dissolved in the discharge medium (Fig. 5). It was also decreased for bacterial suspensions enclosed in cellophan tubing and immersed in the discharge liquids, when u.v.-absorbing material was present in the discharge medium (Fig. 6). Consequently the protective effect of these materials was not a result of intimate contact between bacteria and protective agent but a neutralization of something passing through the discharge medium. Most likely the neutralization was caused by absorption of u.v. radiation, as the extent of the neutralization paralleled u.v. absorbance, and u.v. radiation has a strong microbicidal effect. Since the distance between the electrodes and the cellophan tubes was 65–75 mm., the radiation transmitted to the tubes with bacteria in a liquid with an absorbance/cm. (A/cm.) of 0·1 was 20 % and in one of 0·2 it was 4 % of that in 0·001 M-KCl. In agreement with this were the results (Fig. 6) that the bactericidal effect was moderately decreased at A/cm = 0·1 and almost extinguished at A/cm = 0·2. At the same absorbance coloured dextrans neutralized the bactericidal effect slightly more than the nucleic acids did; this might be due to their stronger absorption at wavelengths longer than 300 m$\mu$. Also *Saccharomyces cerevisiae* suspended in the discharge medium decreased the bactericidal effect. At the same absorbance the decrease by yeast suspensions was less than that of u.v.-absorbing solutions. The absorbance values of the yeast suspensions were not quite comparable to the values of the solutions, however, since light scattering by the yeast particles contributed to the extinction measured in the spectrophotometer. In addition, suspensions of carbon and of calcium carbonate decreased the bactericidal effect. Experiments showing that the bactericidal effect was smaller at greater distances from the spark, and that the killing effect was transmitted through and generated in air (Fig. 9), also supported the initial hypothesis.

However, the kinetics of the inactivation by discharges with an energy content of 620 J. (Figs. 3, 4) did not show the characteristics of u.v.-killing for *Escherichia coli* strain B 17. Assume that more than one u.v. quantum hit was necessary to kill a B 17 bacterium, and that some bacteria were always protected from direct u.v. radiation by the shadow cast by the nylon insulation of the electrodes. When the intensity of the radiation was very strong, almost all bacteria outside the shadow should have been killed. At the next discharge the process was repeated and as the discharge also brought about good mixing of the suspension a nearly straight inactivation curve should result. Since the u.v. radiation probably was very strong with 620 J. per discharge, the generally straight inactivation curves at this energy content (Figs. 3, 4) agreed with u.v. killing, although they did not show the usual u.v. inactivation kinetics. However, since the inactivation of u.v.-sensitive strain B 15 (Figs. 1, 2) exceeded that of u.v.-resistant strain B 17 (Figs. 3, 4) there could not be a well-defined shadow. The size of the arc, reflexion against the walls of the discharge vessel, and the presence of high concentrations of bacteria contribute to make the shadow less absolute. At lower energies (91 J.; Fig. 11), where the light intensity was less, the inactivation kinetics of discharges were more like those of continuous u.v. radiation.

Rentschler, Nagy & Mouromseff (1941) found that the same dose of u.v. radiation (2537 Å) given in periods ranging from a few microseconds to several hours yielded the same bactericidal effect, provided that the time of treatment did not involve an appreciable part of the life-cycle of the organism under exposure. Marcovich (1956), who studied the induction of *Escherichia coli* K 12 ($\lambda$) by u.v. radiation showed that the same dose given in 0·1 or 10,000 sec. had the same effect. In most biological systems the surviving fraction of a given dose is independent of the intensity of the incident radiation (Zelle & Hollaender, 1955). Consequently, the yield of active photons from the arc should be decisive for the microbicidal effect. One may, however, expect different kinds of cell damage by the different wavelengths (Zelle & Hollaender, 1955) which seem to be produced by discharges.

The biological effect of u.v. radiation is dependent on several mechanisms. A large proportion of the effect is caused by thymine dimerization in DNA. Cross-linking between DNA and messenger RNA or DNA and protein may also occur (Wacker, 1963; McLaren & Shugar, 1964). Another mechanism has been postulated (Witkin 1964). Radiation-resistant *Escherichia coli* B strains are probably capable of repairing or getting around the dimer blocks, whereas sensitive bacteria are not (Setlow, Swenson & Carrier, 1963). The repair processes are enhanced by 3000–5000 Å radiation which is called photoreactivation (PR). Two kinds of PR have been described, one of which is almost independent of the dose rate during the PR treatment (Jagger & Stafford, 1965). Assume that the smaller PR of bacteria inactivated by submerged discharges (Figs. 10, 11) was due to the fact that some PR did already take place during the discharge. If this were the case, this mechanism would be still more pronounced with bacteria in cellophan tubing, since, due to preferential absorption of light of shorter wavelengths by the water and the wall of the tube, the proportion of light of longer wavelengths was greater. Since the PR of bacteria inactivated in cellophan tubes often was even greater than that after continuous far ultraviolet (2537 Å) inactivation (Figs. 10, 11), the PR by discharges was probably of little importance.

Consider also the possibility that the smaller PR after submerged discharges was a consequence of uneven distribution of microbicidal photons in the suspension. Under

such conditions bacteria close to the spark might be hit so heavily that they could not be photoreactivated, and some bacteria would not be hit at all. Due to stirring of the suspension by each discharge the number of hits on each bacterium should be more nearly equal after several discharges. The relation between the u.v. radiation dose required for killing a certain fraction of the bacteria without and with PR may be expressed as the dose-reduction factor which in some systems is constant (Rupert, 1964). When *Escherichia coli* strain B 17 was inactivated by u.v. irradiation this factor was not constant but moderately decreasing at higher inactivation rates, being 0·5 at 10 min of u.v. treatment. At inactivation by discharges the factor was moderately increasing, being 0·8 at 10 discharges (Fig. 11). Since the number of photon hits on individual bacteria was more nearly equal after several discharges, these observations suggest that further microbicidal mechanisms, not subject to photoreactivation, exist. The observation that the bactericidal effect on bacteria suspended in 0·001 M-KCl and subjected to one discharge was increased on standing in the refrigerator overnight (Figs. 1, 3), showed that at least under such conditions photon radiation was not the only killing mechanism.

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# Effect of Hypocholesteremic Compounds on Bacterial Multiplication

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SUMMARY

Hypocholesteremic compounds, triparanol, SKF 525-A, SKF 3301-A, SKF 16467-A, inhibited the multiplication of some bacteria and ascomycetes; phycomycetes were not inhibited. Gram-positive bacteria were most sensitive to these compounds. Dimethyl or diethyl aminoethanol and SKF 2314, compounds resembling the major moieties of the SKF compounds, were not inhibitory alone or together; the whole molecule was necessary for inhibition. Inhibition by triparanol of the multiplication of several Gram-positive bacteria was annulled by albumin and ergosterol; oleic acid and squalene were mostly inactive.

## INTRODUCTION

Compounds which inhibit steroid metabolism in mammals ('hypocholesteremics') inhibit the multiplication of a wide variety of micro-organisms, especially Gram-positive bacteria (Aaronson, 1965; Martin-Smith & Sugrue, 1964; Smith, Shay & Doorenbas, 1963). We have examined the effect on bacterial multiplication of several related hypocholesteremic compounds to determine (*a*) whether the Gram-positive bacteria are most sensitive; (*b*) the active moiety of the inhibitory compounds; (*c*) compounds which annul the inhibition; (*d*) the potential of these compounds as antibacterial compounds; (*e*) whether the hypocholesteremic compounds can be used to study bacterial metabolism, especially the role of steroids in bacterial metabolism.

## METHODS

Micro-organisms from the collection of the Biology Department, Queens College, New York, were used. For disc assay, Whatman no. 50 filter-pad discs (0·6 cm. diam.) were soaked in solutions of the test compound at the concentration indicated, dried, and autoclaved. Bacteria were grown on nutrient agar; fungi on Sabouraud's glucose agar. Cultures in triplicate were incubated at 25° for 2 days or until they showed good growth over the surface of the Petri dish. Triparanol (1-[*p*-(4-diethylaminoethoxy)-phenyl]-1-(*p*-tolyl)-2-*p*-chlorophenylethanol) was supplied by Dr F. J. Murray (W. S. Merrell, Cincinanti, Ohio); the SKF compounds by Dr W. L. Holmes (Smith Kline and French Laboratories, Philadelphia, Pa.). All other chemicals were bought from commercial sources. Triparanol, SKF-2314, oleic acid, squalene, and ergosterol were dissolved in 95% ethanol in water. Other compounds were dissolved in water.

Table 1. *Inhibition of bacterial multiplication by hypocholesteremic compounds*

| Bacteria | Triparanol | SKF compounds* 525-A | SKF compounds* 3301–A | SKF compounds* 16467-A | SKF compounds* 2314 | Amino-ethanols† |
|---|---|---|---|---|---|---|
| | Size of zone of inhibition (cm) | | | | | |
| Gram-negative | | | | | | |
| *Aerobacter aerogenes* | 0 | 1·2±0·2 | 2·1±0·2 | 1·7±0·2 | 0 | 0 |
| *Escherichia coli* | 0 | 0 | 0 | 0 | 0 | 0 |
| *Proteus mirabilis* | 0 | 1·1±0·2 | 1·5±0·1 | 0 | 0 | 0 |
| *Pseudomonas putida* | 0 | 0 | 1·1±0·1 | 0 | 0 | 0 |
| *P. reptilia* | 0 | 1·0±0·1 | 1·8±0·1 | 0·8±0·1 | 0 | 0 |
| *P. vulgaris* | 0 | 0 | 1·8±0·2 | 1·4±0·1 | 0 | 0 |
| Gram-positive | | | | | | |
| *Bacillus cereus* | 0·8±0·1 | 1·0±0·2 | 1·8±0 | 0·8±0·1 | 0 | 0 |
| *B. circulans* | 1·4±0·2 | 1·8±0·1 | 3·1±0·1 | 2·1±0·6 | 0 | 0 |
| *B. fusiformis* | 1·0±0·3 | 1·0±0·1 | 1·9±0·4 | 0·8±0 | 0 | 0 |
| *B. mycoides* | 1·1±0·3 | 1·9±0·4 | 1·9±0·5 | 1·4±0·6 | 0 | 0 |
| *B. subtilis* | 1·1±0·6 | 2·0±0·1 | 2·7±0·2 | 0·9±0·1 | 0 | 0 |
| *Micrococcus luteus* | 1·8±0·2 | 1·8±0·1 | 2·6±0·4 | 1·5±0·3 | 0 | 0 |
| *M. lysodeikticus* | 0·8±0·2 | 1·9±0·1 | 2·2±0·2 | 0·7±0·1 | 0 | 0 |
| *M. roseus* | 2·0±0·3 | 1·6±0·1 | 2·1±0·3 | 0·9±0·1 | 0 | 0 |
| *M. sodonensis* | 1·5±0·3 | 1·9±0·5 | 2·2±0·7 | 0·8±0·1 | 0 | 0 |
| *Mycobacterium phlei* | 2·0±0·4 | 2·9±0·6 | 3·3±0·5 | 1·6±0·6 | 0 | 0 |
| *My. smegmatis* | 1·1±0·2 | 2·0±0·5 | 2·4±0·3 | 1·2±0·2 | 0 | 0 |
| *Sarcina flava* | 1·1±0·3 | 1·7±0·4 | 2·1±0·1 | 1·1±0·2 | 0 | 0 |
| *Staphylococcus albus* | 0·9±0·2 | 0·8±0·1 | 1·5±0·1 | 1·1±0 | 0 | 0 |
| *S. aureus* | 1·2±0·2 | 1·3±0·1 | 1·6±0·1 | 0 | 0 | 0 |
| *S. citreus* | 1·5±0·5 | 1·3±0·2 | 2·0±1·0 | 0 | 0 | 0 |

* Discs soaked in solution (0·1 M) of compound.

† Dimethylaminoethanol, diethylaminoethanol, dimethyl-3-aminopropanol, diethyl-3-aminopropanol, dimethyl-2-aminopropanol, diethyl-1-aminopropanol.

Table 2. *Effect of concentration on inhibition*

| Compound | Discs soaked in conc. (M) | *Bacillus subtilis* | *Micrococcus sodonensis* | *Mycobacterium smegmatis* |
|---|---|---|---|---|
| | | Size of zone of inhibition (cm) | | |
| Triparanol | 0·1 | 1·2±0·2 | 1·5±0·2 | 2·3±0·2 |
| | 0·05 | 0·9±0·1 | 1·4±0·6 | 2·0±0·2 |
| | 0·01 | 1·2±0·1 | 1·7±0·3 | 2·2±0·2 |
| | 0·005 | 1·1±0·2 | 1·5±0·3 | 2·4±0·2 |
| | 0·001 | 0 | 0·9±0·2 | 1·4±0·4 |
| SKF 525-A | 0·1 | 1·8±0·2 | 1·9±0·2 | 2·3±0·5 |
| | 0·05 | 1·4±0·3 | 1·2±0·3 | 2·1±0·1 |
| | 0·01 | 1·2±0·5 | 0 | 1·3±0·2 |
| | 0·005 | 0 | 0 | 0·9±0·1 |
| | 0·001 | 0 | 0 | 0 |
| SKF 3301-A | 0·1 | 2·4±0·8 | 2·0±0·1 | 3·6±0·4 |
| | 0·05 | 2·1±0·6 | 1·5±0·4 | 2·8±0·3 |
| | 0·01 | 0 | 0·7±0·1 | 1·0±0·2 |
| | 0·005 | 0 | 0·7±0 | 0 |
| | 0·001 | 0 | 0·7±0 | 0 |

RESULTS

Earlier work (Aaronson, 1965) with two hypocholesteremic compounds (triparanol, benzmalecene) showed that Gram-positive bacteria were more sensitive than Gram-negative bacteria. Triparanol again inhibited only Gram-positive bacteria (present work) while the SKF compounds (525-A, 3301-A, 16467-A) inhibited Gram-positive and Gram-negative bacteria; the Gram-positive bacteria were more sensitive to the SKF compounds (Table 1). The bacteria sensitive to the hypocholesteremic compounds varied in sensitivity to the several compounds (Table 2). As with triparanol (Aaronson, 1965) several of the SKF compounds inhibited ascomycetes but not phycomycetes (Table 3). The inhibitory SKF compounds have aromatic and aliphatic parts (Fig. 1). Neither the aromatic moiety (SKF 2314) nor the aliphatic moiety (dimethyl- or diethyl-aminoethanols or their analogues) inhibited bacterial multiplica-

Table 3. *Inhibition of fungal multiplication by hypocholesteremic compounds*

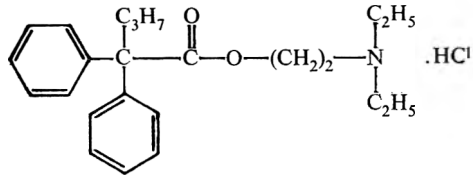
| Fungi | Triparanol | SKF compounds* 525-A | 3301-A | 16467-A | 2314 | Amino-ethanols |
|---|---|---|---|---|---|---|
| | Size of zone of inhibition (cm) | | | | | |
| Ascomycetes | | | | | | |
| *Hansenula saturans* | 1·3±0·1 | 1·0±0·1 | 0·9±0·3 | 0 | 0 | 0 |
| *Neurospora crassa* | 1·5±0·1 | 0 | 0 | 0 | 0 | 0 |
| *Penicillium roqueforti* | 1·6±0·3 | 0 | 0 | 0 | 0 | 0 |
| *Rhodotorula rubra* | 1·6±0·1 | 1·0±0·1 | 0·9±0·2 | 0 | 0 | 0 |
| Phycomycetes | | | | | | |
| *Aspergillus niger* | 0 | 0 | 0 | 0 | 0 | 0 |
| *Synecophalotium nigricans* | 0 | 0 | 0 | 0 | 0 | 0 |

* Discs soaked in 0·1 M solution of compound.

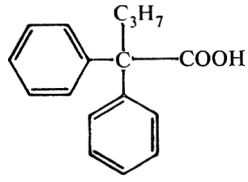
Table 4. *Annulment of inhibition by triparanol*

| Gram-positive | Triparanol | Triparanol + albumin | Triparanol + oleic acid | Triparanol + squalene | Triparanol + ergosterol |
|---|---|---|---|---|---|
| | Size of zone of inhibition (cm.) | | | | |
| *Bacillus cereus* | 0·8±0·1 | 0 | 0·9±0 | . | . |
| *B. circulans* | 1·4±0·2 | 0·8±0·1 | 1·8±0·1 | 1·6±0·2 | . |
| *B. fusiformis* | 1·0±0·3 | 0 | 1·3±0·2 | . | 0 |
| *B. mycoides* | 1·1±0·3 | 0 | 0·9±0·2 | . | 0·7±0 |
| *B. subtilis* | 1·1±0·6 | 0 | 0·9±0·2 | 1·2±0·4 | 0 |
| *Micrococcus luteus* | 1·8±0·2 | 0 | 2·0±0·8 | 1·4±0·1 | 0·7±0·1 |
| *M. lysodeikticus* | 0·8±0·2 | 0 | 1·1±0·2 | 0·7±0 | 0 |
| *M. roseus* | 2·0±0·3 | 0·8±0 | . | 1·7±0·2 | 0 |
| *M. sodonensis* | 1·5±0·3 | 0 | . | 1·3±0·1 | 0·7±0·1 |
| *Mycobacterium phlei* | 2·0±0·4 | 0·8±0·2 | 1·8±0·2 | 2·7±0·3 | 0·9±0 |
| *My. smegmatis* | 1·1±0·2 | 0 | 1·0±0·1 | 1·8±0·4 | 1·0±0·1 |
| *Sarcina flava* | 1·1±0·3 | 0 | 1·1±0·1 | 1·1±0·2 | 0 |
| *Staphylococcus albus* | 0·9±0·2 | 0 | 0 | 1·0±0·1 | . |
| *S. aureus* | 1·2±0·2 | 0·7±0 | 1·1±0·1 | . | 0·8±0 |
| *S. citreus* | 1·5±0·5 | 0 | 1·9±0·7 | 2·0±0·2 | . |

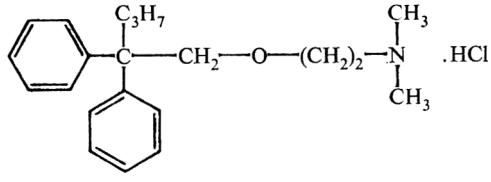
Concentrations used: triparanol, 0·1 M; oleic acid, 0·1 M; squalene, 0·1 M; ergosterol, 0·01 M; bovine serum albumin, 0·2 %.



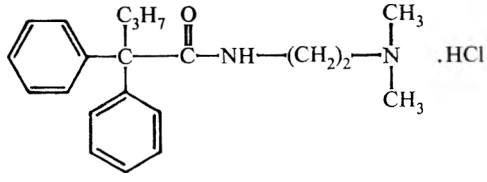
β-Diethylaminoethyl-diphenylpropyl acetate.HCl (SKF 525-A)



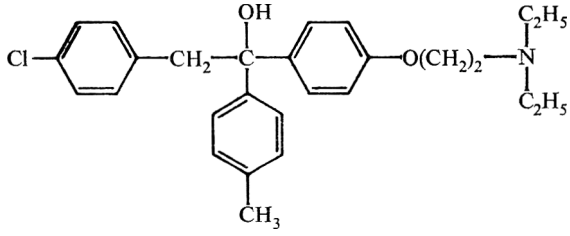
Diphenylpropylacetic acid (SKF 2314)



2,2-Diphenyl-1-(β-dimethylaminoethoxy)pentane.HCl (SKF 3301-A)



β-Dimethylaminoethylamino-diphenylpropylacetate.HCl (SKF 16467-A)



1-[4-Diethylaminoethoxy)phenyl]-1-(*p*-tolyl)-2-(*p*-chlorophenyl) ethanol (Triparanol)

Fig. 1. The structure of several hypocholesteremic compounds.

tion at 0·1 M, a concentration at which the other SKF compounds were inhibitory. Discs containing SKF 2314 or diethylaminoethanol added on top of each other or discs soaked in SKF 2314+diethylaminoethanol were inactive.

In previous work with a photosynthetic bacterium oleic acid, ergosterol and squalene annulled the inhibition by triparanol (Aaronson, 1964). Oleic acid was not as effective

in preventing the inhibition of multiplication by triparanol as were albumin or ergosterol, while squalene seemed to enhance the inhibition by triparanol (Table 4). Alone, none of these lipids or albumin was inhibitory at the concentrations used.

## DISCUSSION

Gram-positive bacteria are more inhibited by hypocholesteremic compounds than are Gram-negative bacteria (Aaronson, 1965; Martin-Smith & Sugrue, 1964; Smith *et al.* 1963). The results here with the SKF compounds (Table 1) bear this out. Of the several microbial groups exposed to the SKF compounds, bacteria, ascomycetes, protozoa (Aaronson, 1966) but not phycomycetes were sensitive. These results are like those obtained with triparanol and benzmalecene (Aaronson, 1965). While the combined aliphatic and aromatic moieties, i.e. SKF 525-A, SKF 3301-A, seem necessary for bacterial inhibition (see Table 1), these compounds acted strikingly differently with the eucaryotic phytoflagellate *Ochromonas danica* (Aaronson, 1966) where the aromatic moiety, i.e. SKF-2314, but not the aliphatic moiety, i.e. diethylaminoethanol, was as inhibitory as SKF-525A. The sensitivity of bacteria to a disc soaked in as little as 1 mM of the compound and to a variety of hypocholesteremic compounds, i.e. azasteroids (Martin-Smith & Sugrue, 1964; Smith *et al.* 1963), triparanol and benzmalecene (Aaronson, 1965) and SKF 525-A, SKF 3301-A, and SKF 16467-A, implies that these compounds may offer leads to compounds which inhibit pathogenic bacteria, especially the Gram-positive ones, as well as other pathogens.

Several workers have reported that unsaturated fatty acids (e.g. oleic and linoleic acids) inhibit multiplication of Gram-positive but not Gram-negative bacteria (Kodicek, 1956; Kodicek & Worden, 1945; McQuillen, 1958). McQuillen (1958) found that this inhibition was prevented by lecithin, several steroids, and $\alpha$-tocopherol for *Lactobacillus helveticus*, while vitamin $D_2$ annulled the inhibition of *L. casei* by linoleic acid. McQuillen (1958) observed that linoleic acid inhibited the uptake in *L. casei* of $^{14}C$-labelled metabolites such as glutamic acid, lysine, glucose, phenylalanine, adenine and uracil in fixed cellular form (proteins of membranes, etc.) but had far less effect on their uptake into the cellular pools. Vitamin $D_2$ prevented the inhibition by linoleic acid of the uptake of the compounds into fixed form. Like the inhibition by unsaturated fatty acids, the inhibition of Gram-positive bacteria by triparanol was annulled by ergosterol and also by albumin. While earlier workers did not report on albumin (a well-known annuller of the inhibition of mitochondrial activity by unsaturated fatty acids) it may well be that triparanol (and perhaps other hypocholesteremics) and unsaturated fatty acids inhibit Gram-positive bacteria at the same site, and that these inhibitions are annulled by albumin or steroids. The nature of this site and the reason for the lack of sensitivity of Gram-negative bacteria remain to be determined. It is provocative that steroids annul the inhibition of multiplication by hypocholesteremics in procaryota, which are not supposed to synthesize sterol, while only unsaturated fatty acids annul the inhibition by hypocholesteremics in the eucaryotic Ochromonas, which does synthesize steroids (Aaronson, 1965, 1966).

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# Giant Carbohydrate-rich Cells of the Dinoflagellate *Gonyaulax monilata*

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(*Accepted for publication* 1 *August* 1967)

SUMMARY

A small number of organisms in cultures of the dinoflagellate *Gonyaulax monilata* attained twice the diameter of normal organisms and showed an excessive accumulation of carbohydrate reserves. The nuclei of these giants degenerated as did the cytoplasm. It is suggested that this was not a response to cultural conditions but represented an altered metabolism of individual organisms.

## INTRODUCTION

During a study of the nuclear structure and mitosis in the dinoflagellate *Gonyaulax monilata*, it was observed that a small percentage of the organisms in actively growing cultures showed an excessive accumulation of carbohydrate reserves and a loss of normal nuclear structure. A comparison between the morphology of normal and carbohydrate-rich forms is presented here.

## METHODS

Cultures of *Gonyaulax monilata* were supplied by Dr L. Provasoli from the Haskins Collection (Haskins Laboratories, New York City) and were maintained axenically on ST3 medium (Tatewaki & Provasoli, 1964) under a 12 hr light, 12 hr dark cycle. The method of culture, staining procedures, preparation of Epon and paraffin sections were as previously described (Chunosoff & Hirshfield, 1967).

## RESULTS

The normal *Gonyaulax monilata* contained a moderate number of large periodic acid–Schiff (PAS)-positive granules situated around the periphery of the organism. Often there was a concentration of these granules between the convex border of the nucleus and the cell wall (Pl. 1, fig. 1). Even when few other PAS-positive granules were present there was usually a crescent-shaped deeply staining area which was flush with the cell wall at the sulcus (Pl. 1, fig. 1). This is the general area of the origins of the flagella.

The PAS-positive granules were easily thrown to one end of the organism by

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centrifugation at moderate speed for a few minutes. Staining of centrifuged organisms for nucleic acids, carbohydrates and protein (Himes & Moriber, 1956) showed that when the carbohydrate granules were few or moderate in number, the organism was filled with a strongly staining cytoplasm (Pl. 1, fig. 2).

The normal interphase nucleus had a shallow U-shape. As is the case with other dinoflagellates (Dodge, 1966) the chromatin material within the nucleus was condensed into thick discrete chromosomes. In *Gonyaulax monilata* these chromosomes run parallel to each other in a tightly packed bundle (Chunosoff & Hirshfield, 1967). A small proportion of the organisms (estimated to be less than 0·5 %) in young as well as older cultures attained a diameter of 100 $\mu$, twice the normal diameter, and were filled with PAS-positive granules (Pl. 1, fig. 3). Feulgen-alkaline fast-green-stained preparations of these giant forms showed that the nuclei had lost their normal morphology and appeared as irregularly shaped masses of chromatin. These were usually pushed to one side of the organism by the excessive number of carbohydrate granules (Pl. 1, fig. 4). Only a small amount of cytoplasm was present in these forms and was concentrated around the nucleus (Pl. 1, fig. 4). In extreme cases the nucleus had completely degenerated and no cytoplasm was detectable. PAS-stained 1 $\mu$ thick sections of Epon-embedded organisms showed that the granules of both normal and giant forms had colourless centres (Pl. 1, figs. 5, 6). Forms with excessive carbohydrate reserves were never observed to divide.

## DISCUSSION

The phenomenon of excessive accumulation of carbohydrate reserves has been observed in several algae when they were grown under heterotrophic conditions and in the presence of glucose (Taylor, 1950; Griffiths, 1965; Rodriguez-Lopez, 1963, 1966). Chlorella grown in a glucose medium increased to two times the normal size and showed a disorganization of chloroplast structure (Rodriguez-Lopez, 1963, 1965). In *Gonyaulax monilata* accumulation of excessive carbohydrate reserves was accompanied by the degeneration of both nucleus and cytoplasm. However, only a few organisms in a culture exhibited this phenomenon, indicating that 'giantism' is not a general response to the carbohydrate source (glucose) in the medium; it seems more probably to be the result of an altered metabolism of individual organisms.

