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# THE JOURNAL OF GENERAL MICROBIOLOGY

## THE PREPARATION OF PAPERS

The more words there are, the more words there are about which doubts may be entertained.'
JEREMY BENTHAM (1748–1832)

What can be said at all can be said clearly; and whereof one cannot speak thereof one must be silent.'
LUDWIG WITTGENSTEIN: *Tractatus Logico-philosophicus* (tr. C. K. Ogden)

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(1) Papers should be written with the utmost conciseness consistent with clarity. The best English for the purpose of the *Journal* is that which gives the sense in the fewest short words, spelt according to the *Shorter Oxford English Dictionary*.

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**Nomenclature of Enzymes.** The system published in *Report of the Commission on Enzymes on the International Union of Biochemistry*, Oxford: Pergamon Press, 50*s*., is used.

**Nomenclature in Bacterial Genetics.** The proposal by M. Demerec, E. A. Adelberg, A. J. Clark and P. E. Hartman published in *Genetics* (1966), **54**, 61 has been reprinted in the *Journal* (1968), **50**, 1 as a useful guide to authors. The general principles laid down should be followed wherever practicable.

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The following books may be found useful:

*Bergey's Manual of Determinative Bacteriology*, 7th edn (1957), edited by R. S. Breed, E. G. D. Murray and A. P. Hitchens. London: Ballière, Tindall and Cox.

V. B. D. Skerman, *A Guide to the Identification of the Genera of Bacteria with Methods and Digest of Genetic Characteristics* (1959). Baltimore, Maryland, U.S.A.: The Williams and Wilkins Company.

S. T. Cowan, *A Dictionary of Microbial Taxonomic Usage* (1968). Edinburgh: Oliver and Boyd.

*Ainsworth and Bisby's Dictionary of the Fungi*, 5th edn (1961). Kew: Commonwealth Mycological Institute.

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# A Mutant in the Initiation of DNA Synthesis in *Salmonella typhimurium*

By B. G. SPRATT AND R. J. ROWBURY
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(*Accepted for publication 30 September 1970*)

SUMMARY

A mutant of *Salmonella typhimurium* was isolated which showed temperature-sensitive synthesis of DNA. The mutant (11G) increased in cell mass at 38° without concurrent DNA synthesis, resulting in loss of viability and the production of long filaments. DNA synthesis at 38° continues for approximately 40 min. at a gradually decreasing rate giving an increase of DNA in Casamino acid minimal medium of about 55 % over that present at the time of the shift. This residual DNA synthesis is not reduced by increasing the temperature to 42°, but can be increased by enriching of the medium in which the organisms are grown before the shift. The effect of high temperature on DNA synthesis in 11G mimics the effect of chloramphenicol, which is known to allow the completion of rounds of replication of DNA without allowing new initiations. The lesion is essentially irreversible and is not corrected by increasing the osmotic pressure of the growth medium. Phage P22 can develop normally in 11G if added at the time of the shift or 30 min. later. Even if the phage is added $3\frac{1}{2}$ h. after the shift substantial multiplication occurs. Cell division continues for several hours after the temperature shift, resulting in the production of DNA-less cells as reported in a separate publication.

## INTRODUCTION

Although considerable advances have been made in our knowledge of DNA synthesis in the last few years, the processes involved in the control of DNA synthesis in bacteria are still poorly understood. The isolation of temperature-sensitive mutants of DNA synthesis in *Escherichia coli* and *Bacillus subtilis* has added to our knowledge of these processes. Several laboratories have isolated mutants of DNA synthesis in *E. coli* (Bonhoeffer, 1966; Fangman & Novick, 1968; Hirota, Ryter & Jacob, 1968; Inouye, 1969; Kuempel, 1969) and *B. subtilis* (Gross, Karamata & Hempstead, 1968; Bazill & Retief, 1969) and these fall into two groups: class I mutants, in which DNA synthesis stops immediately after a shift from low to high temperature; class II, whose DNA synthesis continues for some time before it stops. The first class includes mutants affected in DNA synthesis *per se*, e.g. in an enzyme involved in DNA replication. The second class can be divided into two groups: first, those mutants that allow rounds of replication to be completed at the high temperature but cannot initiate new rounds of DNA synthesis (these are generally called initiation mutants); secondly, mutants that synthesize DNA after a temperature shift but which are not involved in initiation will appear in this group if the protein affected is only slowly inactivated at the high temperature.

The isolation and mapping of initiator mutants of bacteria should give valuable information about DNA synthesis and its control. The isolation of such mutants has been reported in *Escherichia coli* by Hirota *et al.* (1968) and Kuempel (1969) and in *Bacillus subtilis* by Gross *et al.* (1968).

We have isolated a temperature-sensitive strain of *Salmonella typhimurium* which appears to be defective in the initiation of DNA synthesis. It seems to be very similar to the strain CRT46 of Hirota *et al.* (1968) in that it continues cell division at the high temperature with the production of DNA-less cells. Details of cell division in our strain and the properties of the DNA-less cells are the subject of another publication.

## METHODS

*Organisms.* The mutant 11G was isolated as described below from *Salmonella typhimurium* PG154. Strain PG154 has the following genetic constitution: $F^-$, *metA22*, *tryB2*, *ilv-90*, *xyl-1*, *fla-66*, *malA1*, *str A210*, *tre-12*, *h1-b*, *h2-e*, *n*, *x* and was kindly supplied by Dr K. Sanderson (SA98 in Sanderson's collection). Strain 11GP22 is a derivative of the mutant 11G that has been made lysogenic for phage P22. Phage P22L4 is a virulent mutant of the temperate Salmonella phage P22 and was kindly supplied by Dr D. A. Smith.

*Isolation of mutant.* Strain 11G was one of 70 temperature-sensitive mutants isolated from *Salmonella typhimurium* PG154. Organisms grown in nutrient broth were mutagenized using *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine by the method of Adelberg, Mandel & Chen (1965). After mutagenesis organisms were washed free of mutagen, allowed to grow at 25° for several hours in nutrient broth and then plated on nutrient agar at 25°. Colonies which failed to grow when replicated on nutrient agar at 38° were tested for filament formation at this temperature. Five of these mutants formed filaments and were tested for thymidine incorporation at 38°. Two mutants incorporated little thymidine; one of these was 11G.

*Media.* Oxoid nutrient broth no. 2 was used as a rich undefined medium and will be referred to as nutrient broth. Solidified with 2 % Difco Bacto agar it will be referred to as 'nutrient agar'. In one experiment Oxoid brain and heart infusion medium was used. The minimal (MM) was that of Davis & Mingioli (1950) with 0·2 % glucose and 40 μg./ml. of the DL-form of the required amino acids. It was enriched in some experiments with 0·1 % Difco vitamin-free Casamino acids (Casamino MM). Difco Bacto agar (2 %) was added to obtain solid media. Trypticase agar used as basal agar for phage assays consisted of 1 % trypticase agar base, 1·4 % Difco Bacto agar and 0·5 % NaCl. The soft agar overlay consisted of 0·2 % $MgCl_2$, 1 % Oxoid no. 2 broth, 0·3 % NaCl and 0·75 % Difco Bacto agar.

*Growth of organisms.* In all experiments organisms were subcultured from a nutrient agar slope to a small volume of liquid medium, incubated overnight in a shaking water bath, and then diluted and grown for several hours keeping the extinction of the culture below about 0·2 before use. Extinction of the culture was measured in a Hilger photoelectric colorimeter against an uninoculated medium blank (filter 49 for minimal media and filter 55 for nutrient media).

*Measurement of cell viability.* Organisms growing in nutrient broth at the required temperature were diluted into phosphate buffer (0·05 M, pH 7·4) and appropriate dilutions were plated on nutrient agar. The plates were incubated at 25° for 40 h.

*Assay of phage.* In some experiments the multiplication of the virulent phage mutant P22 L4 in strains 11G and PG154 was studied. Organisms growing exponentially in nutrient broth were diluted in broth to 100 $\mu$g. dry wt/ml. and about $10^4$/ml. infective phage added. Incubation was continued and samples taken at zero and after various intervals were shaken with a few drops of chloroform, and the chloroform removed by bubbling air. Phage were estimated in sample dilutions using a soft agar technique with strain PG154 as indicator.

In other experiments induction of phage P22 in a lysogenic derivative of strain 11G was followed. In this case exponentially growing organisms were harvested, suspended in broth and irradiated for 90 sec. using a Philips TUV 6 W tube (854 ergs cm.$^2$/sec.). Organisms were then diluted into nutrient broth and samples taken for phage counts (as above) at intervals during incubation.

*Synthesis of protein, RNA and DNA.* Protein synthesis was measured by the incorporation of [$^{14}$C]phenylalanine. Organisms growing exponentially in MM were diluted to a cell density of 100 $\mu$g. dry wt/ml. in MM containing 0·05 % vitamin-free Casamino acids and [$^{14}$C]phenylalanine (0·1 $\mu$Ci/ml. specific activity 12·6 mCi/mM) and incubated with shaking at the appropriate temperature. Samples (1 ml.) were removed at zero and at intervals into 1 ml. of ice cold 10 % trichloroacetic acid. After standing for at least 1 h. in the cold samples were filtered on Millipore filters (type HA) and the filters after washing with 4 × 2 ml. portions of ice cold 5 % trichloroacetic acid were dried and counted in 15 ml. of scintillation fluid (0·6 % butyl PBD in sulphur-free toluene–methanol, 3:1, v/v) using a Packard Tricarb liquid scintillation spectrometer.

Uracil incorporation was used as a measure of RNA synthesis. Organisms growing exponentially in MM were diluted to a cell density of 100 $\mu$g. dry wt/ml. in MM containing [$^{14}$C]uracil (0·1 $\mu$Ci/5 $\mu$g./ml.). Samples were taken at intervals into an equal volume of ice cold 10 % trichloroacetic acid and then treated as for phenylalanine incorporation.

Since 11G does not require thymine for growth, DNA synthesis was measured by the incorporation of tritiated thymidine. In the strains used in this work as in *Escherichia coli* (Budman & Pardee, 1967) thymidine incorporation occurs at an appreciable rate for only a few minutes, falling off due to the induction of thymidine phosphorylase which breaks down the thymidine to thymine which cannot be utilized. DNA synthesis was therefore measured in the presence of uridine, which inhibits both the induction and the activity of thymidine phosphorylase. Exponentially growing organisms were diluted to 100 $\mu$g. dry wt/ml. in MM containing 1·5 mM uridine and [$^3$H]thymidine (0·2 $\mu$Ci/0·2 $\mu$g./ml.), and samples were taken (during incubation at the appropriate temperature with shaking) into an equal volume of ice cold 10 % trichloroacetic acid containing 40 $\mu$g./ml. unlabelled thymidine. Samples were then treated as previously except that filters were washed with ice cold 5 % trichloroacetic acid containing 40 $\mu$g./ml. unlabelled thymidine.

DNA synthesis in nutrient broth was performed in essentially the same way using nutrient broth plus 1·5 mM uridine and 0·2 $\mu$Ci/ml. [$^3$H]Thymidine (specific activity 5·0 Ci/mM).

*Chemicals.* Radioisotopes were obtained from the Radiochemical Centre, Amersham, Buckinghamshire. Methyl [$^3$H]thymidine was supplied at 5·0 Ci/mM; L-4-[$^{14}$C]phenylalanine at 12·6 mCi/mM and 2-[$^{14}$C]uracil at 54·9 mCi/mM. Chloramphenicol was obtained from Parke, Davis and Co. All other chemicals were of at least analytical grade in purity.

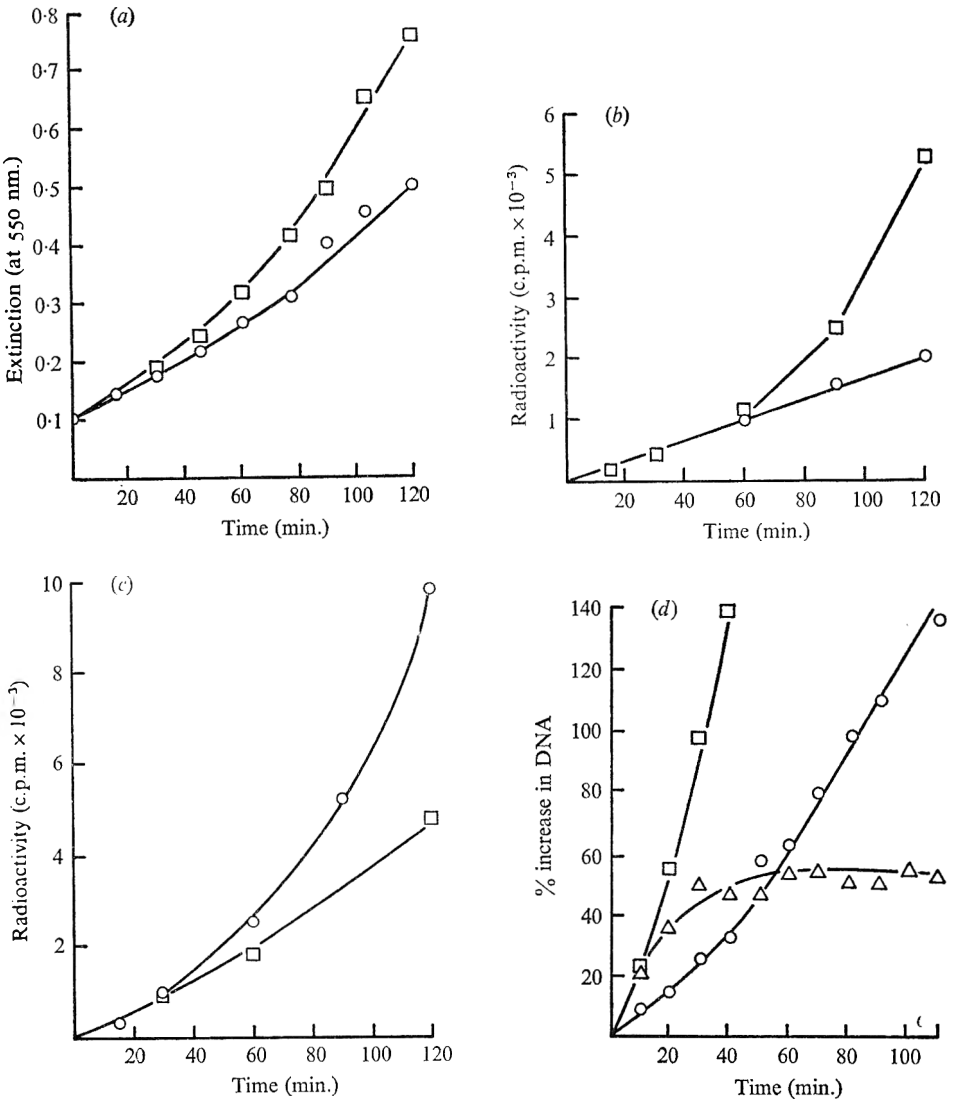


Fig. 1. Growth and macromolecular synthesis in PG 154 and 11G.

(*a*) Growth of PG 154 and 11G at 38°. Organisms growing exponentially in nutrient broth at 25° were diluted to an extinction of 0·1 and the cultures transferred to 38° and the extinctions followed. □, PG 154; ○, 11G.

(*b*) Synthesis of protein in PG 154 and 11G. Organisms growing exponentially in MM at 25° were diluted into MM + 0·05 % vitamin-free Casamino acids. [$^{14}$C]Phenylalanine (0·1 $\mu$Ci/ml.; 12·6 mCi/mM) was added to each and the cultures transferred to 38° and samples taken at intervals for estimates of cold trichloroacetic acid insoluble radioactivity as described in Methods. □, PG 154; ○, 11G.

(*c*) Synthesis of RNA in PG 154 and 11G. Organisms growing exponentially in MM at 25° were diluted to approximately 100 $\mu$g. dry wt/ml. into MM + [$^{14}$C]uracil (0·1 $\mu$Ci/5 $\mu$g./ml.). The cultures were shifted to 38° and samples taken as in (*b*). ○, PG 154; □, 11G.

(*d*) Synthesis of DNA in PG 154 and 11G. Organisms growing exponentially in Casamino acid MM were diluted into Casamino acid MM + 1·5 mM uridine. [$^{3}$H]Thymidine (0·2 $\mu$Ci/0·2 $\mu$g./ml.) was added and samples taken at intervals and treated as described in Methods. □, PG 154 at 38°; ○, 11G at 25°; △, 11G at 38°.

## RESULTS

### *Growth and macromolecular synthesis in strain 11G*

In nutrient broth, strain 11G grew at approximately the wild-type rate at 25° data) not shown). After a shift from 25 to 38° cell mass as measured by extinction increased extensively and continued at a gradually decreasing rate for at least 5 h. (Fig. 1*a*). Viability, on the other hand, increased slightly for the first hour and then fell off exponentially with a half life of *c.* 20 min. Chloramphenicol (CAP) at 150 μg./ml. to a large extent stops the loss of viability of 11G (data not shown). When samples of the culture were examined under the phase contrast microscope it was seen that cell size increased little in the first hour at 38° but then increased more rapidly to produce long filaments together with smaller cells.

Synthesis of RNA and protein (as measured by the incorporation of [$^{14}$C]uracil and [$^{14}$C]phenylalanine) after a shift from 25 to 38° proceeded fairly normally for the first 60 min. and then became approximately linear paralleling the extinction curve (Fig. 1*b*, *c*). DNA synthesis (as measured by the incorporation of [$^{3}$H]thymidine) continued for about 40 min. and then stopped, resulting in an increase in DNA of about 55 % over the amount present at the time of the shift in Casamino MM (Fig. 1*d*).

### *Phage multiplication in strain 11G at 38°*

The behaviour of the mutant at 38° suggested that it was a DNA synthesis mutant of class II. The multiplication of phage P22 was studied to investigate the possibility that the lesion in 11G affected the supply of DNA precursors and that the residual DNA synthesis at 38° was due to the depletion of the remaining pool of DNA precursors. (P22 would presumably be dependent on host-produced precursors.) Furthermore, if 11G is an initiator mutant the development of a phage with its own initiation apparatus should be unaffected.

Table 1. *Multiplication of phage P22L4 in strain PG154 and strain 11G*

Organisms growing exponentially in nutrient broth were shifted from 25 to 38°. Phage P22L4 was added at the time of the shift or subsequently. After adding the phage, incubation was continued for 120 min., taking samples at the time of phage addition (zero), 40 and 120 min. Infective phage was assayed as described in Methods.

| | | Infective phage/ml. | | |
|---|---|---|---|---|
| Strain | Time of phage addition | Zero | 40 min. | 120 min. |
| PG154 | At shift to 38° | $7 \times 10^3$ | $7{\cdot}2 \times 10^5$ | $6{\cdot}5 \times 10^9$ |
| 11G | At shift to 38° | $8{\cdot}5 \times 10^3$ | $5{\cdot}4 \times 10^5$ | $5{\cdot}5 \times 10^9$ |
| 11G | 30 min. after shift | $2{\cdot}1 \times 10^3$ | $1{\cdot}5 \times 10^6$ | $2{\cdot}5 \times 10^9$ |
| 11G | 210 min. after shift | $1{\cdot}7 \times 10^4$ | $1{\cdot}0 \times 10^5$ | $2{\cdot}9 \times 10^6$ |

Phage development in 11G was measured using a virulent mutant (L4) of phage P22. Table 1 shows the results of experiments with the phage. The phage is able to develop normally whether it is added to 11G at the time of the temperature shift or after 30 min. at the high temperature and even if added $3\frac{1}{2}$ h. after the shift there is a 200-fold increase in phage. Similarly, when 11G was made lysogenic for normal phage P22 and induced with u.v. the phage could still develop at 38°, although the phage was not induced by growth at 38° alone (data not shown).

*Stability of DNA in strain 11G*

The cessation of DNA synthesis could result from an uncontrolled breakdown of the DNA in 11G at the high temperature. This was ruled out by measuring the stability of the DNA in 11G at 38°. Cells labelled with [$^3$H]thymidine at 25° were washed free of the label and transferred to 38° and samples taken over a period of $2\frac{1}{2}$ h. The amount of label present in cold trichloroacetic acid insoluble material was constant for the first hour and then fell off slowly (Fig. 2). This loss of label was not found at 25° or at 38° in the presence of 150 μg./ml. of CAP. This very slow loss of label cannot explain the cessation of DNA synthesis in 11G and is probably similar to the slight breakdown of DNA found in several situations where DNA synthesis is inhibited under conditions allowing protein synthesis, representing the induction of nuclease activity (Cook, Deitz & Goss, 1966).

*Attempted reversal of temperature lesion in strain 11G*

The lesion in DNA synthesis in 11G is essentially irreversible. After 90 min. at 38° in Casamino acid MM all DNA synthesis has stopped. If at this time the culture is shifted back to 25°, DNA synthesis only recommences slowly after $1\frac{1}{2}$ h. and doubles in about $2\frac{1}{2}$ h. This slow reversibility is stopped by the addition of 150 μg./ml. of CAP at the time of the shift to 25°. Increasing the osmotic pressure of the growth medium by the addition of 2 % NaCl to the Casamino acid MM does not reverse the lesion in DNA synthesis and allow increased synthesis of DNA at 38°.

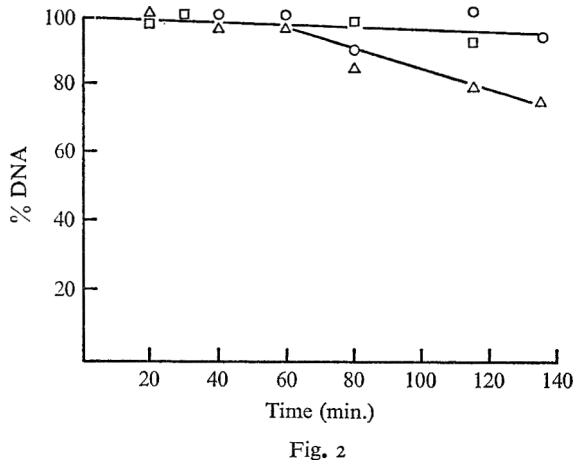


Fig. 2

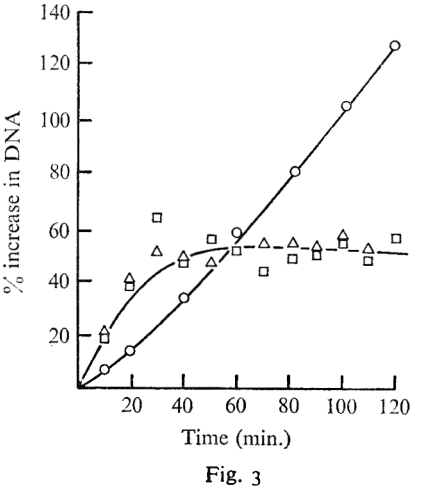


Fig. 3

Fig. 2. Stability of DNA in 11G. Organisms were grown for several generations in Casamino acid MM + 1·5 mM uridine + 0·2 μCi/0·2 μg./ml. [$^3$H]thymidine at 25°. Label was removed by centrifuging and washing twice in 0·05 M tris buffer, pH 7·5. Organisms were then resuspended in Casamino acid MM at a concentration of 100 μg. dry wt/ml. and shaken at 25°, □; at 38° + CAP, ○; and at 38°, △. Samples were taken into cold trichloroacetic acid and counted as described in Methods.

Fig. 3. Effect of temperature on DNA synthesis in 11G. Organisms growing exponentially in Casamino acid MM at 25° were diluted into Casamino acid MM + 1·5 mM uridine. [$^3$H]-Thymidine (0·2 μCi/0·2 μg./ml.) was added and the cultures split into three. One portion was kept at 25°, one at 38° and the third at 42° and samples taken as in Fig. 1(*d*). ○, 25°; △, 38°; □, 42°.

*DNA synthesis in strain 11G*

Several experiments were performed to see if the behaviour of 11G at 38° was compatible with a mutation in the initiation of DNA synthesis. If the residual DNA synthesis after a shift to 38° is due to a mutation in some aspect of DNA synthesis *per se* which takes several minutes to be expressed due to the slow inactivation of some essential enzyme, then on increasing the temperature from 38 to 42° the residual synthesis should be drastically reduced. On the other hand, if this is a mutation in the initiation of DNA synthesis increasing the temperature should have no effect as rounds of replication will still be finished. Fig. 3 shows that the amount of DNA synthesized at 38 and 42° is exactly the same, a result compatible with 11G being an initiator mutant.

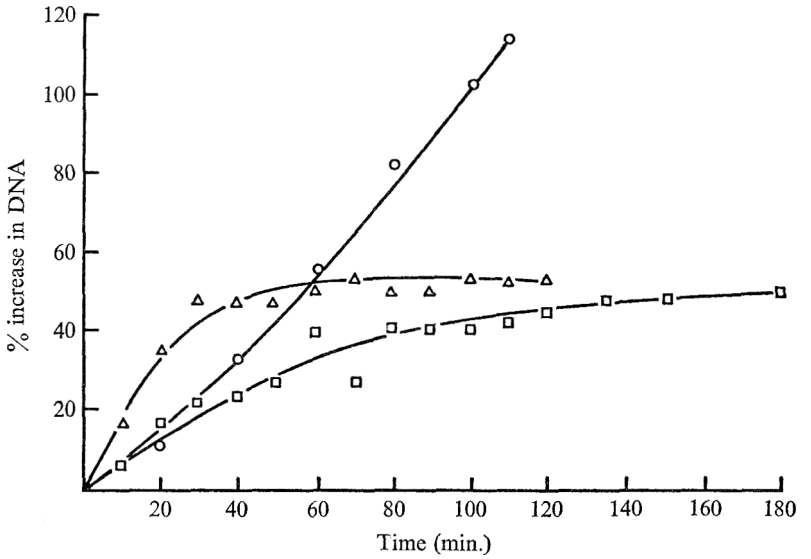


Fig. 4. Effect of CAP on DNA synthesis in 11G. Organisms were grown in Casamino acid MM at 25° and diluted into Casamino acid MM + 1·5 mM uridine. [$^3$H]Thymidine (0·2 $\mu$Ci/0·2 $\mu$g./ml.) was added and the culture split into three portions. One was shaken at 25°, ○; one at 25° + 150 $\mu$g./ml. CAP, □; and the third at 38°, △; and samples taken and treated as in Methods.

As CAP is known to stop the initiation of further rounds of DNA replication without affecting rounds in progress (Maaløe & Hanawalt, 1961), the effect of CAP and the effect of high temperature on DNA synthesis in 11G should be essentially the same. Fig. 4 shows the result of an experiment where a culture of 11G growing in Casamino acid MM at 25° was split into three portions and DNA synthesis measured in one at 25°, in another at 38° and in the third at 25° plus 150 $\mu$g./ml. of CAP. The amount of DNA synthesized at 38° and the amount synthesized at 25° plus CAP are almost identical. In both cases the amount of DNA synthesized is between 50 and 55 %, the amount predicted if cells in this medium at this temperature have multiple replication forks per chromosome for a part of the replication cycle (Helmstetter & Cooper, 1968). Furthermore, if CAP and the high temperature have the same effect, i.e. allow the completion of rounds of DNA replication in the absence of new initiations, then ad-

dition of CAP at 38° should not further reduce the increment. Fig. 5 shows that CAP at 38° only reduces very slightly the amount of DNA synthesized.

Bacteria whose doubling time is less than the time for a round of chromosome replication have multiple replication forks in their DNA (Yoshikawa, O'Sullivan & Sueoka, 1964; Helmstetter & Cooper. 1968). Thus minimal grown cells of *Salmonella typhimurium* contain a single chromosome replicated by a single fork. As the doubling

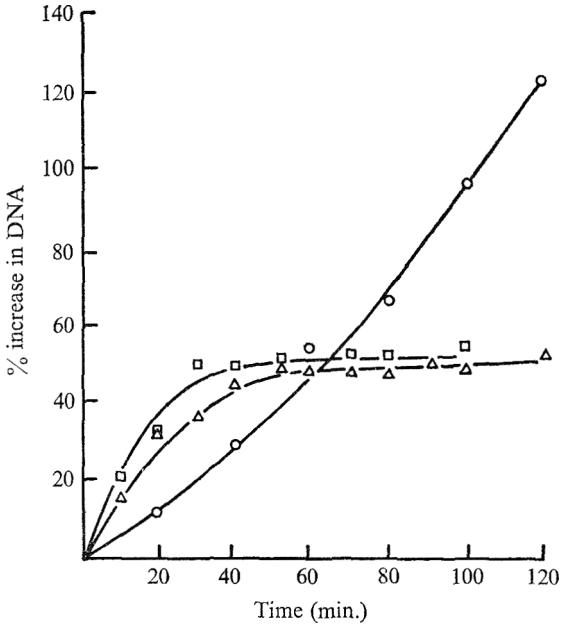


Fig. 5. Effect of CAP on DNA synthesis in 11G at 38°. Organisms growing exponentially in Casamino acid MM at 25° were diluted into Casamino acid MM + 1·5 mM uridine. [$^3$H]-Thymidine (0·2 $\mu$Ci/0·2 $\mu$g./ml.) was added and the culture split into three. One was shaken at 25°, one at 38° and one at 38° + 150 $\mu$g./ml. CAP, and samples taken and treated as in Methods. ○, 25°; □, 38°; △, 38° + CAP.

time permitted by the medium decreases to below about 40 min. at 37° multiple replication forks appear. It is easily seen that if rounds of replication are allowed to be completed in the absence of new initiations the amount of residual DNA synthesis will increase as the average number of replication forks increases. Fig. 6 shows the amounts of DNA synthesized by 11G after a shift from 25 to 38° in various media. Clearly the amount of DNA synthesized increases as the growth rate afforded by the medium increases as predicted for an initiator mutant.

Similarly, the amount of DNA synthesized at 38° in the presence of chloramphenicol increases as the richness of the medium increases.

*Cell division in 11G at 38°*

Observations of 11G growing on nutrient agar by phase contrast microscopy after a shift to 38° show that organisms divide once or twice in the first 90 min. These divisions may be normal ones resulting in the segregation of the newly finished chro-

mosomes. The daughter cells then elongate and after a period of elongation bud off cells at the ends of the short filaments. The details of this division process and the properties of the small cells will be described in a later publication.

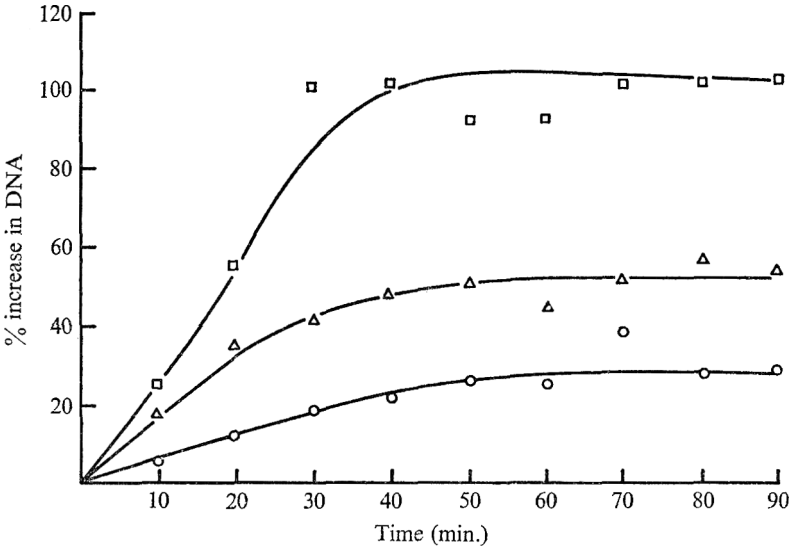


Fig. 6. Effect of growth medium on DNA synthesis in 11G. Organisms growing in various growth media at 25° were diluted into the same media + 1·5 mM uridine. [$^3$H]Thymidine was added and DNA synthesis measured at 38° as described in Methods. Brain and heart infusion (mean generation time at 25° = 50 min.), □; Casamino MM (mean generation time at 25° = 105 min.), △; MM (mean generation time at 25° = 260 min.), ○.

## DISCUSSION

The continuing increase in extinction, RNA and protein synthesis with concomitant loss of viability and formation of filaments is typical of growth in the absence of DNA synthesis, and is shown by all DNA synthesis mutants and in other treatments that stop DNA synthesis without affecting RNA or protein synthesis, e.g. thymine starvation of a thymine-requiring strain or treatment with nalidixic acid. The viable count of 11G at 38° increases for about an hour and this is probably due to normal cell division continuing for one or two divisions in this mutant after a shift from low to high temperature. Following this increase viable count falls exponentially.

All the physiological experiments performed on 11G are suggestive of or compatible with a mutation in the initiation of DNA synthesis. The development of phage P22L4 in 11G at 38° can occur because the phage can control the initiation of its own DNA synthesis as implied by the replicon theory of Jacob, Brenner & Cuzin (1963). The development of phage also suggests that the supply of DNA precursors is not affected at 38° since phage P22 is unlikely to code for enzymes involved in the supply of deoxynucleotide triphosphates. Since phage P1 (though not phage $\lambda$) can multiply in a DNA synthesis mutant of *Escherichia coli* which is not an initiator mutant (165/70 studied by Lanka & Schuster, 1970), the experiments with phage P22 cannot, however, be considered to differentiate conclusively between an alteration in initiation and one in DNA synthesis *per se*.

A direct effect on an enzyme involved in DNA synthesis *per se* but only slowly inactivated is made unlikely by the fact that increasing the temperature from 38 to 42° (which should increase the rate of inactivation of such an enzyme) did not decrease the amount of DNA synthesized after a temperature shift. Increased breakdown of DNA is also ruled out as a cause of the lesion.

The effect of CAP on DNA synthesis after a shift is exactly as predicted. The amount of DNA synthesized in Casamino acids MM is about 50 to 55 % at 25° plus CAP or at 38°, and this is in good agreement with the amount expected if cells in this medium contain two replication forks for a part of their replication cycle. This agreement is not fortuitous (as it was in the mutant of Fangman & Novick, 1968) since the increment in DNA synthesis in II G increases as the growth rate afforded by the medium increases. In brain–heart infusion the amount of DNA synthesized after a temperature shift is about that expected of a very rich medium. Similarly, in MM alone the amount of DNA synthesized by II G after a shift is about 30 %; this is to be expected, as growth of II G in this medium is very slow at 25° (mean generation time of about 260 min.) and there would probably be a gap between rounds of replication. This would mean that some organisms would not be synthesizing DNA at the time of the temperature shift, and this would decrease the amount of DNA synthesized in the absence of new initiations to below the 38 % expected for organisms where DNA synthesis is continuous with a single replication fork (Maaløe & Hanawalt, 1961).

The amount of DNA synthesized plus CAP at 25° and to a greater extent the amount at 38° plus CAP is generally slightly lower than in II G at 38° in the absence of CAP. This would occur if new rounds of replication could be initiated in a few organisms after the temperature shift. Lark & Lark (1964) have dissected the initiation of DNA synthesis in *Escherichia coli* into two steps on the basis of different sensitivities to CAP. One step (the CAP-resistant step) is resistant to low levels of CAP but sensitive to high levels, and the other step (the CAP-sensitive step) is sensitive to low levels of CAP. Lark & Renger (1969) have separated these two steps temporally and find that the CAP-resistant step occurs 15 min. before the initiation event, and the CAP-sensitive step occurs 30 min. before the initiation event. Ward & Glaser (1969) have confirmed these results using more physiological conditions (and using *E. coli* B/r and not *E. coli* 15T⁻) and find that the CAP-resistant step occurs at the time of initiation of DNA synthesis and the CAP-sensitive step occurs about 21 min. before initiation. If a mutant of initiation was affected in this early CAP-sensitive step then the amount of DNA synthesized plus high levels of CAP (e.g. 150 μg./ml. as used in the experiments with II G) should be very much less than the amount synthesized by the mutant at the high temperature, as new initiations could occur for about 20 min. in this kind of mutant. The amount of DNA synthesized by II G at 38° is only slightly more than in the presence of CAP, and so it would appear that II G is not affected in an early stage of initiation.

Several mutants of DNA synthesis can be corrected by increasing the osmotic pressure of the growth medium (Ricard & Hirota, 1969), and this may reflect the involvement of a membrane protein in the DNA lesion. The absence of any effect of increasing the osmotic pressure on the amount of DNA synthesized after a shift argues against the involvement of the membrane in the lesion in II G.

The lesion in II G is completely irreversible when a culture is held at 38° for 90 min. and then transferred back to 25° in the presence of high levels of CAP, and only very

slightly reversible in the absence of CAP. This at first suggested that the protein altered by the temperature shift cannot be reactivated by lowering the temperature, but since synthesis of new initiator at 25° still cannot allow initiation, irreversibility is probably due to the formation of a faulty initiation complex or to the accumulation of irreparable damage to the DNA after 90 min. at the high temperature. Like most processes that stop DNA synthesis but allow RNA and protein synthesis, some degradation of the DNA occurs (Cook *et al.* 1966). Although this is only a few per cent after 90 min. this may be too much to be repaired in most cells and may also contribute to the loss of viability. In some experiments on DNA synthesis this slight breakdown of the DNA was noticed as a loss of counts after about 60 min. at 38°, but as the maximal increment in DNA synthesis in II G at 38° is reached by this time this loss of label probably has no effect on the estimate of the amount of DNA synthesized after the shift.

The critical test for an initiation mutant of DNA synthesis is to show that the DNA synthesized at the high temperature corresponds to the completion of rounds of chromosome replication. The double labelling method of Lark (Lark, Repko & Hoffman, 1963) makes it possible to analyse this DNA. By synchronizing the cells by amino acid starvation for a required amino acid, the origin and terminus regions of the chromosome can be labelled with $^{3}H$ and $^{14}C$ labels during growth at the low temperature. On shifting to 38° in the presence of 5-bromouracil, samples are removed and the DNA analysed by isopycnic centrifugation in caesium chloride. Initiation mutants should show a preferential replication of the terminus label into hybrid density material. Attempts to reinitiate replication in presumed initiator mutants and show the preferential replication of the origin region have failed due either to the lesion being irreversible, e.g. II G, or to the accumulation of a large pool of thymidine triphosphate at 38° making pulse labelling with labelled thymine impossible (Kuempel, 1969). The first kind of double labelling experiments are being done with the mutant II G.

The DNA synthesis mutants of *Escherichia coli* studied by Hirota *et al.* (1968) map in two regions on the *E. coli* genome. One group that maps very close to the *ilv* loci contains mutants that appear to be initiation mutants. The second group, mapping between *malB* and *pyrB*, contains mutants that stop DNA synthesis immediately after a shift from low to high temperature and some that continue DNA synthesis for a short while, but in which the residual DNA synthesis is dependent on the external conditions. Strain II G is very similar to the first group of mutants, but preliminary mapping of the DNA lesion suggests that it maps close to and to the right of *metA* on the *Salmonella typhimurium* map as it is generally represented. More precise mapping and dominance studies are in progress.

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# Decomposition of Thioureas by a Penicillium Species and Soil and Sewage-sludge Microflora

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SUMMARY

A Penicillium species isolated from soil, decomposed up to 0·1 % (w/v) thiourea only with an energy source such as glucose. The fungus released part of the sulphur and nitrogen as sulphate and ammonia which served as S and N sources. The medium became strongly acid due to organic acids originating from glucose; at the acid reactions the amount of sulphate formed was small, but near neutrality most of the sulphur was oxidized to sulphate. Most decomposition of the thiourea and production of sulphate and acid occurred during the lag phase. Ammonium nitrogen promoted growth but reduced decomposition of thiourea. Nitrate was assimilated in the absence of thiourea but not in its presence. Citrate did not support growth but promoted assimilation of nitrogen. Glucose augmented from 0·2 to 2·0 % led to increased growth, decomposition of thiourea and production of sulphate and acid. Aeration also promoted growth and sulphate production. Among the incompletely oxidized decomposition products were sulphate esters and ureides.

Sulphate production indicated that thiourea and four substituted thioureas underwent slow decomposition in soil and sewage sludge. In soil, all of the sulphur of the compounds was oxidized to sulphate in 20 weeks; decomposition was much slower in sewage sludge. Most cultures isolated from treated soil and sewage sludge failed to attack thiourea in culture media on serial transfer.

## INTRODUCTION

According to available evidence, thiourea decomposes slowly in soil and animal manure with production of sulphate and ammonia (Fuller, Caster & McGeorge, 1950; Frederick, Starkey & Segal, 1957; Jensen & Bendixen, 1958; Stotzky & Norman, 1961). Its toxicity is attested by the fact that thiourea reduced the microbial population of a soil more than 99 % at a concentration of 1 % (Frederick *et al.* 1957). Negligible amounts of thiourea decomposed in sewage sludge during normal retention time (Malaney, Lutin, Cibulka & Hickerson, 1967) but some decomposition was noted in acclimatized sludge (Downing, Tomlinson & Truesdale, 1964).

The nitrification process is particularly sensitive to thiourea, more so than ammonification (Fuller *et al.* 1950; Quastel & Scholefield, 1951; Jensen & Sorensen, 1952; Lees, 1952; Jensen & Bendixen, 1958; Jaques, Robinson & Chase, 1959; Downing *et al.* 1964; Tomlinson, Boon & Trotman, 1966). Because of their toxicity to various

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micro-organisms (Nicholas & Nicholas, 1925; Nicholas & Lebduska, 1928) thioureas have potentialities as germicides (Weuffen, Göckeritz & Pohloudek-Fabrini, 1967). Furthermore, thiourea has been proposed as a larvicide in manure (Jensen, 1957; Jensen & Bendixen, 1958), as a soil disinfectant (Frederick *et al.* 1957), and as a fertilizer with slowly available nitrogen (Fuller *et al.* 1950). Thiourea inhibits certain enzymes (Landon, 1934; Mayer, 1958; Bouchilloux, 1961; Yamafuji & Osajima, 1961). Since it is a strong chelating agent (Boeyens & Herbstein, 1966) its toxicity to micro-organisms and inhibition of enzyme action may be due, at least in part, to its chelation of heavy metals.

Little is known about the micro-organisms which attack thiourea or the products formed. Rippel (1925) reported that a small amount of sulphate was produced by *Aspergillus niger*. The most detailed results were obtained by Jensen (1957), who isolated fungi from soil which used thiourea as a source of sulphur and nitrogen when grown in a glucose medium. The two most active fungi converted 15 and 17 % of the thiourea sulphur to sulphate in 50 days. Less than half of the thiourea nitrogen had become assimilated on prolonged incubation when practically all of the thiourea had disappeared; the greater part of the nitrogen was unaccounted for.

Herein are reported results of studies on the decomposition of thiourea by a fungus isolated from soil, the influence of certain cultural conditions on the breakdown, the sulphur and nitrogen products formed, and the course of events in dissimilation of the compound. Included also are results of study of the decomposition of various thioureas in soil and by micro-organisms in sewage sludge.

## METHODS

### *Cultural*

Pure cultures in 250 ml. Erlenmeyer flasks were shaken at 28° on a rotary shaker (240 to 280 cyc./min.) in 100 ml. of: glucose, 20 g. (sterilized separately); thiourea, 0·25, 0·50 or 1·00 g.; $K_2HPO_4$, 1·5 g.; $KH_2PO_4$, 0·75 g.; $CaCl_2.2H_2O$, 0·05 g.; $MgCl_2.6H_2O$, 0·08 g.; $FeCl_3.6H_2O$, 0·01 g.; dist. $H_2O$ to 1000 ml., pH 7·0. Other organic compounds sometimes replaced glucose; phosphate buffer and thiourea concentrations sometimes differed (see Results).

The stock of Penicillium was maintained on a similar medium which contained 0·1 % yeast extract, 0·05 % thiourea and 2 % agar. Pure culture inoculum contained $2 \times 10^6$ conidia in 0·5 ml. sterile water. After incubation the fungus was filtered off, washed, dried and weighed. The culture solution was brought to volume for analyses.

Decomposition of thioureas in 200 g. portions of barnyard soil was tested at 0·02 and 0·1 M. Glucose (0·5 %) was added to soil with and without thioureas. The moisture content was brought to 50 % of the moisture-holding capacity, and water was added periodically to maintain the moisture level during incubation at 28° for several weeks. For tests with activated sludge, 10 ml. portions were added to 90 ml. of a mineral salts medium containing 0·5 % glucose and thioureas at 0·02 and 0·1 M.

### *Analytical*

Sulphate was determined on soil extracts prepared by a procedure adapted from Chesnin & Yien (1950) and Bartlett & Neller (1960): a 50 ml. flask containing 5 g. of soil and 20 ml. aqueous 10 % sodium acetate + 3 % glacial acetic acid was shaken mechanically for 30 min. and centrifuged. After 1·2 g. of 20 to 30 mesh crystals of

$BaCl_2$ had been added to 10 ml., the material was shaken 30 min. and the turbidity due to $BaSO_4$ measured by a nephelometer. Correction was made for colour of the extracts. For culture filtrates, 0·5 ml. of 12 % (w/v) HCl was added to 4 or 8 ml. filtrate in a 50 ml. Erlenmeyer flask, made up to 40 ml. with distilled water and 4·8 g. of 20 to 30 mesh crystals of barium chloride added. The mixture was shaken intermittently for 20 min. and turbidity measured in a Klett colorimeter using a rectangular glass cell (40 mm. outside diam.). No stabilizer was needed to hold the precipitate in suspension, and the thiourea did not interfere with the determination. Corrections were made for colour and turbidity of the culture filtrates.

Thiourea was determined colorimetrically using Grote's reagent (Anonymous, 1960). Values were accurate from 25 to 200 $\mu$g. thiourea. Total nitrogen was determined by a micro-Kjeldahl method (Colowick & Kaplan, 1955–7), ammonium nitrogen by titration of the distillate of the culture solution made alkaline with NaOH or MgO, and glucose by use of anthrone reagent (Umbreit, Burris & Stauffer, 1957). Amino nitrogen was measured colorimetrically according to Danielson (1933) and Hotchkiss (1956). Urea was determined by microdiffusion with urease (Conway, 1950). The colorimetric procedure of Koritz & Cohen (1954) as modified by Bojanowski, Goudy, Valentine & Wolfe (1964) was used to detect ureido compounds. Test for hydroxylamine was made by a method of Czaky (1948) as modified by Klein & Pramer (1962), and for thiocyanate according to Colowick & Kaplan (1955–7). Sulphate released from ethereal sulphates (Colowick & Kaplan, 1955–7) was determined as barium sulphate. Tests were made also for methylthio groups (Segal & Starkey, 1969) and sulphydryl groups (Colowick & Kaplan, 1955–7), and for thiosulphate and polythionates (Starkey, 1934).

## RESULTS

### *Isolation and characterization of a thiourea-decomposing culture*

One of the crude cultures which developed from an inoculum of thiourea-treated soil produced a substantial amount of sulphate in a few weeks in glucose–thiourea medium. A fungus isolated from this culture developed well and produced sulphate in an aerated glucose–thiourea medium with thiourea as the only source of nitrogen and sulphur. Glucose or its equivalent was required as a source of energy and probably of carbon. Thus the transformation appears to be another example of codissimilation (Ruiz-Herrera & Starkey, 1969). The fungus which was used in the following experiments was identified as Penicillium species of the group Biverticillata-Symmetrica. It resembled *Penicillium rugulosum* (Raper & Thom, 1949).

### *Transformation of thiourea by Penicillium*

The fungus developed well initially in the basal medium containing glucose and 0·025 and 0·05 % (w/v) thiourea, but it grew slowly in the presence of 0·10 % thiourea and failed to grow at 0·20 %. On repeated subculture the fungus developed tolerance to increased concentrations of thiourea.

Fig. 1 illustrates changes which were obtained with 0·05 % (w/v) thiourea; similar changes occurred with 0·025 or 0·01 % thiourea. The log phase extended from the second to the fifth day. The medium became strongly acid and the principal drop in pH occurred during the lag phase. By the end of the lag phase all of the thiourea had disappeared and the sulphate content was nearly maximum. After incubation, only 31 % of the thiourea sulphur was present as sulphate and 7 % of the thiourea nitrogen

as ammonia. Thirty-three per cent of the nitrogen had been assimilated but 60 % was unaccounted for. The sulphur and nitrogen not accounted for in this and other experiments probably were present as intermediate products.

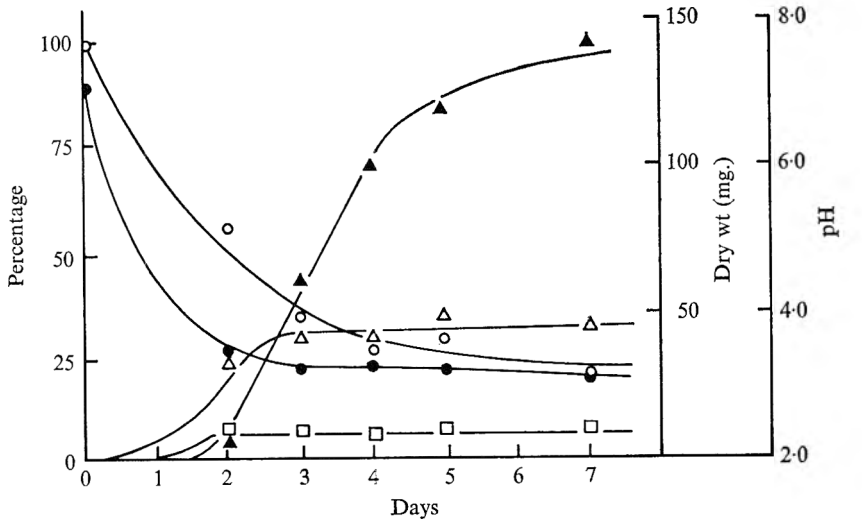


Fig. 1. Changes occurring during transformation of thiourea at 28° by Penicillium. For medium and conditions, see text. ○, (%, w/v) Glucose; △, sulphate sulphur (% of initial thiourea sulphur); □, ammonium nitrogen (% of initial thiourea nitrogen); ●, pH; ▲, growth.

Table 1. *Influence of shaking on breakdown of thiourea by a Penicillium*

Cultures incubated 6 days at 28°, for details see text.

| Condition of incubation | pH | Dry wt of fungus (mg.) | Thiourea decomposed (%) | $SO_4^=$-S, % of initial thiourea-S | $NH_4^+$-N, % of initial thiourea-N |
|---|---|---|---|---|---|
| Static | 4·6 | 10 | 91 | 13 | 22 |
| Shaken | 3·4 | 82 | 91 | 32 | 12 |

*Some growth requirements*

In media prepared with 'reagent grade' salts, the fungus required added magnesium but not calcium, iron, sodium, zinc or manganese. Copper (14 μg./ml.) was toxic but its toxicity was overcome by citrate in a medium containing 0·05 % (w/v) thiourea but not in one with 0·1 %. Yeast extract (50 μg./ml.) increased the amount of growth in 3 days but had no significant effect subsequently. None of nine vitamins increased the amount of growth in 3 and 7 days in the glucose–thiourea medium. The fungus decomposed thiourea in both static and shaken media but more mycelium and more sulphate appeared in shaken media (Table 1). This suggests that the reactions responsible for formation of sulphate were more sensitive to oxygen tension than those effecting the initial transformation of thiourea.

*Replacement of thiourea*

In media containing ammonium sulphate as nitrogen+sulphur source, but no thiourea, the fungus grew well on glucose, fructose, galactose, mannitol, ethanol and

acetate, but only slightly on sorbitol, succinate, or citrate. Only glucose supported good growth with thiourea, which suggests that thiourea either inhibited uptake of the unutilized compounds or blocked their conversion to assimilable substances.