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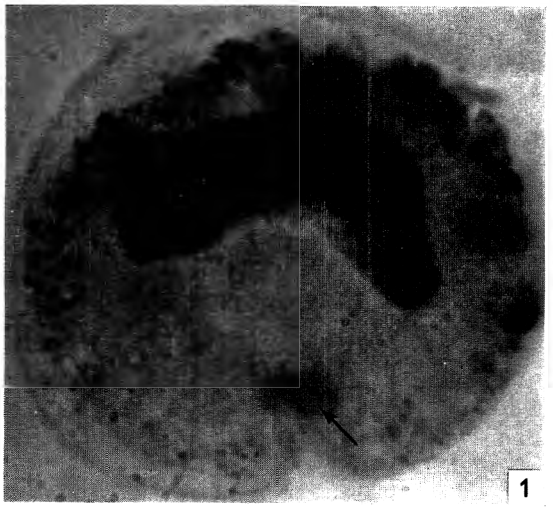


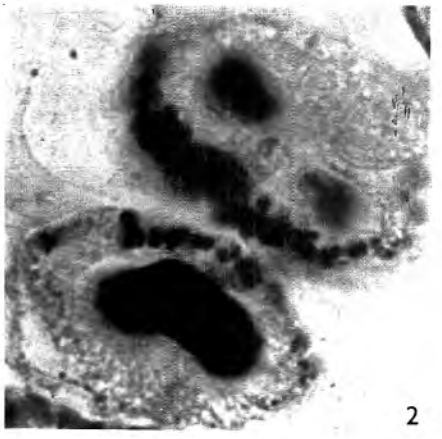


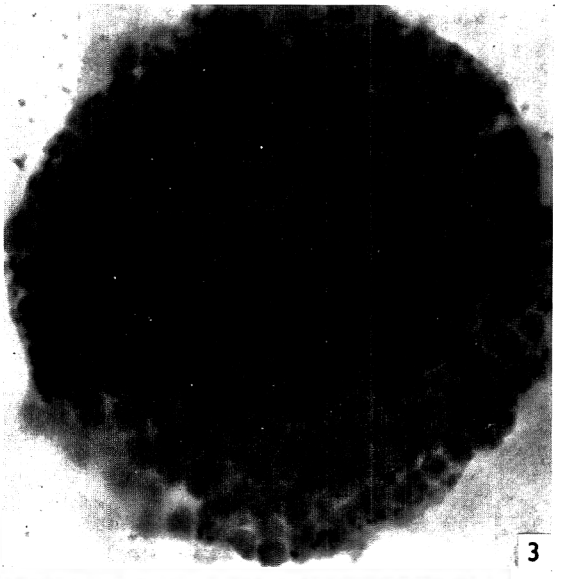


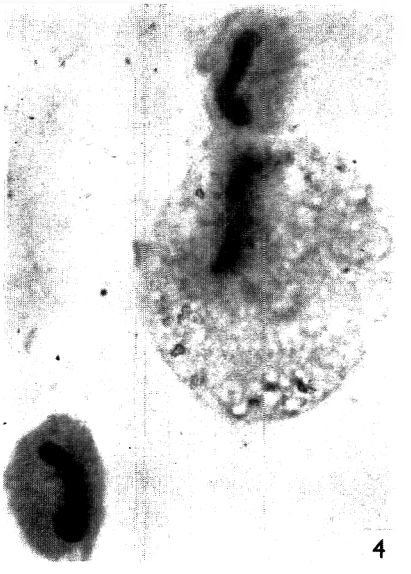


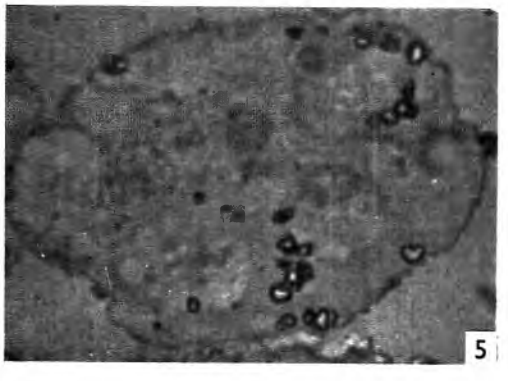


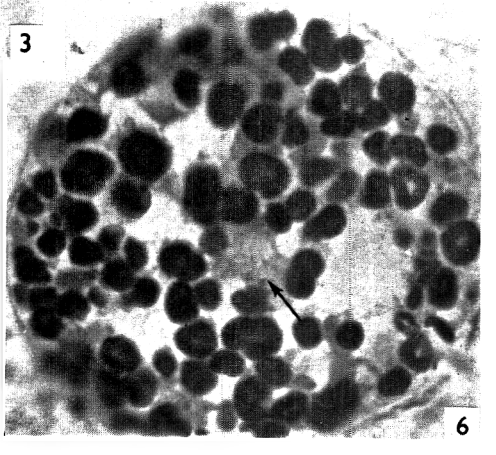


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## EXPLANATION OF PLATE

*Gonyaulax monilata*

Fig. 1. PAS preparation of normal organism showing carbohydrate granules at periphery and at sulcus (arrow). × 1300.

Fig. 2. Organisms in paraffin section stained according to Himes-Moriber technique showing PAS-positive bodies, nuclei and cytoplasm. × 1300.

Fig. 3. Giant carbohydrate-rich organism showing numerous PAS-positive granules. Whole mount. × 750.

Fig. 4. Feulgen-alkaline fast-green preparation of giant form (100 $\mu$ diameter). The cytoplasm is concentrated around the nucleus. Normal forms are above and below it. × 460.

Fig. 5. Section (1 $\mu$) of Epon-embedded normal organism showing cytoplasm and structure of granules. PAS stained. × 1300.

Fig. 6. Section (1 $\mu$) of Epon-embedded giant form showing structure of granules. PAS stained. A fragment of the nucleus is present (arrow); no cytoplasm visible. × 1300.

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# The Effects of Chloramphenicol and Actinomycin D on the Nucleus of the Dinoflagellate *Gonyaulax monilata*

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(*Accepted for publication* 1 *August* 1967)

SUMMARY

The effects of two drugs (chloramphenicol, actinomycin D) which inhibit protein synthesis on the nucleus of the dinoflagellate *Gonyaulax monilata* were examined. Chloramphenicol had no effect on the morphology of the nucleus but caused a diminution of cytoplasmic protein and a suppression of growth; it did not completely suppress mitosis. Treatment with actinomycin D resulted in the loss of chromosomal arrangement within the nucleus, the fraying of the edges of the nucleus and the loss of the nuclear 'band', and suppressed division. The results are thought to support the hypotheses that the matrix functions in maintaining nuclear morphology and that the 'band' is necessary for mitosis.

## INTRODUCTION

In a previous paper (Chunosoff & Hirshfield, 1967) nuclear structure and division in the dinoflagellate *Gonyaulax monilata* were described. Between the arms of the shallow U-shaped nucleus is a band of material which, it was proposed, is similar to the 'central body' described by Dodge (1964) in *G. tamarensis*. During division, the band is a point of attachment for the chromosomes, suggesting that this band is a component of the mitotic apparatus. The band is composed of protein and possibly of RNA. As is the case with other dinoflagellates (Dodge, 1963) the chromosomes remain condensed throughout interphase. In *G. monilata* the chromosomes are arranged parallel to each other in a tightly packed bundle. A protein matrix pervades the nucleus and surrounds the individual chromosome. It was suggested that this matrix is a factor in maintaining the arrangement of chromosomes within the nucleus and, in the absence of a nuclear membrane, nuclear form. To test the hypotheses that the band is a component of the mitotic apparatus and that the matrix functions in maintaining nuclear morphology, the effects on the nucleus of two drugs, which inhibit protein synthesis, namely chloramphenicol and actinomycin D, were investigated.

## METHODS

The methods of growing *Gonyaulax monilata*, fixing and staining of whole organisms with alkaline fast green and according to the Feulgen technique, preparation and staining of extruded nuclei (nuclei removed from cells) and extraction of DNA with

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trichloroacetic acid were as previously described (Chunosoff & Hirshfield, 1967) For the drug studies 10 ml. samples of 10-day stock cultures (this was during the period of rapid division) were distributed with sterile precautions in screw-capped tubes. The drugs were dissolved in culture medium in such concentrations that by adding 0·5 ml. of drug solution to the culture tubes final concentrations of chloramphenicol 32 $\mu$g./ml. and actinomycin D 2 $\mu$g./ml. were obtained. Control tubes were prepared by adding 0·5 ml. sterile medium. The tubes were incubated in a 12 hr light, 12 hr dark cycle for 96 hr. During this time the cultures were observed for division and counts were made. At the end of 96 hr the organisms were fixed and stained.

## RESULTS

### *Normal nuclear morphology*

In Pl. 1, fig. 1 the features of a normal interphase nucleus as described in the introduction are shown. The Feulgen-positive chromosomes are condensed and are parallel to each other. In this organism the alkaline fast-green positive band (arrow) extending between the arms of the nucleus had separated from the chromatin throughout most of its length and was attached to the chromosomes only at its ends. The protein matrix was obscured by the chromosomes.

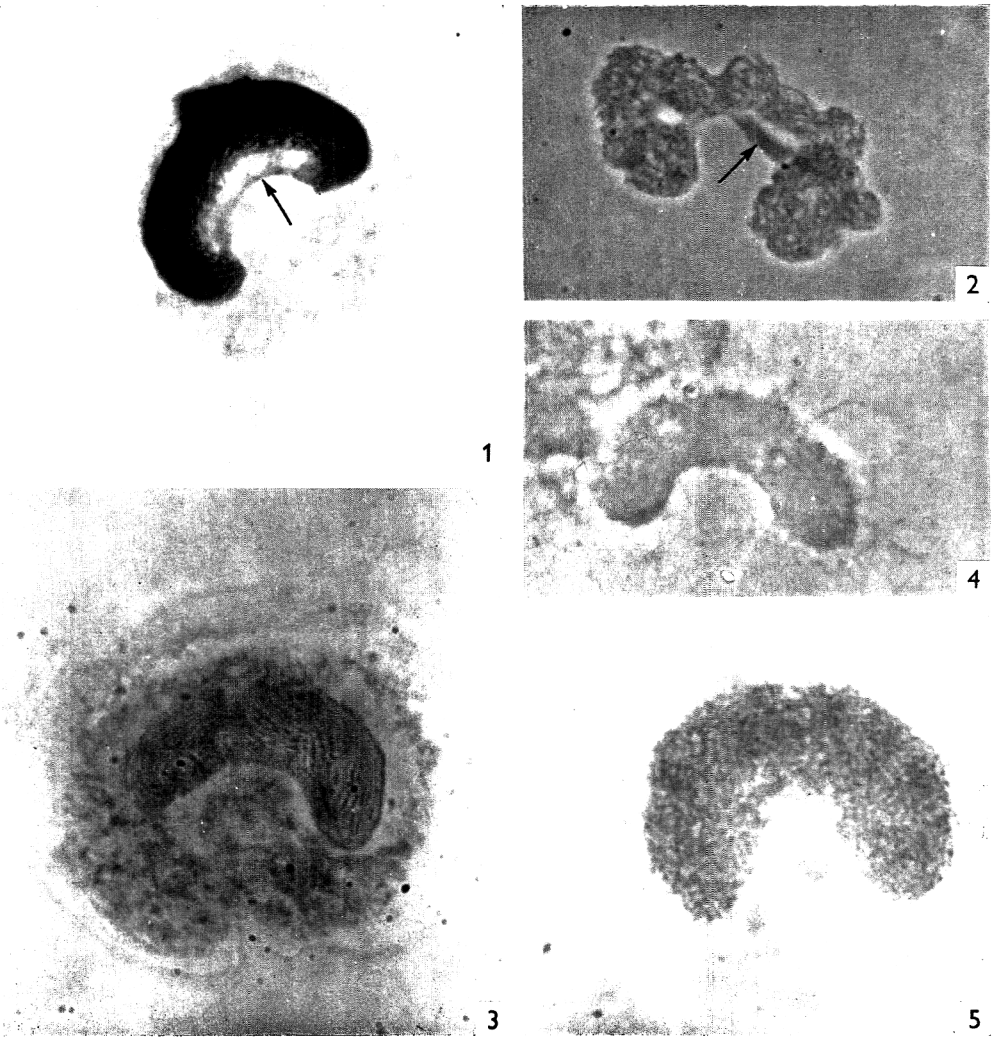
### *Drug studies*

Chloramphenicol caused a depression of growth of *Gonyaulax monilata*. The effect, however, was not primarily due to the inhibition of mitosis since some organisms in division were observed throughout the 96 hr of the experiment. Staining of extruded nuclei (Pl. 1, fig. 2) and whole organisms showed that the protein matrix, the band and chromosomal morphology were unaltered by treatment with chloramphenicol. In many organisms a diminution of cytoplasmic protein was indicated by staining with alkaline fast green. In these organisms (Pl. 1, fig. 3) the protein matrix, plainly reflecting the pattern of the chromosomes, was clearly evident and not, as in control organisms, obscured by cytoplasmic protein.

Cultures treated with actinomycin D showed a slight increase in numbers of organisms within the first 48 hr and then a marked decrease. Unlike cultures treated with chloromaphenicol, no organisms in division were observed after the first 48 hr. Staining of extruded nuclei with alkaline fast green (Pl. 1, fig. 4) indicated that no bands were present and that the protein matrix had lost its patterned appearance and was present as a homogeneous mass. Staining of whole organisms according to the Feulgen technique showed that the chromosomes had lost their parallel arrangement and were distinctly contorted (Pl. 1, fig. 5). Portions of chromosomes were seen to extend into the cytoplasm giving the edges of the nucleus an irregular appearance (Pl. 1, fig. 5).

## DISCUSSION

Chloramphenicol inhibits the synthesis of protein by interfering with the attachment of messenger RNA to the ribosomes (Rendi & Ochoa, 1962). As might be expected, chloramphenicol had the effect of decreasing the amount of cytoplasmic protein and depressing growth of *Gonyaulax monilata*. It did not eliminate the capacity of the organisms to divide and did not affect nuclear morphology. The band, the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matrix and the chromosomes remained intact. This suggests that the protein components of the nucleus (matrix and band) are not dependent on protein produced in the cytoplasm for their maintenance.

Actinomycin D has two modes of action. It suppresses histone protein within the nucleus (Honig & Rabinovitz, 1966) and inhibits synthesis of DNA dependent RNA (Reich, Franklin, Shatkin & Tatum, 1962; Goldberg & Rabinowitz, 1962). The fact that treatment with actinomycin D caused a loss of chromosomal orientation and a fraying of the edges of the nucleus tends to support the suggestion that the protein matrix is a factor in maintaining nuclear morphology. Also, the concomitant loss of the band and suppression of division is consistent with the suggestion that the presence of the band is necessary for division and that it may be a component of the mitotic apparatus. It would be interesting to determine which of the modes of action of actinomycin D caused the alteration of nuclear morphology, i.e. whether it was due to a direct effect on the protein or whether it was a secondary effect caused by the suppression of nuclear RNA. The latter possibility would suggest that the matrix and the band were synthesized in the nucleus itself.

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## EXPLANATION OF PLATE

*Gonyaulax monilata*

Fig. 1. A normal interphase organism stained according to the Feulgen technique and counterstained with alkaline fast green. × 1300.

Fig. 2. Extruded nucleus of a chloramphenicol-treated organism stained with alkaline fast green. The DNA had been extracted with trichloroacetic acid. The band is evident (arrow) and the protein matrix reflects the pattern of the chromosomes. × 1300.

Fig. 3. Chloramphenicol-treated organisms stained with alkaline fast green. The DNA has been removed with trichloroacetic acid. The matrix of the nucleus is visible in the lightly staining cytoplasm. × 1300.

Fig. 4. Extruded nucleus of an organism treated with actinomycin D stained with alkaline fast green. No band is present and the matrix has lost its patterned appearance. × 1300.

Fig. 5. Feulgen preparation of a whole organism treated with actinomycin D. The edges of the nucleus are irregular and the chromosomes have lost their orientation. × 1300.

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# *Aspergillus niger* Technique for the Bioassay of Manganese

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(*Accepted for publication* 3 *August* 1967)

SUMMARY

The rates of growth of five strains of *Aspergillus niger* (M, MUBL I, NRRL 334, NRRL 323, NRRL 346) were followed in an optimum nutrient solution. The time course of growth of all the strains showed a steep decrease after 3 days, except that of strain NRRL 346 which tended to flatten out. A correlation between sporulation and mycelial dry weight was observed. All the five strains showed a marked decrease in mycelial yield in the absence of manganese from the basal medium. Their quantitative responses to graded concentrations of Mn in the medium were compared with spore and mycelial inocula. Statistical analyses of the result suggest that strain MUBL I is the most suitable test organism for the bioassay of Mn. The effective range of concentrations of Mn which coincided with the maximum differences in mycelial dry weights was 0·001–0·006 $\mu$g./ml.

## INTRODUCTION

Many attempts have been made in the past two decades to study the need for trace elements for the growth of micro-organisms in general and fungi in particular. The use of fungi as test organisms for the assay of trace metals has come into vogue because of their specific response to deficiency of individual trace metals in the nutrient medium. Several strains of *Aspergillus niger* have been used in the assay of trace elements under various physical and physiological conditions (Mulder, 1948; Nicholas & Fielding, 1951; Nicholas, 1952; Donald, Passey & Swaby, 1952*a*; Saraswathi-Devi, 1962). The lack of uniformity in experimental conditions has made comparison of the behaviour of various strains to deficiencies of trace metals impossible. Furthermore, since purification of nutrient solutions is an essential prerequisite for obtaining precise deficiency symptoms, attention has been mainly devoted to the methods of purification and little recognition has been given to the fact that comparison between the quantitative response of organisms under physiologically indeterminate conditions would not be of great value. The need for a balanced optimum solution for growth, containing a minimum quantity of constituents and with a composition subject to exact definition on the basis of physiological response was emphasized by Steinberg & Bowling (1939). Such a nutrient solution provides an accurate basis for the comparison of the quantitative response of different strains of the same organism within a defined physical environment. An optimum nutrient solution has been used in the present work to compare the response of five strains of *A. niger* to a graded range of concentrations of manganese in the nutrient solution with a view to selecting a strain for the bioassay of this element.

Bioassay methods are in general based on the observation that in the absence of an essential mineral element growth is retarded as shown by a decrease in mycelial dry

weight. Bioassay of manganese has so far been done by visual comparison of mycelial characters and spore cover between the standards and the unknown since decrease in growth in the absence of manganese could not be obtained (Nicholas, 1952) although Steinberg (1939) reported clear-cut decrease in growth of *Aspergillus niger* in manganese-deficient medium. An attempt was made in the present work to enquire whether it is possible to obtain decreases in mycelial yields in manganese-deficient cultures in an otherwise optimum nutrient solution, by using the purification procedure of Donald *et al.* (1952*b*). and whether the growth of the organism is reproducibly and quantitatively proportional to graded increments in the concentration of manganese. When decrease in growth is considered as the criterion for examining the need for a micronutrient element, a knowledge of the rate of growth and the relationship between growth and reproduction is necessary as comparisons are best made during the phase of maximal growth. Hence the rates of growth of the five strains of *A. niger* were also studied.

## METHODS

The following strains of *Aspergillus niger* were used:

M, Mulder's strain—culture received from Centraal Bureau voor Schimmelcultures, Baarn, Holland.

MUBL 1, isolated from soil at the University Botany Laboratory, Madras and used previously in this Laboratory for micronutrient element deficiency studies.

NRRL 334, the original no. Thom. 4247 used by Steinberg in his studies on physiology of *Aspergillus niger* with special reference to heavy metal nutrition.

NRRL 323, used for soil testing by Niklas & Hirschberger (1924).

NRRL 346, characterized by abundant production of sclerotia.

The basal medium contained (g./l.): ammonium nitrate, 2·06; monopotassium phosphate, 0·55; $MgSO_4.7H_2O$, 0·25; sucrose, 50 (Steinberg, 1939). The trace element composition of the medium was as follows (mg./l.): Fe, 0·20; Zn, 0·20; Cu, 0·04; Mn, 0·02; Mo, 0·01. The basal medium was made up with double-distilled water and was initially purified from trace elements by adsorption with aluminium oxide according to the method of Donald *et al.* (1952*b*). The medium at pH 7·2 with 0·5% (w/v) $Al_2O_3$ (chromatographic analysis grade) was autoclaved at 115° for 20 min. The medium was swirled while still hot and then allowed to stand overnight; it was then filtered through Whatman no. 42 by gravity filtration. The filtered medium was then adjusted to pH 7·5 and distributed into 100 ml. Erlenmeyer flasks; each flask had 15 ml. medium containing the appropriate concentration of trace elements. The trace element stock solutions and dilutions were made in triple-distilled water. The 'minus all' trace-elements medium was the purified basal medium without any added trace element, the 'plus-all' medium contained all the trace elements, and the 'minus Mn' medium had all the trace elements except manganese. The media were autoclaved at 121° for 15 min. The chemicals used were of the Analar or Merck G.R. grade and water was distilled in all-glass Pyrex stills. All the precautions which were essential for the *Aspergillus niger* technique (Saraswathi-Devi, 1958) were observed.

Spore inoculum was prepared by shaking 3 loops of spores from 5-day cultures on a potato glucose agar slope into 5 ml. of sterile triple-distilled water; each flask received three drops of this spore suspension. Mycelial inoculum was prepared by growing the

fungus in 'minus all' medium for 3 days. The mycelium was then filtered, washed and broken with Pyrex glass beads in 10 ml. of sterile distilled water; three drops of this suspension were added to each flask. The flasks were randomized and incubated at $35 \pm 0{\cdot}2°$. The cultures were filtered at appropriate times and the mats dried at 80° to constant weight.

Since only three replicates could be maintained for each concentration of Mn in one experiment, the above experiments were repeated and essentially similar results were obtained.

The rate of growth was measured by recording the mycelial dry weight at 24 hr intervals for 6 days. Statistical analyses were done according to Paterson (1939) and the regression analysis was done according to Bailey (1961) and Freeman (1956). The best-fitting straight line $yr = a + bx$ was constructed by the method of least squares, where $b$ is known as the regression coefficient of $y$ on $x$. The test of significance used was the conventional $F$ test at 5% and 1%.

## RESULTS

The time course of growth of all the five strains was characterized by three distinct phases: (1) a phase of little or no apparent growth extending for 24 h after inoculation; (2) a phase of rapid and approximately linear growth between 24 and 72 h; (3) a phase of no net growth or of decrease in dry weight after 72 h (Fig. 1). This is typical of many filamentous fungi grown in liquid culture (Cochrane, 1958). Sporulation started

Table 1. *Rate of growth of five strains of Aspergillus niger*

| Incubation (days) | Strain | | | | |
|---|---|---|---|---|---|
| | NRRL 346 | NRRL 334 | NRRL 323 | MUBL 1 | M |
| | Mycelial dry weight (mg.) | | | | |
| 1 | 7 ± 6* | 11 ± 5 | 18 ± 7 | 13 ± 7 | 2·4 ± 0·3 |
| 2 | 203 ± 14 | 258 ± 10 | 241 ± 2 | 215 ± 11 | 83 ± 3 |
| 3 | 291 ± 5 | 301 ± 2 | 313 ± 2 | 344 ± 13 | 292 ± 2 |
| 4 | 291 ± 5 | 283 ± 2 | 269 ± 4 | 318 ± 10 | 240 ± 4 |
| 5 | 284 ± 0·3 | 252 ± 4 | 246 ± 2 | 279 ± 19 | 239 ± 4 |
| 6 | 283 ± 3 | 242 ± 3 | 221 ± 2 | 223 ± 3 | 186 ± 2 |

* Standard error of mean.

on the 3rd or 4th day, i.e. at the end of the phase of rapid growth, with the exception of strain NRRL 346, which began to sporulate on the second day. Whether the decrease in dry weight of the mycelium after 72 hr was due to sporulation or to extensive autolysis of the mycelium is not clear. There appeared to be a correlation between sporulation and decrease in mycelial weight, the reproductive phase being presumably stimulated either by exhaustion of the nutrients in the medium or by autolytic products.

Statistical analysis of the data (Table 1) indicated that the mycelial dry weights at the end of 3 days of incubation was significantly greater than on the preceding or the following day. In some of the experiments, however, growth was not significantly different between 3 and 4 days, though the average weight of mycelium at the end of 4 days was always lower. Strain NRRL 346 was an exception in that there was no significant decrease in mycelial weight after reaching the maximum at the end of 3 days.

*Response of two strains of Aspergillus niger to Mn deficiency*

Two drops of spore suspension of the two strains MUBL I and M were added to 'minus all', 'minus Mn' and 'plus all' media and the flasks incubated at 35 ± 0·2°. Each treatment had three replicates. One set of flasks were filtered on the 3rd day and another on the 6th day after inoculation; the results are presented in Table 2.

Both the strains showed a considerable decrease in mycelial yields in the absence of manganese, strain MUBL I showing lesser growth than strain M. In Mn-deficient media both strains produced discontinuous mycelia and there was no sporulation in these Mn-deficient cultures even at the end of 6 days.

Table 2. *Mycelial yield of two strains of Aspergillus niger in Mn-deficient cultures at two different incubation periods*

| | 3 days | | | 6 days | | |
|---|---|---|---|---|---|---|
| Strains | Minus all | Minus Mn | Plus all | Minus all | Minus Mn | Plus all |
| MUBL I | 5·8 | 60·3 | 306·3 | 6·3 | 75·1 | 225·4 |
| M | 4·9 | 96·0 | 261·1 | 8·5 | 139·7 | 188·6 |

Table 3. *Effect of concentration of Mn on the growth of three strains Aspergillus niger*

| | Spore inoculum | | | Mycelial inoculum | | |
|---|---|---|---|---|---|---|
| Concentration of Mn (μg./ml.) | NRRL 346 | NRRL 334 | M | NRRL 346 | NRRL 334 | M |
| | Mycelial weight (mg.) | | | | | |
| 1. 0·000 | 146 | 150 | 89 | 132 | 87 | 59 |
| 2. 0·001 | 216 | 211 | 212 | 204 | 110 | 88 |
| 3. 0·002 | 229 | 202 | 252 | 206 | 165 | 115 |
| 4. 0·004 | 259 | 273 | 271 | 237 | 222 | 162 |
| 5. 0·006 | 275 | 310 | 273 | 255 | 257 | 177 |
| 6 0·008 | 292 | 303 | 259 | 265 | 256 | 185 |
| 7. 0·01 | 291 | 307 | 269 | 263 | 277 | 187 |
| 8. 0·02 ('plus all') | 298 | 301 | 283 | 266 | 277 | 214 |
| Basal medium ('minus all') alone | 5 | 9 | 7 | 11 | 8 | 5 |

Statistical significance by *F* test at 5 %

| Strain | Inoculum: spore suspension | Inoculum: mycelial suspension |
|---|---|---|
| M | 1, 2, 3, 6, 7, 4, 5, 8* | 1, 2, 3, 4, 5, 6, 7, 8 |
| MRRL 346 | 1, 2, 3, 4, 5, 7, 6, 8 | 1, 2, 3, 4, 5, 7, 6, 8 |
| NRRL 334 | 1, 3, 2, 4, 8, 6, 7, 5 | 1, 2, 3, 4, 6, 5, 7, 8 |

* The difference between treatments falling under the same bar are not significant.