*Influence of the nitrogen source on growth and on breakdown of thiourea*

The following compounds, listed in the order in which they promoted growth, served as sources of nitrogen (at 18·4 mg. N/100 ml.) in a glucose medium: ammonium sulphate, glycine > potassium nitrate > oxamic acid > thiourea. The mycelial dry wt with $NH_4$ was more than twice that with thiourea. Jensen (1957) noted also that ammonium nitrogen was preferable to thiourea as the source of nitrogen for cultures

Table 2. *Influence of ammonia and nitrate on fungus growth and decomposition of thiourea*

Cultures shaken 7 days at 28°, for details see text.

| Source of nitrogen (mg.) | Dry wt of fungus (mg.) | N in cell material (mg.) | Thiourea decomposed (%) | Thiourea-S converted to sulphate (%) |
|---|---|---|---|---|
| Thiourea, 52 | 116 | 5·8 | 98 | 50 |
| Thiourea, 26+$NH_4Cl$, 39 | 199 | 8·6 | 51 | 7 |
| Thiourea, 26+$KNO_3$, 50 | 105 | 4·8 | 97 | 76 |

Table 3. *Effect of ammonia and nitrate on transformation of thiourea nitrogen by Penicillium*

Incubated 7 days at 28°, for details see text.

| | Source of N | | |
|---|---|---|---|
| Distribution of nitrogen (mg.) | Thiourea | Thiourea+ $NH_4Cl$ | Thiourea+ $KNO_3$ |
| Uninoculated control | | | |
| Thiourea | 19·2 | 9·4 | 9·4 |
| Nitrate | — | — | 7·0 |
| Ammonia | — | 8·3 | — |
| Inoculated media | | | |
| Mycelium | 5·8 | 8·6 | 4·8 |
| Filtrate | 12·2 | 9·8 | 11·8 |
| N accounted for | 18·0 | 18·4 | 16·6 |
| Ammonia | 2·4 | 0·6 | 0·8 |
| Nitrate | — | — | 7·0 |
| Thiourea | 0·3 | 4·5 | 0·3 |
| N in filtrate other than $NH_4^+$, $NO_3^-$ and thiourea | 9·5 | 4·7 | 3·7 |

of fungi that decomposed thiourea. There was little or no growth with the following sources of nitrogen: cyanamide, potassium thiocyanate, thiosemicarbazide, ethylenediamine dihydrochloride, potassium ferrocyanide, diethylthiourea, dibutylthiourea, phenylthiourea, benzylpseudothiourea.

When approximately equal amounts of nitrogen were supplied as thiourea (0·025 %) and ammonium chloride, the fungus made more growth and assimilated more nitrogen than when all nitrogen was provided as thiourea (Table 2). Ammonium, but not nitrate, nitrogen had an inhibitive effect on decomposition of thiourea and on conversion

of the thiourea sulphur to sulphate. Nearly all of the thiourea disappeared from culture solutions which contained thiourea or thiourea + nitrate as the nitrogen sources, and conversion of the thiourea sulphur to sulphate was greater in the latter. Table 3 shows that practically all the nitrogen provided as ammonium chloride was assimilated, but one-half of the thiourea remained at the end of incubation. Nitrate was not assimilated if the medium contained thiourea. The inhibitive effect of thiourea on nitrate assimilation may have been due to its toxicity to nitrate reductase (Yamafuji & Osajima, 1961).

*Influence of reaction on decomposition of thiourea*

The reaction became strongly acid during development of the fungus in media containing glucose and thiourea. Although some of the acidity was due to sulphate production, most is ascribed to products of glucose breakdown because the change in pH was approximately the same with and without thiourea. The organic acids were not identified, but chromatographic tests excluded formic, acetic, lactic, malonic, oxalic, succinic and citric acids. With the solvent system *n*-butyl alcohol–acetic acid–water (120:30:50) a strong yellow spot developed with bromocresol green at $R_F$ 0·05 to 0·07 and a weak one at $R_F$ 0·20 to 0·25.

When the pH of a medium containing, for example, 0·05 % (w/v) thiourea was controlled by periodic neutralization with NaOH, the thiourea sulphur oxidized to sulphate increased from 37 to 65 %. In other experiments in which the pH was partly or largely controlled, oxidation of from 80 to 100 % of the thiourea sulphur to sulphate took place (Table 4). All thiourea had disappeared from these media and the mycelial dry weights were approximately the same. Only a small portion of the thiourea nitrogen was recovered as ammonia.

Table 4. *Influence of reaction on decomposition of thiourea by Penicillium*

Incubated 7 days at 28°. pH change restricted by increased phosphate buffer or, for last two entries, periodic additions of NaOH.

| Buffer (M) | Final pH | $NH_4^+$-N, % of added thiourea-N | $SO_4^=$-S, % of added thiourea-S |
|---|---|---|---|
| 0·014 | 3·3 | 22 | 33 |
| 0·035 | 3·5 | 11 | 45 |
| 0·042 | 4·0 | 19 | 64 |
| 0·070 | 4·5 | 8 | 100 |
| 0·014 | 7·0 | 19 | 78 |
| 0·042 | 6·6 | 17 | 86 |

*Effect of glucose concentration on decomposition of thiourea*

Fig. 2 shows that increased glucose led to increased growth, thiourea decomposed, sulphate produced and acid. Conversion of thiourea sulphur to sulphate reached a maximum of 72 % with 2 % (w/v) glucose. Irrespective of glucose concentration only a small amount of the thiourea nitrogen was converted to ammonia, the maximum being approximately 12 %. The relatively high buffer content prevented extreme drop in pH; except with 2 % (w/v) glucose, the reaction remained above pH 5·0. In similar experiments with 0·014 M-phosphate buffer, the pH dropped to much lower levels and the conversion of the thiourea sulphur to sulphate was much lower. All of the thiourea

disappeared from the medium which contained 2 % (w/v) glucose. In another experiment, the amounts of fungus and of thiourea transformed were similar with 2 and 3 % glucose.

*Breakdown of thiourea by pregrown mycelium*

Mycelium from thiourea media with various concentrations of glucose and phosphate and with either organic or inorganic sources of nitrogen was harvested during the log phase. It was washed and suspended in 0·014 to 0·042 M-potassium phosphate buffer, pH 7·0 or 5·5, containing 0·01 or 0·05 % (w/v) thiourea. Glucose was present in some media and absent from others. Tests were made for disappearance of thiourea and for production of sulphate and ammonia between 3 to 10 h., and in one experiment at 72 h. In no case was there rapid and complete breakdown of thiourea.

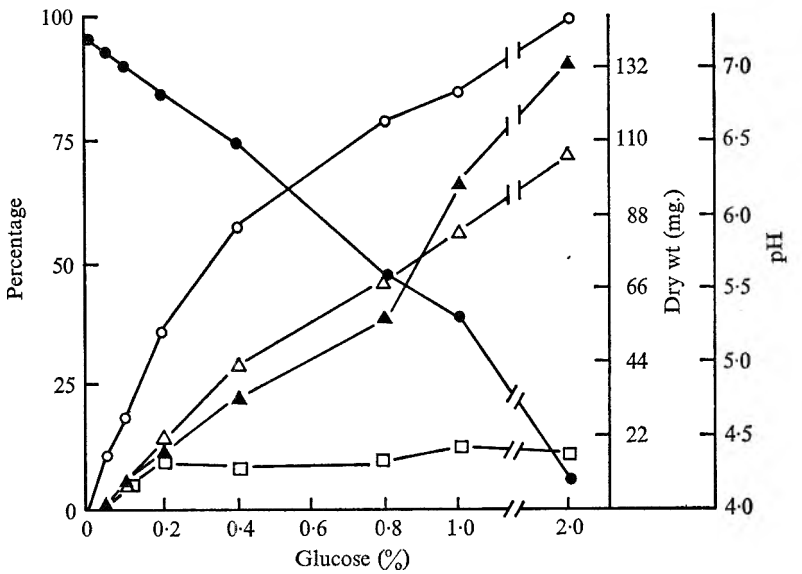


Fig. 2. Effect of glucose concentration on transformation of thiourea by Penicillium after 6 days at 28°; initial pH 7·2; thiourea 0·05 %; phosphate buffer 0·042 M. ○, Loss of thiourea (%); △, increase in sulphate sulphur (% of initial thiourea sulphur); □, increase in ammonium nitrogen (% of initial thiourea nitrogen); ●, pH; ▲, growth.

*Products of thiourea dissimilation*

The only sulphur product identified was sulphate. Other sulphur products must have been formed because only 30 to 50 % of the sulphur of the decomposed thiourea appeared as sulphate in some experiments. Filtrate of a culture with 0·05 % (w/v) thiourea and 0·042 M-phosphate buffer, pH 7·0, incubated 7 days, contained 19 % of the thiourea sulphur as ethereal sulphate and 46 % as sulphate. The identity of the thioether(s) was not established. Compounds with methylthio groups, hydrosulphide groups, thiosulphate, polythionates and hydrogen sulphide were absent.

Ammonia was the only nitrogen product identified. Considerable amounts of nitrogen were assimilated but almost half of the thiourea nitrogen was unidentified material. The culture filtrate from glucose–thiourea media gave a strong positive test for ureido compounds; these were absent from uninoculated thiourea media and from filtrates

of fungus cultured with ammonium sulphate in place of thiourea. Ureido compounds were absent also in culture solutions containing urea, amino nitrogen, primary amines, free and bound hydroxylamines, thiocyanate, biuret, nitrite and nitrate.

*Decomposition of thioureas by natural microbial populations*

Production of sulphate served as the index of thiourea decomposition. All of the compounds underwent decomposition in soil (Table 5) but the breakdown was slow. At the lower concentration of the thioureas there was appreciable decomposition in 1 week and most of the sulphur became transformed to sulphate in 15 weeks. Dibutylthiourea decomposed slowest but all of its sulphur was converted to sulphate in 20 weeks. With 0·1 M-thioureas the percentage conversions of sulphur were much lower, but the actual amounts converted were similar to those at 0·02 M. Decomposition of the thioureas other than benzylthiopseudourea had comparatively little effect on pH of the soil.

Decomposition of the thioureas by the micro-organisms in activated sludge was much slower than by those in soil (Table 6). Even at the lower concentration, the maximum sulphur conversion in 20 weeks was 22 %.

Table 5. *Production of sulphate from thioureas in soil*

Initial pH of soil was 8·5; sulphate analyses corrected for sulphate in untreated soil. For details see text.

| | | pH after | | | % S recovered as sulphate after | | |
|---|---|---|---|---|---|---|---|
| Compound | (M) | 1 week | 2 weeks | 15 weeks | 1 week | 2 weeks | 15 weeks |
| None | — | — | — | 6·4 | — | — | — |
| Thiourea | 0·02 | 7·9 | 7·8 | 7·1 | 22 | 27 | 96 |
| | 0·1 | 7·5 | 7·6 | 6·7 | 4 | 4 | 28 |
| Diethyl- | 0·02 | 7·6 | 7·5 | 7·5 | 40 | 40 | 91 |
| thiourea | 0·1 | 6·9 | 6·6 | 7·1 | 4 | 3 | 12 |
| Dibutyl- | 0·02 | 7·7 | 7·4 | 6·3 | 19 | 26 | 56 |
| thiourea | 0·1 | 7·9 | 7·6 | 7·2 | 4 | 5 | 12 |
| Phenyl- | 0·02 | 7·5 | 7·1 | 5·8 | 41 | 45 | 107 |
| thiourea | 0·1 | 7·3 | 7·0 | 6·4 | 8 | 10 | 26 |
| Benzylthio- | 0·02 | 8·3 | 7·3 | 4·9 | 25 | 44 | 93 |
| pseudourea | 0·1 | 7·6 | 7·4 | 5·7 | 1 | 3 | 45 |

Table 6. *Production of sulphate from thiourea in sewage sludge*

Initial pH 7·0; culture pH kept neutral during incubation by periodic additions of NaOH. $SO_4^{=}$ values corrected for content of controls. For details see text.

| | | pH after | | | % S recovered as sulphate after | | |
|---|---|---|---|---|---|---|---|
| Compound | (M) | 5 weeks | 10 weeks | 20 weeks | 5 weeks | 10 weeks | 20 weeks |
| Thiourea | 0·02 | 7·1 | 7·0 | 7·1 | 6 | 8 | 14 |
| | 0·10 | 7·6 | 7·3 | 7·4 | 1 | 1 | 2 |
| Diethyl- | 0·02 | 7·6 | 7·3 | 7·2 | 5 | 5 | 13 |
| thiourea | 0·10 | 7·9 | 7·8 | 7·6 | 1 | 1 | 3 |
| Dibutyl- | 0·02 | 7·7 | 7·5 | 7·2 | 3 | 3 | 14 |
| thiourea | 0·10 | 7·4 | 7·4 | 7·1 | 2 | 2 | 6 |
| Phenyl- | 0·02 | 7·2 | 7·1 | 7·0 | 3 | 8 | 22 |
| thiourea | 0·10 | 6·9 | 6·8 | 7·0 | < 1 | < 1 | 3 |
| Benzylthio- | 0·02 | 6·9 | 6·6 | 6·6 | 0 | 0 | 5 |
| pseudourea | 0·10 | 6·3 | 5·8 | 5·5 | 0 | 0 | 0 |

*Isolation of thiourea-decomposing micro-organisms*

Dilutions of the soils and solutions containing activated sludge, which had been treated with the thioureas, were plated on agar media modified to contain 0·1 % of the same thiourea as the inoculum source, without and with glucose (0·1 %). Most of the colonies which developed were bacteria, but there were some streptomycetes and filamentous fungi. Growth was slight in media without glucose.

Thirty representative cultures isolated from the plates were inoculated into liquid media containing the appropriate thioureas with and without glucose (1·0 %). Although there was slight growth of some cultures lacking glucose, no sulphate was produced and the cultures failed to develop on serial transfer. Most isolates grew well with glucose and the thioureas but, with few exceptions, none produced sulphate. One culture, a species of the fungus Cephalosporium, produced some sulphate from benzylthiopseudourea in a glucose-containing medium. During growth, the odour of the compound disappeared from the inoculated medium whereas it persisted in the uninoculated one. Thus few micro-organisms seem able to decompose thiourea.

## DISCUSSION

In studies by Jensen (1957), 20 % or less of the thiourea sulphur was converted to sulphate by certain fungi growing in a medium which contained 2 % (w/v) glucose and 0·05 % thiourea. The low production of sulphate may have been due to a pH effect. We noted that the reaction became strongly acidic during breakdown of thiourea and that the acidity impeded conversion of the thiourea sulphur to sulphate. Nevertheless it did not affect the disappearance of the thiourea appreciably.

Sulphate ester(s) was an important sulphur product, particularly in culture solutions which became strongly acid. The fact that the culture solutions gave a strong reaction for ureido compounds suggests that they were the major nitrogen-containing decomposition products. Some ammonium nitrogen was present in culture solutions even when conditions suggested that nitrogen was a limiting factor for growth. Therefore, thiourea or its decomposition products may have interfered with assimilation of nitrogen. Furthermore, even though citrate failed to support growth when it was the only source of energy in a thiourea medium, it promoted growth in a similar medium which contained glucose; a medium with 1·5 % (w/v) glucose and 0·5 % sodium citrate yielded approximately twice as much growth as one with 2 % glucose. It seems likely that citrate neutralized some inhibitive effect of thiourea on nitrogen assimilation.

The data are insufficient to establish the course of events in the dissimilation of thiourea but the detection of ethereal sulphate and ureido compounds as products suggests the scheme in Fig. 3. This assumes that the sulphur of the thiourea became transformed to a hydrosulphide and that the sulphur became oxidized to a sulphate ester which was desulphurated to yield sulphate. The urea produced by desulphuration of the thiourea could have been transformed by a process reported by Cook & Boulter (1964) for *Candida flareri*. This involved combination of the urea with a 2-carbon unit to give hydantoic acid which became converted to its anhydride, hydantoin, or to glycine and a carbamate. In addition to the ureido compounds, hydantoic acid and hydantoin, the carbamyl compound $R'.CO.NH_2$ would react as a ureide if the carbon chain of R′ was short (Koritz & Cohen, 1954). Glucose would have provided the

2-carbon unit. The glycine could have been partly assimilated and partly deaminated to yield ammonia. The residual nitrogen other than ammonia in the culture solution could have been accounted for as the two ureides and the carbamate.

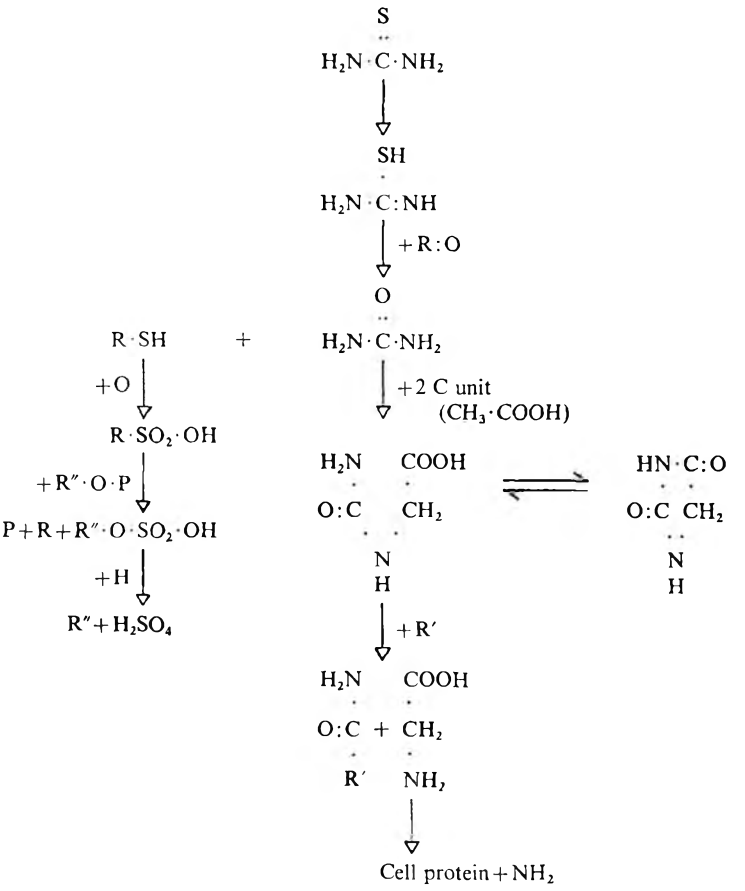


Fig. 3. Proposed scheme of the course of dissimilation of thiourea.

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# The Identification, Taxonomy and Classification of Luminous Bacteria

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SUMMARY

The results of a study of 51 strains of luminous bacteria and 12 strains of non-luminous bacteria from related genera show that the luminous bacteria can be divided into three major groups. These groups span three genera, namely *Vibrio*, *Photobacterium* and a suggested new genus, *Lucibacterium*, to accommodate the '*Photobacterium harveyi*' organisms. Revised descriptions of the species are given.

## INTRODUCTION

Luminous bacteria are not rare in the marine environment and they have occasionally been reported from non-marine sources. In the sea they are found in the water, as saprophytes and parasites on marine animals and living symbiotically in specialized organs in certain fish and cephalopods. Early workers assigned these bacteria to a variety of genera, but Beijerinck (1889) proposed that all luminous bacteria should be placed in one genus, *Photobacterium*, which is based on a single criterion, namely the ability to emit visible light. Later (1916) Beijerinck stated that some species were closely allied to the cholera vibrios, but since then there has been much confusion in the nomenclature and taxonomy of the luminous bacteria. Many species have been named on ecological grounds, e.g. *Micrococcus physiculus*, *Micrococcus* (*Coccobacillus*) *acropomae*, *Achromobacter merluccius*, *Photobacterium sepiae*, etc., and some new species have been described which show only minor differences from existing species.

Much of the literature on luminous bacteria deals with the metabolic systems and biochemical requirements of individual strains, but little has been published on the taxonomy of the group as a whole (see McElroy, 1961, for a review). In 1954, Breed & Lessel pointed out that these bacteria had been assigned to no fewer than 15 genera and numerous specified epithets had been used. *Index Bergeyana* (1966) lists 39 specific epithets that have been used with Photobacterium alone. Breed & Lessel (1954) proposed that all luminous bacteria should be assigned to two genera, *Photobacterium*, which is placed in the family Pseudomonadaceae, and *Vibrio*. Spencer (1955) studied seven named strains and two new isolates and concluded that all luminous bacteria had close affinities with the genera *Vibrio* and *Aeromonas*.

In the seventh edition of *Bergey's Manual* (1957) all luminous bacteria are listed under Photobacterium and Vibrio as proposed by Breed & Lessel (1954). In Photobacterium there are four species, *Photobacterium phosphoreum*, the type species, *P. fischeri*, *P. harveyi* and *P. pierantonii*. These species are fairly well described, but of the four species listed under Vibrio – *Vibrio albensis*, *V. pierantonii*, *V. indicus* and

*V. luminosus* – only *V. albensis*, a non-marine type, has been adequately described. Prévot (1961), in his *Traité de Systématique Bactérienne*, listed the same eight species under the same genera but selected *P. fischeri* as the type species of Photobacterium. Krassil'nikov (1959) listed eight species in Photobacterium with a further eight subspecies included under five of these species, and one species was included in Pseudomonas – *Pseudomonas harveyi*, a species originally placed in Achromobacter by Johnson & Shunk (1936) and in Photobacterium in *Bergey's Manual* (7th edn) and in Prévot's *Traité de Systématique Bactérienne*.

Table 1. *List of organisms used in the study*

| NCMB collection accession number | Species (as received) | Strain and/or other collection numbers | Source |
|---|---|---|---|
| | Luminous strains | | |
| 1 | *Photobacterium splendidum* | 4 | A. J. Kluyver, Delft, via R. Spencer |
| 2 | *P. harveyi* | — | |
| 7 | *P. phosphoreum* | — | |
| 24 | *P. sepiae* | — | |
| 25 | *P. pierantonii* | ATCC 14546 | |
| 41 | *P. albensis* | ATCC 14547 | |
| 42 | *P. fischeri* | | J. Cruickshank, University of Aberdeen, via R. Spencer |
| 60 | *Micrococcus physiculus* | MP | T. Iijima, Osaka, Japan, via R. Spencer |
| 61 | *Coccobacillus macrouri* | CM | |
| 62 | *Photobacterium phosphoreum* | TY | |
| 63 | *Pseudomonas lucifer* | PL | |
| 64 | — | FK | |
| 65 | — | EB | |
| 66 | *Coccobacillus* sp. | HARIDASHIEBISU | Y. Yasaki, Tokyo, Japan, via R. Spencer |
| 67 | *Micrococcus acropomae* | HOTARUJAKO H | |
| 68 | *Coccobacillus macrouri* | TOHJIN | |
| 69 | *C. macrouri* | SOROIHIGE | |
| 70 | — | P 336 | |
| 71 | — | OS | T. Iijima, via R. Spencer |
| 193 | *Vibrio* sp. | 1876 | J. Liston, T.R.S. (Liston, 1955) |
| 390 | — | $I_2$ | N. K. Velankar, Mandapam, South India, via R. Spencer (deposited, 1958) |
| 391 | — | $I_3$ | |
| 392 | — | $I_4$ | |
| 393 | — | $I_6$ | |
| 394 | — | $I_7$ | |
| 395 | *Photobacterium phosphoreum* | | R. Spencer, Humber Laboratory, Hull |
| 840 | — | $I_2B$ | N. K. Velankar, via R. Spencer (deposited 1961) |
| 841 | — | $I_3$ (= 391) | |
| 842 | — | $I_7$ (= 394) | |
| 843 | — | 7g | |
| 844 | — | DLG I | D. L. Georgala, Fishing Industry Research Institute, Rondebosch, South Africa, via R. Spencer |
| 993 | — | 21–I–12 | U. Melchiorri-Santolini, Fiascherino (La Spezia), Italy |

Table 1 (*cont.*)

| NCMB collection accession number | Species (as received) | Strain and/or other collection numbers | Source |
|---|---|---|---|
| 1061 | *Vibrio* sp. | 314 | J. I. W. Anderson, Department of Bacteriology, University of Glasgow |
| 1147 | *Vibrio* sp. | 368 | R. Spencer, Humber Laboratory, Hull |
| 1148 | *Vibrio* sp. | 379 | |
| 1149 | *Vibrio* sp. | 380 | |
| 1150 | *Vibrio* sp. | 381 | |
| 1151 | *Vibrio* sp. | 382 | |
| 1152 | *Vibrio* sp. | 390 | |
| 1198 | — | $I_{34}$ | A. N. Bose, Central Institute of Fisheries Technology, Ernakulam, South India |
| 1199 | — | $I_{52}$ | |
| 1200 | — | $I_{62}$ | |
| 1274 | *Vibrio noctiluca* | U-I | H. Weisglass, Zagreb, Yugoslavia (Weisglass & Skreb, 1963; Weisglass & Gavrilovic, 1963) |
| 1275 | *Photobacterium profundum* | R-I | |
| 1276 | — | DLG 2 | D. L. Georgala, via R. Spencer |
| 1277 | — | DLG 3 | |
| 1278 | — | F 37 | R. Spencer, via J. C. Early |
| 1279 | — | CB 95 | |
| 1280 | *Photobacterium harveyi* | ATCC 14126 | ATCC. Originally deposited in ATCC by F. H. Johnson |
| 1281 | *P. fischeri* | ATCC 7744 | |
| 1282 | *P. phosphoreum* | ATCC 11040 | |
| | Non-luminous strains | | |
| 23 | *Aeromonas formicans* | ATCC 13137, NCIB 9235 | I. P. Crawford (1954) (Pivnick & Sabina, 1957) |
| 72 | *A. hydrophila* | G I | W. Hodgkiss, T.R.S. |
| 87 | *A. liquefaciens* | L 417 | A. A. Miles, who obtained it from A. J. Kluyver (Miles & Miles, 1951) |
| 129 | *Pseudomonas fluorescens* | NC 5 | L. I. Fletcher, T.R.S. via W. Hodgkiss |
| 130 | *Pseudomonas* sp. | NC 6 | |
| 224 | *Pseudomonas* sp. | 139 | J. Liston, T.R.S. |
| 320 | *Pseudomonas* sp. | 4722 | D. L. Georgala, T.R.S. |
| 406 | *P. putida* | A 1005 | W. Hodgkiss, T.R.S. |
| 407 | *Vibrio ichthyodermis* | PL I | W. Hodgkiss & J. M. Shewan, T.R.S. (Hodgkiss & Shewan, 1950; Shewan, Hobbs & Hodgkiss, 1960) |
| 1143 | *V. marinus* | PS 207, ATCC 15382 | R. R. Colwell (Colwell & Morita, 1964) |
| 1144 | *V. marinus* | MP-1, ATCC 15381 | |
| NCIB 9243 | *Aeromonas shigelloides* | C 27, ATCC 14030 | W. H. Ewing (Ferguson & Henderson, 1947) |

NCMB = National Collection of Marine Bacteria; NCIB = National Collection of Industrial Bacteria, both at Torry Research Station, Aberdeen. ATCC = American Type Culture Collection; T.R.S. = Torry Research Station.