*Response of five strains of Aspergillus niger to concentration of Mn*

The effect of graded quantities of Mn on the growth and sporulation of the five strains was studied under the conditions mentioned above. The required micronutrient elements were added, varying only the concentration of manganese. The Mn concentrations were 0·001, 0·002, 0·004, 0·006, 0·008, 0·01 and 0·02 μg./ml. Spore and

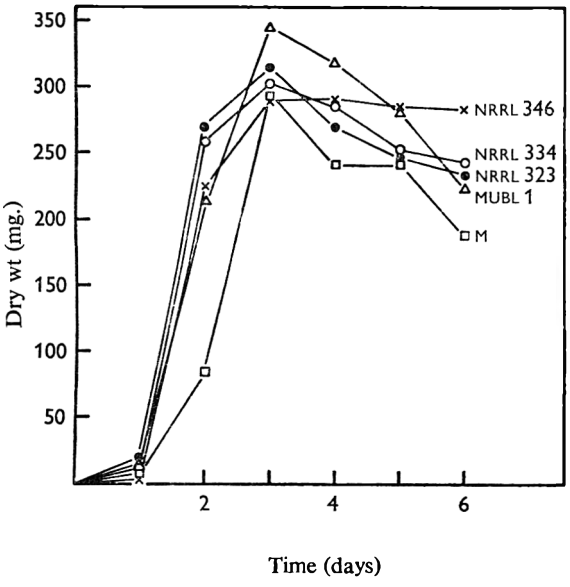


Fig. 1

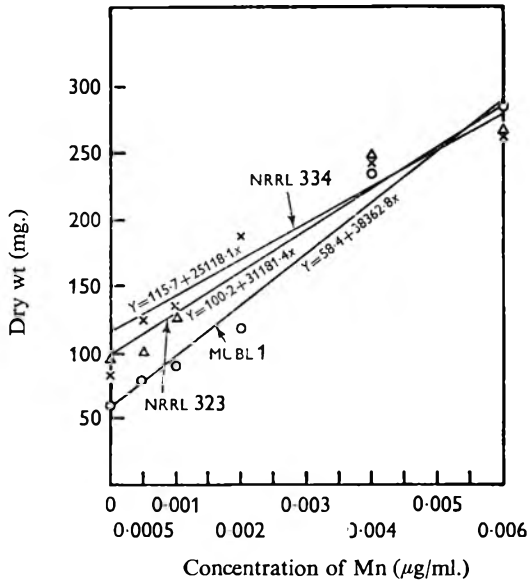


Fig. 2

Fig. 1. The rate of growth of five strains of *Aspergillus niger* in static cultures.

Fig. 2. Regression graphs showing the growth response of *Aspergillus niger* strains MUBS 1, NRRL 323 and NRRL 334 to graded increments in Mn concentration.

Table 4. *Effect of concentration of Mn on the growth of three strains of Aspergillus niger*

| Concentration of Mn (μg./ml.) | Spore inoculum | | | Mycelial inoculum | | |
|---|---|---|---|---|---|---|
| | NRRL 323 | MUBL 1 | M | NRRL 323 | MUBL 1 | M |
| | Mycelial dry weight (mg.) | | | | | |
| 1. 0·0 | 140 | 87 | 153 | 150 | 89 | 173 |
| 2. 0·001 | 183 | 177 | 252 | 220 | 151 | 264 |
| 3. 0·002 | 266 | 264 | 278 | 263 | 276 | 324 |
| 4. 0·004 | 300 | 345 | 295 | 293 | 355 | 336 |
| 5. 0·006 | 311 | 371 | 307 | 299 | 385 | 337 |
| 6. 0·008 | 314 | 375 | 292 | 300 | 381 | 344 |
| 7. 0·01 | 309 | 381 | 303 | 302 | 386 | 338 |
| 8. 0·02 ('plus all') | 309 | 387 | 310 | 288 | 375 | 344 |
| Basal medium ('minus all') alone | 10 | 14 | 10 | 6 | 5 | 4 |

Statistical significance by *F* test at 5%

| Strain | Inoculum: spore suspension | Inoculum: mycelial suspension |
|---|---|---|
| M | 1, 2, 3, 6, 4, 7, 5, 8 | 1, 2, 3, 4, 5, 7, 6, 8 |
| NRRL 323 | 1, 2, 3, 4, 7, 8, 5, 6 | 1, 2, 3, 8, 4, 5, 6, 7 |
| MUBL 1 | 1, 2, 3, 4, 5, 6, 7, 8 | 1, 2, 3, 4, 8, 6, 5, 7 |

mycelial inocula were separately used. In all these experiments the mycelial mats were harvested at the end of 3 days of incubation when maximum mycelial yield was obtained. The effect of Mn on sporulation was observed in separate sets of flasks which

were incubated for 6 days. Three strains were compared in each experiment, using either spore or mycelial inocula. The combination of strains used were as follows: (1) M, NRRL346 and NRRL334, (2) M, NRRL323 and MUBL I. All strains showed a gradual increase in mycelial production with increasing concentration of manganese (Tables 3, 4). Statistical analyses indicated that strain M does not show significant variation in mycelial dry weight with variation in Mn concentration. In a few experiments, however, there was a significant increase in dry weight with 0·001 and 0·002 μg./ml. Strain NRRL346 showed difference in weight only between 'minus Mn' and 0·001 μg. Mn/ml. and between 0·002 and 0·004 μg. Mn/ml. Strain NRRL334 was similar to strain NRRL346 in its response when spore inoculum was used; however, significant differences between various levels of Mn were obtained with mycelial inoculum. Strains NRRL323 and MUBL I showed significant increase in mycelial dry weight with increasing Mn concentration up to 0·004 and 0·006 μg./ml., respectively, when spore inocula were used; mycelial inocula did not improve the response of these two strains.

Table 5. *Effect of concentration of Mn on the growth of five strains of Aspergillus niger (mycelial inoculum)*

| Concentration of Mn (μg./ml.) | Strain: NRRL346 | NRRL334 | NRRL323 | MUBL I | M |
|---|---|---|---|---|---|
| | Mycelial dry weight (mg.) | | | | |
| 1. 0·0000 | 171 | 82 | 94 | 66 | 77 |
| 2. 0·0005 | 152 | 125 | 101 | 78 | 78 |
| 3. 0·001 | 197 | 133 | 127 | 91 | 102 |
| 4. 0·002 | 219 | 187 | 185 | 118 | 164 |
| 5. 0·004 | 244 | 243 | 250 | 236 | 157 |
| 6. 0·006 | 249 | 263 | 265 | 279 | 167 |
| 7. 0·02 ('plus all') | 272 | 288 | 294 | 348 | 191 |
| Basal medium ('minus all') alone | 10 | 8 | 13 | 15 | 9 |

Statistical significance by *F* test at 5 %

| | |
|---|---|
| NRRL346 | Not significant |
| NRRL334 | A, B, C, D, E, F, G |
| NRRL323 | A, B, C, D, E, F, G |
| MUBL I | A, B, C, D, E, F, G |
| M | A, B, C, D, E, F, G |

Two features were observed in the above experiments: (1) there was no marked differences in the mycelial dry weight of any of the strains tested above 0·006 μg. Mn/ml.; (2) in the majority of the experiments there was a significant difference between 'minus Mn' and 0·001 μg. Mn/ml. Hence the response of all the strains was compared for the following manganese concentrations: 0·0005, 0·001, 0·002, 0·004, 0·006 and 0·02 μg./ml. The data presented in Table 5 confirmed the earlier observations on the response of all the strains. In addition, there was no significant difference between the growth obtained at 0·0005 μg. Mn/ml. and 'minus Mn' cultures of strain M, MUBL I, and NRRL323. Strain NRRL334 showed no difference in growth between 0·0005 and 0·001 μg. Mn/ml. On statistical analysis, the relationship

between concentration of Mn and growth exhibited by MUBL 1 and NRRL 323 was found to follow a linear regression (Fig. 2) that is real and highly significant at both 5% and 1% levels, the observed $F$ value being 161·34 and 43·1 respectively (The Table value of $F$ at 5% is 7·71 and at 1%, 21·2) with degrees of freedom 1 and 4. For strain NRRL 334, the linear regression is just significant at 5% level only, the observed value of $F$ being 8·05.

No attempt was made in the present work at a quantitative evaluation of sporulation in relation to Mn concentration. Visual observation indicated that sporulation started only at the concentration of 0·006 μg. Mn/ml. and that there was a good gradation in degree of sporulation from 0·006 to 0·02 μg. Mn/ml. for all the strains.

## DISCUSSION

A study of the growth curves of the five strains of *Aspergillus niger* used in relation to sporulation suggests a correlation between growth and reproduction. Sporulation started towards the end of the phase of rapid growth in all strains except in NRRL 346 and the increase in sporulation was accompanied by a decrease in dry weight of mycelium. Few studies have considered critically the relation of reproduction to growth phases. The data of Robinson (1926) for *Sporodinia grandis* Link indicated that in static culture, spores usually developed at the end of the phase of rapid growth; although spores may appear earlier (Cochrane, 1958).

In the absence of manganese, *Aspergillus niger* strain M showed a reduction in mycelial yields of more than 60–70% of that in 'plus all' cultures; MUBL 1 showed a decrease of more than 75%, whereas the other three strains showed only a 50% decrease in growth. Nicholas & Fielding (1951) and Nicholas (1952) did not obtain decreases in mycelial yields of *A. niger* strain M in the absence of Mn, and Saraswathi-Devi (1954, 1962) obtained 120% growth in Mn-deficient cultures as compared to the 'plus all' cultures. The higher mycelial yields in the absence of Mn might probably be explained by the fact that the mycelial dry weights were measured on the 6th day when 'plus all' cultures had sporulated abundantly and hence gave a lower mycelial dry weight than the Mn-deficient cultures which showed neither sporulation nor decrease in mycelial yields. Donald *et al.* (1952*b*) indicated the inefficiency of the purification procedure adopted by Nicholas (1950, 1952). Presumably this was only one of the contributory factors for masking of the Mn-deficiency symptoms, since Sarawathi-Devi (1954, 1962) had used the more efficient purification method of Donald *et al.* (1962*b*) which was also used in the present work. A feature common to all the investigations in which the absence of Mn gave no decrease in mycelial yields was the use of a high salt concentration in the basal medium (about 8·75–9·5 g./l.) and the addition of very high concentrations of micronutrient elements. A high salt concentration, although resulting in tough twisted mycelial strands instead of smooth fluffy growth, does not prevent the decrease in mycelial yields in the absence of Mn, although Donald *et al.* (1952*b*) did obtain more than 60% decrease with a concentration of 8 g./l. However, it is evident from the data of Steinberg (1936) that increasing the concentration of trace elements beyond an optimum value either at pH 7·35 or at pH 8·04 resulted in the lowering of the growth ratio (yield with element : yield without element) besides having a toxic effect on sporulation. Hence it is probable that a high trace element concentration also contributes to masking the Mn-deficiency symptoms.

The comparison of the response of the five strains of *Aspergillus niger* to Mn concentration indicates that although all the strains showed a graded increase in mycelial yields, the effective range for assay of Mn was greater with strain MUBL I than with the other strains tested, the range lying between 0·001 and 0·006 $\mu$g. Mn/ml. If sporulation could be measured precisely, the range of concentrations between 0·006 and 0·02 $\mu$g. Mn/ml. could also be included for purposes of assay.

*Aspergillus niger* MUBL I strain appears to be an ideal test organism for bioassay of manganese. This method has been found to be 100-fold more sentive than the well known spectrophotometric method with potassium periodate or ammonium persulphate as the oxidizing agent for conversion of Mn into $MnO_4$ (Sulochana, 1967).

Under the same cultural conditions the possible use of strain MUBL I for Cu and Mo assays was also studied; satisfactory results were obtained even for Mo series for which neither $Al_2O_3$ purification nor the $H_2S$ co-precipitation method of Hewitt & Hallas (1951) was reported to be satisfactory (Saraswathi-Devi, 1954).

*Aspergillus niger* MUBL I has been used for assay of Mn in soils, plant tissues, human tissues, sera and blood; the standard series repeated every time with the samples have been consistently reproducible. The results of bioassay of manganese in cotton-growing soils will be reported elsewhere.

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# A New Genus of the Actinomycetales: *Microtetraspora* gen.nov.

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SUMMARY

Two aerobic mesophilic microbial species of a new genus belonging to the family Streptomycetaceae of the order Actinomycetales are described under the name *Microtetraspora* (*Microtetraspora glauca*, type species). The micro-organisms produced a filamentous growth which is differentiated into a vegetative and an aerial mycelium.

The new genus is characterized by the formation of a short and sparsely branched aerial mycelium bearing at the end of short sporophores chains of four spores. Sporulation is not observed to take place on the vegetative mycelium. The genus *Microtetraspora* is widely distributed in the soil and has been isolated from many samples.

## INTRODUCTION

The number of genera of the actinomycetes, or ray fungi, has increased considerably in the last few years reflecting a continued interest in these micro-organisms. Thus, while at the time of the latest edition of *Bergey's Manual* (1957) only three genera had been recognized in the Streptomycetaceae, by 1961 Waksman listed the following six genera: *Streptomyces*, *Thermoactinomyces*, *Microbispora* (Waksmania), *Thermopolyspora*, *Micromonospora* and *Thermomonospora*.

The present paper reports the isolation and description of two strains of actinomycetes which were easily distinguished from the known genera of the Streptomycetaceae by their unusual mode of sporulation: a chain of four spores was formed on short sporophores developing on monopodially branched short strands of aerial mycelium. The strains differed significantly from the previously described genera in the family Streptomycetaceae and it is therefore proposed to include them in a new genus to which the name *Microtetraspora* gen.nov. is given. The type species of the genus is *Microtetraspora glauca* (ATCC 23057) a subculture of which has been deposited with the American Type Culture Collection, together with *Microtetraspora fusca* (ATCC 23058) the second species isolated.

## METHODS

A recently developed novel method for the mass isolation of little-known soil micro-organisms belonging to the Actinomycetales was used (Thiemann, in preparation). With this method the large-scale isolation of *Microbispora*, *Streptosporangium*, *Actinoplanes*, *Microtetraspora*, and species of other not yet described new genera has been made possible. A detailed description of this method will be published separately elsewhere.

*Stains.* The staining procedures recommended by the Society of American Bacteriologists (1957) were used.

*Photographs.* Microphotographs were taken with a Zeiss binocular microscope, model Standard WL, with Optovar and 35 mm. photomicrography attachment. Kodak Plus X Pan film was used. Electron photomicrographs of sporulated mycelium were taken with a Hitachi model HU-II electron microscope by touching collodion films mounted on grids onto cultures growing on soil agar incubated at 30°. Sporulation was studied also by growing the cultures directly on collodion membranes according to the technique described by Grein (1955).

*Culture media.* Unless otherwise stated the media used were prepared according to Waksman (1961).

*Soil agar.* Air-dried garden soil passed through a 10-mesh sieve, 30 g., agar 20 g., tap water to 1000 ml., pH 6·0. Sterilized for 20 min. at 120°.

*Examination of spore germination.* The spores from a fully sporulated agar slant culture were carefully scraped off with a sterile loop and streaked on to the surface of soil extract agar plates and soil agar plates. The plates were incubated at 28° and examined directly under the microscope for the appearance of germ tubes.

*Preparation and analysis of cell walls.* The organisms were grown in the seed medium of Margalith & Pagani (1961) and incubated at 28° on a rotary shaker (250 rev./min.). After 48 hr of growth the cells were collected by centrifugation and thoroughly washed with distilled water. After treatment with ethanolic KOH (0·5%) at 37° for 24 hr the cells were washed with ethyl alcohol until the pH value was neutral. Suspensions of cells were disrupted by agitating them for 48–72 hr on a rotary shaker (250 rev./min.) with an equal volume of glass beads (1·0–1·5 mm. diam.). A few ml. chloroform were added to avoid bacterial contamination. The degree of breakage was checked by phase microscopy. For all the other steps the methods described by Becker, Lechevalier & Lechevalier (1965) were followed.

## RESULTS

### *Description of Microtetraspora Thiemann, Pagani and Beretta gen.nov.*

*Morphology.* The fine mycelium (about 1 $\mu$ diam.) differentiated into: (1) vegetative (primary) mycelium which was profusely branched, grew into the agar medium and formed a compact layer on top of it; (2) aerial (secondary) mycelium arising from the vegetative mycelium which grew into the air away from the agar surface. Spores were formed only on the aerial hyphae and never on the vegetative mycelium. The spores were formed in chains of four on short sporophores, which branched characteristically from the aerial hyphae at an angle of 45°, giving to the sporophores, when examined *en masse*, an appearance reminiscent of an ear of wheat (Pl. 1, fig. 1).

### *Description of Microtetraspora glauca ATCC 23057 and Microtetraspora fusca ATCC 23058 Thiemann, Pagani and Beretta, sp.nov.*

Of the various cultures of *Microtetraspora* isolated, strain T 158 and strain T 457 were selected as the type strain of the species *M. glauca* and *M. fusca* respectively. No qualitative differences were found in the microscopic properties of *M. glauca* and *M. fusca*; the two isolates are, however, easily distinguished by their cultural and biochemical characteristics which are shown in Table 1 and Table 2 respectively. The following general morphological description applies equally to both cultures.

*Vegetative mycelium.* About 0·7–1·0 $\mu$ diam. Filaments long, wavy, branching, penetrating the agar and forming on its surface, compact, raised and tough colonies

like those of typical *Streptomyces* spp. The main (older) mycelium strands are larger (1·0 $\mu$) and have a granulated appearance. The mycelium does not fragment even in old cultures.

*Aerial mycelium.* Approximately 1·0 $\mu$ in diam. The hyphae emerging from the vegetative mycelium are erect, sparsely branched and rather short, usually not exceed-

Table 1. *Cultural characteristics of Microtetraspora glauca strain T158 and M. fusca strain T457*

G. growth; V.m. vegetative mycelium; A.m. aerial mycelium; Sp. sporulation. Soluble pigments were not formed on any of the media examined. Colours were determined according to Maerz & Rea Paul (1950).

| Culture media | *M. glauca* strain T158 (ATCC 23057) | *M. fusca* strain T457 (ATCC 23058) |
|---|---|---|
| Glucose asparagine agar | G. moderate, flat<br>V.m. ash grey (pl. 27, A-2)<br>A.m. sparse to absent<br>Sp. absent | G. good, smooth<br>V.m. hyaline<br>A.m. white grey (pl. 11, A-1)<br>Sp. good |
| Glycerol asparagine agar | G. poor, flat<br>V.m. hyaline<br>A.m. absent<br>Sp. absent | G. good, smooth<br>V.m. hyaline<br>A.m. white grey (pl. 11, A-1)<br>Sp. good |
| Calcium malate agar | G. very poor, flat<br>V.m. hyaline<br>A.m. sparse<br>Sp. poor | G. poor, flat<br>V.m. hyaline<br>A.m. white grey (pl. 11, A-1)<br>Sp. good |
| Hickey and Tresner agar | G. very good, elevated<br>V.m. blue green (pl. 25, J-6) to yellowish green (pl. 21, J-1)<br>A.m. abundant, pearl grey (pl. 44, A-1)<br>Sp. good | G. very good, wrinkled<br>V.m. brown violet (pl. 8, L-8)<br>A.m. good, white grey (pl. 11, A-1)<br>Sp. good |
| Soil agar | G. moderate, flat<br>V.m. hyaline to ash grey (pl. 27, A-2)<br>A.m. white grey (pl. 11, A-1)<br>Sp. very good | G. moderate, flat<br>A.m. white grey (pl. 11, A-1)<br>Sp. very good |
| Bennett agar | G. very good<br>V.m. blue green (pl. 26, K-5) to yellowish green (pl. 20, K-1)<br>A.m. grey (pl. 28, C-1)<br>Sp. moderate | G. very good, wrinkled<br>V.m. amber with reddish tinge (pl. 12, B-9)<br>A.m. white grey (pl. 11, A-1)<br>Sp. very good |
| Oatmeal agar | G. good, flat<br>V.m. blue green (pl. 30, G-1)<br>A.m. blue grey (pl. 28, L-1)<br>Sp. good | G. very poor, flat<br>V.m. hyaline<br>A.m. scarce to absent<br>Sp. absent |
| Nutrient agar | G. moderate<br>V.m. cream (pl. 9, D-2)<br>A.m. absent<br>Sp. absent | G. very good, elevated<br>V.m. amber (pl. 12, F-7)<br>A.m. absent<br>Sp. absent |
| Carrot plug | G. no growth | G. very poor; wrinkled<br>V.m. cream (pl. 9, D-2)<br>A.m. absent |
| Potato plug | G. no growth | G. very poor; wrinkled<br>V.m. cream (pl. 9, D-2)<br>A.m. absent |
| Czapek agar | G. no growth | G. no growth |

ing 50 $\mu$. On rich media, such as Hickey and Tresner agar or even oatmeal agar, coremium-like formations of the aerial mycelium can be seen.

*Sporophores.* The sporophores are formed as thin (0·5 $\mu$) monopodial branches of the aerial hyphae. The sporophores can be relatively long (5·5 $\mu$) but normally are only 1·0–2·0 $\mu$ in length (Pl. 1, fig. 2). Occasionally the sporophores are extremely short, giving the impression that the spores are attached directly to the hypha. The sporophores of *M. fusca* tend to fuse into more or less globular masses of spores as the culture ages (Pl. 1, fig. 3).

Table 2. *Biochemical characteristics of Microtetraspora glauca strain T158 and M. fusca strain T457*

| | *M. glauca strain* T158 (ATCC 23057) | *M. fusca strain* T457 (ATCC 23058) |
|---|---|---|
| Nitrate reduction | Reduced | Not reduced |
| Tyrosine agar | Not hydrolysed; no melanoid pigment | Not hydrolysed; no melanoid pigment |
| Skim milk agar | Casein slowly digested | Casein slowly digested |
| $H_2S$* | Positive | Positive |
| Gelatin | Completely hydrolysed | Not hydrolysed |
| Starch | Slight hydrolysis | Not hydrolysed |
| Litmus milk | No coagulation. no peptonization | No coagulation, no peptonization |
| Peptone iron agar | No melanoid pigment | No melanoid pigment |

* Lead acetate strips, according to Küster & Williams (1964).

*Spores.* Spores are formed in chains of four. Mature spores are spherical (1·5 $\mu$) to slightly oval (1·4 × 1·7 $\mu$). No spines or hairs are present on the spore surface (Pl. 1, fig. 4). Immature spores are rather variable, being somewhat cuboid (0·8 × 0·8 $\mu$) or even triangular, as a result of their mode of formation; the central ones tending to be compressed and cuboid and the ones at the extremities of the spore chain are sometimes triangular (Pl. 1, fig. 5). The immature spores when examined under the oil-immersion lens give the impression of being 'empty' and present a central highly refractile spot; fully developed and mature spores are uniformly dark, opaque, spherical to oval and usually without refractile spots (Pl. 1. fig. 6). Spores are formed only on the aerial hyphae and never on the vegetative mycelium. Occasionally in old cultures on soil agar plates, spores come in contact with the agar surface and germinate giving the impression that they are formed on the vegetative mycelium.

*Spore formation.* Spore formation begins by a terminal enlargement of the aerial hyphae, the cytoplasm of which condenses giving a bead-like appearance. In some preparations, transverse septa separating the spores can be seen (Pl. 2, fig. 7), in others the early separation of the spores is indicated only by an area of higher refringence. Four spores are usually formed in each chain but in some instances chains with only two or three spores are formed and very rarely chains of five spores. The four spores of a sporophore do not always grow uniformly and attain the same size; sometimes mature spores of variable size and immature ones occur in the same chain. Usually the four spores of the sporophore are formed in a single plane; however, occasionally the two spores at the top of the sporophore are placed eccentrically in relation to the other two. Sporulation in the genus *Microtetraspora* appears to proceed from the base of a fertile branch towards its tip, since transverse septa, when observed, were always seen to be formed first at the base of the fertile branch (Pl. 2, fig. 8). The spores when

transferred to fresh medium germinate and produce one to four rather large germ tubes. The germinated spores are clearly visible in the matted mycelium even 72 hr after germination (Pl. 2, fig. 9).

*Staining.* Young mycelium grown under submerged conditions was used for the staining reactions. The mycelium of *Microtetraspora glauca* and *M. fusca* was Gram-positive and was not acid-fast.

*Effect of temperature and pH on growth.* The effect of temperature on growth was investigated by incubating inoculated Petri dishes (Bennett agar, oatmeal agar and Hickey & Tresner agar) at 22°, 28°, 37°, 45°, and 50°. *Microtetraspora glauca* grew equally well on all three media at 22° and 37°, but was optimal at 28°, whereas *M. fusca* grew better at 28° and 37° than at 22°. Neither *M. glauca* nor *M. fusca* developed at 45° or 50°. To test for the effect of pH on growth, the above media were corrected to pH 4, 5, 6, 7 and 8, inoculated and incubated at 28°. Under these conditions *M. glauca* did not grow at pH 4; at pH 5 it was sparsely developed, whereas at pH 6, 7 and 8 good development was observed on all three media. *Microtetraspora fusca* on the other hand, grew equally well at pH 6 and 7, and only sparsely at pH 5 and 8. No growth took place at pH 4.

*Utilization of carbon sources. Microtetraspora glauca* and *M. fusca* did not develop on the standard minimal media employed in the carbon utilization tests. Positive results were obtained using the synthetic medium of Magni & von Borstel (1962) to which a vitamin B mixture (0·2 γ/ml) was added. Since on this medium the control plates (no carbon source) showed a moderate amount of growth as well, carbon utilization was considered negative when the growth was similar to or less than the growth on medium without a carbon source. Under these conditions, arabinose, xylose, glucose, galactose, fructose, mannose and ribose supported good growth of *M. glauca*, whereas rhamnose, maltose, saccharose, mannitol, inositol and starch supported only moderate to sparse growth. No growth was supported by lactose, raffinose, glycerol, sorbitol, dulcitol, inulin and sorbose. On the other hand *M. fusca* grew only in presence of arabinose, xylose, glucose, ribose, inulin and maltose. With all the other carbon sources, no visual difference between the control plates was observed.

Table 3. *Major components of cell-wall preparations of various form genera of Actinomycetales showing some morphological similarity with Microtetraspora*

| | DAP acid | | Sugars and amino-sugars | | | Amino acids | |
|---|---|---|---|---|---|---|---|
| Genera | LL | Meso | Galactose | Galactosamine | Arabinose | Glycine | Lysine |
| *Streptomyces** | + | − | − | − | − | + | − |
| *Microellobosporia** | + | − | − | − | − | + | − |
| *Microbispora** | − | + | − | − | − | (+)† | − |
| *Micropolyspora** | − | + | + | − | + | − | − |
| *Microtetraspora* | ± | + | − | + | − | + | + |

All genera had in common: muramic acid, glucosamine, glutamic acid and alanine.
* Data from Becker, Lechevalier & Lechevalier (1965).
† Only some strains do contain glycine.