Clearly there is still much confusion in the classification of luminous bacteria and many of the descriptions given in current editions of manuals are inadequate for present-day requirements. With the desire to clarify the stiuation, we have made a new study of a collection of these organisms.

## METHODS

*Organisms.* Table 1 lists the 51 strains of luminous bacteria and 12 strains of non-luminous bacteria from 'related' genera that were used.

*Media.* All sea-water media contained natural sea water, aged as recommended by ZoBell (1946) (storage in the dark for a minimum of 3 weeks) and filtered before use and then diluted, 3 parts sea water to 1 part tap or distilled water. Sea-water–yeast peptone (SWYP): yeast extract powder (Oxoid), 0·3 %; peptone (Evans), 0·5 % at pH 7·4. Agar (1·5 %, Difco) was added for the solid medium. Yeast peptone (pH 7·4): yeast extract powder, 0·3 %; peptone, 0·5 %; NaCl, 0·5 % in distilled water (in some tests the NaCl concentration was altered as required). Glycerol calcium carbonate agar: yeast peptone agar with 3 % NaCl, 1 % glycerol and 0·5 % $CaCO_3$. Plaice medium (the late Professor A. J. Kluyver, personal communication): minced fresh plaice (200 g.) was soaked in 4 l. tap water for 2 h., boiled for 1 h. and filtered. Peptone (0·5 %) and NaCl (3 %) were added, the broth adjusted to pH 7·3, boiled and filtered. For a solid medium 2 % agar was added. Sea-water lemco: Lab-lemco, 1 %; peptone, 0·5 % at pH 7·4.

Stock cultures were maintained at 20° on SWYP agar or sea-water lemco agar except *Aeromonas shigelloides*, which was maintained on yeast peptone agar. All tests on *A. shigelloides* were carried out in media containing 0·5 % NaCl as growth in sea water or 3 % NaCl media was unsatisfactory.

*Temperature relationships.* All cultures were streaked on SWYP agar plates, 10 strains per plate, and incubated at 1 and 5° (for 21 days); 15, 20 and 25° (7 days) and 30 and 37° (3 days). The plates were examined at intervals during incubation. As all strains except *Vibrio marinus* grew well at temperatures of 15 to 25° and only some grew at either higher or lower temperatures, all subsequent tests were carried out at 20°, *V. marinus* being tested at 15°.

*Morphology.* Cultures grown in SWYP broth for 24 or 48 h. were examined under a dry phase contrast microscope (×240) for motility and shape of the living cells. Strains which were not observed to be motile in liquid medium were examined from 24 h. SWYP agar slope culture as it had been noted that motile forms could occasionally be detected from a solid medium and not from a liquid medium. Smears for staining were prepared from a 2 day SWYP agar culture by suspending the bacteria in 2 % NaCl solution to prevent uptake of water by the cells, and fixed with methanol. The smears were then desalted by flooding with water for 5 min. and allowed to dry before Gram-staining by Jensen's modification (Mackie & McCartney, 1960).

Bacteria required for electron microscopy came from 24 or 48 h. cultures grown on SWYP agar, plaice agar or glycerol calcium carbonate agar. Suspensions were prepared by the following methods. (*a*) Bacteria were harvested from slopes into 5 % ammonium acetate solution and washed three times by centrifugation at low speed (2000*g*) and then the deposit was suspended in ammonium acetate. A droplet of this suspension was placed on a grid. (*b*) Bacteria were harvested into 2·5 % NaCl con-

taining 5 % (v/v) formalin and fixed for 30 min. A drop of this suspension was placed on a grid, dried and was washed by floating the grid face down on distilled water for 10 min. (*c*) Bacteria were fixed in formalin in sodium chloride solution as in (*b*), washed twice in 5 % ammonium acetate and a suspension in this solution placed on the grids.

Metal-shadowed preparations were made by drying the suspensions on Formvar-coated 200-mesh copper grids and shadowing with gold + palladium (40 + 60) at an angle of 20°. For negatively stained preparations the suspensions were dried on carbon-stabilized Formvar-coated grids and stained with either 1 % phosphotungstic acid (pH 7; Brenner & Horne, 1959) or 1 % ammonium molybdate. After 1 min. the excess fluid was removed by touching the grid with filter paper. Specimens were examined in a Siemen's Elmiskop I with a double condenser system, a condenser aperture of 200 $\mu$m. and an objective aperture of 50 $\mu$m. Micrographs were recorded on Ilford N 50 plates.

Colony appearance was recorded after 3 days incubation on SWYP agar and appearance of growth in liquid medium after 2 days in SWYP broth.

*Effect of NaCl on luminescence.* Agar slopes of SWYP, plaice medium and glycerol–calcium carbonate media with 0·5 and 3 % NaCl and yeast peptone medium containing different amounts of NaCl (0·5, 1, 1·5, 2, 2·5 and 3 %), were inoculated with a drop of a 2 day broth culture and examined in the dark room daily for 4 days and again after 7 days incubation. The observer spent 10 min. in the dark room before examining the cultures to allow the eyes to become accommodated to the dark.

*NaCl requirement and tolerance.* Yeast peptone broth with no NaCl and with 5, 7·5 and 10 % NaCl was inoculated from a 2 day SWYP broth culture. The extent of visible growth was recorded after 4, 7 and 15 days incubation, and the tubes were examined for luminescence daily for 4 days.

*Antibiotic sensitivity.* This was tested by the method of Shewan, Hodgkiss & Liston (1954). The vibriostatic compound '0/129' (2,4-diamino-6,7-diisopropyl pteridine) was applied as filter-paper discs which had been saturated with a 0·1 % solution in dioxan and dried (Schubert, 1962, modified). Yeast peptone agar was used as Spencer (1955) had suggested that sea water interfered with the action of terramycin. Since many strains did not grow enough on this medium to give a satisfactory result, the test was repeated on SWYP agar with compound 0/129 applied in duplicate, as a saturated aqueous solution in cups out from the agar, and as impregnated paper discs.

*Biochemical tests.* Acid production from carbohydrates and alcohols was tested using 1 % of the substrate and 1 % Andrade indicator in a sea-water peptone medium (1 % peptone in 3 : 1 sea water : distilled water). The 28 substrates tested were arabinose, rhamnose, ribose, xylose, fucose, glucose, fructose, galactose, mannose, lactose, sucrose, maltose, cellobiose, raffinose, trehalose, sorbose, cellulose, mannitol, dulcitol, sorbitol, glycerol, inositol, salicin, inulin, dextrin, laminarin, starch and glycogen. Results were recorded at 1, 2, 4 and 7 days and then weekly up to 4 weeks.

Dissimilation of glucose was tested by the methods of Hugh & Leifson (1953) and Leifson (1963), but in the latter medium, with filtered aged natural sea water instead of artificial sea water. Results were read after 1, 2, 4 and 7 days, and at 10 and 14 days for strains showing little change after 7 days.

Oxidase was tested by the method of Kovacs (1956).

Tests for indole production, ammonia production from peptone, nitrate reduction,

acetylmethylcarbinol production, methyl red reaction (*Manual of Microbiological Methods*, 1957), 2,3-butanediol production (Bullock, 1961) and trimethylamine oxide (TMO) reduction (Wood & Baird, 1943) were made on 7 day cultures in sea-water media (indole, ammonia, nitrate) or in media supplemented with 3 % NaCl.

Hydrolysis of Tweens was tested by Sierra's method (1957) using media with 3 % NaCl and read at 1, 2, 4 and 7 days.

Møller's method (1955) was used for the detection of lysine and ornithine decarboxylases and the production of alkaline products from arginine. The medium contained 3 % NaCl and results read at 1, 2, 4, 7 and 9 days.

Gelatin liquefaction was tested in stab cultures in SWYP + 12 % gelatin; growth and changes were recorded at 1, 2, 4 and 7 days and weekly to 5 weeks. Strains showing slow liquefaction or no liquefaction were retained for 7 weeks.

Egg yolk reactions were tested on SWYP agar + egg yolk emulsion (Willis & Hobbs, 1958); after 4 days plates were examined and tested for free fatty acids.

Esterase patterns were examined by starch gel electrophoresis by the method of Cann & Willox (1965).

Base compositions of deoxyribonucleic acid were determined by Professor M. Mandel by the buoyant density in caesium chloride method (Mandel, 1966) for 13 luminous strains representative of all the groups and subgroups differentiated by the morphological and biochemical tests. The base composition of the Pseudomonas species and three named luminous strains were determined by the thermal denaturation method (Marmur & Doty, 1962) after preparation of the DNA by Marmur's method (1961).

## RESULTS

### *Luminous strains*

All luminous strains were Gram-negative rods, and all except three were motile. Gram-stained preparations frequently showed pleomorphic forms. Morphology was most clearly seen in wet preparations under phase contrast and in electron microscope preparations. There were three morphological types.

1. Coccobacilli which, though motile, had a low proportion of motile forms in the culture and frequently appeared non-flagellate. The flagellate forms possessed only a single polar flagellum. Typical of this morphological group is *Photobacterium phosphoreum* (Pl. 1, fig. 1).

2. Motile slender slightly curved rods with a single polar flagellum (strains labelled *Photobacterium splendidum* and *Vibrio albensis*; Pl. 1, fig. 2) or a tuft of polar flagella (strains of *P. fischeri*; Pl. 1, fig. 3).

3. Rods which are often long and unusual in that they have peritrichous flagella, but in addition to the many fine lateral flagella, have a much thicker flagellum at one or both poles. This type of flagellation was seen, for example, in strains of *Photobacterium harveyi* (Pl. 1, fig. 4). Two types of flagella were also seen by Johnson, Zworykin & Warren (1943) in electron microscopic studies of this species, but they did not report the position of attachment.

Preparations of *Photobacterium harveyi* NCMB 1280 showed fascicles of finer flagella (Pl. 2, fig. 5). The difference in the diameters of the thin and thick flagella can be seen in shadowed preparations (Pl. 2, fig. 6). The diameters of the fine flagella were 13 to 18 nm. and those of the thick flagella 30 to 41·6 nm. In negatively stained preparations

the thick flagellum was seen to possess a distinct sheath which was much less stable than the central core (10 to 12 nm. diameter) of the flagellum (Pl. 2, fig. 7, 8). This sheathed structure of the flagellum has been reported for various Vibrio species, *Vibrio metschnikovii* (Follett & Gordon, 1963), *V. cholerae* and *V. parahaemolyticus* (Ogasawara & Kuno, 1964*a*, *b*). Another morphological feature seen in strains of *P. harveyi* was the presence of fimbriae.

Strains of *Photobacterium fischeri* and *Vibrio noctiluca* produced pale yellow pigmented colonies; all others were non-pigmented. No strains produced diffusible pigments.

Only one strain – *Vibrio albensis* – grew in salt-free broth and none grew in 10 % NaCl. Most strains grew in 5 % NaCl and growth in 7·5 % NaCl was confined to strains of *Photobacterium harveyi* and *P. splendidum*. Luminous strains were observed to emit light in media with NaCl concentrations from 0·5 to 3 %, and many were also luminous in yeast peptone broth containing 5 % NaCl. All strains were capable of anaerobic growth.

All strains were insensitive to penicillin, but sensitive to streptomycin, chloramphenicol and polymyxin B; most were sensitive to terramycin. There did not appear to be any definite pattern in the strains insensitive to terramycin. Strains of *Photobacterium harveyi* and *P. splendidum* were insensitive to the vibriostatic compound 0/129: all other strains showed some degree of sensitivity to this compound.

Glucose was metabolized fermentatively by all strains in the Leifson (1963) marine OF medium. In the Hugh & Leifson (1953) medium 24 strains were fermentative: the remaining 27 strains did not grow satisfactorily in this medium.

Nitrate was reduced to nitrite by 49 of the 51 strains. The methyl red reaction was positive in 50 strains, four giving a weak result. Ammonia was produced from peptone by all 51 strains. Trimethylamine oxide was reduced by 41 strains, three were negative, the remaining seven strains were not tested.

Of the 28 'sugars' tested, no acid or gas was produced from nine of these; namely, arabinose, rhamnose, xylose, sorbose, raffinose, cellulose, dulcitol, inositol, inulin. A further three sugars were occasionally attacked: ribose (by 12 strains), lactose (by two strains) and fucose (by one strain).

The results of the other tests are summarized in Table 2.

Esterases were seldom detected in strains falling into groups I and II (Table 2) except group I*d*; but they were detected in strains in groups III and IV. The pattern of esterases within a group was not homogeneous.

### *Non-luminous strains*

The non-luminous strains were all motile Gram-negative rods possessing polar flagella. The Aeromonas and Vibrio species metabolized glucose fermentatively, were methyl red-positive (except *Aeromonas shigelloides*) and reduced nitrate to nitrite. The Pseudomonas species either attacked glucose by the oxidative pathway (NCMB 129, 320, 406) or did not attack glucose (NCMB 130, 224), were negative in the methyl red test and variable in reduction of nitrate. All strains produced ammonia from peptone. The results of the other tests are given in Table 2.

Table 2. *Summary of results for luminous and non-luminous strains*

| Morphology | Peptone water 'sugars' | | | | | | | | | | | | | | | | Oxidase (Kovacs) | Gelatin liquefaction | Indole production | VP | 2,3-Butanediol | Species included | Group |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Glucose | Fructose | Galactose | Mannose | Sucrose | Maltose | Cellobiose | Trehalose | Mannitol | Sorbitol | Glycerol | Salicin | Dextrin | Laminarin | Starch | Glycogen | | | | | | | |
| | AG | AG | AG | AG | – | AG | – | – | – | – | – | – | – | – | – | – | – | – | – | – or ± | – or + | *P. phosphoreum* | I*a* |
| Coccobacilli, motile with a single polar flagellum, or non-motile | AG | AG | AG | AG | – | AG | – | – | – | – | – | – | – | – | – | – | – | – (2+) | – | + | + | *P. profundum*<br>*C. macrouri*<br>*M. acropomae*<br>*M. physiculus* | I*b* |
| | A | A | A | A | – | A | – | – | – | – | – | – | – | – | – | – | – | – or + | – | + | + | *Ps. lucifer* | I*c* |
| | AG | AG | AG | AG | – | – | – | – | – | – | – | – | – | – | – | – | + | + or – | – | + | – | | I*d* |
| | A | A | A | A | A | A | A | – | A | – | A | A | A | – | A | – | + | + | – | – | – | *P. pierantonii* | II*b* |
| Rods, motile with a tuft of polar flagella | A | A | A | A | – | A | A or – | – or A | – or A | – | – or A | A or – | A | – | A | A or – | + | + or – | – | – | – | *P. fischeri*<br>*V. noctiluca* | II*a* |
| Rods, motile with 1 to 4 polar flagella | A | A | – | – | A | A | A | – | A | a | – | – | – | a | a | – | + | ++ | + | + | + | *V. albensis* | IV |
| Rods, motile with a single polar flagellum | A | A | A | A | – | A | A | A | A | A | – | – | A | A | A | A | + | ++ | + | – | – | *P. splendidum* | III*b* |
| Rods, motile with peritrichous flagella | A | A | A | A | A or – | A | A | A | A | A | – or A | A or – | A | A | A | A | + | ++ | + | – | – | *P. harveyi*<br>*P. sepiae* | III*a* |
| Rods, motile with a single polar flagellum | A | A | A | A | A | A | A | A | A | A | a | – | A | – | A | A | + | + | + | – | . | *V. ichthyodermis* | |
| Rods, motile with one or more polar flagella | A | A | A | A | A or – | A | A | A or – | – | – | A or – | a | A | – | a | – | + | (+ or –) | – | – | . | *V. marinus*[4] | |
| Rods, motile with many polar flagella | A | A | a | – | – | A | – | A | – | – | – | – | – | – | – | – | + | – | + | – | . | *A. shigelloides* | |
| Rods, motile with a single polar flagellum | A | A | A | A | A | A | A | A | A | A | a | A | A | – | A | A | + | ++ | – | – | . | *A. formicans* | |
| Rods, motile with a single polar flagellum | AG | AG | AG | AG | AG | AG | – | AG | AG | – | AG | AG | AG | – | AG | AG | + | ++ | + | + | . | *A. liquefaciens*<br>*A. hydrophila* | |
| Rods, motile with 1 to 8 polar flagella | A | – | A | A | – | – | – | – | – | – | – | – | – | – | – | – | + | ++ | – | – | . | *Ps. fluorescens* Gp I[3] | |
| | A | – | A | A | – | – | – | – | – | – | – | – | – | – | – | – | + | – | – | – | . | *Ps. putida* Gp. I | |
| | A | – | A | A | – | – | – | – | – | – | – | – | – | – | – | – | + | – | – | – | . | *Pseudomonas* sp. Gp II | |
| Rods, motile with 1 to 4 polar flagella | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | + | + | – | – | . | *Pseudomonas* sp. Gp III[3]/IV[3] | |

Table 2 (*cont.*)

| Morphology | Hydrolysis of: Tween 20 | Tween 40 | Tween 60 | Tween 80 | Egg yolk agar: Opalescence | Free fatty acid | Møller medium: Arginine | Lysine | Ornithine | Compound o/129 sensitivity | Growth at 5° | Growth at 37° | Esterases detected | No. of strains | DNA base ratio of representative strain(s) | Species included | Group |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Coccobacilli, motile with a single polar flagellum, or non-motile | + | + | − | − | − | − | + | + | − | + | + | − | − (1+) | 5 | 42·0 | *P. phosphoreum* | Ia |
| | − (2+) | − (2+) | − | − | − | − | + (1−) | + | − (1+) | + or ± | + | − | − (1+) | 13 | 40·5<br>40·5 | *P. profundum*<br>*C. macrouri*<br>*M. acropomae*<br>*M. physiculus* | Ib |
| | − | − | − | − | − | − | + | + | − | + | + | − | − | 3 | 39·5 | *Ps. lucifer* | Ic |
| | + | + | + or − | + or − | − | − | + | (1−) | − | ± | − | + | + | 4 | 42·0 | | Id |
| | + | + | + | + | + | − | − | + | + | + | − | − | − | 1 | 39·0 | *P. pierantonii* | IIb |
| Rods, motile with a tuft of polar flagella | + | + | + (2−) | + (2−) | + | − (1+) | − | + | − | + | + (2−) | − (2+) | − | 10 | 43·5<br>45·5 | *P. fischeri*<br>*V. noctiluca* | IIa |
| Rods, motile with 1 to 4 polar flagella | + | + | − | − | − | − | − | + | + | + | − | + | + | 1 | 48·5 | *V. albensis* | IV |
| Rods, motile with a single polar flagellum | + | + | + | + | + | + | − | + | + | − | + | − | + | 1 | 45·0 | *P. splendidum* | IIIb |
| Rods, motile with peritrichous flagella | + | + | + | + | + | + | − (1+) | + | + (1−) | − | − | + | + (2−) | 13 | 46·0<br>46·0 | *P. harveyi*<br>*P. sepiae* | IIIa |
| Rods, motile with a single polar flagellum | + | + | + | + | + | + | + | − | − | + | + | − | . | 1 | 44·5[1] | *V. ichthyodermis* | |
| Rods, motile with one or more polar flagella | + | + | + | + | + | + | − | + | − | + | + | − | . | 2 | 40·0[2]<br>42·0[2] | *V. marinus*[4] | |
| Rods, motile with many polar flagella | + | + | + | + | n.t. | n.t. | + | + | + | + | − | + | . | 1 | 51·0[2] | *A. shigelloides* | |
| Rods, motile with a single polar flagellum | + | + | + | + | − | − | + | − | − | − | − | + | . | 1 | 59·0[2] | *A. formicans* | |
| Rods, motile with a single polar flagellum | + | + | + | + | − | − | + | − | − | − | + | + | . | 2 | 59·0[2]<br>62·0 | *A. liquefaciens*<br>*A. hydrophila* | |
| Rods, motile with 1 to 8 polar flagella | + | + | − | − | + | + | + | − | − | − | + | − | . | 1 | 64·2 | *Ps. fluorescens* Gp I[3] | |
| | + | + | − | − | − | − | + | − | − | − | + | − | . | 1 | 63·2 | *Ps. putida* Gp I | |
| | + | + | − | − | − | − | + | − | − | − | + | − | . | 1 | 60·8 | *Pseudomonas* sp. Gp II | |
| Rods, motile with 1 to 4 polar flagella | + | + | + | + | − | − | − | − | − | − | + | − | . | 2 | 42·2<br>41·9 | *Pseudomonas* sp Gp III[3]/IV[3] | |

A, acid; a, acid weak and late; G, gas; +, positive; + +, strong or rapid positive; ±, weak or doubtful positive; n.t., not tested; [1], results of Véron & Sebald, 1964; [2], from Hill, 1966; [3], the Pseudomonas groupings of Shewan, Hobbs & Hodgkiss, 1960; [4], one strain of *V. marinus* (NCMB 1144) did not grow in some of the Tween 80 and Møller media. Figures in parenthesis indicate the number of strains giving a different result.

## DISCUSSION

Table 2 shows that 44 of the luminous strains fall into three major groups (I*a*, *b*, *c*, II*a*, III*a*) which correspond well with the three morphological divisions. Of the remaining seven strains, *Vibrio albensis*, the only non-marine strain, is unique, whereas the other six strains appear to be transitional between the main morphological and biochemical types, particularly the two strains labelled *Photobacterium pierantonii* and *P. splendidum*.

Groups I*a*, *b* and *c* are all very similar and such differences as do exist appear to be of minor importance and to represent variation of strains within a single species, e.g. group I*b* produces acid and gas from carbohydrates whereas group I*c* is anaerogenic, otherwise they are identical. The only differences between group I*a* and groups I*b* and *c* are the variability in the VP and 2,3-butanediol tests in I*a* and their ability to hydrolyse Tweens 20 and 40, but two strains in I*b* also hydrolyse these Tweens. All group I strains examined have DNA base ratios of 39·5 to 42·0 moles % guanine + cytosine (% G+C). We recommend that all 21 strains in groups I*a*, *b* and *c* should be combined in the species *Photobacterium phosphoreum*. Spencer (1955) proposed that *P. phosphoreum* should be assigned to the genus *Vibrio* as it was sensitive to the vibriostatic compound 0/129 and dissimilated glucose fermentatively, but all the strains in this group (*P. phosphoreum*) are Kovacs' oxidase-negative and are usually aerogenic in the breakdown of carbohydrates whereas Vibrio species are oxidase-positive and never aerogenic. The species *P. phosphoreum* should include species previously labelled *P. profundum* (Weisglass & Gavrilovic, 1963), *Pseudomonas lucifer*, *Coccobacillus macrouri*, *Micrococcus physiculus* and *Micrococcus* (or *Coccobacillus*) *acropomae*.

The position of group I*d* is further away from groups I*a*, *b* and *c* than these are from each other. Although the group I*d* strains have much in common with the other group I strains, they attack hexoses only, are Kovacs' oxidase-positive, hydrolyse Tweens 20 and 40 (some strains also Tweens 60 and 80) and although VP-positive, they are 2,3-butanediol negative. The groups I*a*, *b* and *c* strains attack maltose in addition to hexoses, are oxidase-negative and never hydrolyse Tweens 60 and 80. The DNA base ratio of the one strain from I*d* examined was identical with a strain of *Photobacterium phosphoreum* in group I*a* (42·0 moles % G+C). This group appears to form a separate species, and we propose the name *P. mandapamensis*, since the first strain of this group was isolated at Mandapam.

The 10 strains in group II*a* fit closely to the second morphological type and are different from the organisms of group I in being oxidase-positive, not giving an alkaline reaction with arginine, and attacking a much wider range of carbohydrates anaerogenically. They are also the only pigmented group, the colonies being pale yellow. The DNA base ratios of the strains examined are 43·5 and 45·5 moles % G+C. All strains in this group should be combined under the specific epithet *fischeri* which should be assigned to the genus *Vibrio* as suggested by Spencer (1955) as they are polar flagellate rods, fermentative in their action upon glucose, sensitive to compound 0/129 and also have a DNA base ratio within the range for that genus (40 to 50 moles % G+C; Colwell & Mandel, 1964). Furthermore, they are very similar to the working neotype strains of the non-luminous species *Vibrio marinus* (Colwell & Morita, 1964). Included in the species *V. fischeri* is the recently described species *V. noctiluca* (Weisglass & Skreb, 1963).

The 13 strains in group III*a* also form a single species which differs from group I

and group II*a* in being insensitive to the vibriostatic compound 0/129, indole-positive, much more actively proteolytic in gelatine, having an ornithine decarboxylase (except *Photobacterium harveyi* NCMB 1280, the type strain) and lipases which are active in egg yolk agar, in addition to having the peritrichous flagella of the third morphological type. The DNA base ratios of the strains examined were 45·5 to 46·5 moles % G+C. The strains in this group should be combined under the specific epithet *harveyi* and should include the strain labelled *P. sepiae*, a name which is not considered to be legitimate (*Index Bergeyana*, 1966), and NCMB 42 which was originally labelled *P. fischeri*. Spencer (1955) believed that this group had affinities with the genus *Aeromonas* as it was insensitive to compound 0/129. However, the *harveyi* group is peritrichate and has a DNA base ratio (45·5 to 46·5 moles % G+C) which is well outside the range of the genus *Aeromonas* (59 to 62 moles % G+C) (Hill, 1966; Rosypal & Rosypalova, 1966). We recognize that this group (III*a*) has some affinities with the genus *Vibrio*, having a similar DNA base ratio and the sheathed structure of the thick polar flagellum seen in some strains is similar to that described for some Vibrio species (Follett & Gordon, 1963; Ogasawara & Kuno, 1964*a*, *b*). However, the possession of peritrichous flagella and insensitivity to compound 0/129 leads us to suggest that the species should be assigned to a new genus for which we propose the name *Lucibacterium* (Latin: *lux*, *lucis*, light) for the genus with *Lucibacterium harveyi* (Johnson & Shunk) comb.nov. as the type species.

The strain labelled *Photobacterium splendidum* (group III*b*) is identical with group III*a* except that it has a single polar flagellum and a lower temperature range. This strain has been maintained in artificial culture for many years and it is possible that some of its properties may have been irretrievably lost, as has happened with some strains of the *Vibrio fischeri* group which are no longer luminous, although this property was observed by us when they were freshly isolated. The DNA base ratio of *P. splendidum* (45·0 moles % G+C) is also near that of the group III*a* strains and we believe that this organism may be a degenerate *Lucibacterium harveyi*.

The organism labelled *Photobacterium pierantonii* (group II*b*) is morphologically similar to those in group I, being a non-motile coccobacillus and having a low DNA base ratio (39·0 moles % G+C) similar to other strains in this group. Biochemically this organism is almost identical with those of group II*a*. In the seventh edition of *Bergey's Manual* (1957) there are two luminous species described which have the specific epithet *pierantonii* – *Photobacterium pierantonii* and *Vibrio pierantonii*. Our strain does not fit exactly the description of either species. Biochemically it is close to II*a* *V. fischeri*, but morphologically is more like the Photobacterium species and its temperature range did not reach the value quoted for *V. pierantonii*. This might possibly be a single representative of a species but for the present we propose to group it as an atypical strain of *V. fischeri*.

The non-marine strain *Vibrio albensis* (group IV) is distinct from all the others. Morphologically it is similar to group II*a*, being a slender rod with one or more polar flagella. It is similar to group III in being actively proteolytic, indole-positive, but it is the only strain outside group I which is VP and 2,3-butanediol-positive. It has decarboxylases for lysine and ornithine like many other Vibrio species (Baan & Shewan, 1968; Carpenter, Hart, Hatfield & Wicks, 1968). The DNA base ratio was the highest of all the strains examined at 48·5 moles % G+C and is close to that of *V. cholerae*. Its similarity to *V. cholerae* has long been recognized (*Bergey's Manual*, 1957).

It is therefore suggested that luminous bacteria should be classified into three genera, namely *Photobacterium*, *Vibrio* and a new genus, *Lucibacterium*, to accommodate organisms currently called *P. harveyi* and *P. sepiae*. The species to be included in the genus *Vibrio* are *V. albensis* and *V. fischeri*, and in the genus *Photobacterium*, *P. phosphoreum*, the present type species, with the addition *P. mandapamensis* as a further distinct species. The genus *Lucibacterium* would include *P. splendidum* as a degenerate form of *L. harveyi*.

We thank Professor M. Mandel for his help. The DNA base ratio estimations by the thermal denaturation method were done by Mr D. G. McLeod and the starch-gel electrophoresis examination for esterase patterns by Mrs Margaret E. Taylor of Torry Research Station.

## APPENDIX

Our proposals for (i) modified and extended descriptions of the species named in this appendix and (ii) the generic descriptions of Photobacterium and Lucibacterium, are based on information from the present study, from the seventh edition of *Bergey's Manual* (1957) and from the work of Spencer (1961) on the chitinoclastic activities of luminous bacteria.

### Species included in the genus *Vibrio* Pacini, 1854

*Vibrio albensis* Lehmann & Neumann, 1896

(Synonyms: Elbe vibrio, Dunbar, 1893; *Microspira dunbari* Migula, 1900.)

Rods, 1·5 to 3·0 × 0·4 to 0·7 μm., occurring singly and in pairs, sides parallel, ends rounded, axis frequently curved. Motile by means of polar flagella, usually one per cell, but occasional cells may have 2 or 4 flagella. Not encapsulated. Gram-negative.

Agar colonies: Off-white, translucent, circular, convex, entire margin, smooth, shiny, 1·0 to 2·0 mm. diam. Good growth. Luminous. On sea-water agar the colonies are punctiform and luminous.

Broth: Good growth, thin pellicle, uniform turbidity, viscid deposit. Luminous 2 to 3 days.

Grows at 37° but not at 5°. Grows in media containing 0 to 5 % NaCl, no growth in 7·5 % NaCl, luminescent in media with 0·5 to 3 % NaCl. Growth in media at pH 6 to pH 9, no growth at pH 5.

Aerobic, facultatively anaerobic.

Sensitive to chloramphenicol, streptomycin, aureomycin, terramycin, polymyxin B and 2,4-diamino-6,7-diisopropyl pteridine. Insensitive to penicillin.

Indole produced, acetylmethylcarbinol produced, 2,3-butanediol produced, nitrite produced from nitrate, ammonia produced from peptone, trimethylamine oxide reduced to trimethylamine. Methyl red test negative.

Oxidase- and catalase-positive.

Possesses decarboxylases for lysine and ornithine but does not produce alkaline products from arginine.

Hydrolyses Tweens 20, 40, 60 and 80.

No opalescence or free fatty acids produced on egg yolk agar.

Gelatin liquefied.

Starch hydrolysed.

Chitin digested.

Acid but no gas formed in glucose, sucrose, maltose, trehalose; weak acid production in fructose, mannose, mannitol, sorbitol, dextrin, starch and glycogen. No acid or gas in arabinose, rhamnose, xylose, ribose, fucose, galactose, lactose, sorbose, raffinose, cellobiose, cellulose, dulcitol, glycerol, inositol, adonitol, salicin, inulin, aesculin or laminarin.

Carbohydrate metabolism fermentative.

DNA base ratio 48·5 moles % G+C.

Habitat: Fresh water, in human faeces and bile.

*Vibrio fischeri* (Beijerinck) Lehmann & Neumann, 1896.

(Synonyms: Die einheimischen Leuchtbacillen, Fischer, 1888; *Photobacterium fischeri* Beijerinck, 1889; *Bacillus fischeri* (Beijerinck) Trevisan, 1889; *Bacillus phosphorescens indigenus* Eisenberg, 1892; *Bacterium phosphorescens indigenus* Chester, 1897; *Microspira fischeri* (Beijerinck) Chester, 1901; *Spirillum phosphorescens* Holland, 1920; *Vibrio phosphorescens* Holland, 1920; *Achromobacter fischeri* (Beijerinck) Bergey *et al.* 1930; *Vibrio noctiluca* Weisglass & Skreb, 1963.)

Short rods, 0·5 × 1·0 to 1·5 μm, occurring singly and in pairs, sides parallel, ends rounded, axis straight or slightly curved. Stained preparations frequently show pleomorphism and polar staining. Motile by means of a tuft of polar flagella. Gram-negative.

Sea-water agar colonies: Off-white in young cultures, becoming pale yellow, translucent, circular, convex, entire margin, smooth, shiny 1·0 to 2·0 mm. diam. Usually luminous at 1 to 3 days. Growth moderately good.

Sea-water broth: Abundant growth, ring or thin pellicle, uniform turbidity, small deposit, usually luminous at 2 to 3 days.

Usually grows at 5° but not at 37°. Growth and visible light usually emitted in media containing 0·5 to 5 % NaCl, some strains grow in 7·5 % NaCl; no growth without NaCl. Growth from pH 6 to pH 9.

Aerobic, facultatively anaerobic.

Sensitive to chloramphenicol, streptomycin, polymyxin B and 2,4-diamino-6,7-diisopropyl pteridine. Insensitive to penicillin. Sensitivity to terramycin and aureomycin varies from strain to strain.

Nitrite produced from nitrate; trimethylamine oxide reduced to trimethylamine; ammonia produced from peptone. Indole, acetylmethylcarbinol and 2,3-butanediol not produced.

Methyl red test positive.

Oxidase- and catalase-positive.

Usually has a lysine decarboxylase but not an ornithine decarboxylase nor produces alkaline products from arginine.

Tweens 20 and 40 hydrolysed, Tweens 60 and 80 usually hydrolysed.

Opalescence produced in egg yolk agar, production of free fatty acids variable.

Gelatin liquefied.

Starch hydrolysed.

Chitin may be digested.

Acid but no gas formed in glucose, fructose, galactose, mannose, maltose, dextrin and starch, and by some strains in cellobiose, trehalose, mannitol, glycerol, salicin and

glycogen. No acid or gas formed in arabinose, rhamnose, ribose, xylose, fucose, lactose, sucrose, sorbose, raffinose, cellulose, dulcitol, sorbitol, inositol, inulin or laminarin.

Metabolism of carbohydrates fermentative.

DNA base ratio 43 to 45 moles % G+C.

Habitat: Sea water.

### *Photobacterium* Beijerinck, 1889

Coccobacilli or occasional rods, pleomorphic forms frequently observed in adverse conditions of growth, motile by means of polar flagella or non-motile. Many strains appear to be non-motile as only a low proportion of the cells in a culture are seen to move actively and detection of flagella is difficult. Chemo-organotrophic, aerobic and facultatively anaerobic. Acid and gas or acid only produced from glucose and other carbohydrates but not lactose, do not attack complex carbohydrates or alcohols. Carbohydrate metabolism is fermentative. Nitrite produced from nitrate. Usually luminescent. Growth and luminescence occur on media containing 0·5 to 5 % NaCl; optimum concentration 3 % NaCl. Growth at pH 6 to pH 9. Most strains grow at 5 but not at 37°. Usually sensitive to 2,4-diamino-6,7-diisopropyl pteridine. DNA base ratio 39 to 42 moles % G+C. Found living symbiotically in the tissues of luminous organs of cephalopods and deep-sea fishes and on the skin and in the intestine of some marine fish.

The type species is *Photobacterium phosphoreum* (Cohn) Ford.

Key to the species of the genus *Photobacterium*:

Coccobacilli which produce acid and usually gas from glucose.

A. Oxidase-negative, acid and usually gas from maltose, no growth at 37°. *Photobacterium phosphoreum.*

B. Oxidase-positive, no acid or gas from maltose, growth at 37°. *Photobacterium mandapamensis.*

*Photobacterium phosphoreum* (Cohn) Ford, 1927

(Synonyms: *Micrococcus phosphoreus* Cohn, 1878; *Bacillus phosphorescens* II Baumgarten, 1888; *Photobacterium phosphorescens* Beijerinck, 1889; *Bacillus hermesi* Trevisan, 1889; *Streptococcus phosphoreus* (Cohn) Trevisan, 1889; *Bacillus phosphoreus* (Cohn) Macé, 1901; *Photobacter phosphorescens* Beijerinck, 1901; *Pseudomonas lucifera* Molisch, 1904; *Bacterium phosphoreum* (Cohn) Molisch, 1912; *Photobacter phosphoreum* (Cohn) Beijerinck, 1916; *Micrococcus physiculus* Kishitani, 1930; *Coccobacillus acropoma* Yasaki & Haneda, 1936; *Coccobacillus macrouri*; *Acinetobacter phosphorescens* Brisou, 1955; *Photobacterium profundum* Weisglass & Gavrilovic, 1963.)

Short rods or coccobacilli 1·0 to 2·5 × 0·4 to 1·0 μm., occurring singly and occasionally in pairs, axis straight. Motile by a single polar flagellum, but only a small proportion of the cells in a culture are observed in motion. In stained specimens many pleomorphic cells are observed and polar staining frequently occurs. Gram-negative.

Sea-water agar colonies: Off-white, translucent, circular, convex, entire margin, smooth, shiny, 1 to 2 mm. diameter. Luminous 1 to 3 days. Moderately good growth.

Sea-water broth: Moderately good growth, ring of dense growth at surface, slight uniform turbidity, granular deposit.

No growth at 37°, most strains grow at 5°. Growth and visible light usually emitted in media containing 0·5 to 5 % NaCl. No growth in 7·5 % NaCl or without NaCl. Growth at pH 6 to pH 9.

Aerobic, facultatively anaerobic.

Sensitive to chloramphenicol, streptomycin, polymyxin B and 2,4-diamino-6,7-diisopropyl pteridine; sensitivity to terramycin and aureomycin is variable. Insensitive to penicillin.

Nitrite produced from nitrate, acetylmethylcarbinol and 2,3-butanediol produced, ammonia produced from peptone, trimethylamine oxide reduced to trimethylamine. Indole not produced.

Methyl red test positive.

Oxidase-negative, catalase-positive.

Usually produces alkaline products from arginine and possesses a lysine decarboxylase but does not possess an ornithine decarboxylase.

Tweens 20 and 40 occasionally hydrolysed, Tweens 60 and 80 not hydrolysed.

No opalescence or free fatty acids produced on egg yolk agar.

Gelatin not usually liquefied.

Starch not hydrolysed.

Chitin usually digested.

Acid and usually gas formed in glucose, fructose, galactose, mannose and maltose and occasionally in ribose and fucose. No acid or gas formed in arabinose, rhamnose, xylose, lactose, sucrose, sorbose, cellobiose, trehalose, raffinose, cellulose, mannitol, dulcitol, glycerol, sorbitol, inositol, dextrin, salicin, inulin, laminarin, starch or glycogen.

Metabolism of carbohydrates fermentative.

DNA base ratio 39·0 to 42·0 moles % G+C.

Habitat: Sea water.

*Photobacterium mandapamensis* sp.nov.

Short rods or coccobacilli 1·0 to 2·5 × 0·4 to 1·0 $\mu$m., occurring singly and occasionally in pairs, axis straight. Motile by polar flagella. In stained specimens pleomorphic cells and polar staining may be observed. Gram-negative.

Sea-water agar colonies: Off-white, translucent, circular, convex, entire margin, smooth, shiny 1 to 2 mm. diameter. Luminous 1 to 3 days. Moderately good growth.

Sea-water broth: Moderately good growth, thin pellicle, uniform turbidity, powdery deposit. Luminous 2 to 3 days.

Grows at 37° but not at 5°. Growth and visible light usually emitted in media containing 0·5 to 5 % NaCl. No growth without NaCl or in 7·5 % NaCl. Growth at pH 6 to pH 9.

Aerobic, facultatively anaerobic.

Sensitive to chloramphenicol, streptomycin, polymyxin B and 2,4-diamino-6,7-diisopropyl pteridine. Sensitivity to terramycin and aureomycin is variable. Insensitive to penicillin.

Nitrite produced from nitrate, trimethylamine oxide reduced to trimethylamine, ammonia produced from peptone, acetylmethylcarbinol produced but 2,3-butanediol not produced. Indole not produced.

Methyl red test positive.

Oxidase- and catalase-positive.

Usually produces alkaline products from arginine and possesses a lysine decarboxylase, but not an ornithine decarboxylase.

Tweens 20 and 40 hydrolysed, Tweens 60 and 80 may be hydrolysed.

No opalescence or free fatty acid produced on egg yolk agar.

Gelatin may be slowly liquefied.

Starch not hydrolysed.

Chitin may be digested.

Acid and gas formed in glucose, fructose, galactose and mannose. No acid or gas formed in arabinose, rhamnose, ribose, xylose, fucose, lactose, sucrose, maltose, trehalose, sorbose, cellobiose, raffinose, cellulose, mannitol, dulcitol, sorbitol, glycerol, inositol, salicin, dextrin, inulin, laminarin, starch or glycogen.

Metabolism of carbohydrates fermentative.

DNA base ratio 42·0 moles % G+C.

Habitat: Sea water.

*Lucibacterium* gen.nov.

Rods, motile by peritrichous flagella. In some strains the flagellation is of an unusual type with a thick polar flagellum in addition to the finer lateral flagella. Occasionally only the polar flagellum can be detected. Chemo-organotrophic. Aerobic, facultatively anaerobic. Carbohydrate metabolism is fermentative without the production of gas. Lactose not usually attacked. Gelatin liquefied. Nitrite produced from nitrate. Oxidase-positive. Insensitive to 2,4-diamino-6,7-diisopropyl pteridine. Usually luminescent. Growth and luminescence occurs on media containing 0·5 to 5 % NaCl, optimum concentration 3 % NaCl. Growth at pH 6 to pH 9. Usually grows at 37° but not at 5°. DNA base ratio 45 to 46 moles % G+C. Found on the surface of dead fish and in sea water.

The type species is *Lucibacterium harveyi* (Johnson & Shunk) comb.nov.

*Lucibacterium harveyi* (Johnson & Shunk)

(Synonyms: *Achromobacter harveyi* Johnson & Shunk, 1936; *Photobacterium splendidum* (Beijerinck) Eymers & van Schouwenburg, 1937; *Photobacterium sepiae* Kluyver, 1938; *Photobacterium harveyi* (Johnson & Shunk) Breed & Lessel, 1954; *Pseudomonas harveyi* (Johnson & Shunk) Krassil'nikov, 1959.)

Rods, 1·2 to 4·0 × 0·3 to 1·0 μm., straight or slightly curved, sides parallel, ends rounded, occurring singly and in pairs. Pleomorphic form may be present. Motile by means of peritrichous flagella. Flagellation may be of an unusual type showing a thick polar flagellum in addition to many fine lateral flagella. In some strains only the polar flagellum can be detected. Gram-negative.

Sea-water agar colonies: Off-white, translucent, circular, convex, entire margin, smooth, shiny, 1 to 3 mm. diameter. Colonies frequently mucoid and tend to spread. Abundant growth. Luminous after 1, 2 or 3 days.

Sea-water broth: Good growth, thin pellicle, uniform turbidity, flocculent deposit. Luminous.

Usually grows at 37° but not at 5°. Grows in media containing 0·5 to 7·5 % NaCl,

but not in media without NaCl. Visible light emitted in media containing 0·5 to 5 % NaCl. Growth at pH 6 to pH 9.

Aerobic, facultative anaerobic.

Sensitive to chloramphenicol, streptomycin, aureomycin, may be sensitive to terramycin. Insensitive to penicillin and 2,4-diamino-6,7-diisopropyl pteridine.

Indole produced, nitrite produced from nitrate, ammonia produced from peptone, trimethylamine oxide reduced to trimethylamine.

Acetylmethylcarbinol and 2,3-butanediol not produced.

Methyl red test positive.

Oxidase- and catalase-positive.

Usually possesses decarboxylases for lysine and ornithine, but does not usually produce alkaline products from arginine.

Tweens 20, 40, 60 and 80 hydrolysed.

Opalescence and free fatty acids produced in egg yolk agar.

Gelatin liquefied.

Starch hydrolysed.

Chitin digested.

Acid but no gas produced from glucose, fructose, galactose, mannose, maltose, cellobiose, trehalose, mannitol, sorbitol, dextrin, laminarin, starch and glycogen. Acid may be produced from ribose, sucrose, glycerol, salicin and occasionally from lactose (weak). No acid or gas from arabinose, rhamnose, xylose, fucose, sorbose, raffinose, cellulose, dulcitol, inositol or inulin.

Metabolism of carbohydrates fermentative.

DNA base ratio 45 to 46 moles % G+C.

Habitat: Sea water.

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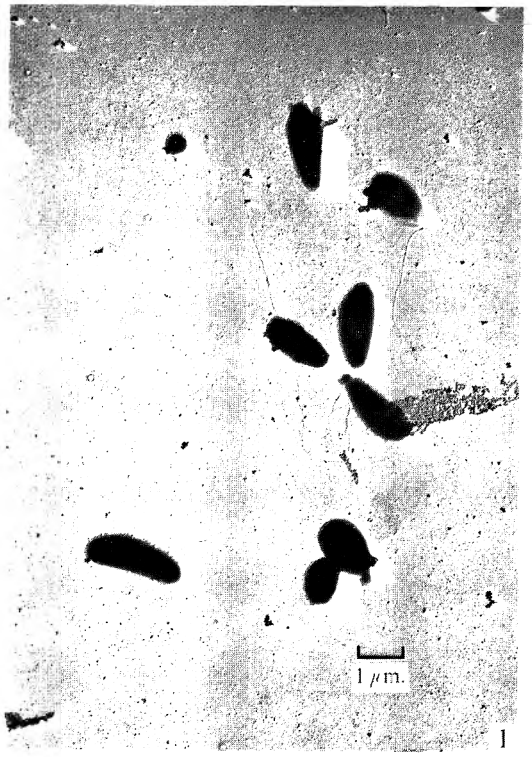
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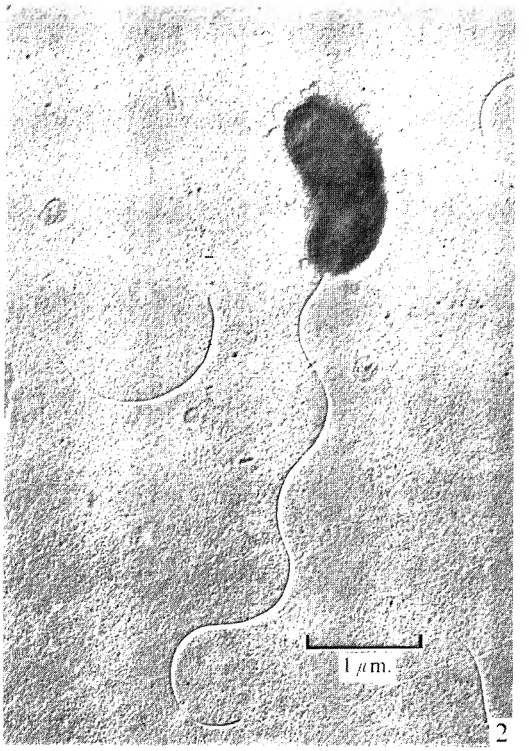
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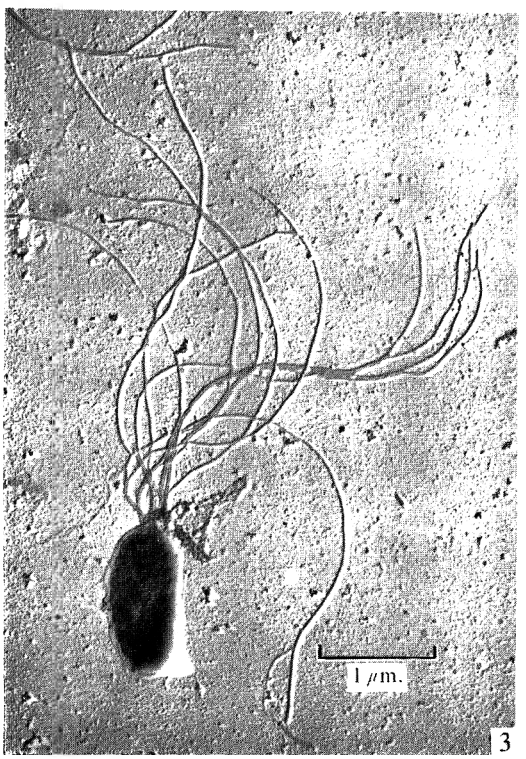
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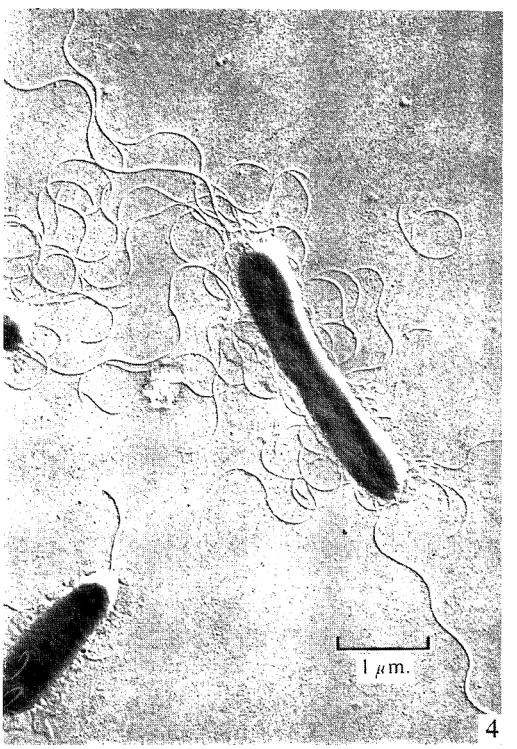
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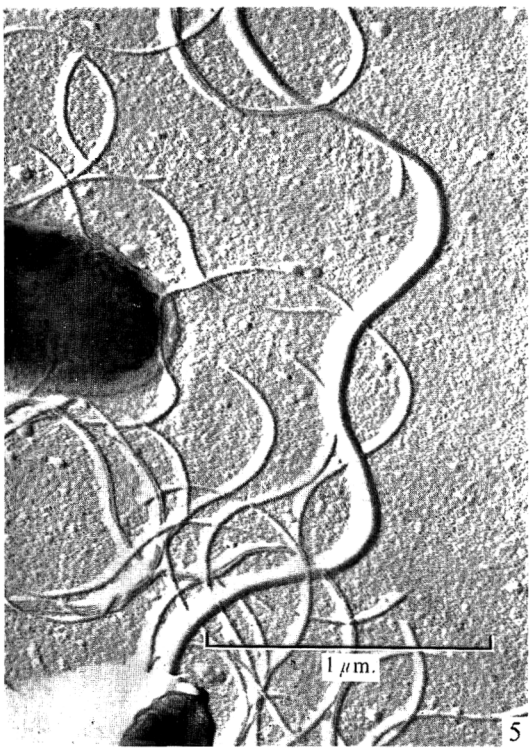
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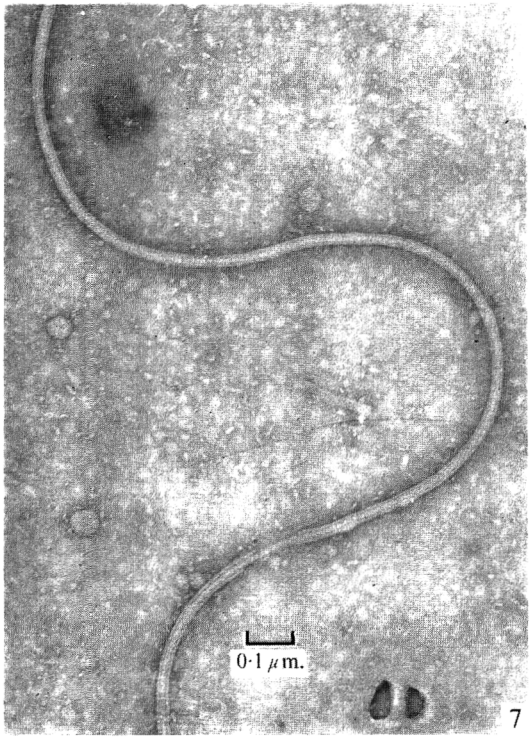
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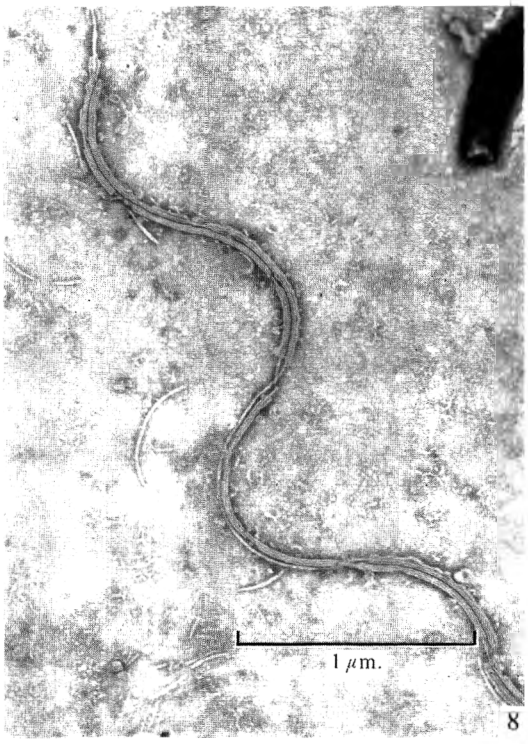
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## EXPLANATION OF PLATES

All the photographs are electron micrographs of preparations from cultures incubated at 20° on SWYP agar for 24 h. and fixed in 5 % (v/v) formalin in 2·5 % NaCl. Fig. 1 to 6 inclusive are of gold + palladium shadowed preparations. Fig. 7 and 8 are of specimens negatively stained with phosphotungstic acid.