*Cell-wall composition.* Chemical analysis of cell-wall preparations of *Microtetraspora* showed them to contain, as major components, meso-diaminopimelic acid, glucosamine, galactosamine, muramic acid, glycine, glutamic acid, alanine and lysine.

LL-diaminopimelic acid was always present, however, in small amounts. In Table 3 the cell-wall components of the various form-genera of the Actinomycetales showing some morphological similarity with *Microtetraspora* are given. As can be seen from this table, the cell walls of *Microtetraspora* differ considerably in their composition from the cell walls of the other genera described in the literature and with which *Microtetraspora* has some morphological affinity.

*Source. Microtetraspora glauca* was first isolated from a soil sample collected from the vicinity of carrot roots at Appiano Gentile, Como, Italy, and *M. fusca* from a sample received from Thailand and collected on a rubber plantation in the Province of Amphur Sadao, Songkhla. Species of *Microtetraspora* are, however, widespread in nature and have been isolated from many of the soil samples so far examined, received from various parts of Italy, Thailand and Brazil.

## DISCUSSION

The growth characteristics on different nutrient media and the mode of spore formation indicate that the new genus *Microtetraspora* should be included in the family Streptomycetaceae. The characteristic production of a linear chain of four spores on the aerial mycelium differentiated these cultures from the previously described species that we have found in the literature and from those that we have isolated and studied up till now.

The genus *Micropolyspora* described by Lechevalier, Solotorovsky & McDurmont (1961) and characterized by the formation of chains of spores both on the aerial and on the vegetative mycelium differs from *Microtetraspora* not only in the formation of spores on the vegative mycelium but also in the overall morphology of the sporophores and aerial mycelium. Whereas in *Microtetraspora* as a rule, chains of 4 spores are formed, attached to the hypha by short sporophores, in *Micropolyspora* spore chains of up to 10 spores are not uncommon. Stolon-like structures formed by the long and branching hyphae of the aerial mycelium have also been described for *Micropolyspora* as well as for the genus *Microbispora*. Two new genera recently described having a short row of spores are *Microellobosporia* (Cross, Lechevalier & Lechevalier, 1963) and *Microechinospora* (Koniev, Tsyganov, Minbayev & Morozov, 1965). *Microellobosporia* which belong to the Actinoplanaceae is characterized by the production of large (1·5–3·5 $\mu$) and non-motile sporangiospores arranged in a single straight row inside a sporangium formed both on the aerial and on the vegetative mycelium. The same genus was described simultaneously by Russian workers under the name *Macrospora* (Tsyganov, Zhukova & Timofeeva, 1963). The genus *Microechinospora* characterized by the formation on the aerial as well as on the vegetative mycelium of a straight row of large spherical spores (2·5–3·5 $\mu$) enclosed in a club-shaped sporangium having a characteristically spiny surface is also included in the Actinoplanaceae. The genus *Microechinospora* closely resembles the genus *Microellobosporia* from which it is differentiated mainly by the spiny surface of the sporangia. The mode of sporulation, size of the spores, and sporangia formation clearly distinguish these genera from *Microtetraspora*. Thorough examination of stained and unstained preparations of *Microtetraspora* with the optical as well as electron microscope (Pl. 1, fig. 4; Pl. 2, fig. 10) failed to reveal any indication of sporangia formation.

Recently, Lechevalier & Lechevalier (1965) proposed a classification of the aerobic

actinomycetes based on their morphology as well as on the chemical composition of their cell walls. On the basis of their data these authors grouped the euactinomycetes as follows. Family Dermatophilaceae, one genus, *Dermatophilus*; Family Actinoplanaceae, 6 genera, *Actinoplanes*, *Ampullariella*, *Spirillospora*, *Streptosporangium*, *Amorphosporangium* and *Microellobosporia*; Family Streptomycetaceae, one genus, *Streptomyces*; Family Micromonosporaceae one genus, *Micromonospora*; Family Nocardiaceae Fam.nov., 6 genera, *Thermoactinomyces*, *Thermomonospora*, *Microbispora*, *Micropolyspora*, *Pseudonocardia* and *Nocardia*. In the genus *Streptomyces sensu lato* of Lechevalier & Lechevalier (1965) were included the various forms described as *Streptoverticillium*, *Chainia* and *Actinopycnidium*. The cell-wall preparations from the organisms belonging to this genus contained only glucosamine, muramic acid, alanine, glutamic acid, glycine and LL-diaminopimelic acid. In recent years the cell-wall composition of the actinomycetes has been frequently used as an aid in their classification. Whether the classical taxonomic approach of Waksman (1961) to the classification of the actinomycetes is to be preferred to the new and undoubtedly also more objective approach of Lechevalier & Lechevalier (1965) is beyond our scope. It suffices to mention, however, that when atypical *Streptomyces* and *Nocardia* are examined only for their morphological and cultural characters it is often entirely a matter of personal preference whether an isolate is placed in one genus or the other (Waksman, 1961). In these dubious cases, the chemical approach to the classification has already proved to be of great value (Becker, Lechevalier, Gordon, & Lechevalier 1964; Becker *et al.* 1965).

Analysis performed on cell-wall preparations of *Microtetraspora* brought to light some interesting data. The presence of small but reproducible amounts of LL-diaminopimelic acid, the presence of glycine, and the absence of characteristic sugars in the cell-wall hydrolysates of *Microtetraspora* is an interesting feature shared also by the cell walls of *Streptomyces* spp.

On the other hand, the composition of the cell walls of *Microtetraspora* shows also some similarities with the one of *Microbispora*; the presence of galactosamine and lysine, apart from the small amount of LL-diaminopimelic acid, distinguishes nevertheless the cell walls of these two genera. On the basis of these overall similarities it is possible to think of *Microtetraspora* as forming a link between the *Streptomyces* and *Microtetraspora*. Apart of this hypothetical phylogenetic relationship between *Microtetraspora* and some of the other genera of the Actinomycetales, it is important to bear in mind that in addition to the morphological features which distinguish *Microtetraspora* from the genera already described, it differs from them also in the cell-wall composition.

The following revision of the existing key to the family Streptomycetaceae *sensu* Waksman (1961) is proposed to accommodate *Microtetraspora*:

1. Aerial (secondary) mycelium formed
   a. Spores formed in chains mainly on the aerial mycelium, rarely also on the substrate mycelium (Becker, Lechevalier & Lechevalier, 1965).
      *Streptomyces* Waksman & Henrici
   b. Spores formed in chains, both on the aerial and substrate mycelium.
      *Micropolyspora* Lechevalier, Solotorovsky & McDurmont
   c. Spores formed in chains of four on short sporophores on the aerial mycelium only.
      *Microtetraspora* gen.nov. Thiemann, Pagani & Beretta

d. Spores occurring singly, in pairs or in chains
  a 1. Mesophilic forms, spores in pairs on sporophores on the aerial mycelium and with rare exception also on the substrate mycelium (Becker, Lechevalier & Lechevalier, 1965). *Microbispora* Nonomura & Ohara
  b 1. Thermophilic forms:
    1. Spores single on sporophores both on the aerial and substrate mycelium. *Thermoactinomyces* Tsiklinsky
    2. Spores single on aerial mycelium only. *Thermomonospora* Henssen
2. Aerial (secondary) mycelium not produced.
  a. Spores occurring singly on short sporophores. . *Micromonospora* Orskov

The genus *Thermopolyspora* (Henssen, 1957), of questionable validity (Krasilnikov, 1964; Becker *et al.* 1965; Lechevalier, 1965), has been omitted since its characterization was based on cultures contaminated with bacteria of unknown nature and her original cultures of *Thermopolyspora polyspora* are now dead, and according to Rule 24 g of the *International Code of Nomenclature of Bacteria and Viruses* (1958): 'A name of a taxon is illegitimate. . . if the characterization of the group was based upon an impure or mixed culture.' The new thermophilic genus *Actinobifida*, isolated by Krasilnikov & Agre (1964), has also been omitted. This genus is characterized by the formation of single spores borne on the tip of dichotomously branching sporophores arising from the hyphae of the aerial mycelium or from the surface of the colony. This genus was included by Krasilnikov & Agre (1964) in the family Micromonosporaceae, it is felt, however, that it would be more properly included among the thermophilic forms (*Thermoactinomyces*, *Thermomonospora*). Where *Actinobifida* will finally be placed has to await the results of more detailed comparative studies between *Actinobifida* and the other actinomycetes which form single isolated spores on the aerial mycelium.

Twelve soil samples out of a total of 53 different ones so far examined and received from various parts of the world yielded strains of *Microtetraspora*. A total of 134 strains were isolated. This relatively large number of strains isolated can be taken as an indication of the cosmopolitan distribution of this genus. At present no indications of the possible role these micro-organisms might play in the soil are at hand.

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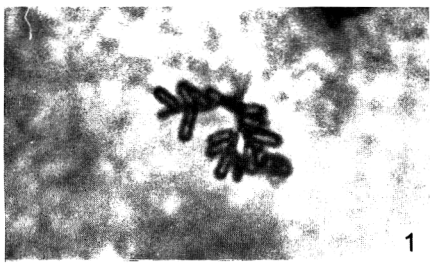
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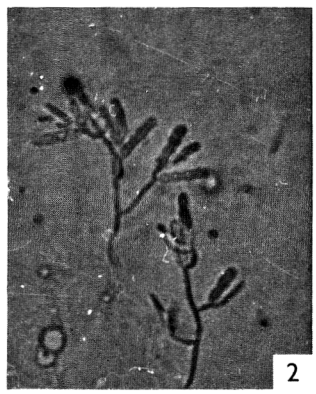
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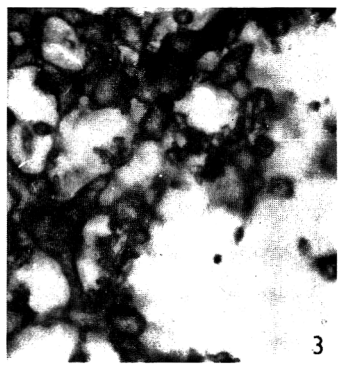
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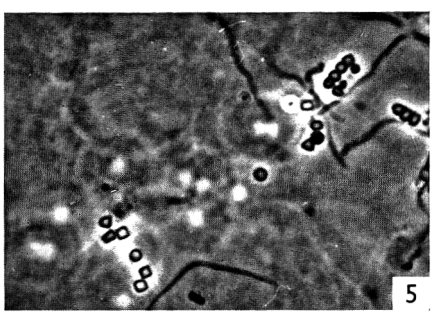
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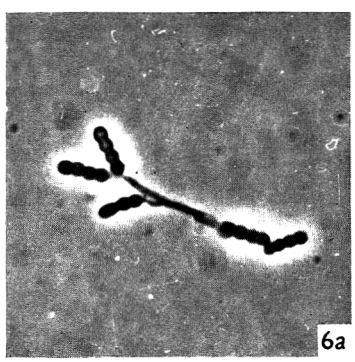
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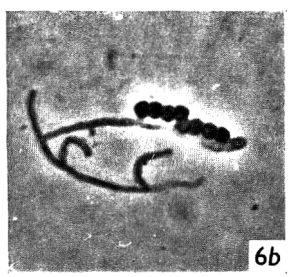
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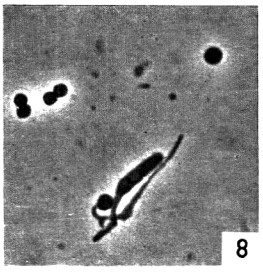

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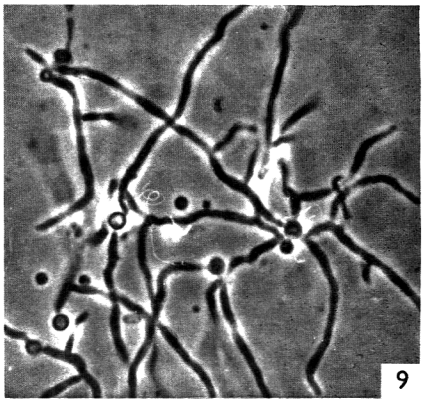

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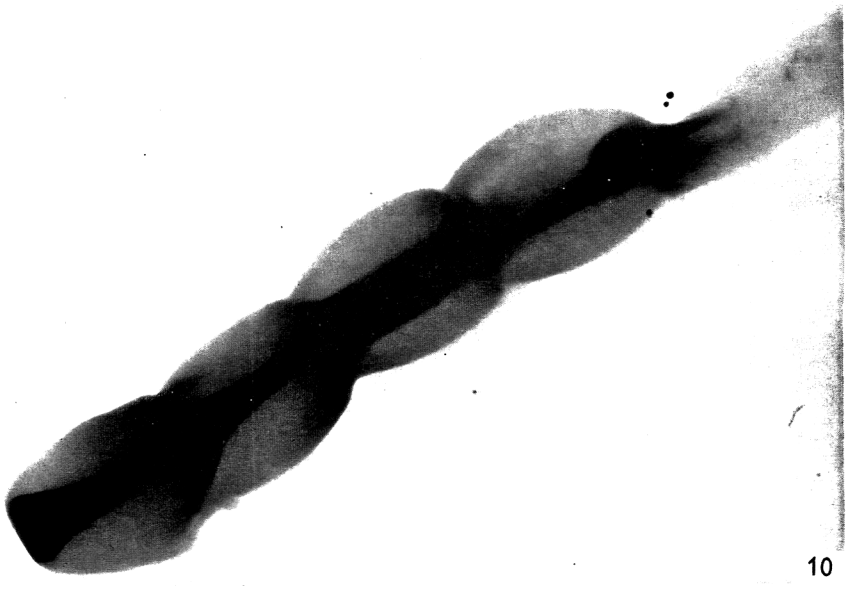

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## EXPLANATION OF PLATES

### PLATE 1

Fig. 1. *Microtetraspora glauca* on soil agar plates. Low-power magnification (× 1120) of the aerial mycelium and spore bearing hyphae.

Fig. 2. *M. glauca*; aerial mycelium showing mature spores as well as spore chains in different stages of development (soil plate-direct microscopical examination, × 3500).

Fig. 3. *M. fusca*; spore-fusion into globular masses; Ca malate agar.

Fig. 4. *M. glauca*; electron micrograph of aerial mycelium with sporophores (soil plate, × 10370).

Fig. 5. *M. fucsa*; disrupted chains of spores showing the variable morphology of the still immature spores (× 3500.)

Fig. 6. *M. glauca*; aerial mycelium with fully developed spores (soil plates, × 3500).

### PLATE 2

Fig. 7. *Microtetraspora glauca*; different stages of spore formation. Transverse septa are clearly seen (soil plates, × 3500).

Fig. 8. *M. glauca*; formation of transverse septa at the base of the sporophore giving rise to spore formation (× 3500).

Fig. 9. *M. glauca*; spore germination on soil extract agar after 38 hr at 28° (× 3500).

Fig. 10. *M. glauca*; electron micrograph of isolated spore chain (soil plate, × 22,100).

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# The Chemical Composition of the Nucleic Acids and the Proteins of Some Mycoplasma Strains

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SUMMARY

The base compositions of the deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) of three strains of *Mycoplasma laidlawii* and one of *Mycoplasma mycoides* var. *mycoides* have been determined. The adenine (A) + thymine (T) content of all the samples examined was much greater than the guanine (G) + cytosine (C) content, the values for % GC ranging from 34·4 to 32·5 for the sample of *M. laidlawii* to 30·0 for *M. mycoides* var. *mycoides*. The adenine + uracil/guanine + cytosine value for the RNA of all the samples was in the range 1·17–1·20.

The amino acid content of the protein of *Mycoplasma laidlawii* was also determined. This agrees closely with the values obtained by Sueoka (1961) for the amino acid composition of the protein for a bacterium having the same DNA base composition.

The values given in a previous communication (Jones, Tittensor & Walker, 1965) for the amino acid composition of the protein of *Mycoplasma mycoides* var. *capri* were incorrect since a protein fraction had precipitated from the medium during the growth of the organism because of the decrease in pH value of the culture which occurred. The correct values are now given and they agree closely with the values predicted from the work of Sueoka (1961).

## INTRODUCTION

In a recent compilation of the base compositions of the deoxyribonucleic acids (DNA) of bacteria (Hill, 1966) which included the literature published before 1966, the analyses of the DNA of several Mycoplasma strains were listed. Most of the DNA specimens had extreme adenine + thymine (AT) compositions, the guanine + cytosine (GC) values ranging from 24% for *Mycoplasma mycoides* var. *capri* and strains from calf and young goat, to 46·5 for *M. hominis*.

DNA from one strain of *Mycoplasma gallinarum* and from one of *M. gallisepticum* have been analysed by the melting point (Tm) method and shown to have values of 28% and 32·5% GC respectively (Rogul, McGee, Wittler & Falkow, 1965). DNA from a strain of *M. laidlawii*, *M. pneumoniae* and strains from calf and young goat were isolated and analysed by the Tm method and by CsCl density-gradient centrifugation and gave values of 31–35·5%, 39–41%, 23·5–26·5% and 24–25·5% GC respectively (Neimark & Pène, 1965). The wide range of these values for individual strains shows the difference obtained by the two methods of analysis and hence throws some doubt on analyses of the DNA of Mycoplasma strains done solely by physical methods, as differences in GC content of 10% as obtained here are usually

too great to be acceptable. The values for *M. gallisepticum* DNA of 33 % GC obtained by CsCl density-gradient centrifugation and by chemical analysis are inconsistent, as the cytosine content obtained by the latter method is some 25 % lower than the guanine content, although the DNA is known to be double-stranded (Morowitz *et al.* 1962). The other values obtained for the DNA from *M. gallisepticum* obtained by Tm and CsCl density-gradient centrifugation studies agree fairly well at a value of 34–35 % GC (Marmur & Doty, 1962; Schildkraut, Marmur & Doty, 1962). The figure for *M. hominis* DNA, the GC content of which is considerably higher than that quoted for the DNA from any other mycoplasma, is questionable as the A:T and G:C ratios were not unity and the analysis was not performed on a pure sample of DNA (Lynn & Smith, 1957).

In a previous paper (Jones *et al.* 1965) we presented our findings on the nucleic acid base composition of *Mycoplasma mycoides* var. *capri* and the amino acid composition of the protein. The DNA was isolated in a pure form and gave an analysis for base composition (by the method of Wyatt & Cohen, 1953) which was consistent with a double-stranded DNA. The composition of this DNA has also been determined by CsCl and $Cs_2SO_4$ density-gradient centrifugation and a value of 25 % GC was obtained in both cases (Dr W. Szybalski, personal communication) which agrees well with the value of 24·8 % obtained by chemical analysis.

The present communication corrects the value obtained for the amino acid composition of the protein of *Mycoplasma mycoides* var. *capri* (Jones *et al.* 1965) and presents further work on the base composition of the DNA and RNA from three strains of *M. laidlawii* and one strain of *M. mycoides* var. *mycoides*. The amino acid composition of the protein of the strains of *M. laidlawii* is also given.

## METHODS

### *Organisms*

*Mycoplasma mycoides* var. *capri* PG 3; *Mycoplasma laidlawii* PG 9 and *Mycoplasma mycoides*, var. *mycoides* T 3 were obtained from Dr D. G. ff. Edward (Wellcome Research Laboratories, Beckenham, Kent). *Mycoplasma laidlawii* 544A and 545B were obtained from Dr P. Pease (Bacteriology Dept., The University, Edgbaston, Birmingham 15).

### *Media*

The following media were used for growing *Mycoplasma mycoides* var. *capri*: (1) the 'normal medium' of Jones *et al.* (1965); (2) the 'dilute medium' of Jones *et al.* (1965); (3) a medium similar to the 'dilute medium' but with disodium hydrogen phosphate (2 %, w/v) instead of the previous concentration of 0·25 %. The pH of the medium was adjusted to 7·4 with 0·1 N-HCl.

The following medium was used for growing *Mycoplasma laidlawii*: Difco-Tryptose (2 %, w/v), NaCl (0·5 %, w/v), thiamine hydrochloride ($5 \times 10^{-4}$ %, w/v), trihydroxymethylaminomethane (0·5 %, w/v), glucose (1 %, v/v), Difco-PPLO serum fraction (1 %, w/v).

The following medium was used for growing *Mycoplasma mycoides* var. *mycoides*. Difco-beef heart infusion (1·6 %, w/v), Difco peptone (0·3 %, w/v), NaCl (0·15 %, w/v), Burroughs Wellcome horse serum (20 %, v/v), glucose (1 %, w/v), DNA (Na salt from hymus, British Drug Houses Ltd., 1 % (v/v) of 0·2 % (w/v)), Difco-yeast

extract (10% (v/v) of 25% (w/v)), dipotassium hydrogen phosphate (0·25%, w/v), thallous acetate (0·01%, w/v), penicillin (100 units/ml.).

*Determination of the amino acid composition of the proteins*

The organisms were grown in 1·5 l. of the appropriate medium at 37° for 48 hr. The organisms were harvested by centrifugation at 23,000*g* (High speed no. 18 Refrigerated Centrifuge, Measuring and Scientific Equipment Ltd.) for 30 min., the organisms broken in a Mickle tissue disintegrator (H. Mickle, Mill Works, Gomshall, Surrey). for 15 min. suspended in a tris + magnesium buffer ($10^{-2}$ M tris, $10^{-3}$ M-magnesium, pH 7·3 10 ml.) in the presence of Ballotini beads no. 5 and the mixture dialysed against distilled water and freeze-dried. The amino acid composition of the protein of the samples was determined as previously described (Jones *et al.* 1965).

*Isolation of the nucleic acids*

(*a*) The organisms from *Mycoplasma laidlawii* strains PG 9, 544 A and 545 B after incubation at 37° for 48 hr in 1·5 l. of the medium detailed above were harvested at 23,000*g*. They were suspended in a mixture of phenol and sodium *p*-aminosalicylate solution (6%, w/v; 5 ml.) saturated with phenol (1 ml.) and broken for 15 min. in a Mickle tissue disintegrator in the presence of glass beads (1 ml., Junior size, 0·152 mm. diameter; Prismo Safety corporation, Huntingdon, Pennsylvania, U.S.A.; Kolbe, 1960) with cooling (Chelton, 1963). The supernatant liquid was removed from the beads, which were washed with phenol-saturated salicylate solution until the washings were clear. The combined mixture was then added to an equal volume of phenol saturated with salicylate solution and shaken for 5 min. The layers were separated by centrifugation and the nucleic acids precipitated from the aqueous layer by adding ethanol (3 vol.). The phenol layer and any cell debris were re-extracted by shaking with more salicylate solution until no more material precipitable with 3 vol. ethanol was obtained; usually two extractions only were necessary. The combined ethanol precipitates were dissolved in water (5 ml.) and centrifuged at 25,000*g* (M.S.E. Major centrifuge, superspeed head) for 30 min. to remove debris. The supernatant fluid was then made M with respect to NaCl and allowed to stand at 0° for 18 hr. The precipitate was removed by centrifugation at 25,000*g* for 30 min., washed with M-NaCl, dissolved in water, dialysed and freeze-dried to give RNA (5–6 mg.).

The RNA and DNA were separated from any polysaccharide as previously described (Jones *et al.* 1965) and the DNA fractionated from the RNA by the fractional precipitation of their cetyltrimethylammonium salts at 0·6 M NaCl such that the final volume of the solution was less than 4 ml.

Owing to the small quantity of organisms obtained, the isolation procedure involved working with rather dilute solutions of nucleic acids (< 1 mg/ml.) and thus complete separation of the DNA from RNA soluble in M-NaCl could not be achieved by one fractionation with cetyltrimethylammonium bromide. Due to the inevitable losses at each precipitation a second fractionation could not be attempted. Thus the DNA was finally purified from RNA by digesting a solution of the nucleic acids (2 mg. in 5 ml. of 0·01 M acetate buffer pH 6·0) with ribonuclease (0·01 mg.) at 37° for 18 hr. The protein was denatured with chloroform, the aqueous solution dialysed against 2 M-

NaCl solution to remove the ribonuclease resistant 'core', dialysed against distilled water and freeze-dried to give DNA (≃ 1 mg.).
The weights of material obtained were:

| Strain of *M. laidlawii* | PG 9 | 544 A | 545 B |
|---|---|---|---|
| RNA (mg.) | 5 | 5·2 | 6 |
| DNA (mg.) | 1 | 1·1 | 1·4 |

(*b*) The organisms from *Mycoplasma mycoides* var. *mycoides* strain T 3 after incubation at 37° for 4 days in 10 l. of the medium detailed above were harvested in a continuous-flow refrigerated centrifuge (M.S.E. Ltd.) at 22,000*g*, washed with phenolized physiological saline (phenol 0·2 % (w/v), pH 7·4) and resuspended in phosphate-buffered physiological saline (pH 7·4; 5 ml.). This mixture was made to 6 % (w/v) with respect to sodium *p*-aminosalicylate, added to an equal volume of phenol saturated with *p*-amino salicylate solution and the nucleic acids isolated as described for *M. laidlawii*. The weights obtained were: DNA, 2·5 mg; RNA, 13·0 mg.

*Base composition of the nucleic acids*

These were carried out as previously described (Jones *et al.* 1965).

## RESULTS AND DISCUSSION

*Amino acid composition of the protein of Mycoplasma mycoides var. capri—A correction*

In a previous communication (Jones *et al.* 1965) values were given for the amino acid composition of the proteins of *Mycoplasma mycoides* var. *capri*. We have since discovered that organisms harvested from growth in the dilute medium were contaminated with a protein fraction which had precipitated from the medium during growth. The dilute medium is not sufficiently buffered to prevent changes in pH value during growth of the organism, consequently the pH value decreased (7·4 to 6·4 in normal medium, 7·4 to 4·9 in dilute medium) causing the precipitation of a protein fraction from the dilute medium. When the organisms were grown in medium (3) above, which is similar to the dilute medium but with added phosphate, the decrease in pH of the medium was only from pH 7·4 to 6·7 and no precipitation of a fraction from the medium occurred. The results of the amino acid composition of the protein of the organism (the average for the organisms grown in the normal medium and medium 3 is given as the results were almost identical) are shown in Table 1. Also given are the results previously quoted (Jones *et al.* 1965) and the results given by Sueoka (1961) for the amino acid composition of the protein of *Tetrahymena pyriformis* which has a DNA with a similar base composition to that of the DNA of *M. mycoides* var. *capri*.

From the results in Table 1 it can be seen that the proline content of the protein was close to the expected value as predicted from Sueoka's (1961) results. Other experiments have indicated that the 'organisms' previously isolated from the dilute medium contained at least 50 % (w/w) of protein contaminant. It was also found that the use of a continuous-flow centrifuge (High Speed 18 Refrigerator Centrifuge, Continuous Action Rotor, Measuring and Scientific Equipment Co. Limited) for harvesting the organisms was very inefficient even with a flow rate of less than 2 l./hr. Thus only

16 mg. of DNA were isolated from the organisms harvested by a continuous-flow centrifuge from 20 l. of culture medium (i.e. 0·8 mg./l.), whereas 3 mg./l. were obtained when the organisms from an identical culture were harvested by using an angle-head centrifuge at 23,000*g*, although the *g* value for the two methods of centrifugation were almost identical.