### PLATE 1

Fig. 1. *Photobacterium phosphoreum* NCMB 7.

Fig. 2. *Lucibacterium harveyi* NCMB 1 (*Photobacterium splendidum*).

Fig. 3. *Vibrio fischeri* NCMB 1281 (*Photobacterium fischeri*).

Fig. 4. *Lucibacterium harveyi* NCMB 1280 (*Photobacterium harveyi*).

### PLATE 2

Fig. 5. *Lucibacterium harveyi* NCMB 1280 (*Photobacterium harveyi*). Thick and thin flagella.

Fig. 6. *Lucibacterium harveyi* NCMB 1280 (*Photobacterium harveyi*). Fascicles of thin flagella.

Fig. 7. *Lucibacterium harveyi* NCMB 1280 (*Photobacterium harveyi*). Thick (polar) flagellum showing core and sheath.

Fig. 8. *Lucibacterium harveyi* NCMB 1280 (*Photobacterium harveyi*). Two thick (polar) flagella showing disintegration of the sheath.

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# Influence of Environment on the Content and Composition of Microbial Free Amino Acid Pools

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SUMMARY

The free amino acid pool contents of Gram-negative bacteria (*Aerobacter aerogenes*, *Erwinia carotovora*, *Pseudomonas fluorescens*) were studied as functions of the growth environment and were compared with those from correspondingly grown cultures of Gram-positive bacteria (*Bacillus subtilis* var. *niger*, *B. megaterium*, *B. polymyxa*) and the yeast *Saccharomyces cerevisiae*.

Although the pools of the Gram-positive bacteria and the yeast contained five to 20 times the concentration of free amino acids present in the pools of Gram-negative bacteria, all pools were similar in containing only a limited range of detectable amino acids. Glutamate invariably predominated and generally accounted for over 50 % of the total amino acid content of the pool. The contents and composition of pools from micro-organisms maintained in steady states in chemostat cultures did not vary with time, but changed significantly with changes in either growth rate or the nature of the growth limitation. However, these pool variations were small compared with those resulting from addition of 2 % (w/v) NaCl to a culture of growing bacteria. With cultures of Gram-negative bacteria, sudden changes in medium salinity effected marked and rapid changes in free glutamate content; with Gram-positive bacteria, similar changes occurred, but extremely slowly. Addition of 4 % (w/v) NaCl to growing yeast cultures brought about no observed changes in pool size or composition. These results are discussed with reference to the involvement of free amino acids in synthesis and functioning of micro-organisms.

## INTRODUCTION

The macromolecular composition and metabolic activity of micro-organisms vary to a considerable extent with changes in their growth environment (Herbert, 1961; Neidhardt, 1963; Brown & Rose, 1969*a*, *b*; Tempest, 1970). These phenotypic changes in cell structure and functioning reflect changes in genetic expression and are presumably mediated by some environmentally linked mechanism. Thus, changes in the growth condition must affect primarily the intracellular concentrations of substances reacting directly with the genetic control mechanisms; those most likely to act in this capacity within a microbial cell being the low molecular weight 'pool' constituents. Therefore in attempting to analyse the relationship between environment and microbial

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physiology it is logical to examine quantitatively the effects of specific environmental changes on the composition of microbial pools.

One of the most obvious conclusions to be drawn from a survey of the literature on microbial pools – particularly amino acid pools – is that they are extremely variable and markedly dependent on the nutritional complexity of the growth medium (Holden, 1962). Thus, in order to rationalize observed changes in the content and composition of such pools in organisms grown in different environments, as many variables as possible must be closely controlled. To fulfil this requirement we have used a chemostat and have limited our investigations to organisms growing in a series of simple salts media in which the sole sources of nitrogen and carbon were $NH_3$ and glucose, respectively. Thus we have avoided complications associated with the transport and accumulation of exogenously-supplied amino acids (see Britten & McClure, 1962).

Previous studies, using a chemostat to provide both specifically defined and closely regulated environments (see Tempest, 1969, 1970), revealed that the parameters most extensively affecting microbial physiology were (i) the chemical nature of the environment, particularly the nature of the growth-limiting component of the medium, (ii) the growth rate, and (iii) the medium osmolarity (e.g. NaCl concentration). Consequently we have systematically examined the effects of each of these parameters on the amino acid pools of different organisms growing in environments that were, in all other respects, rigidly controlled. A preliminary account of some of the results obtained has been published previously (Tempest, Meers & Brown, 1970).

## METHODS

*Organisms. Aerobacter aerogenes* (NCTC 418), *Pseudomonas fluorescens* (KB 1), *Erwinia carotovora*, *Bacillus subtilis* var. *niger*, *B. megaterium* (KM) and *B. polymyxa*. Each was maintained by monthly subculture on Tryptic meat-digest, agar slopes. *Saccharomyces cerevisiae* (described by Brown & Hough, 1965) was subcultured monthly on yeast extract (1 %, w/v), peptone (2 %, w/v), glucose (2 %, w/v), agar.

*Growth conditions.* The organisms were grown in 0·5 l. Porton-type chemostats, with automatic pH control (Herbert, Phipps & Tempest, 1965). Media used for the growth of bacteria were as follows. Glucose-limited: $Na_2HPO_4$, 1·0 mM; $NH_4H_2PO_4$, 10 mM; $(NH_4)_2SO_4$, 6 mM; $K_2SO_4$, 2 mM; citric acid, 1 mM; $MgCl_2$ 0·6 mM; $CaCl_2$ and $FeCl_3$, each 0·1 mM; $MnCl_2$ and $ZnCl_2$, each $2{\cdot}5 \times 10^{-5}$M; $CuCl_2$, $CoCl_2$ and $Na_2MoO_4$, each $5 \times 10^{-6}$M; glucose, 14 mM. $NH_3$-limited: $(NH_4)_2SO_4$, 5 mM; $NaH_2PO_4$, 20 mM; $K_2SO_4$, 2 mM; citric acid, 1 mM; $MgCl_2$, 0·6 mM; $CaCl_2$, $FeCl_3$, $MnCl_2$, $ZnCl_2$, $CuCl_2$, $CoCl_2$ and $Na_2MoO_4$, as specified above; glucose, 60 mM. $PO_4^{3-}$-limited: $Na_2HPO_4$, 1 mM; $K_2SO_4$, 2 mM; $(NH_4)_2SO_4$, 10 mM; citric acid, 1 mM; $MgCl_2$, 0·6 mM; $CaCl_2$, $FeCl_3$, $MnCl_2$, $CuCl_2$, $CoCl_2$ and $Na_2MoO_4$, as specified above; glucose, 60 mM. $Mg^{2+}$-limited: $Na_2HPO_4$, 1 mM; $NH_4H_2PO_4$, 10 mM; $(NH_4)_2SO_4$, 6 mM; $K_2SO_4$, 2 mM; citric acid, 0·5 mM; $MgCl_2$, 0·2 mM; $CaCl_2$, $FeCl_3$, $MnCl_2$, $ZnCl_2$, $CuCl_2$, $CoCl_2$ and $Na_2MoO_4$, as specified above; glucose, 60 mM. $K^+$-limited: $Na_2HPO_4$, 1 mM; $NH_4H_2PO_4$, 10 mM; $(NH_4)_2SO_4$, 6 mM; $K_2SO_4$, 0·5 mM (for Gram-negative bacteria) or 1 mM (for Gram-positive bacteria); citric acid, 1 mM; $MgCl_2$, 0·6 mM; $CaCl_2$, $FeCl_3$, $MnCl_2$, $ZnCl_2$, $CuCl_2$, $CoCl_2$ and $Na_2MoO_4$, as specified above; glucose, 60 mM. With all cultures, except that which was ammonia-limited, the pH was controlled automatically by the addition of 4M-$NH_3$, as required.

The basal growth medium for the yeast had the following composition: $KH_2PO_4$, 15 mM; $MgSO_4$, 4 mM; $CaCl_2$, 1 mM; inositol, $1{\cdot}1 \times 10^{-4}$M; pyridoxine HCl, $1{\cdot}0 \times 10^{-5}$M; calcium pantothenate, $1{\cdot}0 \times 10^{-5}$M; thiamine HCl, $1{\cdot}2 \times 10^{-6}$M; biotin, $4 \times 10^{-9}$M; with trace amounts of $Zn^{2+}$, $MoO_4^{2-}$, $Ca^{2+}$, $Fe^{3+}$ and $Mn^{2+}$. For glucose limitation, glucose (12 mM) and $(NH_4)_2SO_4$ (15 mM) were added, while for $NH_3$ limitation, glucose (60 mM) and $(NH_4)_2SO_4$ (2 mM) were added.

*Analytical procedures.* Bacterial concentration (mg. equiv. dry wt organisms/ml. culture) was determined by the method of Tempest, Hunter & Sykes (1965). The free amino acid pool content and composition was assessed as follows: a volume of culture containing approximately 30 mg. equiv. dry wt organisms (in the case of Gram-negative bacteria), or 5 mg. equiv. dry wt organisms (in the case of Gram-positive bacteria), was centrifuged (3000*g*, 3 min.) and the supernatant fluid separated and discarded. The pellet of organisms was then dispersed in ice-cold 0·25 N-$HClO_4$ (final volume of 5·0 ml.) and was kept at 4° for about 10 min. before again being centrifuged (3000*g*, 5 min.) and the supernatant fluid collected. Yeast concentration was determined by the membrane filter method of Brown & Rose (1969*a*). The free amino acid pool was extracted from cells with boiling water as described by Dawson (1965). Standard volumes (0·4 ml.) of amino acid extract were applied to the columns of a Technicon automatic amino acid analyser, along with a known amount (0·05 $\mu$mole) of norleucine as the internal standard and the individual amino acids were separated, assayed with ninhydrin reagent and recorded automatically. The amounts of each amino acid in the extracts were determined from the strip chart recordings by measurement of peak areas and subsequent reference to standard curves (prepared from data obtained with mixtures of purified amino acids in 0·25 N-$HClO_4$). With each chromatogram the individual peak areas were corrected for variations in the peak areas of the internal standard; the standard errors were then all within $\pm$ 10 % of the mean values.

*A note on the extraction of bacterial pools.* Since the amino acid pool must be in a highly dynamic state in growing bacteria, large changes in pool content and composition could occur during the manipulation period (< 5 min.) prior to the addition of $HClO_4$. However, no differences were detected between samples that were rapidly cooled after removal from the growth vessel and processed at 4° and those that were not chilled and processed at room temperature. Indeed, holding the sample at 20° for 5 min. after removal from the growth vessel and before centrifuging had only a small effect on the concentration of individual free amino acids in the extracted pool. Differences in the amino acid pool content and composition of bacteria, when they occurred, were therefore truly representative of differences in the growing bacteria and not artefacts produced on processing samples.

## RESULTS

As mentioned above, no difference was observed between extracts prepared from cultures that had been processed either at 4° or at 20°; the free amino acid content and composition of the pool seemingly did not change appreciably during the short period between sampling and $HClO_4$ extraction. Similarly, the pool amino acids extracted from three separate $NH_3$-limited cultures of *Aerobacter aerogenes*, grown at a fixed rate (0·3 $h.^{-1}$), temperature (35°) and pH value (6·8) were markedly similar. Thus the pool concentrations of glutamic acid and alanine (the two predominant amino acids; Table 1) were 5·4, 5·3, 5·8 mM and 1·5, 1·6, 1·4 mM, respectively.

*The content and composition of bacterial amino acid pools*

Large differences in the intracellular free amino acid contents of Gram-positive and Gram-negative bacteria were found by Taylor (1947). Indeed, using specific amino acid decarboxylases to assay the intracellular free amino acids (released by heating the organisms at 100°) she concluded that the Gram-negative organisms totally lacked an amino acid pool. Although this conclusion was erroneous (see Mandelstam, 1958), gross differences between the pool contents of Gram-positive and Gram-negative bacteria have frequently been observed (see Holden. 1962) and are apparent here (Table 1). However, in contrast to previous data, the data shown in Table 1 were

Table 1. *Pool free amino acid contents of different bacteria grown in chemostat cultures ($D = 0{\cdot}3\ h.^{-1}$, 35°, pH 6·8), in simple salts media, with growth limited by the availability of $NH_3$*

Organisms were grown, and samples processed, as described in the Methods. The concentrations of each amino acid in extracts was assessed by measurement of peak areas on the traces from the automatic amino acid analyser. These concentrations were related to the intracellular free amino acid concentrations by assuming the cell-bound water content to be four times the bacterial dry weight.

| | Gram-negative bacteria | | | Gram-positive bacteria | | |
|---|---|---|---|---|---|---|
| | *Aerobacter aerogenes* | *Erwinia carotovora* | *Pseudomonas fluorescens* | *Bacillus subtilis* | *B. megaterium* | *B. polymyxa* |
| Amino acid | (Intracellular free amino acid concentration, mM) | | | | | |
| Glutamate | 5·8 | 4·7 | 9·6 | 103 | 62 | 82 |
| Glutamine/ threonine | 0·1 | 0·4 | 1·4 | 2·6 | 0·5 | 21 |
| Proline | n.d. | n.d. | 0·7 | 1·1 | 0·9 | n.d. |
| Aspartate | n.d. | 0·1 | 1·2 | 0·3 | 0·1 | 3·1 |
| Lysine | n.d. | 0·8 | 0·9 | 2·0 | 1·3 | 16·5 |
| Isoleucine | n.d. | 0·2 | n.d. | 0·8 | 0·3 | 3·3 |
| Serine | n.d. | 0·2 | 1·0 | 0·6 | 0·2 | 0·5 |
| Glycine | 0·3 | 0·5 | 0·3 | 3·4 | 1·5 | 7·1 |
| Alanine | 1·4 | 0·9 | 0·5 | 3·1 | 0·6 | 20 |
| Valine | 0·4 | 0·9 | 0·5 | 4·2 | 2·4 | 2·8 |
| Leucine | n.d. | 0·2 | n.d. | 0·9 | 0·3 | n.d. |
| Total free amino acid pool (mM) | 8·0 | 8·9 | 16·1 | 122·0 | 70·1 | 156·3 |
| Glutamate portion of pool (%) | 72 | 53 | 59 | 84 | 89 | 52 |

n.d. = Not detectable (invariably less than 0·1 mM).

derived from cultures that were grown in media of identical composition and at rates, temperatures and pH values that were uniform throughout. Furthermore, since the growth of the cultures was limited by the availability of the nitrogen source ($NH_3$) these pools should contain the minimum concentrations of each amino acid necessary for the organisms to function (that is, grow) in the prescribed medium, at the imposed rate.

Significantly, not all the amino acids present in the proteins of bacteria were present in the pools in detectable concentrations (that is, greater than 0·1 mM). In particular, the pools of both Gram-positive and Gram-negative bacteria generally lacked tryptophan, tyrosine, phenylalanine, histidine, arginine, cysteine and methionine. Indeed, the

only amino acids that were present in appreciable amounts were glutamate and alanine; valine, glutamine and lysine generally were present in lesser amounts. Invariably, glutamic acid was the predominant amino acid and accounted for between 52 and 89 % of the total amino acid content in these $NH_3$-limited bacteria (Table 1).

*Influence of growth rate, and the nature of the growth limitation, on the pool free amino acid content and composition of Aerobacter aerogenes*

It is logical to suppose that when the growth of an organism is limited by the availability of either glucose (the sole carbon source) or $NH_3$ (the sole nitrogen source) its pool would contain minimal levels of the various amino acids. But clearly (Table 2) the total concentrations of free amino acids present in *Aerobacter aerogenes* grown in media containing an excess of glucose and $NH_3$ (for example $Mg^{2+}$-limited or $PO_4^{3-}$-limited) were not markedly different from those in organisms that were either $NH_3$- or glucose-limited. However, there were obvious differences in the contents of the individual amino acids, particularly glutamate and alanine. Thus glucose- or $NH_3$-limited *A. aerogenes* contained about four times as much free glutamate as free alanine; $Mg^{2+}$-limited organisms had about equimolar amounts of each, and $PO_4^{3-}$-limited organisms contained three times as much free alanine as free glutamate (Table 2). Again, it should be pointed out that the temperature, pH value and growth rate were uniform, and maintained constant throughout; only the relative concentrations of glucose:$NH_3$: $Mg^{2+}$: $PO_4^{3-}$ in the simple salts medium feeding the chemostat cultures were different (see Methods).

Table 2. *Influence of the nature of the growth-limiting component of the medium on the free amino acid pool content and composition of Aerobacter aerogenes growing in a chemostat culture* ($D = 0{\cdot}3\ h.^{-1}$, $35°$, *pH 6·8*)

The conditions of growth and treatment of samples were as specified in Table 1. Concentrations expressed as mM, assuming a cell water content four times the dry weight.

| Amino acid | Growth condition | | | |
|---|---|---|---|---|
| | Glucose-limited | $NH_3$-limited | $Mg^{2+}$-limited | $PO_4^{3-}$-limited |
| Glutamate | 3·4 | 5·8 | 3·5 | 1·1 |
| Glutamate/threonine | n.d. | 0·1 | 0·3 | 0·2 |
| Proline | n.d. | n.d. | 0·3 | 0·1 |
| Aspartate | n.d. | n.d. | 0·3 | 0·1 |
| Lysine | n.d. | n.d. | 0·2 | 0·1 |
| Isoleucine | n.d. | n.d. | n.d. | n.d. |
| Serine | n.d. | n.d. | n.d. | 0·1 |
| Glycine | 0·7 | 0·3 | 0·6 | 0·4 |
| Alanine | 0·8 | 1·4 | 3·1 | 3·0 |
| Valine | 0·8 | 0·4 | 1·1 | 0·6 |
| Leucine | n.d. | n.d. | n.d. | n.d. |
| Total free amino acid pool (mM) | 5·7 | 8·0 | 9·4 | 5·8 |

n.d. = Not detectable (that is, less than 0·1 mM).

Although the effect of the growth-limiting component of the medium on the pool amino acid content of *Aerobacter aerogenes* was not as anticipated, the influence of growth rate (that is, culture dilution rate) on the content of free amino acids was completely predictable (Table 3). Increasing the dilution rate from 0·1 to 0·3 to 0·7 $h.^{-1}$ (which would provoke a progressive increase in the rate of protein synthesis) effected

a progressive increase in the total concentration of intracellular free amino acids from 5·5 to 8·0 to 14·7 mM. Not all the detectable amino acids increased proportionately in concentration; the overall increase was principally due to changes in the levels of free glutamate and alanine (Tables 2, 3).

Table 3. *Influence of growth rate on the free amino acid pool content and composition of Aerobacter aerogenes, growing in a chemostat (35°, pH 6·8) in a simple salts medium in which growth is limited by the availability of* $NH_3$

The conditions of growth and treatment of samples were as specified in Table 1. Concentrations are expressed as mM, assuming a cell water content four times the dry weight.

| | Dilution rate ($h.^{-1}$) | |
|---|---|---|
| Amino acid | 0·1 | 0·7 |
| Glutamate | 4·0 | 9·3 |
| Glutamine/threonine | 0·2 | 0·2 |
| Proline | 0·1 | 0·1 |
| Aspartate | n.d. | 0·2 |
| Lysine | 0·2 | 0·2 |
| Isoleucine | n.d. | 0·1 |
| Serine | 0·1 | 0·1 |
| Glycine | 0·3 | 0·5 |
| Alanine | 0·4 | 3·1 |
| Valine | 0·2 | 0·9 |
| Leucine | n.d. | n.d. |
| Total free amino acids (mM) | 5·5 | 14·7 |

n.d. = Not detectable (that is, less than 0·1 mM).

*Influence of medium osmolarity on the content and composition of bacterial amino acid pools*

Though the various media used (see Methods) differed principally in the proportions of glucose, $NH_3$, $Mg^{2+}$ and $K^+$ they were also not of equivalent osmolarity. Indeed, when glucose was in excess of requirement much acid was produced and much titrant was required to maintain the culture pH constant, so it was not possible to prescribe or control the osmolarity. Instead, we decided to study this parameter independently and chose to effect osmolarity changes by adding graded amounts of NaCl to the various media.

With an $NH_3$-limited culture of *Aerobacter aerogenes*, increasing NaCl content of the medium from < 0·1 to 2 to 4 % (w/v) effected a dramatic increase in the content and composition of the amino acid pool (Table 4 and, for comparison, Tables 2, 3). The effect was progressive and varied with growth rate: at a dilution rate of 0·1 $h.^{-1}$, addition of 4 % (w/v) NaCl to the medium caused a ninefold increase in the pool free amino acid content; at a dilution rate of 0·3 $h.^{-1}$, a similar addition of NaCl effected a 17-fold increase. Again, not all of the different amino acids present in the pool increased proportionately; the gross changes in pool amino acid content were due almost entirely to changes in its glutamic acid content (Table 4).

The data contained in Table 4 are steady state values derived from samples of organisms that had grown for several generation-times in the particular environment. In order to assess the nature of the mechanisms regulating pool amino acid levels (particularly pool glutamate levels) kinetic data were required. Thus changes in the

pool content and composition of $Mg^{2+}$-limited *Aerobacter aerogenes* (cultures of which contained an excess of $NH_3$ and glucose) were followed immediately after addition of NaCl to 2 % (w/v). The data obtained are plotted in Fig. 1 *a*. There was an immediate large increase in pool glutamate, which reached a maximum level within 20 min. (that is, one-seventh of the culture doubling time at a dilution rate of 0·3 $h.^{-1}$); the pool glutamine also increased initially but then diminished, and all other amino acids either did not vary in concentration or increased only slowly.

Table 4. *Influence of the medium NaCl concentration on the free amino acid pool content of $NH_3$-limited Aerobacter aerogenes growing in a chemostat culture (35°, pH 6·8) at different dilution rates*

The conditions of growth and treatment of samples were as specified in Table 1. Concentrations are expressed as mM, assuming a cell water content of four times the bacterial dry weight.

| | Dilution rate ($h.^{-1}$) | | | | | |
|---|---|---|---|---|---|---|
| | 0·1 | | 0·3 | | 0·7 | |
| | 2 % NaCl | 4 % NaCl | 2 % NaCl | 4 % NaCl | 2 % NaCl | 4 % NaCl |
| Glutamate | 19·1 | 47·4 | 30·2 | 113·5 | 72·9 | No growth |
| Glutamine/threonine | 0·3 | 0·6 | 0·4 | 16·9 | 4·7 | No growth |
| Proline | 0·1 | 0·2 | 1·0 | 1·3 | 2·2 | No growth |
| Aspartate | n.d. | n.d. | n.d. | 0·5 | 0·5 | No growth |
| Lysine | 0·4 | 0·3 | 0·4 | 1·2 | 0·6 | No growth |
| Isoleucine | n.d. | n.d. | n.d. | n.d. | n.d. | No growth |
| Serine | 0·1 | 0·1 | 0·1 | 0·3 | 0·1 | No growth |
| Glycine | 0·6 | 0·6 | 1·1 | 1·3 | 0·8 | No growth |
| Alanine | 0·4 | 1·1 | 1·5 | 3·0 | 5·2 | No growth |
| Valine | 0·1 | 0·1 | 0·3 | 1·4 | 2·7 | No growth |
| Leucine | n.d. | n.d. | n.d. | 1·2 | n.d. | No growth |
| Total free amino acids (mM) | 21·1 | 50·4 | 35·0 | 140·6 | 89·7 | No growth |

n.d. = Not detectable (that is, less than 0·1 mM).

Since the pool glutamate level changed extremely rapidly on adding NaCl to the environment, it was important to establish whether the change was caused by an increase in the rate of synthesis *de novo* of glutamate, or from turnover of intracellular constituents (presumably not protein since glutamate increased disproportionately to the other amino acids). With washed suspensions of $Mg^{2+}$-limited *Aerobacter aerogenes* suspended in NaCl-containing media that were either complete, or lacked added $NH_3$, the rate of glutamate accumulation greatly depended on the presence of free $NH_3$. Thus addition of NaCl to the environment directly affected the rate of glutamate synthesis *de novo*.

Irrespective of the nature of the growth-limiting component of the medium, the pool glutamate level increased substantially upon addition of NaCl. Thus in the presence of 2 % (w/v) NaCl the steady state pool glutamate of *Aerobacter aerogenes* (growing at a dilution rate of 0·3 $h.^{-1}$, 35°, pH 6·8) were: glucose-limited, 37 mM; $NH_3$-limited, 30 mM; $Mg^{2+}$-limited, 40 mM; $PO_4^{3-}$-limited, 54 mM (cf. Table 2). Surprisingly, the $PO_4^{3-}$-limited organisms (which normally had a very small glutamate pool) responded to the greatest extent to changes in the medium NaCl content; whereas in the absence of added NaCl free glutamate accounted for less than 20 % of the total pool amino

acid concentration, in the presence of 2 % (w/v) NaCl glutamate accounted for over 80 %.

The concentrations of free amino acids (particularly glutamate) within the pools of other Gram-negative bacteria (*Erwinia carotovora*, *Escherichia coli*, *Pseudomonas fluorescens*) were also markedly influenced by the NaCl content of the growth medium. But Gram-positive bacteria behaved differently. Thus when NaCl (2 %, w/v) was added to a $Mg^{2+}$-limited culture of *Bacillus subtilis* var. *niger* (dilution rate of 0·3 $h.^{-1}$,

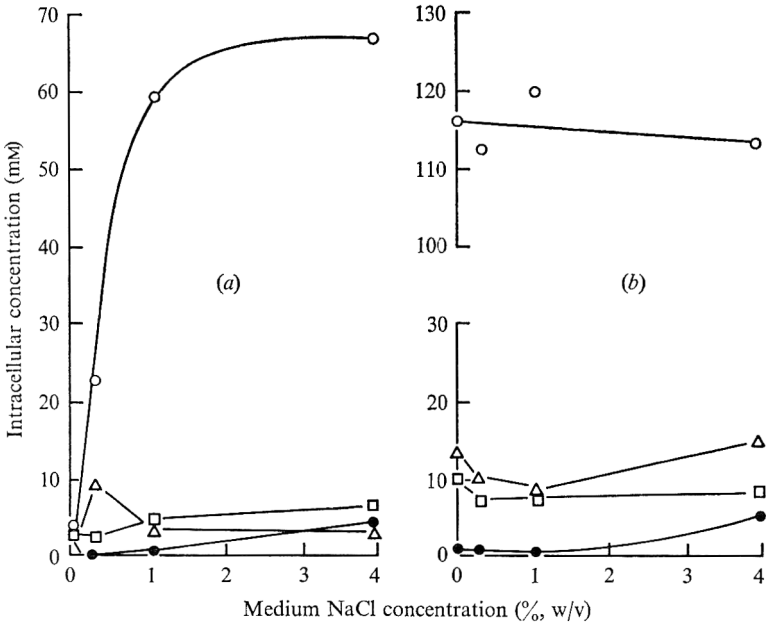


Fig. 1. Changes in the pool concentrations of (○) free glutamate, (●) proline, (△) glutamine and (□) alanine following the addition of NaCl (2 %, w/v, final concentration) to steady state $Mg^{2+}$-limited cultures of (*a*) *Aerobacter aerogenes* and (*b*) *Bacillus subtilis* var. *niger*, growing in chemostats at a dilution rate of 0·3 $h.^{-1}$ (35°, pH 6·8).

35°, pH 6·8) there was no immediate increase in the content of free glutamic acid, or of any other amino acid (Fig. 1*b*). After 60 min. exposure to 2 % (w/v) NaCl, in the growth medium, the pool free amino acid concentration had increased only from 160 to 163 mM; but after a further 72 h. growth in the presence of 2 % (w/v) NaCl, the steady state total intracellular free amino acid concentration was 190 mM.

Similar results were obtained when NaCl (2 %, w/v) was added to glucose-limited, $NH_3$-limited or $PO_4^{3-}$-limited cultures of *Bacillus subtilis* var. *niger*. In every case the pool amino acid concentration was not immediately affected but ultimately increased; invariably the increase was due largely to increases in intracellular glutamate, glutamine and proline (Table 5). The effect of medium NaCl content on the pool proline content was progressive, and particularly marked at the higher NaCl concentrations (Fig. 2).

Epstein & Schultz (1965) and Tempest & Meers (1968) reported that addition of NaCl to growing cultures of Gram-negative bacteria (*Escherichia coli*, *Aerobacter aerogenes*) effected a large increase in their cellular $K^+$ content and concluded that this

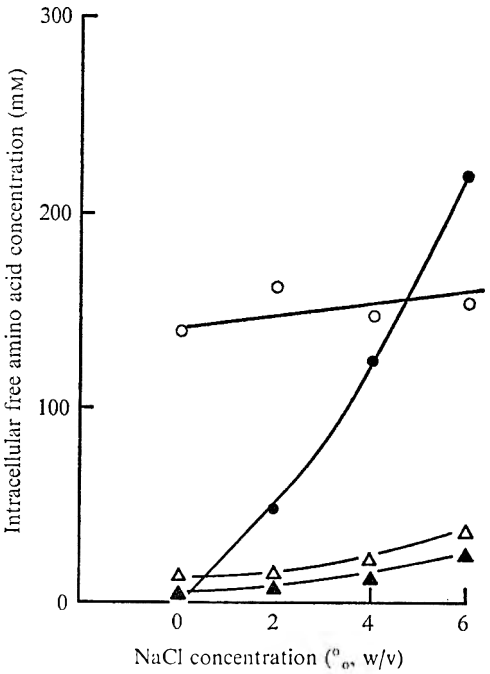


Fig. 2. Influence of medium NaCl concentration on the steady state pool concentrations of (○) free glutamate, (●) proline, (△) glutamine and (▲) alanine in $PO_4^{3-}$-limited *Bacillus subtilis* var. *niger* growing in a chemostat at a dilution rate of 0·2 $h.^{-1}$ (35°, pH 6·8).

Table 5. *The influence of medium NaCl content on the steady state free amino acid pool content and composition in variously limited cultures of Bacillus subtilis var. niger, growing in simple salts media in chemostats (D = 0·3 $h.^{-1}$, 35°, pH 6·8)*

The conditions of growth and processing of samples were as described in Table 1. Amino acid concentrations are expressed as mM, assuming a cell water content equal to four times the dry weight of organisms.

| | *Bacillus subtilis* var. *niger* pool compositions | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Glucose-limited | | $NH_3$-limited | | $Mg^{2+}$-limited | | $PO_4^{3-}$-limited | |
| Amino acid | 0 | 2 % NaCl | 0 | 2 % NaCl | 0 | 2 % NaCl | 0 | 2 % NaCl |
| Glutamate | 130 | 145 | 103 | 113 | 103 | 138 | 131 | 148 |
| Glutamine/ threonine | 1·7 | 9·5 | 2·6 | 4·7 | 9·3 | 21·8 | 7·7 | 32·7 |
| Proline | n.d. | 18·9 | 1·1 | 33 | 0·6 | 5·8 | 0·8 | 32·7 |
| Aspartate | 4·3 | 7·5 | 0·3 | 1·8 | 2·9 | 2·1 | 6·8 | 8·0 |
| Lysine | 0·5 | n.d. | 2·0 | 1·3 | 1·8 | 1·2 | 1·8 | 2·2 |
| Isoleucine | 0·2 | n.d. | 0·8 | 0·7 | 0·6 | 0·7 | 0·9 | 1·5 |
| Serine | 0·5 | 1·4 | 0·6 | 1·6 | n.d. | 0·5 | 0·9 | 1·0 |
| Glycine | 0·5 | 2·0 | 3·4 | 1·9 | 0·8 | 2·1 | 1·8 | 1·8 |
| Alanine | 1·0 | 2·0 | 3·1 | 4·1 | 6·0 | 8·1 | 5·5 | 6·8 |
| Valine | 0·5 | 0·7 | 4·2 | 4·2 | 7·5 | 4·8 | 2·2 | 4·1 |
| Leucine | 0·2 | n.d. | 0·9 | 1·2 | 2·9 | 2·1 | 2·1 | 2·6 |
| Total free amino acid pool (mM) | 139·4 | 187·0 | 122·0 | 167·5 | 135·4 | 187·2 | 161·5 | 241·4 |

n.d. = Not detectable (that is, less than 0·1 mM).

'additional' potassium served an osmoregulatory function. However, no corresponding increase in the cellular contents of inorganic anions was found. Our results (Table 4; Fig. 1*a*) suggest that glutamic acid may, in part, fulfil this requirement. In this connexion, it may be significant that *Bacillus subtilis* var. *niger*, which appeared always to contain much $K^+$ (Tempest, Dicks & Ellwood, 1968; Tempest, 1969) was always found to contain much free glutamic acid (Tables 1, 5).

With cultures of *Bacillus megaterium* and *B. polymyxa* we have observed (Tempest, 1969) that the cell-bound $K^+$ content was considerably lowered when growth was limited by the availability of $K^+$; unlike *B. subtilis* var. *niger* (but like the Gram-negative bacteria), when NaCl was added to these $K^+$-limited cultures the cellular $K^+$ content increased markedly. Examination of the pools of $K^+$-limited *B. megaterium* and *B. polymyxa* grown in the presence and absence of NaCl (2 %, w/v, final concentration) and, for comparison, $Mg^{2+}$-limited organisms, revealed (Table 6) that changes in bacterial $K^+$ content were accompanied either by changes in bacterial free glutamate content or by changes in the intracellular levels of free lysine and DAP (two basic amino acids). However, the changes in cellular $K^+$ content were invariably much greater than the corresponding molar changes in free glutamate, lysine and DAP. Nevertheless, it is quite clear (Tables 4, 6) that environmental conditions that prescribe an increased intracellular $K^+$ content also effected changes in the content and composition of bacterial free amino acid pools such that the net anion concentration was increased.

Table 6. *The influence of medium NaCl content on the free amino acid pool content and composition in $K^+$-limited Bacillus megaterium and Bacillus polymyxa growing in simple salts media, in chemostats ($D = 0{\cdot}3\ h.^{-1}$, 35°, pH 6·8)*

The conditions of growth and processing of samples were as described in Table 1. Amino acid concentrations are expressed as mM, assuming a cell water content of four times the dry weight of organisms.

| | | $K^+$-limited | | | $K^+$-limited | |
|---|---|---|---|---|---|---|
| Amino acid | $Mg^{2+}$-limited | 0 % NaCl | 2 % NaCl | $Mg^{2+}$-limited | 0 % NaCl | 2 % NaCl |
| Glutamate | 56·9 | 30·6 | 87·0 | 82·3 | 44·7 | 41·7 |
| Glutamine/threonine | 0·4 | 0·4 | 0·8 | 20·9 | 13·5 | 38·5 |
| Proline | 0·6 | 0·6 | 1·0 | n.d. | 0·8 | 0·5 |
| Aspartate | 0·4 | 0·8 | 2·2 | 3·1 | 2·7 | 4·0 |
| Lysine | 0·6 | 9·4 | 5·3 | 16·5 | 67·7 | 2·1 |
| Isoleucine | 0·5 | 1·3 | 1·8 | 3·3 | 0·9 | n.d. |
| Serine | 0·3 | 0·3 | 1·4 | 0·5 | n.d. | 0·5 |
| Glycine | 1·2 | 0·8 | 1·2 | 7·1 | 3·5 | 1·4 |
| Alanine | 24·8 | 36·0 | 15·8 | 19·8 | 9·6 | 7·5 |
| Valine | 3·0 | 3·0 | 6·8 | 2·8 | 2·9 | 1·2 |
| Leucine | 1·2 | 0·5 | 0·7 | n.d. | n.d. | n.d. |
| DAP | n.d. | 16·4 | 7·3 | 1·8 | 3·2 | n.d. |
| Total free amino acid pool (mM) | 89·9 | 100·1 | 131·3 | 158·1 | 149·5 | 97·9 |

n.d. = Not detectable (that is, less than 0·1 mM).

*Influence of environment on the content and composition of the amino acid pool of Saccharomyces cerevisiae*

Data obtained for amino acid pools of *Saccharomyces cerevisiae* are shown in Table 7. $NH_3$-limited yeast cells contained an amino acid pool of total size similar to that of the Gram-positive bacteria. Many differences, however, were apparent be-

tween the composition of this yeast pool and that of any of the bacteria studied. Unlike its bacterial counterparts the yeast pool contained appreciable amounts of lysine, arginine and histidine while glutamic acid, although remaining the dominant constituent, accounted for a lesser proportion of the total pool. Yeast cells grown in glucose-limiting cultures contained a larger pool than those grown in $NH_3$-limiting conditions. While all the pool constituents with the exception of aspartic acid and leucine were higher in these glucose-limited cultures, the largest increases were found in the concentrations of glutamic acid, alanine, valine, arginine and histidine.

Increasing the growth rate from 0·1 to 0·25 $h.^{-1}$ (near to the maximum growth rate in this medium) resulted in an increased pool size in glucose-limited cells (Table 7). In this case the concentrations of all the amino acids increased, with the exception of histidine, the largest increases being found in lysine, serine, glycine and arginine.

Cultures of *Saccharomyces cerevisiae* grew at a dilution rate of 0·1 $h.^{-1}$ in glucose-limited media containing up to 4 % (w/v) NaCl. Such concentrations of NaCl, however, did not affect the size of the amino acid pool (assayed with ninhydrin) nor its composition.

Table 7. *The pool free amino acid contents of Saccharomyces cerevisiae grown in chemostat cultures (25°, pH 4·5) in simple salts media*

Organisms were grown, and samples prepared, as described in Methods. The concentration of each amino acid in extracts was assessed by measurement of peak areas on the traces from the amino acid analyser. The concentrations were related to the intracellular free amino acid concentrations by assuming a cell water content of four times the yeast dry weight.

| | Intracellular free amino acid concentration (mM) | | |
|---|---|---|---|
| | | Glucose-limited | |
| Amino acid | $NH_3$-limited $D$ = 0·1 $h.^{-1}$ | $D$ = 0·1 $h.^{-1}$ | $D$ = 0·25 $h.^{-1}$ |
| Glutamate | 35·6 | 135·4 | 146·8 |
| Threonine | 13·5 | 25·8 | 30·6 |
| Proline | n.d. | n.d. | n.d. |
| Aspartate | 10·7 | 11·2 | 16·3 |
| Lysine | 11·5 | 25·8 | 76·5 |
| Isoleucine | 5·7 | n.d. | 10·0 |
| Serine | 15·1 | 13·2 | 34·8 |
| Glycine | 8·6 | 12·0 | 40·1 |
| Alanine | 17·9 | 54·9 | 63·9 |
| Valine | 9·3 | 24·0 | 26·4 |
| Leucine | 7·2 | 1·9 | 7·9 |
| Arginine | 6·4 | 45·5 | 67·5 |
| Histidine | 2·8 | 11·2 | 8·4 |
| Total free amino acid pool (mM) | 144·3 | 380·9 | 529·2 |

n.d. = Not detectable.

## DISCUSSION

Free amino acids lie directly on the pathway of synthesis of proteins from carbohydrates and $NH_3$; therefore it is reasonable to suppose that significant amounts of these intermediary substances would always be present within the growing cell. But the distribution of amino acids in the pools which we examined bore no relationship to their contribution to microbial proteins. In particular, the bacterial pools invariably

lacked detectable amounts of tryptophan, tyrosine, phenylalanine and cysteine while only the yeast pools contained histidine and arginine. Of the amino acids regularly present in bacterial pools, leucine, isoleucine, serine, glycine, aspartate, valine, lysine and proline were frequently present in low concentrations. In fact, only glutamate and alanine appeared always to be present in substantial amounts in bacterial pools, and glutamate nearly always predominated (Tables 1 to 6). Thus, since the content and composition of bacterial amino acid pools could not be related, directly or indirectly, to the protein content and composition of the cells, they must therefore have some other significance.

Clearly (Table 1) there are large differences between the free amino acid pool contents of Gram-positive and Gram-negative bacteria, even when these various organisms are grown at identical rates in identical media. However, in every case shown in Table 1 glutamate was the predominant amino acid and accounted for between 52 and 89 % of the total pool free amino acid contents of these $NH_3$-limited organisms. Furthermore, when large changes in pool amino acid contents were provoked (by varying the growth rate or the medium NaCl content) almost invariably there were correspondingly large changes in pool glutamate levels. In Gram-negative organisms particularly, the pool glutamate levels were most sensitive to changes in environment. Thus, in attempting to rationalize the effects of environment on pool content and composition, it is necessary to account principally for (*a*) the large differences between the pool glutamate contents of Gram-positive and Gram-negative bacteria, and (*b*) the gross effects of medium osmolarity on the intracellular levels of free glutamate in Gram-negative bacteria.

In bacteria, glutamate is generally synthesized from 2-oxoglutarate and $NH_3$ by glutamate dehydrogenase (EC 1.4.1.4). Therefore, changes in the free glutamate content of bacteria, growing at a fixed rate, must be mediated by changes in either the cellular content or activity of this enzyme. In fact, rapid changes in bacterial pool glutamate levels (such as occurred when NaCl was added to the growth environment) could only have resulted from a change in enzyme activity. In this connexion, a detailed examination of glutamate dehydrogenase from *Aerobacter aerogenes* showed it to have a marked pH dependence (Fig. 3). Now, addition of NaCl to suspensions of *A. aerogenes* invariably caused a rapid efflux of $H^+$ from the organisms which could be measured as a decrease in extracellular pH; thus, presumably, the intracellular $H^+$ decreased correspondingly. This would effect a marked increase in glutamate dehydrogenase activity and an increase in the rate of glutamate synthesis. The synthesis of glutamate *de novo* (from glucose and $NH_3$) would tend to restore the intracellular $H^+$ concentration and thereby decrease the rate of glutamate synthesis to a new steady state value. Thus changes in glutamate dehydrogenase activity would provide a compact feedback control system for maintaining the intracellular $H^+$ concentration constant, and it probably is this maintenance of intracellular $H^+$ concentration that dictates the levels of glutamate in Gram-negative bacteria.

On the other hand, the marked differences between the glutamate contents of Gram-negative and Gram-positive bacteria cannot be accounted for in terms of their glutamate dehydrogenase contents or activities (see Meers, Tempest & Brown, 1970). In some respects, Gram-positive bacteria resemble Gram-negative bacteria that have been grown in the presence of much NaCl (for example, in having much cell-bound potassium and free glutamate) but in other respects the organisms are quite different.

Thus addition of NaCl to growing cultures of *Bacillus subtilis* var. *niger* caused no plasmolysis and no immediate increase in bacterial glutamate and potassium contents (Fig. 1*b*). Ultimately the pool glutamate (and potassium) contents did increase (Table 5), but the slowness of this change suggested that it had resulted more from some progressive change in cell structure (for example, in their wall content and composition; see Meers & Tempest, 1969) rather than in the functioning of the organisms.

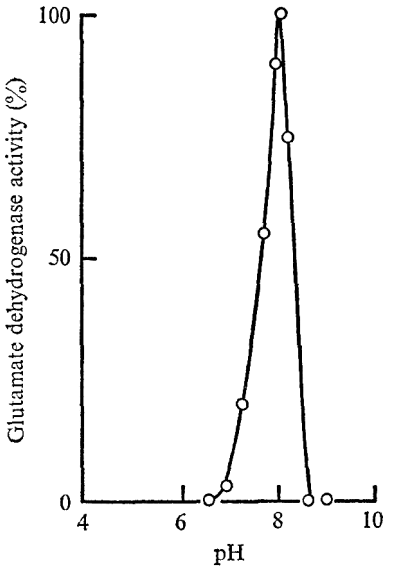


Fig. 3. Influence of pH on the activity of glutamate dehydrogenase extracted from glucose-limited *Aerobacter aerogenes* grown in a chemostat at a dilution rate of 0·3 $h.^{-1}$ (35°, pH 6·8).

It is clear that, in growing bacteria, some correlation exists between their contents of potassium and free glutamate (see Tables 1 to 6 and the papers of Tempest & Dicks, 1967; Tempest & Meers, 1968). But whether changes in bacterial potassium content effect changes in pool glutamate level, or vice versa, is not obvious. Potassium contributes substantially to the net cation content of bacteria, and free glutamate presumably contributes (although not equivalently) to the net anion content. Clearly, in $K^+$-limited *Bacillus megaterium* and *B. polymyxa* cultures the pool basic amino acids (lysine and DAP) spared to some extent the bacterial requirement for $K^+$. Significantly, however, these organisms did not respond to changes in medium NaCl content by increasing their intracellular concentrations of basic amino acids; instead, the rates of synthesis of these basic amino acids were decreased whilst glutamate and $K^+$ accumulated (Table 6).

The amino acid pools of *Saccharomyces cerevisiae*, unlike those of any of the bacteria studied, showed large changes with variations in the nature of the growth-limiting substrate. It appears likely that the supply of $NH_3$ limits the size of the yeast pool and in particular the concentrations of those amino acids containing more than one nitrogen atom per molecule (for example, arginine and lysine). In all cases studied, increasing the growth rate of a culture (and hence the rate of protein synthesis) resulted in a swelling of the amino acid pool thus reflecting the precursor nature of such pools

The limited distribution of the amino acids present in detectable amounts in the pools of micro-organisms, growing in a simple salts environment, is surprising. However, examination of the pathways by which the various amino acids regularly present in these pools arise shows that in the majority of cases they lie close to intermediary metabolites formed in the oxidation of glucose; that is, their synthesis requires only a few additional enzyme-catalysed reactions. It is clear (Table 5; Fig. 2) that with cultures of *Bacillus subtilis* var. *niger* increases in pool glutamate content, following addition of 2 to 6% (w/v) NaCl to the culture, were accompanied by substantial increases in the metabolically associated amino acids, glutamine and, particularly, proline. But no proportionality is apparent between the concentrations of these three amino acids in bacterial pools, nor between other groups of amino acids that arise from a common precursor.

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*Printed in Great Britain*



# 'Glutamine(amide): 2-Oxoglutarate Amino Transferase Oxido-reductase (NADP)', an Enzyme Involved in the Synthesis of Glutamate by Some Bacteria

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SUMMARY

Ammonia-limited *Aerobacter aerogenes*, *Erwinia carotovora*, *Pseudomonas fluorescens*, *Bacillus subtilis* and *B. megaterium* synthesized glutamate from $NH_3$ and 2-oxoglutarate by a process that involved first the synthesis of glutamine and then the reductive transfer of the glutamine amide-nitrogen to the 2-position of 2-oxoglutarate. The latter step required the recently reported enzyme 'glutamine(amide): 2-oxoglutarate amino-transferase oxido-reductase (NADP)', some further properties of which are described here. This enzyme, from different organisms, always had a well-defined maximum activity at a pH value between 7·5 and 8·0; it had an apparent $K_m$ for 2-oxoglutarate between 0·1 and 2·0 mM and an apparent $K_m$ for glutamine between 0·2 and 1·8 mM. Glutamate (the metabolic end-product) and $Mg^{2+}$ strongly inhibited the enzyme from Gram-negative bacteria but less so that from Gram-positive species. Synthesis of glutamate by this enzyme required NADPH, and NADH was inactive; pyruvate, oxaloacetate, 2-oxobutyrate and 2-oxoisovalerate could not substitute for 2-oxoglutarate, nor could the requirement for glutamine be met by asparagine, citrulline, arginine or urea. Although conditions that favoured the synthesis of this enzyme generally also favoured synthesis of glutamine synthetase and caused suppression of glutamate dehydrogenase formation, a close correlation between the bacterial contents of these different enzymes was not apparent.

## INTRODUCTION

In order to grow in a simple salts medium in which $NH_3$ provides the sole source of utilizable nitrogen, micro-organisms must possess some mechanism for the synthesis of amino acids from $NH_3$ and intermediary metabolites. In many bacteria this requirement can be met solely by glutamate dehydrogenase (EC 1.4.1.4), which reductively aminates 2-oxoglutarate to glutamate. This enzyme was found to be present in substantial amounts in glucose-limited *Aerobacter aerogenes* but, surprisingly, was almost absent from these organisms when they were grown in media that lacked sufficient $NH_3$ to allow growth to occur at its maximum rate. Examination of these $NH_3$-limited organisms showed that they synthesized glutamate by a two-step process that involved first the amidation of endogenous glutamate to glutamine and then the

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reductive transfer of the glutamine amide-nitrogen to the 2-position of 2-oxoglutarate (thereby producing a net synthesis of glutamate) (Meers, Tempest & Brown, 1970; Tempest, Meers & Brown, 1970*a*). The latter step involved a novel enzyme, 'Glutamine (amide): 2-oxoglutarate amino-transferase oxido-reductase (NADP)' (for convenience designated GOGAT), and the present communication provides a more detailed description of its properties. We also report on the distribution of this enzyme in other bacteria and on the differing relationships found between this and the metabolically associated enzymes, glutamine synthetase and glutamate dehydrogenase, in different bacteria.

## METHODS

*Organisms. Aerobacter aerogenes* (NCTC 418), *Erwinia carotovora* (MRE 604), *Pseudomonas fluorescens* (KB-1), *Bacillus subtilis* var. *niger* (ATCC 9372), *B. subtilis* W23 and *B. megaterium* KM were maintained on tryptic meat digest agar slopes by monthly subculture at 35°.

*Cultural conditions.* Organisms were grown in 500 ml. chemostats (Herbert, Phipps & Tempest, 1965) in simple salts media that contained growth-limiting concentrations of either glucose or $NH_3$ (see Evans, Herbert & Tempest, 1970). The dilution rate was maintained at 0·3 $h.^{-1}$, pH 6·8 and temperature 35°. Culture effluent was collected in an ice-cooled receiver and the organisms subsequently harvested by centrifugation.

*Extraction of enzymes.* Extracts were prepared by disrupting thick suspensions of organisms (containing about 50 mg. equivalent dry wt organisms/ml., in 50 mM-tris buffer, pH 7·6, plus 10 mM mercaptoethanol) in a Hughes press (Hughes, 1951). The crushed and thawed paste was diluted with about 3 volumes of the above tris buffer and centrifuged (30 min. at 10,000*g*, 4°) to sediment the unbroken cells and debris. The clear supernatant fluid, which contained between 5 and 10 mg. protein/ml., contained the required enzymes.