Table 1. *Amino acid composition of the total protein of Mycoplasma mycoides var. capri strain P. G. 3*

The protein was hydrolysed with 5 N-HCl *in vacuo* at 110° for 22 hr. The amino acid composition was determined with a Technicon Automatic Amino Acid Analyser. The results are expressed in the same form as those quoted by Sueoka (1961) and his values for the amino acid composition of the protein of *Tetrahymena pyriformis* are given for comparison. The value of the amino acid composition of the protein of *M. mycoides* var. *capri* when grown in two media is given: (1) the results quoted before (Jones *et al.* 1965); (2) the correct value which is the average of the values obtained from the cells grown in the normal medium and the dilute medium with extra phosphate. Molar amounts of amino acids, lysine, histidine, arginine, aspartic acid and asparagine, glutamic acid and glutamine, proline, alanine, valine, leucine, tyrosine and phenylalanine which are known to be stable and to be well recoverable in the analysis were summed. The other amino acids are classified as unstable amino acids. The amount of each amino acid both stable and unstable is expressed by its proportion to the sum of the stable amino acids.

| | *Mycoplasma mycoides* var. *capri* | | *Tetrahymena pyriformis* |
|---|---|---|---|
| % GC content of DNA. | 24·8 | | 25·0 |
| | (1) Jones *et al.* (1965) | (2) Present paper | |
| | Stable amino acids (average of 3 determinations) | | |
| Lysine | 14·0±0·2 | 14·3±0·2 | 11·9 |
| Histidine | 3·0±0·1 | 2·8±0·1 | 2·8 |
| Arginine | 4·3±0·2 | 4·0±0·1 | 6·1 |
| Aspartic acid | 13·4±0·1 | 16·7±0·3 | 16·5 |
| Glutamic acid | 16·2±0·2 | 15·7±0·2 | 17·6 |
| Proline | 11·0±0·2 | 5·4±0·1 | 5·1 |
| Alanine | 10·0±0·2 | 9·9±0·2 | 10·3 |
| Valine | 7·2±0·2 | 8·9±0·2 | 8·0 |
| Leucine | 11·8±0·1 | 12·1±0·1 | 11·7 |
| Tyrosine | 3·6±0·1 | 4·5±0·2 | 4·3 |
| Phenylalanine | 5·5±0·1 | 5·6±0·1 | 6·0 |
| Total | 100·0 | 100·0 | 100·0 |
| | Unstable amino acids | | |
| Glycine | 6·0±0·1 | 12·8±0·2 | 11·0 |
| Threonine | 6·8±0·2 | 7·9±0·2 | 7·7 |
| Serine | 7·3±0·2 | 7·3±0·2 | 8·1 |
| Isoleucine | 5·6±0·2 | 10·4±0·2 | 8·8 |
| Methionine | 1·3±0·3 | 3·2±0·3 | 1·6 |
| Cysteine | 2·7±0·3 | — | — |
| Total | 29·9 | 41·6 | 37·2 |

The decrease in pH value of the dilute medium during the growth of the organism possibly also accounted for the fact noticed previously (Jones *et al.* 1965), that the DNA from organisms grown in the dilute medium was always degraded unless precautions were taken to cool the medium before centrifugation. It seems probable that enzymic activity was much enhanced at pH 5 as compared with pH 6·4.

*Amino acid composition of the protein of Mycoplasma laidlawii*

The results for the amino acid composition of the protein of *Mycoplasma laidlawii* are given in Table 2 and for comparison the values given by Sueoka (1961) for the amino acid composition of the protein of *Bacillus cereus* which has a DNA of similar base composition to that of *M. laidlawii*. The amino acid analysis of the protein of *M. laidlawii* agrees well with the values quoted by Sueoka (1961) for the amino acid content of the protein of *B. cereus* and with those predicted from his results for a bacterium with a DNA of GC content of 33–34%.

Table 2. *Amino acid composition of the total protein of Mycoplasma laidlawii strains P. G. 9, 544A and 545B*

The protein was hydrolysed as given in Table 1 and the results are quoted in the same form. The results obtained by Sueoka (1961) for the amino acid composition of the protein of *Bacillus cereus* are shown for comparison.

| Organism | *Mycoplasma laidlawii* | | | *Bacillus cereus* |
|---|---|---|---|---|
| Strain | PG 9 | 554 A | 545 B | ATCC 6464 |
| % GC content of DNA | 33·3 | 32·5 | 34·4 | 35·0 |
| Stable amino acids (average of 2 determinations) | | | | |
| Lysine | 10·7 | 11·4 | 11·1 | 10·4 |
| Histidine | 2·6 | 2·8 | 3·2 | 2·9 |
| Arginine | 6·0 | 6·0 | 6·3 | 5·9 |
| Aspartic acid | 13·6 | 14·4 | 13·4 | 12·7 |
| Glutamic acid | 17·5 | 16·2 | 17·2 | 17·4 |
| Proline | 5·0 | 5·4 | 5·0 | 4·9 |
| Alanine | 12·0 | 10·8 | 11·6 | 14·6 |
| Valine | 10·5 | 11·0 | 10·3 | 10·7 |
| Leucine | 11·7 | 12·3 | 11·8 | 11·6 |
| Tyrosine | 4·9 | 4·7 | 4·9 | 3·8 |
| Phenylalanine | 5·5 | 5·2 | 5·2 | 5·0 |
| Total | 100·0 | 100·0 | 100·0 | 99·9 |
| Unstable amino acids | | | | |
| Glycine | 12·0 | 12·8 | 12·4 | 13·7 |
| Threonine | 8·4 | 7·9 | 8·3 | 7·9 |
| Serine | 7·3 | 7·1 | 7·1 | 6·1 |
| Isoleucine | 8·5 | 9·1 | 8·4 | 8·4 |
| Methionine | 3·1 | 2·9 | 3·4 | 4·0 |
| Cysteine | — | — | — | 0·5 |
| Total | 39·3 | 39·8 | 39·9 | 39·0 |

No attempt was made to determine the amino acid composition of the protein of *Mycoplasma mycoides* var. *mycoides* because (*a*) with the large amount of horse serum in the medium it was very likely that some precipitation of a serum protein fraction would occur; (*b*) as such a small quantity of organisms was obtained from such a large volume of medium, any small amount of precipitate from the medium would involve a considerable percentage error in the amino acid composition of the protein, and it was therefore thought that no reliance could be placed on the results.

*Composition of the nucleic acids*

The base compositions of the DNA and RNA are shown in Tables 3 and 4 respectively.

Table 3. *Base composition of the DNA of Mycoplasma laidlawii strains P. G. 9, 544 A and 545 B and M. mycoides var. mycoides strains T 3*

The amounts of bases present were determined from the ultraviolet absorption of the eluate from paper chromatograms after hydrolysis of the DNA with formic acid. The amounts are given in moles base/100 g. atoms P.

| Species | Guanine (G) | Adenine (A) | Cytosine (C) | Thymine (T) | $\frac{A+T}{G+C}$ |
|---|---|---|---|---|---|
| *M. laidlawii* PG9 | 16·3 | 33·0 | 17·0 | 33·7 | 2·00 |
| *M. laidlawii* 544 A | 15·7 | 33·2 | 16·8 | 34·3 | 2·08 |
| *M. laidlawii* 545 B | 17·0 | 32·8 | 17·4 | 32·8 | 1·91 |
| *M. mycoides* var. *mycoides* T3 | 15·2 | 35·5 | 14·8 | 34·5 | 2·33 |

Table 4. *Base composition of the RNA of Mycoplasma laidlawii strains P. G. 9, 544 A and 545 B and M. mycoides var. mycoides T 3*

The amounts of bases present were determined from the ultraviolet absorption of the eluate from paper chromatograms after hydrolysis of the RNA in hydrochloric acid. The amounts are given in moles base/100 g. atoms P.

| Species | Guanine (G) | Adenine (A) | Cytosine (C) | Uracil (U) | $\frac{A+U}{G+C}$ | $\frac{G+U}{A+C}$ |
|---|---|---|---|---|---|---|
| *M. laidlawii* PG9 | 27·0 | 30·5 | 19·0 | 23·5 | 1·17 | 1·02 |
| *M. laidlawii* 544 A | 26·0 | 31·0 | 19·5 | 23·5 | 1·20 | 0·98 |
| *M. laidlawii* 545 B | 26·7 | 30·5 | 19·0 | 23·7 | 1·18 | 1·02 |
| *M. mycoides* var. *mycoides* T3 | 26·4 | 31·0 | 19·4 | 23·2 | 1·18 | 0·99 |

It is noteworthy that the base composition of the DNA of *Mycoplasma laidlawii* given in the present paper lies within the range of values previously obtained and that the DNA from the two species of Mycoplasma analysed here are both of the extreme AT type, thus indicating the possibility that the mycoplasmas may be closely related. It would be of interest to attempt DNA/DNA hybridization between mycoplasmas to see whether the similarity in base composition is matched by a similarity in base sequence. The only work of this type to have been reported is on the hybridization between the DNA of a mycoplasma and its supposedly related bacterium; no DNA homology was demonstrated (Rogul *et al.* 1965).

The base compositions of the RNA of the two species of mycoplasma are very similar to those previously determined for *Mycoplasma mycoides* var. *capri*. The A + U : G + C ratios are in the range 1·17–1·20 and the moles of 6-amino bases are equal to the moles of 6-keto bases.

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# Characterization of *Corynebacterium acnes*

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SUMMARY

Thirty-eight strains of *Corynebacterium acnes* were used in a systematic study of biochemical and physiological reactions in an effort to define and clarify some of the characters of this species. Strains were isolated both from lesions in acne vulgaris and from normal skin.

The characters which were positive for all 38 strains were: gelatin liquefaction within 20 days, catalase production, ammonia production in peptone broth, tributyrin hydrolysis, α-haemolysis (green) in blood agar containing human or rabbit red blood cells, acid production from fructose, glucose, galactose, mannose, glycerol and trehalose.

## INTRODUCTION

*Corynebacterium acnes* (*Propionibacterium acnes*) is a predominant member of the normal flora of human skin (Evans, Smith, Johnston & Giblett, 1950). This anaerobic diphtheroid was initially isolated from lesions in acne vulgaris (Sabouraud, 1897), and the controversy as to its role in this disease is still a matter of interest (Kirschbaum & Kligman, 1963; Puhvel, Barfatani, Warnick & Sternberg, 1964; Puhvel, Hoffman, & Sternberg, 1966; Strauss & Kligman, 1960). The literature on the characterization of *C. acnes* strains presents a very confusing and inconclusive picture. This was pointed out by Brzin (1964) who studied 15 strains of *C. acnes* and attempted to clarify the criteria for classifying this organism. The present paper is a report on the systematic study of 38 strains of *C. acnes*, some of which were isolated from lesions in patients with acne vulgaris, and others from healthy skin.

## METHODS

Thirty-eight strains of *Corynebacterium acnes* were used for a comparative study of biochemical and biological properties; 19 of these strains were isolated from lesions in patients with severe to moderate comedo-papular, pustular or cystic acne. The sources of the strains are listed in Table 1. Of the other 19 strains, 16 were isolated from healthy skin by swabbing the area around the folds of the nose with sterile swabs saturated in Brain Heart Infusion Broth (Difco) enriched with 1% (w/v) glucose. The remaining 3 strains were obtained from the American Type Culture Collection and were *C. acnes* strains 6921, 6922 and 11827 (Seeliger). The procedure used for isolation and identification of *C. acnes* was as follows. The material to be cultured was swabbed on to Brain Heart Infusion Agar (BHIA) supplemented with 1% glucose and incubated anaerobically in Brewer jars at 37° for a minimum of 3 days. Presumptive *C. acnes* colonies were picked out and stained by Gram's method. Colony types which grew

anaerobically in *C. acnes*-like colonies and were Gram-positive short rods were isolated in pure culture anaerobically. These were tested for aerobic growth on BHIA medium supplemented with 1 % glucose. When no growth occurred aerobically final identification was made by positive slide agglutination tests of the organisms with commercially produced specific antisera against *C. acnes* (Difco). Stock cultures were maintained at 4° and were cultured monthly. Cultures were grown on BHIA medium supplemented with 1 % glucose, and incubated anaerobically for 4–5 days at 37°. It was found that best growth occurred on fresh media.

Table 1. *Source of Corynebacterium acnes strains used*

| No. of strains | Source |
|---|---|
| 3 | American Type Culture Collection |
| 16 | Normal skin (nasal area) |
| 3 | Comedo-papular acne |
| 10 | Pustular acne |
| 6 | Cystic acne |

Table 2. *Properties tested for the characterization of 38 strains of Corynebacterium acnes*

1. Liquefaction of gelatin
2. Catalase production
3. Indole production
4. Reduction of litmus milk
5. Haemolysis of blood agar
6. Reduction of nitrate
7. Hydrolysis of starch
8. Hydrolysis of tyrosine
9. Methyl red test
10. Voges-Proskauer reaction
11. Production of hydrogen sulphide
12. Hydrolysis of tributyrin
13. Production of cytochrome oxidase
14. Utilization of citrate
15. Production of ammonia
16. Survival at 60°
17. NaCl tolerance
18. Hydrolysis of urea
19. Fermentation of arabinose, adonitol, cellobiose, fructose, galactose, glucose, glycerol, inositol, lactose, maltose, mannitol, mannose, melibiose, raffinose, rhamnose, salicin, sorbitol, sorbose, sucrose, trehalose, xylose

The properties used for the characterization of 38 strains of *Corynebacterium acnes* are listed in Table 2. Unless otherwise stated, all incubation was at 37° under anaerobic conditions. The methods used were as follows.

*Liquefaction of gelatin.* Tubes of nutrient gelatin (Difco) were stab-inoculated and cultures were observed every fourth day up to 20 days by placing tubes in the refrigerator at 4° for 3 hr.

*Catalase.* Freshly prepared hydrogen peroxide (5 %, v/v) was flooded on 3 to 4-day-old colonies of *Corynebacterium acnes* on BHIA plates and the production of effervescence noted.

*Indole.* Cultures were grown for 3–5 days at 37° in 5 ml. tryptone broth (Difco) supplemented with 1 % glucose. For determination of indole production 0·3 ml.

Kovacs reagent (Ewing, 1960) was added to the cultures; production of a red ring on the surface of the medium was observed in positive tests.

*Reduction of litmus milk.* Cultures in litmus milk broth (Difco) were examined daily, and after noting no changes at the end of 20 days of incubation the tests were discarded as negative.

*Haemolysis* was tested on blood agar base supplemented with 1 % glucose and 5 % (v/v) human or rabbit red blood cells. Plates were examined daily for a maximum of 6 days.

*Reduction of nitrate to nitrite.* Initially organisms were inoculated on nitrate agar slants (Difco) and incubated for a maximum of 7 days. Because of poor results by this method, nitrate broth (Difco) cultures supplemented with 1 % glucose were used instead. Cultures were observed for nitrate reduction at the end of 2 and 4 days, by the addition of 1 ml. of reagent A (8 g. sulfanilic acid in 1 l. 5 N-acetic acid) and 1 ml. reagent B (5 g. α-naphthylamine in 1 l. 5 N-acetic acid). Negative mixtures were reduced by the addition of zinc dust to test whether reduction of nitrate had not proceeded beyond nitrite. The presence of nitrate was shown in this way in all mixtures which gave negative results for nitrite. Further testing of nitrate-negative strains was repeated by using the medium of Douglas & Gunter (1946).

*Hydrolysis of starch.* Plates of BHIA medium supplemented with 0·5 % (w/v) potato starch were inoculated and incubated for 4–7 days. Subsequent flooding with Lugol's iodine solution (Dowson, 1957) showed whether or not starch had been hydrolysed.

*Hydrolysis of tyrosine.* Originally plates of BHIA medium supplemented with 0·5 % tyrosine were inoculated and incubated for 4–7 days; because of poor growth of *Corynebacterium acnes* on this medium 1 % glucose was added to the agar.

*Methyl red–Voges Proskauer test.* MR–VP medium (Difco) inoculated with *Corynbacterium acnes* was incubated for 4 and 7 days and the MR–VP tests done by Ewing's method (1960).

$H_2S$. Triple sugar iron slopes (Difco) were stab-inoculated with *C. acnes* and incubated for 4–7 days.

*Hydrolysis of tributyrin.* Tributyrin agar was made as follows: tributyrin was sterilized by millipore filtration and added in a final concentration of 1·5 % to sterile medium consisting of 1 % peptone, 0·5 % yeast extract and 2 % agar. Plates were surface-inoculated and incubated for 6 days. A zone of clearing around the colonies constituted a positive test.

*Oxidase.* Tests for oxidase production were performed as recommended for Neisseria (*Gradwohl's Clinical Laboratory Methods and Diagnosis*, 1963, p. 497).

*Utilization of citrate as a carbon source.* Organisms were inoculated on Bacto Simmon's citrate medium and slopes were incubated for a minimum of 10 days, but the medium was nutritionally inadequate for all the strains studied.

*Production of ammonia.* Peptone broth supplemented with 1 % glucose was inoculated and incubated for 5 days and 12 days. Equal volumes of Nessler reagent (Dowson, 1957) were added to each tube and the development of an orange precipitate was observed in positive tests.

*Heat resistance.* Turbid suspensions of fresh (4-to-5-day-old) cultures of *Corynebacterium acnes* in 0·85 % (w/v) NaCl solution were prepared and incubated in a 60° water bath for periods of 10, 20 and 30 min. Before incubation, and at 10 min. intervals

0·1 ml. samples of the suspensions were inoculated on plates of BHIA supplemented with 1 % glucose to test for survival of the organisms.

*Salt tolerance.* Ability to grow in the presence of various concentrations of sodium chloride was initially examined in broth medium (2 % peptone, 0·5 % yeast extract, 1 % glucose with 2·5–25 % sodium chloride). Incubation for 10 days produced inconclusive results. For better results BHIA medium supplemented with 1 % glucose and concentrations from 2·5 % to 25 % of sodium chloride were prepared.

*Urease activity.* Inoculated slopes of urea agar (Difco) were examined for positive reactions (pink coloration) after 3–7 days of incubation.

*Carbohydrate fermentation.* This was tested for with 21 different sugars. The basal medium consisted of 2 % peptone, 0·5 % yeast extract, 0·1 % sodium thioglycollate, bromcresol purple (*Gradwohl's Clinical Laboratory Methods and Diagnosis*, 1963, p. 21). Sugars were sterilized in 10 % (w/v) solution in distilled water by millipore filtration, and suitable volumes added to the basal medium to give a final concentration of 1 %. All cultures were observed daily for a minimum of 20 days before discarding.

Table 3. *Results of biochemical tests on 38 isolates of Corynebacterium acnes*

| Test | No. positive | Test | No. positive |
|---|---|---|---|
| 1. Liquefaction of gelatin | 38 (100)* | 19. Fermentation of | |
| 2. Production of catalase | 38 (100) | arabinose | 0 |
| 3. Production of indole | 34 (85) | adonitol | 31 (81) |
| 4. Reduction of litmus milk | 0 | cellobiose | 0 |
| 5. Haemolysis of blood agar (alpha) | 38 (100) | fructose | 38 (100) |
| 6. Reduction of nitrate | 29 (76) | galactose | 38 (100) |
| 7. Hydrolysis of starch | 0 | glucose | 38 (100) |
| 8 Hydrolysis of tyrosine | 0 | glycerol | 38 (100) |
| 9. Methyl red test | 0 | inositol | 0 |
| 10. Voges-Proskauer test | 0 | lactose | 0 |
| 11. Production of hydrogen sulphide | 0 | maltose | 29 (76) |
| 12. Hydrolysis of tributyrin | 38 (100) | mannitol | 35 (92) |
| 13. Production of cytochrome oxidase | 0 | mannose | 38 (100) |
| 14. Citrate utilization | 0 | melibiose | 0 |
| 15. Production of ammonia | 38 (100) | raffinose | 0 |
| 16. Survival for 10 min at 60° | 0 | rhamnose | 0 |
| 17. Tolerance of | | salicin | 0 |
| 2·5 % NaCl | 38 (100) | sorbitol | 21 (55) |
| 5·0 % NaCl | 0 | sorbose | 0 |
| 18. Hydrolysis of urea | 0 | sucrose | 0 |
| | | trehalose | 38 (100) |
| | | xylose | 0 |

* Percentages in brackets.

## RESULTS

Results of the biochemical tests are summarized in Table 3, from which it can be seen that the characteristics which were positive for all 38 strains of *Corynebacterium acnes* were: gelatin liquefied with 20 days, catalase-positive, ammonia produced in peptone broth, tributyrin hydrolysed, $\alpha$-haemolysis (green) produced in blood agar containing human or rabbit red blood cells, acid produced from fructose, glucose, galactose, mannose, glycerol, trehalose.

Characteristics which were negative for all strains were: cytochrome oxidase, urease, and hydrogen sulphide not produced, starch and tyrosine not hydrolysed, no

growth on BHIA plus 1 % glucose incubated aerobically, litmus milk not reduced, the methyl red and Voges-Proskauer reactions negative, and citrate not utilized as carbon source, lactose, raffinose, arabinose, xylose, sucrose, salicin, cellobiose, melibiose, inositol, rhamnose and sorbose not fermented. No strains survived 60° for 10 min., none grew on media containing more than 2·5 % sodium chloride.

The characteristics which differed with different strains were indole production, nitrate reduction and fermentation of mannitol, maltose, sorbose, adonitol. Since previous reports have suggested that reduction of nitrates is a characteristic of *Corynebacterium acnes* (e.g. Douglas & Gunter, 1946; Prévot, 1948, 1966; Hauduroy, 1953; Beerens, 1953; King & Meyer, 1957), particular attention was paid in double-checking all negative nitrate-reduction tests. Nitrate broth inoculated with *C. acnes* was tested for nitrate daily by testing 1 ml. samples of the culture over a period of 5 consecutive days, until heavy growth was present in all tubes. The addition of powdered zinc to negative tests at this point always showed the presence of unreduced nitrate. Thus of the 38 strains tested, only 15 were strong nitrate reducers, 14 reduced nitrate very weakly and 9 not at all. This property could not be related to the source of the strain, nor to any other variable property demonstrated.

All 38 strains were agglutinated by (Difco) hyperimmune *Corynebacterium acnes* antisera in slide agglutination tests. The same occurred when agglutination tests were made with rabbit antiserum prepared against *C. acnes* strains isolated in this study.

Table 4. *Comparison of results obtained in sugar fermentation studies of Corynebacterium acnes*

| Author... | Sudmerson & Thompson | Douglas & Gunter | Prévot | Hauduroy | King & Meyer | Bouisset *et al.* | Brzin | Present results |
|---|---|---|---|---|---|---|---|---|
| Date... | 1909 | 1946 | 1948, 1966 | 1953 | 1957 | 1960 | 1964 | — |
| No. of strains | 2 | 34 | . | . | 4 | . | 15 | 38 |
| Glucose | + | + | + | + | + | + | + | + |
| Galactose | + | +1* | + | + | + | + | + | + |
| Fructose | . | + | + | . | + | + | + | + |
| Mannose | . | + | . | . | . | . | + | + |
| Glycerol | . | +3* | . | . | . | . | + | + |
| Trehalose | . | . | . | . | . | . | + | + |
| Maltose | + | −1* | + | + | + | + | V | V |
| Mannitol | . | V | . | . | + | . | V | V |
| Lactose | − | − | + | V | − | − | −1* | − |
| Raffinose | V | − | . | . | V | . | V | − |
| Arabinose | + | − | . | . | . | . | V | − |
| Xylose | . | . | . | . | − | . | V | − |
| Sucrose | . | − | + | . | + | . | . | − |
| Salicin | . | − | . | . | − | . | V | − |
| Rhamnose | . | . | . | . | . | . | − | − |
| Adonitol | . | . | . | . | . | . | V | V |
| Sorbitol | . | . | . | . | . | . | V | V |
| Cellobiose | . | . | . | . | . | . | − | − |
| Inositol | . | . | . | . | . | . | −2* | − |
| Melibiose | . | . | . | . | . | . | V | − |
| Sorbose | . | . | . | . | . | . | − | − |
| Inulin | + | − | . | . | . | − | − | . |
| Starch | . | − | . | . | . | . | V | − |

* Exception. V = variable.

## DISCUSSION

The results of previous investigations characterizing *Corynebacterium acnes* are summarized in Tables 4 and 5. Some of these are the results of studies comparing biochemical reactions of *C. acnes* with those of other organisms (King & Meyer, 1957). The results of the present work with 38 strains, together with the studies of Douglas & Gunter (1946) with 34 strains and Brzin (1964) with 15 strains, represent the findings with a total of 87 different isolates of *C. acnes*. With minor exceptions the

Table 5. *Comparison of results of biochemical studies of Corynebacterium acnes*

| Author... Date... | Douglas & Gunter 1946 | Prévot 1948, 1966 | Hauduroy 1953 | Beerens 1953 | King & Meyer 1957 | Bouisset *et al.* 1960 | Brzin 1964 | Present results |
|---|---|---|---|---|---|---|---|---|
| Catalase test | + | + | . | + | + | . | . | + |
| Gelatin liq. | + | − | − | + | − | − | . | + |
| Nitrates reduced | + | + | + | + | + | + | V | V |
| Indole prod. | 50% | V | − | + | + | + | 50% | 85% |
| Haemolysis (on human RBC) | $\beta$ | . | . | $\beta$ | . | . | $\beta$* | $\alpha$ |
| Litmus milk red | . | . | . | + | Slight | + | . | − |
| Ammonia | + | + | . | . | . | . | . | + |

* One of 15 strains non-haemolysis

results agree that *C. acnes* consistently ferments glucose, fructose, mannose, galactose, trehalose and glycerol, but not sucrose, raffinose, arabinose, salicin, xylose, rhamnose or lactose. Mannitol, maltose, adonitol, and sorbitol fermentation varies with the strain. The carbohydrate fermentation reactions on these 87 strains were done with sodium thioglycollate as a reducing agent. Moore & Cato (1963) showed that the degree of anaerobiosis may be important in establishing the fermentative capacity of *C. acnes*; they found that under extremely stringent anaerobic conditions, *C. acnes* fermented lactate, whereas the same organism did not ferment lactate in deep broth culture.

Gelatin was always liquefied by *Corynebacterium acnes*, and catalase produced. Catalase production suggests that *C. acnes* is not an obligate anaerobe, and this is confirmed by the fact that the organism can be grown aerobically when a very heavy inoculum is used (Douglas & Gunter, 1946; Brzin, 1964). However, aerobic growth is always slower and poorer than after anaerobic incubation.

Production of indole by *Corynebacterium acnes* varies with the strain (see Table 5). Of the 38 strains in the present study a slightly higher percentage (85%) produced indole than reported in previous studies (50% by Douglas & Gunter, 1946; Brzin, 1964). However, Brzin's observation (1964) that there was an inverse correlation between indole production and ability to ferment maltose was not confirmed in the present work; nor could we confirm Brzin's more generalized observation that *C. acnes* strains can be divided into saccharolytic and indologenic types. As regards nitrate reduction we believe that, contrary to previous reports (see Table 5), this is not a definitive property of *C. acnes*. Repeated tests of *C. acnes* strains have shown that nitrate reduction varies with the strain and cannot be used for classifying the organism.

The three strains obtained from the American Type Culture Collection were among those which did not reduce nitrate.

Beta haemolysis on blood agar containing human red blood cells has been reported as a property of *Corynebacterium acnes* by several investigators (see Table 5). Attempts to grow *C. acnes* on nutrient agar containing both human and rabbit red blood cells showed that all 38 strains of the present study produced a green or alpha-type haemolysis. This failure to demonstrate beta haemolysis perplexed us. Medium identical to that described by Douglas & Gunter (1946) was prepared, but again there was never any evidence of beta haemolysis. Since the three registered strains of *C. acnes* from the American Type Culture Collection produced exactly the same green haemolysis on blood agar as those isolated for our study, there was no reason to doubt the authenticity of our isolates.

Table 6. *Sources of Corynebacterium acnes strains giving negative reactions*

| Negative test | No. of strains from acne vulgaris (total of 20) | No. of strains from normal skin (total of 15) | No. of strains from ATCC (3) |
|---|---|---|---|
| Indole production | 3 | 2 | 0 |
| Nitrate reduction | 1 | 5 | 3 |
| Maltose fermentation | 7 | 2 | 0 |
| Mannitol fermentation | 3 | 0 | 0 |
| Sorbitol fermentation | 8 | 9 | 0 |
| Adonitol fermentation | 5 | 2 | 0 |

Of the 39 properties tested in the present work, there were only 6 which varied with the strain (indole production, nitrate reduction, fermentation of maltose, mannitol, adonitol, sorbitol). These results confirm those of Douglas & Gunter (1946) in that there were no basic differences between strains of *Corynebacterium acnes* isolated from lesions in acne vulgaris and those strains isolated from normal skin (see Table 6). Nor could any pattern of reactions be found which could be used to group strains of *C. acnes*. Of the 38 strains used, only 9 were positive in all of the six characters which varied with the strains. The other 29 strains produced 15 different patterns of reactions. Of these, 7 produced indole, fermented maltose, adonitol, sorbitol and mannitol, but did not reduce nitrate; 6 reduced nitrate, produced indole, fermented maltose, mannitol, adonitol, but did not ferment sorbitol. The remaining 16 strains produced 13 different combinations of reactions.

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# The Pathogenicity of *Aeromonas salmonicida* (Griffin) in Sea and Brackish Waters

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SUMMARY

The experimental pathogenesis of *Aeromonas salmonicida* for the sea trout (*Salmo trutta*) and the brown trout (*Salmo fario*) in sea and brackish waters was examined by contact between infected and healthy fishes. Sea trout and brown trout both acquired infection by contact with infected *S. fario* at salinity values from 2·54% to 3·31% (w/w) when water temperatures ranged from 5·6° to 14·5°. The species differed in their resistance to infection. About 90% of the sea trout became infected and died within 7 days, developing superficial lesions typical of the disease. The brown trout appeared to be more resistant, about 75% developing and dying from an atypical form of the disease.

## INTRODUCTION

*Aeromonas salmonicida* is the causal agent of furunculosis, a specific infective disease of fish. Mackie *et al.* (1930) stated that the genus *Salmo*, which contains both freshwater and migratory species of fish, was most frequently affected. In their Final Report, the Furunculosis Committee stated that the degree of susceptibility varied between different species, salmon (*Salmo salar*), sea trout or salmon trout (*Salmo trutta*), and American brook trout (*Salmo* or *Salvelinus fontinalis*) were very susceptible, whilst the rainbow trout (*Salmo irideus*) was more resistant. Investigation of the dissemination of the disease was made under the direction of the Furunculosis Committee between the years 1928 and 1934. As a result of these investigations Mackie *et al.* (1935) reported that furunculosis was acquired exclusively in fresh water and that migratory Salmonidae became infected after they had left the sea. These widely held views (McCraw, 1952) are based mainly on field observations. McCraw (1952) stated that 'it is difficult to conceive the propagation of furunculosis among salmon or other fish in the sea as it has been shown that the specific organism survives for only a short time in sea water'. Field observations reported by Mackie *et al.* (1930, 1933, 1935) indicated that active spread of furunculosis did not occur to any extent where the volume of water per single fish was great. Furthermore, temperature conditions in the sea would generally be unfavourable to the development and spread of the disease. However, although it may be argued that the volume of water per single fish is great in the ocean, prevailing conditions such as temperature and volume of water in certain tidal areas may be comparable with those upstream in fresh water. Migratory Salmonidae tend to congregate in pools in tidal water for considerable periods before moving upstream (Jones, 1959), such a condition predisposing transmission of infection. Smith (1962) stated that investigations (unpublished) had shown that the viability of *Aeromonas salmonicida* in sea

water was not negligible, and that bacteriological examination of smolts for the organism revealed that these fish might be infected. Therefore *A. salmonicida* may not only pass from one adult host to another, it may also pass from an adult host to a juvenile host and survive a period in the sea, with the consequence that at least part of the returning migrants are infected before they reach fresh water. Thus, if the longevity of *A. salmonicida* is comparable in sea water, brackish water and fresh river water, it would appear that furunculosis might also be acquired in tidal waters. There is an obvious need for detailed information about the pathogenesis of *A. salmonicida* in saline waters. The following is an account of such an investigation planned as a set of small-scale epizootological experiments in which transmission of infection in sea and brackish water was attempted between sea trout (*S. trutta*) and also land-locked trout (*S. fario*) by contact with brown trout which had died from furunculosis.

## METHOD

*Aeromonas salmonicida.* Two strains of *A. salmonicida*, morphologically and biochemically identical but isolated from different sources, were used for the experiments. Strain 5 was isolated from a brown trout, strain 7 from a salmon smolt. Both cultures were examined for purity, consistency of biochemical activity and pathogenicity at regular intervals during the period of experimentation.

*Culture of bacteria from fish.* Fish were seared on the undersurface before opening and sampling. Bacterial specimens for plating were taken with a platinum wire loop from heart-blood, spleen, liver, kidney and intestine. Several loopfuls were taken from each organ; inoculum tissue was macerated with a sterile scalpel. Spreading of the inoculum on solid Blood Agar Base No. 2 (Oxoid) in Petri dishes was done by using a sterile glass spreader. The plates were incubated aerobically at 20° for at least 48 hr. The brown pigment which is generally accepted to be characteristic of *A. salmonicida* developed in the agar in this time.

*Suspensions of Aeromonas salmonicida.* Ten ml. of sterile physiological saline were added in small portions to a well-grown 48 hr slope culture of the organism, the growth being emulsified with the aid of a wire loop. The suspension was then decanted and allowed to stand for 30 min. until bacterial clumps and fragments of agar had sedimented. The suspension was then standardized to tube 2 of Brown's opacity standards (*Mackie & McCartney's Handbook*, 1960).

*Experimental infection tanks.* Three polythene-lined galvanized tanks enclosed by movable wire-netting covers were set up under natural conditions as experimental tanks. Brown trout inoculated intramuscularly with *Aeromonas salmonicida* were housed in one tank, capacity 16·25 cu.ft., (0·46 m.$^3$) which was open to sunlight and provided with a regulated supply of well-water at constant temperature (4·4°). The other tanks were placed in a shaded position, a smaller tank, capacity 16·25 cu.ft., which contained sea water being set above the level of the larger tank, capacity 24·1 cu.ft. (0·68 m.$^3$) which contained fresh well-water. The lower tank was used as the experimental tank.

*Fish.* Ten experiments were made, using one sea trout in each; the average length of these fish was 45 cm., giving a population density of approximately 1 fish/24 cu. ft. (0·68 m.$^3$) water. Two experiments were done with 12 land-locked brown trout in each, giving a population density of 1 fish/2 cu.ft. (0·05 m.$^3$) water; these fish ranged in size

from 10 to 23 cm. in length. The experimental fish were adapted to brackish water before the beginning of each experiment by introducing sea water at the rate of approximately 4 gallons/hr into the lower tank.

Once the required degree of salinity minimum 2·5 % (w/w) was attained (Harvey, 1960) the sea-water supply was regulated to a slightly slower rate for the beginning of the experiment, the flow being such that aeration was required.

*The experiments.* The sequence for each experiment was to inject intramuscularly six brown trout (10–13 cm. long) with 0·25 ml. of a suspension of *Aeromonas salmonicida.* Strain 5 of *A. salmonicida* was used for Expts. 1–5 and Expt. 11, and strain 7 for Expts. 6–10 and Expt. 12. The inoculated fish developed a well-defined 'boil' at the site of injection and died within 4–5 days, after which they were introduced into the sea-water tank containing the experimental sea trout or brown trout already adapted to the requisite degree of salinity. Sea trout and brown trout kept in a hatchery race-way during each experiment served as controls. At the conclusion of each experiment the sea-water tank was emptied and sterilized with hypochlorite solution, and thoroughly rinsed by pumping well-water through for 1 hr, the water then being tested to ensure that no residual chlorine remained (Windle Taylor, 1958). During the experimental period a regular check was kept on water temperature, degree of salinity and dissolved oxygen content of the water. As each experimental fish died, it, together with the control fish, was examined for external lesions indicative of disease. Heart blood, kidney, liver, spleen, intestine and reproductive organs were examined for the presence of *A. salmonicida.*

## RESULTS

The pathogenicity of *Aeromonas salmonicida* for sea trout and brown trout was successfully demonstrated in sea and brackish waters (Tables 1, 2). Infection was transmitted at salinity values from 2·54 % to 3·31 % (w/w) at water temperatures ranging from 5·6° to 14·5°. While the strains of *A. salmonicida* used appeared to be identical, some variation occurred in the infectivity rate. Strain 5 of *A. salmonicida* was transmitted and caused the subsequent deaths of all of the sea trout and 10 of 12 brown trout. *Aeromonas salmonicida* strain 7, however, infected and caused the deaths of only 3 of 5 sea trout and 7 of 12 brown trout.

The sea trout died within 7 days of contact with the infected fish. They did not develop 'boil-like' lesions, but superficial lesions were noted at the base of the pelvic fins, occasionally at the base of the pectoral fins, and around the vent; a brownish discharge from the vent was observed in several of the fish. The pathological picture in the sea trout was typical of the disease, most of the fish having developed congestion and haemorrhages in the internal organs, frequently with necrosis of kidney and spleen. Haemorrhages in the reproductive organs were common and in most cases the intestine was grossly congested. *Aeromonas salmonicida* was not recovered from control sea trout. It is interesting to note that one of the sea trout died within a few hours of being put into the experimental tank. This fish was found to be harbouring *A. salmonicida* which was recovered from kidney and heart blood; it is improbable that it had acquired the organism on re-entry to fresh water, since the fish had been removed from the river immediately on leaving tidal water, and had then been confined in well-water until required for experimental purposes.

Brown trout appeared to be more resistant than sea trout, remaining alive for up to

14 days after contact with infected fish. Again, 'boil-like' lesions were absent, but superficial lesions were noted at the base of the pelvic fins, occasionally at the base of the pectoral fins and around the vent. No brownish discharge from the vent was observed with any of these fish. The disease in brown trout was atypical in that there were few internal lesions; those lesions which did occur were found in the hind gut, reproductive organs and kidney. All control fish appeared to be healthy and did not yield *Aeromonas salmonicida.*

Table 1. *Infection of sea trout in sea water by contact with brown trout previously infected with Aeromonas salmonicida strain 5 (Expts. 1–5) and strain 7 (Expts. 6–10)*

| Salinity (%) | Average water temp. (°C) | Sex | Sea trout Length (cm.) | Sea trout Weight (g.) | Time in days between contact and death | Isolate result | Site of isolation |
|---|---|---|---|---|---|---|---|
| | | | Experiments 1–5 | | | | |
| 3·01 | 14·0 | Male | 48 | 1049 | 7 | + | Heart, liver, kidneys, intestines, spleen, milt |
| 2·82 | 14·5 | Male | 51 | 1467 | 7 | + | Heart, liver, spleen, kidneys, milt |
| 2·72 | 13·5 | Female | 41 | 624 | 7½ | + | Heart, liver, kidneys, spleen, muscle |
| 2·54 | 13·0 | Male | 43 | 829 | 6 | + | Heart, liver, kidneys, spleen, intestine, muscle and testes |
| 2·63 | 11·0 | Female | 42 | 780 | 7 | + | Kidneys only |
| | | | Experiments 6–10 | | | | |
| 2·96 | 9·0 | Male | 45 | 985 | 7 | + | Heart, liver, spleen, kidneys, intestines, muscle and testes |
| 2·90 | 9·6 | Female | 43 | 794 | 5 | − | All organs negative |
| 3·31 | 8·3 | Female | 50 | 1417 | 7 | + | Kidneys only |
| 0·16 | 5·6 | Female | 40 | 567 | 5 hours | + | Kidneys, heart, 'Carrier'? |
| 2·79 | 8·3 | Female | 42 | 673 | 6 days | − | All organs negative |

+ = *A. salmonicida* isolated. − = *A. salmonicida* not recovered.

Death following infection acquired by injection of *Aeromonas salmonicida* suspension was more rapid for brown trout in fresh water than death following infection acquired by contact with infected fish in sea water. Injected fish died within 5 days; *A. salmonicida* was recovered from the heart blood and all other organs except the reproductive organs, and was also isolated from the skin lesions, together with *Saprolegnia ferax* which appeared as a secondary infection in the 'boil' tissue. One interesting observation recorded for all the injected brown trout was that when they were allowed to remain in fresh water the boil tissues remained ragged, deep red, and intact, whereas, in sea water the lesions became clear-cut and their contents were washed away, leaving undamaged muscle tissue. Davis (1946) reported that this type of lesion is typical of Ulcer Disease.

Table 2. *Experiments 11 and 12: infection of brown trout in sea water by contact with brown trout previously infected with Aeromonas salmonicida*

| Brown Trout | | | | | |
|---|---|---|---|---|---|
| Sex | Length (cm.) | Weight (g.) | Time in days between contact and death | Isolate result | Site of isolation |
| Experiment 11: infection with *A. salmonicida* strain 5; water temperature 6·0°; salinity 3·01 % | | | | | |
| Female | 17 | 50 | 10 | + | Heart, liver, kidneys, ovary |
| Male | 23 | 99 | 10 | + | Heart, kidneys, testis, intestine |
| Male | 19 | 71 | 11 | + | Heart, liver, kidneys, spleen, testis |
| Female | 14 | 28 | 12 | + | Heart, kidneys |
| Male | 18 | 71 | 12 | + | Heart, liver, kidneys, testis, intestine |
| Female | 18 | 57 | 13 | + | Heart, liver, kidneys, spleen, ovary |
| Female | 10 | 14 | 13 | + | Kidneys |
| Female | 18 | 64 | 13 | + | Heart, kidneys, intestine |
| Male | 19 | 64 | 13 | + | Heart, liver, spleen, kidneys, testis |
| Male | 15 | 28 | 14 | + | Heart, kidneys |
| Female | 17 | 64 | Fish killed* | − | All organs negative |
| Female | 15 | 28 | Fish killed* | − | All organs negative |
| Experiment 12: infection with *A. salmonicida* strain 7; water temperature 5·6°; salinity 2·99 % | | | | | |
| Male | 24 | 120 | 7 | + | Heart, liver, spleen, kidneys, testis |
| Male | 11 | 7 | 7 | + | Heart, liver, spleen, kidneys, testis, intestine |
| Female | 20 | 57 | 7 | + | Heart, liver, spleen, kidneys, ovary |
| Male | 19 | 85 | 9 | + | Heart, kidneys, intestine |
| Male | 20 | 85 | 9 | + | Heart, kidneys, testis, intestine |
| Female | 15 | 28 | 11 | + | Kidneys |
| Female | 20 | 78 | 13 | + | Heart, kidneys, ovary |
| Female | 17 | 50 | 14 | − | All organs negative |
| Male | 19 | 64 | Fish killed* | − | All organs negative |
| Female | 19 | 64 | Fish killed* | − | All organs negative |
| Male | 18 | 71 | Fish killed* | − | All organs negative |
| Male | 10 | 7 | Fish killed* | − | All organs negative |

+ = *A. salmonicida* isolated. − = *A. salmonicida* not recovered.

* Fish killed denotes those fish remaining alive after 18 days, killed for bacteriological examination.

## DISCUSSION

The main purpose of this work was to examine the pathogenicity of *Aeromonas salmonicida* for sea trout and brown trout in sea and brackish waters. Preliminary work had indicated that the longevity of *A. salmonicida* was similar in fresh water, brackish water and sea water; Smith (1962) reported that the viability of the organism in sea water is not negligible. Consequently it was necessary to consider the possibility of infection occurring in other than fresh water.

The experiments indicated that infection by contact with infected fish is possible in sea and brackish waters. Transmission of infection was studied at salinity values of

2·54–3·31 %, simulating the salt content of estuarial waters and coastal sea waters, respectively. It appeared that infection by contact in these waters produced a generalized type of infection without localized skin lesions. Sea trout appeared to be more susceptible to this type of infection than did brown trout. However, environmental changes may have accounted for a weakening of resistance to infection. The sea trout had already been subjected to one complete change in osmotic pressure having been removed for experimental purposes from the river immediately on their re-entry to fresh water. Mackie *et al.* (1930) reported that on entering a river in which the disease existed fish became rapidly infected because of environmental changes. Although adequate concentrations of dissolved oxygen were maintained, it may be that in the experiments reported here water temperatures played an important part, being considerably higher during the experiments with sea trout than those with brown trout. Mackie *et al.* (1935) recorded the optimum temperature for the development of the disease as 13–19°, but when fish are weakened disease may occur at a considerably lower temperature, e.g. 7–10° and, exceptionally, at or below 4°. They reported that oxygen deficiency was not one of the determining factors in the incidence of the disease.

It is difficult to compare the present results with those of previous work, because no reference has been found about the pathogenicity of *Aeromonas salmonicida* in brackish water or sea water. The implications of the present findings, however, could be interesting because Smith (1962) reported the possibility of furunculosis in salmon smolts. Her findings are fully supported by the results of preliminary work for this present investigation when 1·7 % of migrating smolts taken from the River Coquet during one season were found to be harbouring *A. salmonicida*. Furthermore, field observations by Mr J. T. Percival in 1964 (personal communication) suggested there was little evidence that salmon and sea trout which entered the River Coquet contracted furunculosis after entering fresh water. Percival observed only a small incidence of furunculosis in the River Coquet during the years 1946–57. However, after the removal of the commercial net in the estuary in 1958, Percival observed an alarming increase of migratory fish dying from the disease the following season. As Percival pointed out, it is impossible for sick fish to fall back into tidal areas from fresh water in the Coquet because they are obstructed by a dam and fish pass which define the tidal limit. Hence these sick fish may have been returning migrants harbouring the organism which had survived a period in the sea or had become infected in the tidal water. The present findings contradict the concept that furunculosis is only transmitted in fresh water. It would also appear that migratory fish might play an important role both in transmitting the infection and as reservoirs of the disease.

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# The Starch-Gel Electrophoresis of Glucose-6-phosphate Dehydrogenase and Glyceraldehyde-3-phosphate Dehydrogenase of *Streptococcus faecalis*, *S. faecium* and *S. durans*

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SUMMARY

Thirty-six strains of enterococci were examined by standard physiological tests. The soluble proteins of cell-free extracts were separated by electrophoresis in starch gels. Specific staining technique for glucose-6-phosphate dehydrogenase and glyceraldehyde-3-phosphate dehydrogenase were applied to the gels. The results of electrophoresis supported the subdivision of the group into two major divisions represented by *Streptococcus faecalis* and *S. faecium*, with *S. durans* as a variant of *S. faecium*. It was possible tentatively to identify three strains which did not fit well into the taxonomic scheme used.

## INTRODUCTION

The enterococci have been subdivided into several named subgroups of which two, *Streptococcus faecalis* and *S. faecium*, are regarded by some workers (Deibel, Lake & Niven, 1963; Deibel, 1964; Whittenbury, 1965) as separate species. *Streptococcus zymogenes* and *S. liquefaciens* are considered to be varieties of *S. faecalis*, and *S. durans* to be a variety of *S. faecium*. Colobert & Blondeau (1962) and Defayolle & Colobert (1962) found that none of the physiological properties used in taxonomy were exclusive to any of the named types, and therefore preferred to regard the enterococci as one species, *S. faecalis*, with two major subdivisions, *S. faecalis proprium* and *S. faecium*. The techniques of electrophoresis and protein or enzyme staining have recently been applied to the differentiation and taxonomy of micro-organisms. Wagman, Pollack & Maio (1959) found that the pattern of soluble proteins after electrophoresis in liquid media differed amongst several micro-organisms studied. The more convenient technique of gel electrophoresis has been used to show differences in protein pattern amongst the pleuropneumonia-like organisms (Fowler, Coble, Kramer & Brown, 1963) and in streptomyces (Gottlieb & Hepden, 1966). The pattern of esterase bands on starch gels was used by Norris (1964) to classify strains of *Bacillus thuringiensis*. Heterogeneity of glyceraldehyde-3-phosphate dehydrogenase (Ga3PD) was observed amongst the enterococci (Williams, 1964) and considerable strain variation of glucose-6-phosphate dehydrogenase (G6PD) was also found in this group (Williams, 1965). Little variation was found in the case of 6-phosphogluconate dehydrogenase, or with a stain designed to locate pyruvate kinase that has since been

proved to reveal adenylate kinase and has been simplified for this purpose. The lactic dehydrogenase pattern in these organisms showed some strain variation, but bands could not be detected at all in some strains by the staining method used. Techniques similar to those of Norris have since been applied to mycobacteria (Cann & Willox, 1965; Cann, Hobbs & Shewan, 1966) and to the enterococci (Lund, 1965). In the present work the two enzymes found to vary most in the earlier work (Ga3PD, G6PD) have been re-examined for electrophoretic heterogeneity by using 36 enterococcal strains, which were also tested by normal physiological methods.

## METHODS

*Organisms.* The strains used and their sources are listed in Table 1. All were grown on blood agar plates aerobically at 37° for 24 hr and the whole crop of organisms suspended in 1 ml. of Oxoid Nutrient Broth no. 2 containing glucose 5 g./l. and bovine serum (Oxoid) 50 ml./l. One drop (0·03 ml.) quantities of suspension were freeze-dried and the ampoules stored at 4° until required.

*Media.* Peptone water for the determination of growth at 50° contained (g./l.): 20, peptone (Evans); 5, yeast extract (Difco); 5, sodium chloride; 1, disodium hydrogen phosphate; 1, glucose.

Table 1. *Sources of strains of enterococci used*

| Strain | Source |
|---|---|
| Z93, S161, H69D5, S41, HGH511, 2703, 60, S32, S43, 98D | R. Whittenbury |
| R26, K2A, FB82, 1GAU, K8A, R5 | R. H. Deibel |
| P17/8, 5422, C3 | B. M. Lund |
| 8886, 2707, 370, 8274, 8191, 6991 | NCIB |
| 588, 580, 942, 943, 582, 712, 596 | NCDO |
| 775, 8131, 8619 | NCTC |
| 680, isolated from human dental plaque | G. H. W. Bowden |

The basal medium for the physiological tests was (g./l.): 5, Lab-Lemco (Oxoid); 5, peptone (Evans); 5, yeast extract (Difco); 0·5, Tween 80; 0·1, $MnSO_4.4H_2O$; 0·02, bromcresol purple. This solution was adjusted to pH 6·5 and autoclaved at 121° for 15 min.

Nutrient broth for harvesting organisms was prepared from a solution containing (g./l.): 20, proteose peptone (Oxoid); 10, yeast extract (Difco); 20, glucose. This solution was made to pH 3·0 with hydrochloric acid, filtered through Whatman no. 4 and no. 1 papers together, neutralized with sodium hydroxide, diluted to twice the original volume and autoclaved at 108° for 30 min. on two successive days.

*Fermentation of carbohydrates.* The basal medium was adjusted to the correct volume to provide the final concentrations stated above, by adding sufficient 100 g./l. aqueous carbohydrate solution, previously sterilized by filtration, to give a final concentration of 5 g./l. of arabinose, sorbitol, melezitose, melibiose or mannitol. Three Pasteur pipette drops (0·1 ml.) of a growing culture were added to 5 ml. medium and incubated at 37° for 72 hr. A positive result was indicated by a distinct yellow colour corresponding to a decrease of one pH unit or more. Some of the cultures recorded as negative decreased the pH value by 0·5–0·7 units.

*Reduction, of 2,3,5-tetrazolium chloride (tetrazolium).* Basal medium containing glucose 5 g./l. but no bromcresol purple was adjusted to pH 6·0. An aqueous solution of tetrazolium was heated to 100° for 15 min. and added to the basal medium to a final concentration of 0·1 g./l. Cultures were incubated for 8 hr at 30° after inoculation as above. A deep magenta colour was regarded as a positive result; negative cultures were slightly pink or colourless.

*Tolerance of potassium tellurite.* Basal medium was prepared containing (g./l.): 15, agar; 5, glucose; 10, instead of 5 Lab-Lemco (Whittenbury, 1965); no bromcresol purple. A 10 g./l. aqueous solution of potassium tellurite was sterilized by filtration and added to the medium at 50° to give a final concentration of tellurite 0·4 g./l. Growing cultures were plated on tellurite agar and incubated at 37° for 72 hr. A positive result was indicated by grey or black colonies; negative cultures did not grow at all.

*Growth at 50°.* One drop (0·03 ml.) of a 17 hr culture was inoculated into 5 ml. peptone water and incubated in a 50° water bath and examined daily for 7 days. An uninoculated tube was included to serve as a blank for extinction readings. However, this was never necessary since positive cultures gave an increase in turbidity easily perceptible to the eye.

*Preparation of cell-free extracts.* Freeze-dried specimens were plated on blood agar and incubated aerobically at 37° for 24 hr. A colony was picked off and inoculated into 100 ml. nutrient broth and incubated for 17 hr under the above conditions. A smear was examined for purity by plating out and Gram-staining and the bulk culture centrifuged at 4000 *g* for 20 min. at 5°. The cocci were washed twice by suspension in 25 ml. water and centrifugation at 4000 *g* for 10 min. then suspended in 1 ml. water in a bijou bottle and shaken for 20 min. with 1 ml. ballotini beads (Jencons, grade 11) by using a Griffin flask shaker. The beads were separated by centrifugation at 1100 *g* over a Hemmings filter (Gallenkamp, Ltd.,) containing Whatman no. 1 filter paper. The homogenate was centrifuged at 7000 *g* for 10 min. at 5° and the clear or slightly opalescent supernatant fluid was used for starch-gel electrophoresis.

*Starch-gel electrophoresis.* A suspension of starch (Connaught Medical Research Labs., Toronto, Canada) in 0·01 M-phosphate buffer (pH 7·0) was boiled for 2 min. and the dissolved gases removed under reduced pressure. The concentration of starch used was that recommended by the manufacturer, being 102 g./l. for one batch and 120 g./l. for a second batch. The volume of the solution was adjusted with freshly distilled water to that before boiling and the liquid then poured into a mould (11·5 × 17·5 × 0·7 cm.) lined with 'Melinex' (I.C.I., Ltd.), allowed to set, and the whole placed in the refrigerator for at least 1 hr.

Samples of bacterial extract (0·06 ml.) were pipetted on to 0·5 × 1·5 cm. strips of Whatman no. 17 filter paper. These strips were placed in a transverse slot 5 cm. from the cathodic end of a starch gel, and the gaps between filled with molten gel of the same composition as the main slab. Six sample strips could be used on each gel. The gel and its Melinex sheets were removed from the mould and placed between metal-faced cooling plates through which was pumped water at 5°. The ends of the gel were linked to reservoirs containing 0·10 M-phosphate buffer (pH 7·0) by cloth bridges and approximately 10 V./cm. applied along the gel for 1·5 hr. At the end of this time the voltage gradient in the gel was measured with a Model 40 Avometer.

The gel slabs were sliced horizontally and the cut surfaces stained for the dehydrogenases G6PD or Ga3PD.

*Location of dehydrogenases in electrophoregrams.* The oxidation of a substrate catalysed by its dehydrogenase can be coupled to the reduction of tetrazolium in the presence of phenazine methosulphate as mediator. Treatment of an electrophoretrogram with a reagent mixture which includes an appropriate substrate will thus give rise to blue bands (due to the insoluble reduced tetrazolium salt) over areas which contain the dehydrogenase for this substrate. The glucose-6-phosphate (G6P) dehydrogenase was located by using G6P as the substrate (Fildes & Parr, 1963) and glyceraldehyde-3-phosphate (Ga3P) dehydrogenase was located by using fructose-1,6-diphosphate + aldolase (Williams, 1964). An extract of streptococcus NCIB8886 was used on every gel as a standard. The value $R_{8886}$ was calculated for all the bands of G6PD and Ga3PD of other strains and also for the minor bands of NCIB8886, where

$$R_{8886} = \frac{\text{distance travelled by a given band} \times 100}{\text{distance travelled by the major band of NCIB8886}}.$$

## RESULTS

### *Physiological tests*

Each strain was examined on three or four different occasions for each of the tests except the ability to produce acid from mannitol and the ability to grow at 50°; these tests were done twice. The results obtained are listed in Table 2. In several instances the results of a given test with a given strain were not uniform. In these cases a positive (or negative) result obtained on two of three, or three of four, occasions was judged to be positive (or negative) rather than variable. Only the tolerance of tellurite by strain 775 and the growth at 50° by strains 8886 and P17/8 were variable on this basis.

Fifteen strains were diagnosed as *Streptococcus faecalis* of which 8 were unequivocal and 3 showed consistently atypical reactions. These latter were 370 (−ve sorbitol and tellurite resistance) 580 and H69D5 (−ve melezitose). Eleven of the 15 strains were able to grow at 50°, which is anomalous for *S. faecalis* (Sharpe, Fryer & Smith, 1966), and the results of this test were therefore regarded with caution.

Of the 12 strains allotted to the *Streptococcus faecium* group 7 had all the properties typical of the group. The other 5 were anomalous in the fermentation of one or two carbohydrates: 8191 and 8619 (−ve arabinose), S32 (−ve arabinose and mannitol), IGAU (−ve melibiose) and 582 (+ve sorbitol).

Six strains were judged to be *Streptococcus durans* but of these 4 gave one positive reaction each.

Three strains could not be allotted to any of the three groups on the basis of the tests used. Strain 8274 gave 4 positive results and was definitely not *Streptococcus durans*, but many of the tests were variable with this strain and it could not be allotted to either *S. faecium* or *S. faecalis*. For the same reason S161 was not *S. durans* but might be regarded as *S. faecalis*, which had failed to resist tellurite and to reduce tetrazolium. Strain 943 could either be regarded as *S. durans* with the atypical ability to ferment mannitol and to grow at 50°, or as *S. facium* that failed to ferment arabinose and melibiose.

### *Electrophoresis*

Extracts of all 36 strains were prepared, electrophoresed and the dehydrogenases stained by the techniques described above. The most deeply coloured bands were

invariably detected, but this was not the case with many of the minor bands. The minor bands which most commonly occurred had a slightly slower migration than the major bands. The main finding in the present work was that the major band of both enzymes had a markedly lower mobility in strains of *Streptococcus faecium* and *S. durans* than in *S. faecalis* strains. Examples of these differences are shown in Pl. 1, fig. A for glucose-6-phosphate dehydrogenase, and Pl. 1, fig. B for glyceraldehyde-3-phosphate dehydrogenase.

The 15 strains that were designated *Streptococcus faecalis* by physiological tests all showed major bands of G6PD with $R_{8886}$ values between 88 and 104, and main bands of Ga3PD of $R_{8886}$ from 97 to 104. The enzymes of the unclassified strain S161 were also of this fast-migrating type; this strain is probably *S. faecalis*.

Table 2. *Results of physiological tests on 36 strains of enterococci*

Each symbol (+ or −) represents one test. Where consistent results were obtained in repeated tests, the numeral in the table indicates the number of repetitions. Where the results of repeat tests differed, the individual results are all scored.

| | | | Acid production from sugars | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Strain | Reduction of tellurite | Reduction of tetrazolium | Arabinose | Sorbitol | Melezitose | Melibiose | Mannitol | Growth at 50° | Diagnosis |
| 8886 | 4+ | 4+ | 4− | +−++ | 4+ | 4− | 2+ | +− | |
| 2707 | 3+ | 3+ | 3− | 3+ | 3+ | 3− | 2+ | 2− | |
| 370 | −−+− | 3+ | 3− | 3− | 3+ | 3− | 2+ | 2− | |
| 588 | 3+ | 3+ | 3− | 3+ | 3+ | 3− | 2+ | 2+ | |
| 580 | 3+ | 3+ | −+−− | 4+ | 4− | 4− | 2+ | 2+ | |
| 775 | +−−+ | 4+ | 3− | 3+ | 3+ | 3− | 2+ | 2+ | |
| 8131 | 3+ | 3+ | 3− | 3+ | 3+ | 3− | 2+ | 2+ | |
| Z93 | 3+ | 3+ | 3− | 3+ | 3+ | 3− | 2+ | 2+ | 'faecalis' |
| H69D5 | 3+ | 3+ | 4− | +−++ | +−−− | 4− | 2+ | 2+ | |
| S41 | 3+ | +−+ | 3− | 3+ | 3+ | 3− | 2+ | 2+ | |
| R26 | 4+ | 4+ | 4− | 4+ | 4+ | 4− | 2+ | 2+ | |
| K2A | 3+ | 3+ | 3− | 3+ | 3+ | 3− | 2+ | 2+ | |
| FB82 | 3+ | 3+ | 3− | 3+ | 3+ | 3− | 2+ | 2+ | |
| 680 | 4+ | 4+ | −−+− | 4+ | +−++ | 4− | 2+ | 2− | |
| R5 | 3+ | 3+ | 3− | 3+ | 3+ | 3− | 2+ | 2+ | |
| 8274 | +−++ | +−−− | −−+ | +−− | +−− | −++ | 2+ | 2+ | |
| S161 | 3− | 3− | 3− | ++− | 3+ | 3− | 2+ | 2+ | ? |
| 943 | 4− | 4− | 4− | 4− | 4− | 4− | 2+ | 2+ | |
| 98D | 4− | 4− | 3− | 3− | 3− | 3− | 2− | 2− | |
| 6991 | 3− | −−+ | 3− | 3− | 3− | 3− | 2− | 2− | |
| P17/8 | 3− | 3− | +−− | 3− | 3− | 3+ | 2− | −+ | 'durans' |
| C3 | 3− | 3− | 3− | 3− | 3− | 3+ | 2− | 2− | |
| 712 | 3− | 3− | 3− | 3− | 3− | 3− | 2+ | 2− | |
| 596 | 3− | 3− | 3− | 3− | 3− | 3− | 2− | 2+ | |
| 8191 | 3− | 3− | −−+ | −+− | 3− | 3+ | +− | 2+ | |
| 8619 | 3− | 3− | −−+ | 3− | 3− | −++ | 2+ | 2+ | |
| HGH511 | 3− | 3− | −++ | 3− | 3− | 3+ | 2+ | 2+ | |
| 2703 | 4− | 4− | −+++ | 4− | 4− | 4+ | 2+ | 2+ | |
| 60 | 4− | 4− | −++ | 3− | 3− | 3+ | 2+ | 2+ | |
| S32 | 3− | 3− | −+− | 3− | +−− | 3+ | 2− | 2+ | 'faecium' |
| S43 | 4− | 4− | −++ | −+− | 3− | −++ | 2+ | 2+ | |
| IGAU | 3− | 3− | +−+ | 3− | 3− | 3− | 2+ | 2+ | |
| K8A | 4− | 4− | 3+ | 3− | 3− | 3+ | 2+ | 2+ | |
| 582 | +−−− | +−−− | −+++ | +++− | 4− | −+++ | 2+ | 2+ | |
| 5422 | 4− | 4− | +−++ | +−−− | 4− | 4+ | 2+ | 2+ | |
| 942 | 3− | 3− | −+++ | 3− | 3− | −++ | 2+ | 2+ | |

Eleven of the 12 strains identified as *Streptococcus faecium*, 5 of the 6 *S. durans* strains, and the two unclassified strains 8274 and 943 had G6PD enzymes with mobilities between $R_{8886}$ 62 and 75. Strain 583, which was *S. faecium* according to the physiological tests, and strain 6991 which was identified as *S. durans* both had a G6PD pattern of the *S. faecalis* type however. All the *S. faecium* and *S. durans* strains, together with strains 8274 and 943 were consistent with regard to the mobility of Ga3PD, having $R_{8886}$ values between 67 and 83.

In two strains of the *Streptococcus faecalis* group (8886, *R*26) there appeared a second band of Ga3PD of comparable density of colour to the major band typical of the group (Pl. 1, fig. B). This band had a much lower mobility ($R_{8886} = 57$) than the enzyme fractions characteristic of *S. faecium* and *S. durans*; its significance is not known.

In the case of both enzymes the *Streptococcus faecalis* strains were closely similar to one another in band pattern, while the *S. faecium* and *S. durans* strains showed minor variations in mobility and frequently had double major bands (Pl. 1, fig. A, B). These minor variations were shown not to be electrophoretic artifacts by electrophoresis of mixtures of pairs of extracts. In twenty such experiments the G6PD and Ga3PD enzymes of two *S. faecalis* strains could not be separated in such mixtures. When *S. faecalis* and either *S. durans* of *S. faecium* extracts were mixed, a marked separation of the characteristic forms was obtained; but *S. faecium* could not be distinguished from *S. durans* in this way. The small differences in $R_{8886}$ value frequently gave rise to separations in such mixtures, but neither the degree nor the frequency of these separations was more marked in *S. faecium* + *S. durans* mixtures than in mixtures of two *S. faecium* extracts or two *S. durans* extracts.

## DISCUSSION

Recent work has shown the value of electrophoretic separation of esterases in the characterization of micro-organisms. Location of dehydrogenases in electrophoretograms, such as those described here, by coupling their action with the reduction of a tetrazolium salt (Williams, 1964), provides another character which can be revealed by a technique having several apparent advantages over the esterase method. The natural substrate of each enzyme is used in the dehydrogenase reaction and several different dehydrogenases can be located by including the appropriate substrate in the same basal reaction mixture, whereby the blue reduced tetrazolium locates the specific dehydrogenase band. The comparative simplicity of the band patterns for glucose-6-phosphate and glyceraldehyde-3-phosphate dehydrogenases reported here is in contrast with more complex patterns of esterase bands reported by other workers. The number of enzymes demonstrable by tetrazolium reduction can be further extended by coupling the action of pure dehydrogenases, used as reagents in the stain, to other enzymes in the electrophoretograms which produce the substrates for the reagent dehydrogenases. For example, hexokinase may be located by using glucose-6-phosphate dehydrogenase as a reagent (Brown, Miller, Holloway & Leve, 1967). The availability of a number of different stains, each of high specificity for one of the enzymes of carbohydrate metabolism, should prove the value in taxonomic studies. Furthermore, much information is already available on the comparative aspects of dehydrogenase enzymes, including amino acid analyses, coenzyme and coenzyme-

analogue specificities, electrophoretic mobilities and serological specification (Kaplan, 1965). To compare this existing information with data on the properties of microbial enzymes obtained during taxonomic studies would be both desirable and economical.

The results reported here support the division of the Lancefield Group D streptococci into two subgroups comprising *Streptococcus faecalis* and its varieties on the one hand, and *S. faecium* and *S. durans*. The electrophoretic similarity amongst strains within each subgroup does not imply identity of structure in the enzyme molecules concerned. Dance & Watts (1962) showed that electrophoretically identical creatine phosphotransferases gave rise to different peptides on tryptic digestion. In spite of this and of the anomalous mobility of enzymes in a small minority of strains, the large difference in enzyme mobility between the two subgroups and the high degree of similarity between the strains comprising each subgroup makes the electrophoretic patterns highly significant taxonomically.

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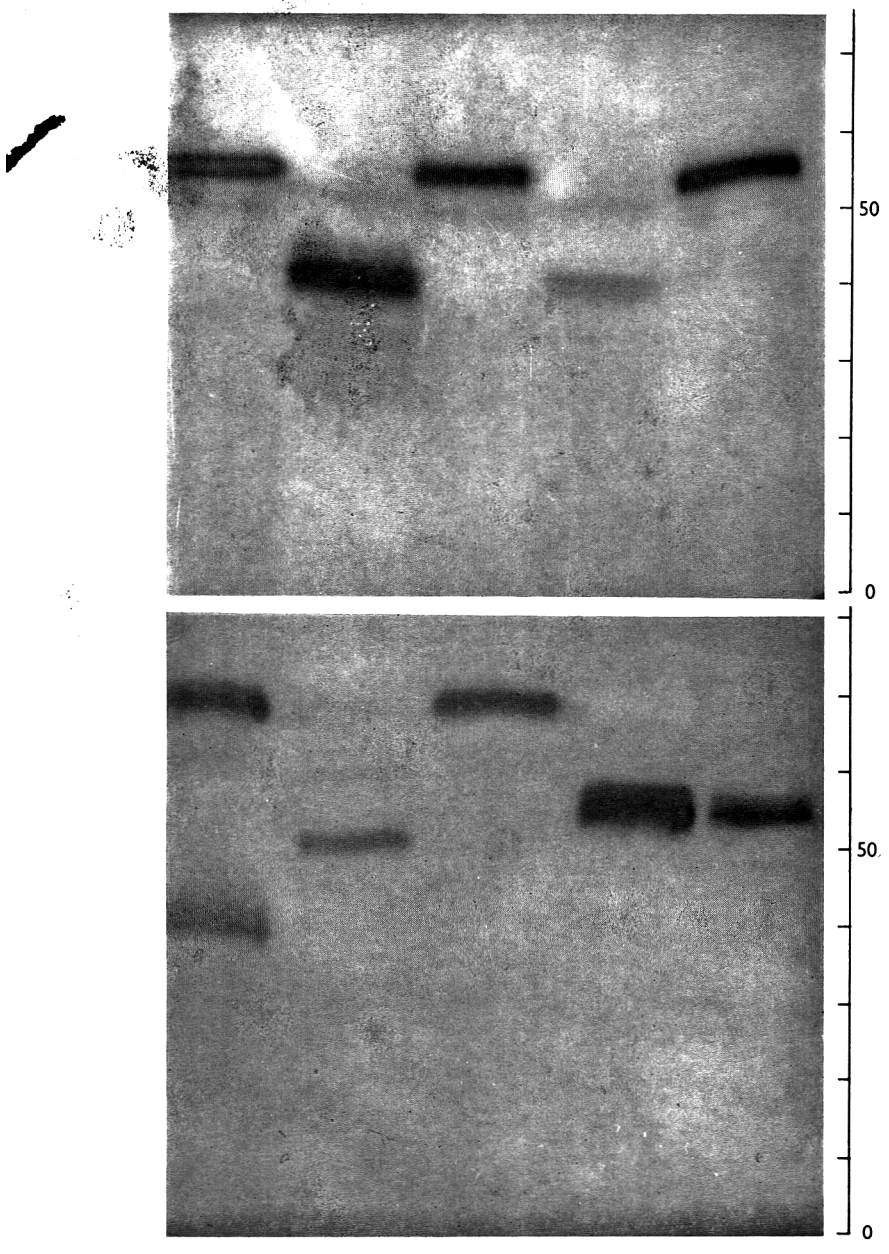
## EXPLANATION OF PLATE

The two gels shown are stained for glucose-6-phosphate dehydrogenase (A) and glyceraldehyde-3-phosphate dehydrogenase (B). In each the bacterial extracts used in each track are identified from left to right.

Fig. A. *Streptococcus faecalis* 8886; *S. faecium* HGH 511; *S. faecalis* 689; *S. faecium* 2703; *S. faecalis* R 26. The major bands of the three *S. faecalis* strains are all typical ($R_{8886}$ = 100). Strains HGH 511 and 2703 had a single and a double band respectively ($R_{8886}$ = 64) at a position characteristic of *S. faecium* and *S. durans*. In other preparations the double major band of 2703 merged into one band as straining proceeded.

Fig. B. *S. faecalis* 8886; *S. durans* C 3; S 161 (unclassified strain); 943 (unclassified strain); *S. faecium* 5422. This enzyme migrated faster in all strains than did G6PD. Strains 8886 and S 161 had fast-migrating components characteristic of *S. faecalis*. The densely staining second band ($R_{8886}$ = 58) of 8886 was unique to this strain and R 26. The migration of the major bands of the other three strains shows the minor variations ($R_{8886}$ = 74–83) within the range characteristic of *S. faecium* and *S. durans*.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# Somatic Cell Variation during Uninterrupted Growth of *Neurospora crassa* in Continuous Growth Tubes

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SUMMARY

Continuous growth of *Neurospora crassa* was studied to determine whether the organism was capable of uninterrupted hyphal elongation, whether there was any evidence of homeostatic mechanisms controlling growth over extended periods and, particularly, whether spontaneous mutants with suppressive phenotypes were capable of being expressed. Parallel cultures, A and B, having a common origin, were incapable of uninterrupted growth but permanent cessations of growth were not encountered. The A-culture showed frequent stops of long duration whereas B had infrequent stops of short duration. This stop–start growth behaviour in the A- and B-cultures was determined by two different cytoplasmic factors. Both extranuclear mutants show decreased cytochrome-*c* oxidase activity (Bertrand & Pittenger, unpublished). These stopper phenotypes may be similar to 'vegetative death' in Aspergillus and 'senescence' in Podospora. Both A- and B-cultures showed several major increases and decreases in rate, in addition to more frequent minor fluctuations. The general constancy of growth, however, and the routine restoration of growth after cessations, as well as following either transient or long-term growth rate decreases, indicated that homeostatic mechanisms were capable of buffering the organisms against most deleterious intracellular effects. Two spontaneous nuclear mutants with decreased growth rates as homokaryons also accumulated during growth. These altered nuclear types, whose growth rates were clearly non-adaptive, nevertheless had selective values. Their proportions increased significantly during growth to the extent of either completely displacing the original type or of reaching a high enough proportion so that growth rates were decreased and the morphology of the cultures altered. These nuclear and extranuclear mutants appeared to have a selective advantage because of suppressive characteristics.

## INTRODUCTION

The use of a continuous growth tube for the study of uninterrupted mycelial growth of *Neurospora crassa* is strictly a laboratory innovation since comparable conditions are never expected in nature. Under such controlled conditions in a rapidly growing organism such as Neurospora, where growth by hyphal elongation in a wild-type strain often exceeds 120 mm./day, the hyphal tips are continually advancing on to fresh medium as they progress down a growth tube. Homeostatic mechanisms are expected to maintain a constant growth rate by buffering the organism against deleterious effects that might accrue from spontaneous mutations and unfavourable physiological conditions. Continuous growth in a constant environment thus provides an unusual opportunity to observe the efficiency of such mechanisms over extended

periods of vegetative growth. Such a study also provides a basis for comparing the effects of long-term growth in Neurospora with those observed in other fungi, such as Aspergillus and Podospora. In the latter, continuous hyphal elongation invariably results in a cytoplasmically determined irreversible cessation of growth described as 'vegetative death' (Jinks, 1959) and 'senescence' (Marcou, 1961). In fact it was the results of such studies with Podospora and other slow-growing fungi which suggested that indefinite hyphal propagation is impossible in those fungi (Marcou, 1961). And lastly, such a study should enable the investigator, through systematic sampling and analyses of asexual spores of the culture, to determine whether the somatic cell variation expected to occur during the continuous asexual growth of such cultures has a genetic basis. Two observations in this regard by Jinks (1958, 1959)—that cytoplasmic elements are involved in naturally occurring variation in aged cultures and our own that many cytoplasmic mutants are characterized by a stop-and-start growth phenotype recognized only in growth tubes—originally prompted the present work.

It is clear from a variety of experiments that the coenocytic nature of the mycelium provides conditions that would allow the accumulation of a variety of spontaneous recessive mutants, which would be rapidly eliminated in a population of uninucleate cells. It can be assumed that spontaneous mutations which occur during growth of the original homokaryotic culture would provide, with time, a heterokaryon and heteroplasmon composed of many genetically different types, and that the effect of the majority of such recessive mutants on the continuous growth of the culture would be negligible. Since Neurospora grows by hyphal elongation and branching, and since nuclear ratios in heterokaryons appear to be relatively stable, it appears that protoplasmic streaming and hyphal fusions should aid in continuous mixing and dilution of spontaneously occurring mutant nuclei and cytoplasmic determinants with their normal homologues within the mycelium. However, because of those factors, it would be expected that mutations which occur in one member of a large population of cellular organelles rarely would be expected to accumulate and be maintained in sufficient numbers to alter the phenotype of the entire culture. Consequently, it could be anticipated that genetically determined changes in the growth rate or morphology of a growing culture might occur only when the mutant type had unusual properties, as being either a dominant mutation or one that suppresses both the action and division of its normal counterpart. Since many of the extranuclear mutations in fungi have that property of suppressiveness which Jinks (1964) believes can be explained best on the basis of superior division rates of the mutant determinant, it was believed that continuous growth might provide both extranuclear and nuclear mutants with unique properties.

## METHODS

*Organisms.* The strains of *Neurospora crassa* used were: *ad-4al-2A* (F4), adenine-4; 15300, albino-2), *pan-1al-1* (5531, pantothenic-1; 4637T, albino-1); *nic-1al-288a* and *nic-1al-255A* (3416, nicotinic-1; 15300, albino-2), and a standard wild-type strain, *74-OR-8-1a*. All the strains were heterokaryotically compatible with one another, except for *74a* and the effect of mating type *a*. Except in cases where the complete genotype seems necessary for emphasis or clarity, the following abbreviations are used: *ad*, *pan*, *nic*, and *74a*.

*Conditions used for continual hyphal propagation.* Special continuous growth tubes were designed to study the effects of perpetual hyphal propagation (McDougall &

Pittenger, 1962). The Pyrex growth tubes were 615 mm. long with inside diameters of about 25 mm. Four cotton-stoppered sampling ports (8 mm. diameter) were spaced along the tops of the tubes for aeration and to facilitate removal of conidia, which formed in abundance under the openings. Ends of the tubes had 24/40 standard-taper ground-glass interchanges. Closures made of the same type of glass joints were used for sealing the ends which were not joined to other tubes. Each 615 mm. section was filled with 100 ml. medium.

An *ad-4al-2A* strain was inoculated at the proximal end of the initial section. Before the culture reached the end of a section, the distal closure was removed and another section filled with medium joined to the first one. Once the mycelium was well established in the new section, the tubes were separated and sealed with sterile closures. Subcultures were prepared from masses of aerial hyphae and conidia removed from at least one sampling port before the proximal section was discarded. The mature subcultures in slopes were stored at 4°. Repeated additions of sections permitted uninterrupted growth by hyphal elongation for more than 4 years with no need to subculture the organism.

The main culture was called the A-culture and sections of the corresponding growth tube were numbered from A 1 to A 41. Because of a significant change in growth rate, a parallel culture, the B-culture, was started from the proximal end of section A 16, in addition to continuing the A-culture from the distal end of the same section. The sections of the growth tube corresponding to the B-culture were numbered B 16 to B 160.

The position of the frontier of the culture was marked daily on the growth tube. Daily average growth rates were determined from these markings.

*Types of media used.* Vogel medium (1956) with 1·5 % (w/v) sucrose as carbon source and solidified with 1·5 % (w/v) of unwashed Difco Bacto-Agar was used in all growth experiments. All experiments were made at 30°. Routine growth-rate determinations of subcultures or single conidial isolates were done in 500 mm. growth tubes (Ryan, Beadle & Tatum, 1943) containing 30 ml. medium. The medium of Westergaard & Mitchell (1947) was used for crosses and for plating of conidia. Supplements at the following concentrations were used: adenine, 0·2 mg./ml., and nicotinamide and calcium pantothenate, 0·005 mg./ml. Complete medium was prepared by adding 0·25 % (w/v) Casamino acids and 0·25 % (w/v) yeast extract to basal medium containing a carbon source.

Methods used to plate conidia, to prepare heterokaryons, to recover homokaryotic conidial isolates from heterokaryons, and for their analyses can be found elsewhere (McDougall & Pittenger, 1966; Bertrand & Pittenger, unpublished).

*Selection of mutants for analysis.* The only criteria used to select cytoplasmic and nuclear mutants for analysis during continuous growth were: (1) prominent changes in growth rate; (2) visible changes in morphology of the culture, such as those related to abundance of aerial growth, conidiation, and lysis of the culture; (3) changes in both sexual and asexual reproductive capacity; (4) changes in morphology of colonies formed when conidia were plated on sorbose medium. No attempt was made to determine the frequency of auxotrophic or visible mutants which may have accumulated during growth. Consequently, the genetic analysis was confined to changes in growth rate and morphological mutants first observed in the A- and B-cultures themselves, rather than analysis of somatic cells routinely isolated from these cultures.

## RESULTS

Space is not available nor would it be particularly meaningful to present a single growth curve for either the A- or B-cultures over a 4-year period which would include all the daily minor variations in growth, including times when growth progressively slowed and finally ceased temporarily. Instead, average maximum growth rates in each section were calculated and plotted against the sections of the continuous growth tube. Such calculations adequately picture the major increases and decreases, as shown in Fig. 1. Because of the variable daily rates observed in A23 to A41, the maximum 24 hr rates or average maximum rates in these sections were used in calculating the data presented in Fig. 1 rather than the average of all daily rates. This procedure avoids minor fluctuations in rate preceding cessation of growth. It was adopted to express more accurately the potential growth rate of the culture in any particular section of the growth tube.

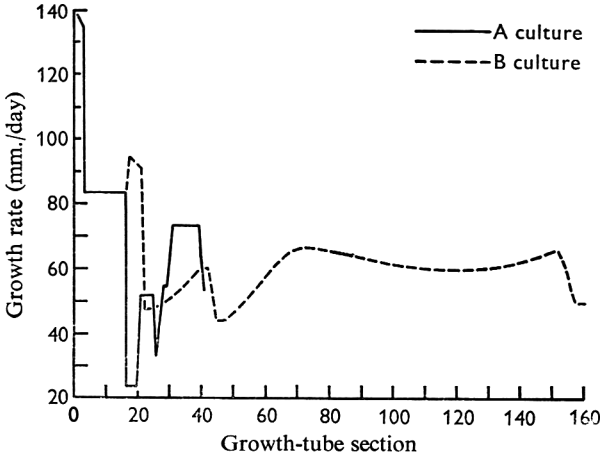


Fig. 1. Growth rates of the *adal-2 A* culture of *Neurospora crassa* during continuous hyphal elongation in growth tubes A and B over a 4-year period. For sections where growth was erratic, or stopped completely, the maximum 24 hr growth rate, rather than the average growth rate, has been plotted to avoid minor fluctuations and to express more accurately the potential rate of the culture.

Although the data presented in Fig. 1 show several significant increases and decreases in the rate of growth of the A- and B-cultures, the most unusual feature, but not shown in the growth curve, was the fact that growth stopped completely 68 different times, 48 in the A-culture and 20 in the B-culture. The growth behaviour in the A- and B-cultures before stopping and after resuming is shown in some representative 'growth maps' in Table 1, but no attempt was made to present such data for the entire period.

### *Description of growth of the A-culture of strain adal-2A*

The *ad al-2A* culture grew 137 mm./day through the first 1200 mm. of the A-growth tube, which was surprising since the *adal-2A* strain normally grows at only 80–85 mm./day. Although this initial rate of 5·7 mm./hr was extremely high, even for a wild-type strain, it could not be repeated either with subcultures from sections A1 and A2,

single conidial isolates from those sections, or with cultures of the strain used to initiate section A 1. Nevertheless, the high average rate was real, for it represented nearly 9 days of growth. It was assumed that factors determining the initial vigour either were not heritable and were eliminated during growth and conidial reproduction, or that the rate was affected by unspecified environmental conditions which could not be reproduced.

A significant change in growth rate was observed in A 16 (Fig. 1). Subsequent growth was slow and erratic until it ceased temporarily in A 23. By then the culture had grown nearly 14,200 mm. in 305 days. After the stop in A 23, the culture grew in highly irregular cycles consisting of a period of active hyphal elongation followed by temporary cessation. For example, growth resumed in A 23 at 38 mm./day after 8 days of no growth. However, 3 days later the culture again stopped, this time for 10 days, which in turn was followed by 31 days of active growth and 15 days of no growth. The stop–start growth pattern was maintained until the culture was discarded in A 41 when it became contaminated with bacteria, about 1065 days after it stopped for the first time. During this irregular growth, periods of active elongation ranged from 1 to

Table 1. *Representative 'growth maps' selected from the 41 and 160 sections of the corresponding continuous growth tubes of the A- and B-cultures to illustrate the variation encountered in average daily growth prior to and following temporary cessation of growth in the adal-2 cultures maintained by continuous hyphal elongation*

| Growth-tube section | Growth (mm.) in successive 24-hr periods* |
|---|---|
| A 16 | 40–50–70–70–62–56–55–48–49–32–15–34 |
| A 17 | 49–21–21–20–28–23–14–15–15–23–22–24–17–8–14–21–39–38**22**–11–25 |
| A 20 | 26–22–20–17–16–18–19–20–18–14–22–24–22–19–18–15–22–18–18–17–18–19 |
| A 22 | 41–45–43–48–49–50–47–49–55 |
| A 23 | 51–52–48–41–43–35–31–31–28–28–13–10–6–6–3–**8-day stop**–40–33–30 |
| A 24 | **10-day stop**–16–23–32–34–40–36–30–34–42–35–37–33–28–26–30–35 |
| A 31 | 68–82–82–80–39–14–8–3–**5-day stop**–79–80 |
| A 32 | 88–86–80–74–62–33–17–**16-day stop**–8–68 |
| B 16 | 105–99–95–87–84–83 |
| B 22 | 81–70–70–77–60–66 |
| B 23 | 46–54–40–10–**11-day stop**–41–50–46–8–**27-day stop**–42–50–41–41–41 |
| B 24 | 40–41–44–47–50–56–55–52–58–49 |
| B 70 | 64–52–17–12–5–**2-day stop**–59–68–66–55–20–16–6–**2-day stop**–33 |
| B 71 | 68–79–70–67–39–12–7–7–**2-day stop**–56–67–62 |

* The constant and normal growth in sections immediately preceding A 16 averaged 83 mm./day.

31 days, during which the hyphal tips advanced as little as 5 mm. or as much as 1050 mm. The periods of no measurable growth ranged from 1 day to over 3 months. During individual periods of active growth daily average rates ranged from 4 to 91 mm./day; the latter rate was comparable to the maximum the culture attained in sections previous to section A 16. The culture stopped 48 times for a total of 780 days, and active growth was observed only during 285 days after the culture first stopped in A 23. The 48 stops in the final 18 sections are evidence of the highly degenerative condition of the culture. It is impossible to estimate how long such aberrant growth would have continued had the culture not become contaminated; there is no reason to expect that it would not have continued after 3 years of stop-start growth.

Observation of the A-culture showed that growth slowed considerably before stopping (Table 1), and that during this period the mycelial frontier often was composed of only a few scattered hyphae. When it was possible to observe the resumption of hyphal elongation, new growth always started at points 10–140 mm. proximal to the stopped frontier.

After stopping first occurred in A23, a growth tube of complete medium was connected to the proximal end of that section to see whether the enriched medium would affect growth. The culture stopped three times in this section of complete medium for 7, 29 and 27 days, compared with one 8-day stop in the original A23. Subsequent tests with complete medium at both 25° and 35° did not show any effect in decreasing either the number of times a culture stopped or the duration of the stops. In fact, complete medium usually more adversely affected growth than did minimal medium supplemented with adenine.

This unusual stop–start phenotype associated with the A-culture has been studied extensively in subcultures and heterokaryons. Its genetic basis was found to be an extranuclear factor, *stp-A*. The experimental evidence for these findings will be reported elsewhere.

### *Morphological changes in the A-culture*

The decrease in growth rate in section A16 was accompanied by several changes in the morphology of the culture. For example, the production of conidia was greatly decreased in sections A16 and A17 and none could be detected microscopically in samples of mycelium removed from sections A31 to A41. This response appeared to be largely environmental, since many of the subcultures from these sections formed some conidia on agar slopes, but were again aconidial when grown in growth tubes. During the same period the A-culture also lost ability to function as a protoperithecial parent in crosses, and subsequent to A16 all subcultures were female-sterile. The appearance of a brown pigment in ageing mycelium and a brown discoloration of the culture medium was another change which appeared in the culture after it had slowed down. Discoloration of the medium was particularly noticeable in regions where the culture ceased to grow temporarily.

### *Cytoplasmic involvement in growth changes in the A-culture*

Following section A16 growth was very slow, but single conidial isolates from these sections showed a wide variation in growth rates. For example, the growth rate of the main culture in A19 was less than 24 mm./day, but single conidial isolates from the same section had rates ranging from 13 to 63 mm./day and averaged 39 mm./day. To determine whether these rates were due to nuclear or cytoplasmic heterogeneity, conidia from section A19 were heterokaryotically combined 1:9 with conidia from a normal *panal-1* strain using the technique of Pittenger, Kimball & Atwood (1955). This heterokaryon was assumed to have a high proportion of normal cytoplasm. It was predicted that *adal-2* homokaryotic isolates recovered from the heterokaryon would have normal growth rates if the previously observed heterogeneity were due to the involvement of an abnormal cytoplasm but not if the heterogeneity were nuclear. From this heterokaryon, 18 *adal-2* homokaryons were recovered. Their growth rates ranged from 69 to 86 mm./day and averaged 81 mm./day—a rate comparable to that

found in early sections of the A-growth tube. Since such heterokaryotic rejuvenation indicated that the *adal-2* nuclei were capable of normal growth in a normal cytoplasm, it appeared that the decreased growth rates of single conidial isolates from the A-culture resulted from extranuclear factors rather than nuclear factors.

Additional evidence for involvement of extranuclear factors was that *panal-1* homokaryons recovered from a heterokaryon between *panal-1* and *adal-2* from section A20 exhibited decreased growth rates. Normally, *panal-1* homokaryons grow at approximately 102 mm./day. Of 33 *panal-1* homokaryotic isolates from the heterokaryon, five had growth rates of less than 48 mm./day, two had stop–start growth phenotypes and the remainder had rates ranging between 48 and 91 mm./day. This was interpreted to mean that the integrity of the *panal-1* component, with respect to growth rate, had been altered by heterokaryotic association with the *adal-2* strain. Presumably, the change was controlled by extranuclear factors.

*Description of growth of the B-culture of adal-2A*

The rapid decrease in growth rate in A16 (Fig. 1; Table 1), which continued until A23, where the culture temporarily stopped, suggested that some genetic change had taken place. Consequently, in addition to the usual procedure of adding a new section to the distal end of A16, one was also added to the proximal end; the mycelium, of course, was severed in separating section A15 from A16. The mycelium in the proximal end of A16 resumed growth immediately when brought in contact with the fresh medium. The new culture was subsequently called the B-culture. Because of its interesting phenotype it was followed from B16 to B160 when the experiment was terminated. Interestingly enough, both the A- and B-cultures continued to grow, although at very different rates, until sections A23 and B23. There the respective cultures stopped temporarily for the first time after 14,140 and 13,720 mm. of growth respectively.

In sections B16 to B19 the B-culture grew approximately 92 mm./day, a rate well above the 58 mm./day observed in the proximal half of A16, but after B19 a slow decrease in rate occurred within the subsequent 2000 mm. until the culture grew only 48 mm./day in the proximal half of B23. Growth ceased in B23 after nearly 4500 mm. of growth in 55 days; it resumed 11 days later and stopped again for 27 days after progressing only 145 mm. in 4 days (Table 1). After growth resumed the second time, there were no significant increases in rate and one significant decrease (Fig. 1) during the following 28,740 mm. of growth in 519 days prior to B70. In section B70 and continuing to B116, there were four series of stops with each series consisting of from 1 to 8 stops. These series of stops and their duration are presented in Table 2. While the two initial stops of the culture were for 11 and 27 days, the 18 subsequent stops averaged slightly more than 2 days in duration and ranged from 1 to 4 days. Although the stops in sections B23 and B70 occurred almost $1\frac{1}{2}$ years apart with no intervening stops, the genetic determinant of stopping, *stp-B*, which Bertrand & Pittenger (unpublished) have shown to be extranuclear in nature, appeared to be present, although not expressed, during the entire period. This evidence comes from isolating and growing conidia from subcultures of the intervening sections. Of 236 single colony isolates analysed from 10 intervening sections, 66, nearly 28 %, stopped during growth in 500 mm. growth tubes.

Other major changes in rate that appeared during growth of the B-culture are shown

in Fig. 1. Several of the rate changes were accompanied by alterations in morphology. A description and genetic analysis of the off-types follows.

Table 2. *A summary of the location and duration of the 20 temporary cessations of growth observed during 4 years of continuous hyphal propagation in the 160 sections of the continuous-growth tube of the B-culture, together with the number of days and mm. of growth between the five series of stops*

| Growth-tube section in which stopping occurred and distance and days of growth between series of stops | No. of times culture stopped in each section | Duration of each stop (days) |
|---|---|---|
| 13,720 mm. of growth in 156 days | | |
| B23 | 2 | 11, 27 |
| 28,740 mm. of growth in 520 days | | |
| B70 | 2 | 2, 2 |
| B71 | 1 | 2 |
| B72 | 2 | 2, 4 |
| B73 | 1 | 3 |
| B75 | 2 | 3, 2 |
| 6300 mm. of growth in 98 days | | |
| B86 | 1 | 2 |
| B88 | 3 | 2, 1, 1 |
| B89 | 1 | 3 |
| 9570 mm. of growth in 158 days | | |
| B104 | 2 | 3, 3 |
| B105 | 2 | 4, 2 |
| 6690 mm. of growth in 108 days | | |
| B116 | 1 | 1 |

### *The variant-1 phenotype*

The decline in growth rate from 84 to 48 mm./day in B19 to B23 was accompanied by marked alterations in morphology of the culture. For example, in B23 the usual abundant production of aerial hyphae was decreased so the culture had a somewhat 'shaven' appearance and conidia were formed only sparsely. Likewise, functional protoperithecia were no longer formed when subcultures were grown on crossing medium. Irregularly shaped 'lysed' areas were also apparent in 2- to 3-week cultures. They were phenotypically recognized primarily by disappearance of aerial hyphae resulting in the appearance of clear areas. Lysis started only in aged mycelium as small discrete areas scattered throughout the length of a growth tube and progressively encompassed most of the surface as shown in Plate 1. Although this trait is inherited, the biochemical mechanisms involved are not known.

The variant-1 phenotype persisted in the B-culture from B23 to B160, suggesting that it resulted from a permanent genetic change. The phenotype was not subject to heterokaryotic rejuvenation, and segregated in crosses in which *adal-2 var-1* was used as a conidial parent. A total of 29 complete asci and 75 random spores from three crosses between *74a* and *adal-2 var-1* from B82 were analysed. Half of the ascospore cultures from each tetrad were *var-1* with respect to aerial growth, decreased conidiation, growth rate and female sterility. The progeny from six tetrads were checked for appearance of lysis. In all cases the cultures which showed the other properties of variant-1 were also lytic. The variant-1 phenotype segregated independently of mating

type, requirement for adenine and albinism. These results are consistent with variant-1 being controlled by a single gene mutation.

Apparently both the *ad* and *var-1* mutants individually decreased the growth rate and the effect was additive. This could be shown by growth rates of isolates from the above cross. The normal isolates from the cross averaged 111 mm./day, 79 individual *ad* strains averaged 85 mm./day, 54 *var-1* isolates averaged 69 mm./day and 89 double-mutants had an average growth rate of only 45 mm./day.

That the variant-1 phenotype from various sections of the B-growth tube was the result of the same genotype was demonstrated as follows. An *adal-2 var-1* isolate from B19 was crossed to a *nic-1 al-2 88a* strain and 21 of 46 random spores isolated were phenotypically variant-1 as expected if *var-1* were determined by a single genetic factor. A *nic-1 al-2 var-1A* culture with an average growth rate of 63 mm./day was selected and heterokaryotically combined with *adal-2 var-1* subcultures from sections B42, 62, 82, 120 and 140. All the resultant heterokaryons, with growth rates averaging 61 mm./day showed sparse conidiation, were female-sterile and showed lysis on ageing. This complete expression of the variant-1 phenotype in all heterokaryons indicated genetic identity of the *var-1* genotype in the various sections of the B-culture.

Table 3. *Neurospora crassa. Number of adal-2 and adal-2 var-1 single colony isolates from subcultures of various sections of A- and B-growth tubes*

| Section | Total isolates tested | Phenotypes of isolates* | |
|---|---|---|---|
| | | *adal-2* | *adal-2 var-1* |
| A13 | 30 | 30 | 0 |
| A14 | 30 | 30 | 0 |
| A15 | 30 | 9 | 21 |
| A19 | 20 | 20 | 0 |
| B16 | 29 | 13 | 16 |
| B19 | 30 | 3 | 27 |
| B20 | 30 | 0 | 30 |
| B21 | 30 | 0 | 20 |
| B23 | 30 | 0 | 30 |

* Heterokaryons (*adal-2* + *adal-2 var-1*) assumed to have *adal-2* phenotype.

The presence in the A- and B-cultures of *var-1* nuclei long before the variant-1 phenotype was expressed was demonstrated by isolating homokaryotic *var-1* conidia from subcultures of the two growth tubes. Homokaryotic *adal-1 var-1* cultures could be distinguished easily from both heterokaryotic and *adal-2* isolates by differences in conidiation in mature cultures on agar slants. The results in Table 3 show that only the *adal-2* type was found in subcultures from sections A13 and A14, but both *adal-2* and *adal-2 var-1* isolates appeared in sections A15, B15, B19 and B20. Finally, only *adal-2 var-1* homokaryons were obtained from subcultures of B21 and B23, and from all subsequent B-cultures. Since these determinations were made from subcultures and not directly from conidia of the B-culture, the proportions of the homokaryotic *adal-2 var-1* isolates do not necessarily provide a good estimate of their frequency in the B-growth tube. Nevertheless the complete displacement of *adal-2* by *adal-2 var-1* indicates a selective mechanism that favours *var-1*.

*The variant-2 phenotype*

The second distinct change in morphology of the B-culture was observed at the same time that a significant change in growth rate occurred between sections B151 and B157. The culture became aconidial in section B156 and aerial hyphae were largely absent by the end of B157. Excessive hyphal branching in the growing tips of the culture was one of the characteristics noted. The phenotype of germinating conidia was also quite unique. One or two hyphae extended from the conidia and then branched extensively to give a fan-like layer of growth on the surface of the sorbose medium. When transferred to growth tubes, the cultures grew extremely slowly and never more than 7 mm./day. The mycelium had the appearance of a flat thin rope with small lateral branches strung along the middle of the agar surface of the growth tube; there was no aerial growth and conidia were completely lacking. That this phenotype, called variant-2 (*var-2* is the corresponding genotype), arose from *adal-1 var-1* was suggested not only because it carried the same mutant genes, but also from crossing data.

Information available about the genetic basis of the variant-2 phenotype is limited, since this altered type gave extremely infertile crosses even when used as the male parent. Among cultures from 14 viable spores from a cross of *74a* with a single conidial isolate of variant-2, segregating for both *ad* and *al-2*, 8 gave rise to cultures that were neither variant-1 nor -2, 2 were phenotypically like variant-1, and 4 had the variant-2 phenotype. Since we do not know whether variant-2 (presumably of the genotype *var-1*, *var-2*) can be distinguished from *var-2* alone, we can only say that the variant-2 phenotype is heritable.

That *var-2* was present in the B-culture before any morphological or growth-rate changes were observed was easily demonstrated. Conidia taken directly from B145 and plated formed two distinct types of colonies. About 96% were normal in size; the remaining 4% were extremely small and, when transferred to agar slope, had the phenotype described for variant-2. The proportion of such colonies, which represents the proportion of homokaryotic *var-2* conidia and not *var-2* nuclei, gradually increased to about 17% in B150 through B156 and then increased to 25% in B157. Since conidia were absent from subsequent sections, no further estimates of the proportion of variant-2 nuclei are available. However, a comparison of the growth rate of variant-2 homokaryons with the B-culture made it clear that the B-culture was still heterokaryotic through section B160 where the experiment was terminated. Since the extremely decreased growth rate of *var-2* compared with *var-1* was clearly non-adaptive, the increased proportion of *var-2* nuclei was unexpected, and some selective mechanism must be postulated.

## DISCUSSION

The growth rate achieved by a culture in a continous growth tube at any given time is determined by the interaction of genetic and environmental factors. It is obviously impossible in a culture with a decreased growth rate to assess which interacting factor limits growth rate unless one plays a dominant role that is reproducible in a subculture. Consequently, reproducibility of altered growth rates was a prerequisite in our attempts to determine whether spontaneous mutants were responsible for changes in growth rates of the A- and B-cultures. As expected, many significant increases and decreases

in growth rates shown in Fig. 1 were not reproducible in subcultures. On the other hand, most of the reproducible rate changes observed in the A- and B-growth tubes not only had a genetic basis but also had the predicted suppressive phenotype. Although the completely different behaviour of the A- and B-cultures, which had a common origin through section A 16, emphasizes the problem of trying to generalize about growth behaviour of such continually growing cultures, both series acquired certain similar abnormal phenotypes which should be mentioned.

Both the A- and B-cultures, as well as several experimentally produced heterokaryons later studied (Grindle & Pittenger, unpublished) during 3 or more months of continuous growth, were similar in that over long periods of time their growth rates were clearly non-adaptive. In all of these continuous cultures, significant increases in growth rate were short-lived and no evidence was found to indicate that they resulted from genetic alterations. However, since there are some genetic phenomena—for example, transient changes in nuclear or cytoplasmic proportions in heterokaryons and heteroplasmons—which might temporarily effect growth rates but would not be expected to be reproducible in subcultures, we can only speculate that the majority of non-reproducible rate changes were not caused by demonstrable genetic changes.

Clearly the most unusual aspect of the long-term growth study was that growth was not continuous but ceased repeatedly in both the A- and B-cultures, and resumed again in characteristically different ways. That is, the period of absence of growth was quite different in the two cultures, averaging slightly over 2 days for the B-culture and 16 days for the A-culture. The corresponding genetic determinants of stopping, *stp-A* and *stp-B*, are cytoplasmically inherited (Bertrand & Pittenger, unpublished).

It also seems clear that there were homeostatic mechanisms affecting growth rates, not only because of the general constancy of growth but also because every major decrease in rate was followed by an increase. Growth rates restored to values previously achieved were routinely noted after cultures had stopped temporarily, as well as in many cases of minor fluctuations. However, only rarely following a major decline was growth ever restored to the value observed before the decline.

One can also generalize that the naturally occurring genetic changes which took place during long-term vegetative growth of Neurospora resulted not only in an overall decrease in growth rate, but also an overall degeneration in both sexual and asexual reproductive capacity. For example, female sterility and decreased conidiation controlled by genetic factors occurred early in the growth of both A- and B-cultures. In this respect, our observations paralleled those reported by Jinks (1959) in Aspergillus except that in Neurospora the phenotypic changes were due to permanent genetic changes at both the cytoplasmic and nuclear levels. Jinks, on the other hand, ascribed them to cytoplasmic changes resulting from a readjustment in the equilibrium of normal cytoplasmic constituents. Furthermore, there was no indication in Neurospora for an irreversible cessation of growth as the final condition of degenerative processes associated with prolonged vegetative propagation, as observed for Aspergillus (Jinks, 1959) and Podospora (Marcou, 1961). In fact, Marcou's work with Podospora and other fungi suggested to her that indefinite hyphal propagation may be impossible in fungi. That generalization apparently does not apply to Neurospora.

The extranuclear determinants of the stopper A and B phenotypes, and the death of stopped hyphal tips, suggest that possibly these phenotypes and 'vegetative death' in Aspergillus and 'senescence' in Podospora are related. Occasional resumption of

growth has been observed in dying cultures of Aspergillus, but the recoveries were short-lived and apparently did not occur repeatedly in the same culture (Jinks, 1959). That is not the case with Neurospora stopper mutants, where temporary cessations were the rule rather than the exception and recoveries were often long-lived. Consequently, if there is a relationship between the stopper phenotypes of Neurospora and irreversible cessation of growth in Aspergillus and Podospora, then Neurospora may have biological properties that permit it to survive deleterious cytoplasmic changes which are lethal in some other fungi. The extremely rapid growth of Neurospora compared with Podospora and Aspergillus immediately suggests that growth rate itself could be indirectly involved. That is, over extended periods of growth, the slower growing species may simply be unable to escape the unfavourable intracellular or extracellular environments that are a byproduct of their own growth. Similarly, the ability of Neurospora to resume growth might be nothing more than a simple difference among the organisms to invade media already occupied by their own dead mycelium. It is equally likely, however, that more subtle physiological phenomena are involved. It will be interesting to find out what biological properties are responsible for such differences between species of fungi.

We can also generalize to some extent about the unique properties of the detectable nuclear and extranuclear alterations that accumulated during growth. We have already referred to the fact that the unusual phenomena of stopping was associated with two extranuclear mutants. It was possible to demonstrate that the extranuclear determinants of stopping were present before stopping was first observed in the A- and B-cultures. This was done by isolating single conidial isolates from sections of the A- and B-growth tubes long before the cultures stopped in sections A 23 and B 23 and showing that some stopped in 500 mm. tubes. The almost continual expression of the stopper phenotype in the A-culture plus the dominance of *stp-A* in heterokaryons shows that this mutant has the property of suppressiveness predicted as characteristic of mutants expressed in continuously growing cultures. The same suppressiveness was manifested periodically for the *stp-B* extranuclear mutant. Although it stopped only periodically and the mutant could be demonstrated to be present 3 years after it was first observed, its behaviour is hardly suppressive in the way described for the *stp-A* mutant.

Obviously, to see a culture stop growing gives no clue to the mechanism involved and we have no evidence to help us distinguish among several hypotheses. Two extreme possibilities are that either the culture 'runs out' of something essential to its growth like an energy synthesizing system, or it accumulates something like an abnormal structural protein (Woodward & Munkres, 1966) that is detrimental to growth. Since the genetic basis of stopping is clearly extranuclear and because both *stp-A* and *stp-B* have defective cytochrome-*c* oxidase systems, it is assumed that the mitochondria are involved in some way. Other than this, however, we have no direct evidence for the mechanism which causes cessation or resumption of growth.

In addition to the cessation of growth associated with the extranuclear mutants, two other significant changes in growth rate deserve some consideration. Discussing only the B-culture, the presumptive suppressive phenotypes of the two nuclear mutants, *var-1* and *var-2*, were of special interest. The complete displacement of the original *adal-2* nuclear type by the *adal-2 var-1* seemed significant since the nuclear type with the highest selective value in a mixed population was the one with the slowest growth rate as a homokaryon (approximately 2·5 and 3·5 mm./hr for *adal-2 var-1* and *adal-2*,

respectively). It is also worth noting that the *adal-2 var-1* type, which became established in the B-culture but not in the A-culture, was first present in A before B was even started in A16. There was no evidence, however, for the persistence of *var-1* nuclei in the A-culture in sections distal to A23. The inability of *var-1* nuclei to become established in the A-culture is inconsistent with its postulated superior division rate, but it may be that the intracellular conditions of the A-culture were sufficiently different, due to the presence of extranuclear factors demonstrated to affect growth of the A-culture in A19 and A20, that the *var-1* was unable to become established.

The *var-2* mutant, which was first detected in B145, and was undoubtedly present much earlier in a lower frequency, also increased significantly during subsequent growth. Since the culture later failed to form conidia, we were unable to obtain further estimates of the proportion of *var-2* nuclei in the population. Nevertheless the 25% homokaryotic *var-2* conidia found in B157 indicated the proportion of *var-2* nuclei in the heterokaryotic culture was considerably higher than 25% and possibly approached 50%. Since the growth rate of *var-2* homokaryons never exceeded 7 mm./day, the only two nuclear mutants isolated that affected growth both had significantly lower homokaryotic growth rates than the nuclear types simultaneously present in the B-culture. Despite the decreased homokaryotic growth rates, *var-1* and *var-2* had selective advantages in heterokaryons as measured by their increase in the population during growth. The simpler explanation of such selection, which was clearly non-adaptive in terms of growth rate, is that it was achieved as a result of an increased division rate of the mutant types; controlled reconstruction experiments have not yet been done. Since this behaviour phenotypically is suppressive, the similarity of these nuclear mutants to the suppressive extranuclear mutants, *stp-A* and *stp-B*, is obvious. *Var-1* and *stp-A* were similar in that both apparently caused a nearly complete displacement of the corresponding nuclear and extranuclear homologues of the culture. The analysis of *var-2* was not complete enough to determine whether it would have eventually displaced *var-1*. The general significance of the observed increases in the proportion of slower-growing components of heterokaryons is unknown. While such slow-growing types would be rapidly eliminated in a population of uninucleate cells, they appear to have some selective value in a coenocytic heterokaryotic mycelium. Grindle & Pittenger (unpublished) have also observed that in a number of experimental heterokaryons there was a significant increase in the proportion of the slower-growing nuclear component during extended periods of growth, this phenomenon would not appear to be completely unique.

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## EXPLANATION OF PLATE

The upper photograph (actual size) of an *adal-2A* strain of *Neurospora crassa* in a continuous-growth tube shows the appearance of the initial stage of 'lysis' which encompasses a relatively small area. The culture had been growing continuously for over 2 years. The two lower photographs, at slightly higher magnifications, show two areas from the same growth tube as the upper photograph after 'lysis' had progressed throughout the culture during the following week. Note the small remaining white patches of 'unlysed' mycelium. The vertical structure in the middle left of the upper photograph and at the right in the two lower ones is a cotton-stoppered sampling port of the growth tube.