*Assaying of enzymes.* The activities of glutamate dehydrogenase and GOGAT were measured spectrophotometrically by recording the rate of oxidation of NADPH (indicated by a change in $E_{340\ nm.}$) following addition of the extract (0·05 to 0·3 ml.) to a solution containing 2-oxoglutarate (5 mM), NADPH (0·25 mM) and either $NH_4Cl$ (40 mM) or glutamine (5 mM) in 3·0 ml. final volume of tris buffer (50 mM), pH 7·6. Measurements were made with a Spectronic-20 spectrophotometer (Bausch & Lomb Inc., New York, U.S.A.) using tubes of 1·1 cm. internal diameter. The results, corrected for a small endogenous NADPH oxidase activity, are expressed as nmoles NADPH oxidized (37°, pH 7·6)/min./mg. protein. pH/activity curves were determined by adding graded amounts of either NaOH or HCl to the above test solutions and measuring the pH immediately following the assay of the enzyme activity. $K_m$ values for ammonia, glutamine and 2-oxoglutarate were determined by conventional methods (Dixon & Webb, 1964).

*Analytical procedures.* In some experiments glutamic acid formation was determined directly by precipitating the protein from the reaction mixture with 0·25 N-$HClO_4$ (15 min., 4°) and applying the clear extract to the columns of a Technicon automatic amino acid analyser. Concentrations of glutamate were assessed by measurement of peak areas, on the recorder charts, and reference to standard curves prepared with pure solutions of glutamic acid in 0·25 N-$HClO_4$. Protein was estimated by the biuret method of Stickland (1951).

## RESULTS

### *Assay and properties of GOGAT from Aerobacter aerogenes*

Initially GOGAT in extracts of $NH_3$-limited *Aerobacter aerogenes* was detected by the formation of glutamic acid when mixtures containing the enzyme plus glutamine (5 mM) and 2-oxoglutarate (5 mM) were incubated at 37°, and pH 7·6, with and without the addition of 0·25 mM NADPH. Some formation of glutamate from glutamine occurred in the absence of NADPH due, presumably, to the action of a glutaminase (EC 3.5.1.2), but considerably more glutamate was formed, without concomitant production of $NH_3$, when NADPH was present. Extracts of glucose-limited *A. aerogenes* possessed a small glutaminase activity but would not catalyse a reaction producing a net synthesis of glutamate from glutamine and 2-oxoglutarate. However, these extracts, unlike those from $NH_3$-limited *A. aerogenes*, readily and rapidly formed glutamate when incubated with 2-oxoglutarate (5 mM), $NH_4Cl$ (20 mM) and NADPH (0·25 mM). Clearly, glucose-limited organisms possessed an active glutamate dehydrogenase whereas $NH_3$-limited organisms possessed the alternative glutamate-synthesizing enzyme—GOGAT. Since the $NH_3$-limited bacterial extracts lacked

Table 1. *The distribution of GOGAT and glutamate dehydrogenase in bacteria grown in different media*

The organisms were grown in chemostat cultures in media that contained growth-limiting concentrations of the nutrients specified. The dilution rate was 0·3 $h.^{-1}$ (35°, pH 6·8) and the organisms were collected and extracted as described in Methods. The activities quoted are the values determined under optimum conditions, and are expressed as nmoles NADPH oxidized/min./mg. protein. Pool glutamate levels were determined by extracting organisms with 0·25 N-$HClO_4$ (4°, 10 min.), separating the amino acids and assaying them automatically with a Technicon automatic amino acid analyser.

| Organism | Growth condition | Pool glutamate level (mM) | Specific activities: GOGAT | Specific activities: Glutamate dehydrogenase |
|---|---|---|---|---|
| *Aerobacter aerogenes* | Glucose-limited | 3·4 | < 1 | 560 |
| | $NH_3$-limited | 5·8 | 66 | 19 |
| | $NH_3$-limited + 2 % NaCl | 30·2 | 32 | 36 |
| | *N(glutamate)-limited | 5·0 | < 1 | < 1 |
| | Phosphate-limited | 1·1 | < 1 | 600 |
| | *P-limited + glutamate | > 20 | < 1 | 10 |
| *Erwinia carotovora* | Glucose-limited | 5·5 | 50 | < 1 |
| | $NH_3$-limited | 4·7 | 26 | < 1 |
| | *N(glutamate)-limited | 4·6 | < 1 | < 1 |
| | Phosphate-limited | 3·0 | 25 | < 1 |
| | *P-limited + glutamate | > 30 | < 1 | < 1 |
| *Pseudomonas fluorescens* | Glucose-limited | 4·0 | 77 | 480 |
| | $NH_3$-limited | 9·6 | 64 | 10 |
| *Bacillus subtilis* var. *niger* | Glucose-limited | 130 | < 1 | 800 |
| | $NH_3$-limited | 103 | 140 | 30 |
| *B. megaterium* | Glucose-limited | 57 | 750 | 10 |
| | $NH_3$-limited | 62 | 140 | 10 |
| *B. subtilis* W23 | $Mg^{2+}$-limited | 142 | 78 | 2 |
| | $NH_3$-limited | 125 | 43 | 5 |

* Glutamate was the sole source of utilizable nitrogen in these cultures.

glutamate dehydrogenase activity, the possible formation of glutamate from glutamine, 2-oxoglutarate and NADPH via a two-step process involving deamidation of glutamine (with glutaminase) followed by amination of 2-oxoglutarate (with glutamate dehydrogenase) could be discounted. The only possible interpretation of the above results is that $NH_3$-limited *A. aerogenes* contained an enzyme (GOGAT) that catalysed the direct transfer of the glutamine amide-nitrogen to the 2-position of 2-oxoglutarate. Thus, although this enzyme, in conjunction with glutamine synthetase, synthesized glutamate from $NH_3$ and 2-oxoglutarate, it did so by a pathway that did not involve glutamate dehydrogenase (Tempest *et al.* 1970*a*); the enzymes involved in this pathway were seemingly only synthesized by *A. aerogenes* under conditions where glutamate dehydrogenase was either not present or not functioning (Table 1).

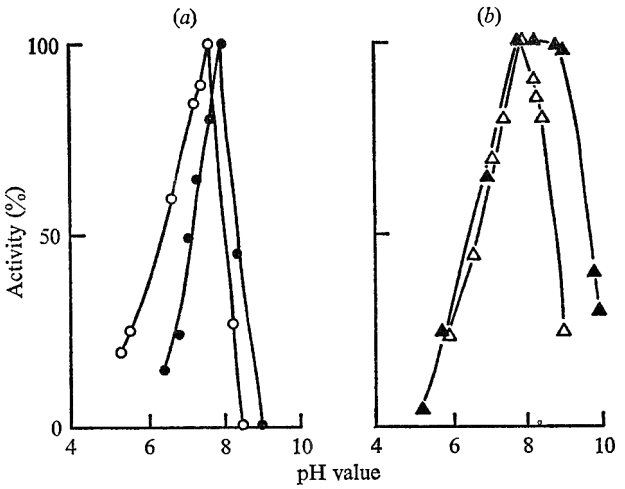


Fig. 1. Influence of pH on the activity of GOGAT extracted from (*a*) Gram-negative bacteria —*Aerobacter aerogenes* (○) and *Erwinia carotovora* (●)—and (*b*) Gram-positive bacteria— *Bacillus subtilis* var. *niger* (△) and *B. subtilis* W23 (▲)—grown in $NH_3$-limited chemostat cultures (dilution rate = 0·3 $h.^{-1}$, 35°, pH 6·8).

To assay GOGAT activity, use was made of the fact that oxidation of NADPH to NADP accompanied the transfer of the glutamine amide-nitrogen to 2-oxoglutarate. This oxidation of NADPH could be followed spectrophotometrically at 340 nm.

*Influence of pH on GOGAT activity.* This was studied by using the spectrophotometric assay described above. The enzyme from $NH_3$-limited *Aerobacter aerogenes* had a sharp optimum activity at pH 7·6; at a pH value of 6·8 the activity was less than 70 % of the maximum value, and at pH 8·4 it was almost nil (Fig. 1*a*). Similar sharp pH/activity profiles were observed with the GOGAT enzymes extracted from $NH_3$-limited cultures of *Erwinia carotovora* and *Bacillus subtilis* var. *niger* (Fig. 1*a*, *b*). The enzyme from *B. subtilis* W23 was different in that its activity was much less affected by high pH values; it showed near-maximal activity over a range of pH from 7·7 to 9·0 (Fig. 1*b*).

*Influence of glutamine and 2-oxoglutarate concentrations on GOGAT activity.* Again, this was studied using the rate of oxidation of NADPH as a measure of GOGAT activity (endogenous NADPH oxidase activity being subtracted). Varying the concentration of either glutamine or 2-oxoglutarate in the incubation mixture (see

Methods) caused the GOGAT activity to change in a manner characteristic of a Michaelis–Menten-type function. With the enzyme from $NH_3$-limited *Aerobacter aerogenes*, the concentrations of glutamine and 2-oxoglutarate supporting half-maximal GOGAT activity were 1·8 and 2·0 mM respectively (Table 2). The $K_m$ values for glutamine and 2-oxoglutarate of enzymes extracted from several different Gram-positive and Gram-negative bacteria are also shown in Table 2; invariably they were less than the corresponding $K_m$ values for the *A. aerogenes* enzyme.

Table 2. *Apparent Michaelis constants for glutamine and 2-oxoglutarate of GOGAT extracted from several different Gram-positive and Gram-negative bacteria*

The organisms were grown in $NH_3$-limited chemostat cultures at a dilution rate of 0·3 $h.^{-1}$ (35°, pH 6·8). Bacterial extracts, prepared as described in Methods, were incubated at 37° and pH 7·6 with varying concentrations of substrate, and the rates of NADPH oxidation were measured spectrophotometrically. The values given below are those concentrations of each substrate supporting half-maximal GOGAT activity, all other reactants being provided in concentrations assumed to be in excess of requirement.

| Organism | Apparent $K_m$ values of GOGAT for: Glutamine (mM) | 2-Oxoglutarate (mM) |
|---|---|---|
| *Aerobacter aerogenes* | 1·8 | 2·0 |
| *Pseudomonas fluorescens* | 0·4 | 0·1 |
| *Erwinia carotovora* | 1·6 | 0·1 |
| *Bacillus subtilis* var. *niger* | 1·7 | 0·1 |
| *B. subtilis* W23 | 0·2 | 0·04 |
| *B. megaterium* | 0·3 | 0·04 |

*Substrate specificity of GOGAT.* The enzyme from *Aerobacter aerogenes* displayed maximal activity in an assay mixture containing glutamine (5 mM), 2-oxoglutarate (5 mM) and NADPH (0·25 mM) in 50 mM tris, pH 7·6. Substituting NADH (0·25 mM) for NADPH decreased the activity almost to zero. Similarly, pyruvate, oxaloacetate, 2-oxobutyrate or 2-oxoisovalerate (each at 5 mM) would not substitute for 2-oxoglutarate, nor would asparagine, citrulline, arginine or urea (each at 5 mM) substitute for glutamine. The requirements for NADPH, glutamine and 2-oxoglutarate were seemingly absolute.

*Inhibitors.* The activity of GOGAT was not appreciably affected when 25 mM-Na/K phosphate buffer (pH 7·6) replaced the tris buffer. However, higher concentrations of $Na^+$ and, to a lesser extent, $K^+$ did lower the activity of the enzyme and $Mg^{2+}$ was markedly inhibitory (Fig. 2).

*Regulation of GOGAT synthesis and activity*

With $NH_3$-limited and glucose-limited cultures of *Aerobacter aerogenes*, a reciprocal relationship seemingly existed between the cellular contents of GOGAT and glutamate dehydrogenase. Thus conditions that favoured the synthesis of GOGAT caused suppression of glutamate dehydrogenase synthesis, and vice versa. In fact, with all the bacteria examined, $NH_3$-limitation invariably caused repression of glutamate dehydrogenase synthesis (in those organisms which otherwise produced this enzyme) but permitted GOGAT synthesis (Table 1). Some organisms (particularly *Erwinia carotovora*, but also, possibly, *Bacillus megaterium* and *B. subtilis* W23) seemingly lacked

a glutamate dehydrogenase but could still grow readily in simple salts media in which $NH_3$ provided the sole source of utilizable nitrogen; in these cases GOGAT was constitutively synthesized. As with glutamate dehydrogenase, however, synthesis of GOGAT was totally repressed when L-glutamate (50 mM) was added to the growth medium (Table 1). Inexplicably, using L-glutamate as the N-limiting component of

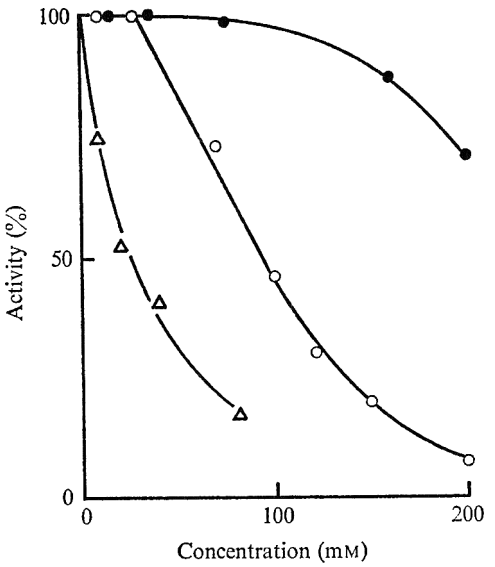


Fig. 2. Influence of various concentrations of $Na^+$ (○), $K^+$ (●) and $Mg^{2+}$ (△) on the activity of GOGAT extracted from $NH_3$-limited *Aerobacter aerogenes* organisms.

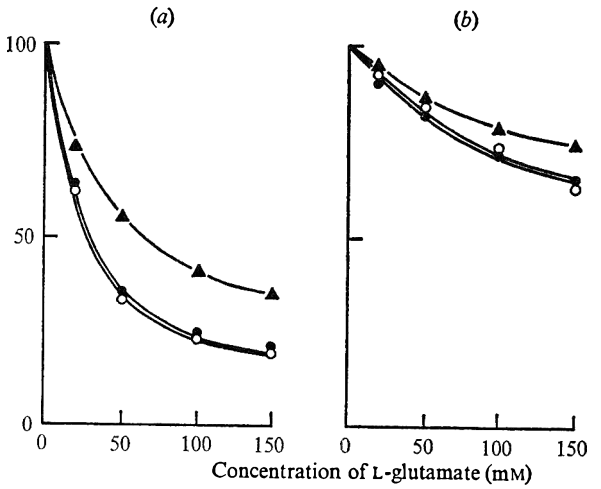


Fig. 3. Influence of L-glutamate concentration on the activities of GOGAT and glutamate dehydrogenase extracted from different bacteria. (*a*) Glutamate dehydrogenase (▲) and GOGAT (●) from *Aerobacter aerogenes* (glucose-limited and $NH_3$-limited, respectively), and GOGAT from $NH_3$-limited *Erwinia carotovora* (○). (*b*) Glutamate dehydrogenase (▲) and GOGAT (●) from *Bacillus subtilis* var. *niger* (glucose-limited and $NH_3$-limited, respectively), and GOGAT from $NH_3$-limited *B. subtilis* W23 organisms (○).

the medium also caused suppression of GOGAT synthesis in *E. carotovora* and *A. aerogenes*, even though their intracellular free glutamate levels were only marginally different from those of $NH_3$-limited organisms. Presumably, in these cases at least, it could not have been glutamate *per se* that was the repressor molecule.

As well as affecting the synthesis of glutamate dehydrogenase and GOGAT, glutamate also markedly influenced their activities (Figs. 3*a*, *b*). In this respect there were obvious differences between Gram-positive and Gram-negative bacteria; enzymes from the former were much less sensitive to end-product inhibition; this correlated with their having a much greater 'pool' content of free glutamate (Table 1; see also Tempest, Meers & Brown, 1970*b*).

## DISCUSSION

Glutamate occupies a central position in bacterial amino acid metabolism, acting as an amino donor in the synthesis of practically all other amino acids. Thus control of glutamate synthesis is fundamental to the control of amino acid synthesis generally and, thereby, to the control of protein synthesis and growth. Glutamate can be formed directly from 2-oxoglutarate and $NH_3$ by glutamate dehydrogenase and, in most bacteria, no other route of synthesis from $NH_3$ was known until the finding by Tempest *et al.* (1970*a*) that $NH_3$-limited *Aerobacter aerogenes* could effect synthesis by a two-step process involving a novel enzyme, 'glutamine (amide):2-oxo-glutarate aminotransferase oxido-reductase (NADP)'. It is now evident that this enzyme is not peculiar to $NH_3$-limited *A. aerogenes*, nor, indeed, to $NH_3$-limited bacteria. Although GOGAT was present in all of the $NH_3$-limited bacteria which we examined, some (particularly *Erwinia carotovora*, which seemingly totally lacks a glutamate dehydrogenase) synthesized it 'constitutively'. It is reasonable to conclude, therefore, that in bacteria the potential to synthesize glutamate from $NH_3$ and 2-oxoglutarate, via reactions catalysed by glutamine synthetase and GOGAT, is not uncommon; indeed, it may be a regular pathway of $NH_3$ assimilation in most, if not all, bacteria that are able to grow in media in which $NH_3$ provides the sole source of utilizable nitrogen.

No simple model for the co-ordinated control of glutamate dehydrogenase and GOGAT syntheses can be suggested. Addition of L-glutamate (50 mM) to phosphate-limited cultures of *Aerobacter aerogenes* and *Erwinia carotovora* suppressed the synthesis of both enzymes but clearly glutamate *per se* was not the repressor. Thus, when glutamate was provided as the sole N-source to an N-limited culture of *A. aerogenes*, the 'pool' free glutamate level was only slightly changed (Table 1) yet the synthesis of GOGAT was still totally repressed. Again, when NaCl (2 %, w/v) was added to the $NH_3$-limited *A. aerogenes* culture, the 'pool' glutamate level was greatly increased but the cellular contents of GOGAT and glutamate dehydrogenase were only partially affected (Table 1).

Large differences were apparent both in the amount of GOGAT present in various $NH_3$-limited bacteria (Table 1) and in their $K_m$ values for glutamine and 2-oxoglutarate (Table 2), but again no common patterns are evident among the organisms examined. For example, although, on average, the Gram-positive bacteria contained the greater amounts of GOGAT and had the lower $K_m$ values for glutamine and 2-oxoglutarate, there were notable exceptions; for example, the enzyme from the Gram-positive organism, *Bacillus subtilis* var. *niger*, had a high $K_m$ for glutamine whilst that from Gram-negative *Pseudomonas fluorescens* had a low $K_m$ for the same substrate. On the

other hand, however, in every case the GOGAT activity was markedly affected by pH and had a common optimal activity in the range pH 7·6 to 8·0, and invariably GOGAT from the Gram-negative organisms was more sensitive to glutamate inhibition than the enzyme from the Gram-positive species.

The synthesis of glutamate from $NH_3$ and 2-oxoglutarate, via glutamine and GOGAT, requires the participation of an active glutamine synthetase. Significantly, bacterial glutamine synthetases generally are found in considerable amounts in organisms whose growth has been limited by $NH_3$ deprivation (Woolfolk, Shapiro & Stadtman, 1966; Wu & Yuan, 1968; Pateman, 1969). Glutamine synthetases characteristically have a low $K_m$ for ammonia, and this fact led Umbarger (1969) to suggest that they may act as 'scavengers' for $NH_3$ (which would provide a functional basis for their derepressed synthesis in $NH_3$-limited environments). The results reported in this paper support and extend Umbarger's hypothesis in that they show that glutamine synthetase (in conjunction with GOGAT) facilitates the synthesis of glutamate from $NH_3$ under conditions where glutamate dehydrogenase (by virtue of its high $K_m$ for $NH_3$) would be functionally inadequate. In this connexion, it was apparent (Table 1) that, with all the organisms examined, $NH_3$-limitation effected an enormous decrease in their glutamate dehydrogenase contents. Ammonia-limited bacteria contained exceedingly little free ammonia (Tempest *et al.* 1970*b*) so that, even if present, glutamate dehydrogenase would be grossly subsaturated; but how $NH_3$-limitation could effect cessation of glutamate dehydrogenase synthesis is not obvious.

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# The Influence of Oxygen, Glucose and Nitrate upon the Formation of Nitrate Reductase and the Respiratory System in *Bacillus licheniformis*

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SUMMARY

When a culture of *Bacillus licheniformis* was shifted from aerobic to anaerobic conditions, lysis occurred unless a fermentable carbon source or a system for nitrate respiration was present. Nitrate reductase was primarily induced by partial or complete anaerobiosis and partially repressed by glucose. The enzyme was repressed and inactivated by high oxygen concentrations. Respiration of bacteria grown anaerobically was 30 to 40 % of that of bacteria grown aerobically. Glucose decreased respiration of other substrates in both aerobically and anaerobically grown organisms. No cytochrome $a_1$ was present after anaerobic growth. Cytochrome $c_1$ was repressed by glucose; under anaerobic conditions this repression was antagonized by nitrate.

## INTRODUCTION

Most of the work in the regulation of nitrate reductase has been done with coliform bacteria, in which it is induced by anaerobiosis in the presence of nitrate. The enzyme is repressed, inhibited and inactivated by oxygen (Pichinoty, 1965; Van't Riet, Stouthamer & Planta, 1968; De Groot & Stouthamer, 1970*a*)

Hackenthal (1966) found parallel induction of nitrate and nitrite reductase by nitrate, nitrite and some other monovalent anions in *Bacillus cereus*. In *B. stearothermophilis* nitrate reductase was induced by nitrate and repressed and inactivated by oxygen. When cells were adapted to nitrate, oxygen respiration decreased by 50 to 70 %; the main change in the cytochrome spectrum was the disappearance of cytochrome $a_3$ (Downey, 1966; Downey & Nuner, 1967; Downey & Kiszkiss, 1969; Downey, Kiszkiss & Nuner, 1969).

The Bacillus species in which nitrate reductase formation has been studied so far are obligate organisms. This paper describes a study of the regulation of nitrate reductase formation and of the influence of nitrate, glucose and oxygen on respiratory activity and cytochrome content in the facultative organism *Bacillus licheniformis*.

## METHODS

*Organism: Bacillus licheniformis* S244 (originally designated *B. licheniformis* 430) was obtained from Dr J. W. Woldendorp, Agricultural University, Wageningen, The Netherlands. It was maintained on slopes of Oxoid brain heart infusion agar and stored at 4°.

*Media.* NBY medium contained Oxoid nutrient broth no. 2, 8 g./l., and Oxoid yeast extract, 3 g./l. NBYG was made by adding glucose (5 g./l.); $NBYGNO_3$ by adding glucose (5 g./l.) and $KNO_3$ (2 g./l.); and $NBYNO_3$ by adding $KNO_3$ (10 g./l.). Glucose and nitrate were added from sterile stock solutions. The pH of all media was 7·0.

*Cultural conditions.* Bacteria were grown at 37° in a New Brunswick MicroFerm Laboratory fermentor in a 7 l. jar containing 3 l. of medium either with vigorous aeration (10 l. air/min., agitation 650 rev./min.), with weak aeration (2 l. air/min., agitation 200 rev./min.) or anaerobically (nitrogen, agitation 100 rev./min.). The medium in the jar was inoculated with 2 × 100 ml. of culture in NBY, shaken overnight at 250 rev./min. at 37° in a 500 ml. bottle. If required, the pH of the medium was maintained at pH 7 by a New Brunswick automatic pH controller using 0·5 N-NaOH. Dissolved oxygen concentration was measured with a New Brunswick oxygen electrode and is expressed as percentage of the dissolved oxygen concentration in the oxygen-saturated medium. Bacterial density was determined by measuring the extinction at 660 nm. ($E_{660}$) in a Unicam SP 600 spectrophotometer.

*Harvesting of bacteria and preparation of cell-free extracts.* Bacteria were harvested by centrifugation in an MSE 18 high speed centrifuge at 10,000*g* at 2° for 15 min. For cell-free extracts, they were washed twice with cold phosphate buffer, 0·065 M, pH 7, resuspended in this buffer and sonicated in an MSE ultrasonic disintegrator, 60 W, 20 kc./sec. for 10 min. at 0°. Cellular debris was not removed, as this caused fluctuations in nitrate reductase activity.

Suspensions in phosphate buffer lysed rapidly at 37° and failed to respire. Lysis was prevented by adding $MgSO_4.7H_2O$ (10 %, w/v). Bacterial suspensions were therefore washed and resuspended in $MgSO_4.7H_2O$ (100 g./l.), tris (1·21 g./l.), KCl (2·47 g./l.), adjusted to pH 7 with HCl. Throughout harvesting, chloramphenicol (30 $\mu$g./ml.) was present.

*Measurement of acid formation.* Cultures were grown anaerobically in NBYG or $NBYGNO_3$ to $E_{660} \approx 1{\cdot}4$. Four hundred ml. of culture was chilled by addition of ice. The bacteria were harvested by centrifugation and resuspended in 200 ml. of fresh cold NBYG medium with chloramphenicol (30 $\mu$g./ml.). Samples (50 ml.) in a beaker were gently stirred and bubbled with nitrogen in a 37° water bath. The pH was measured continuously with a Philips pH meter model PR 9403/01 and at 2 min. intervals adjusted to pH 7 with NaOH (*c.* 0·1 N) previously titrated with 0·1 N-oxalic acid. At zero time 0·5 ml. of distilled water or 20 % $KNO_3$ solution was added. Samples (0·5 ml.) were taken for glucose determinations at 5 min. intervals.

*Cytochromes.* Cytochromes were measured in dithionite-reduced suspensions using a Unicam SP 800 spectrophotometer. Cytochrome $c_1$ content was expressed as the difference in extinction between the peak at 552 nm. and the line connecting 540 and 570 nm.; cytochrome $a_1$ content as the difference in extinction between the peak at 595 nm. and the line connecting 580 and 610 nm. Specific cytochrome content was expressed as $E_{552}$ or $E_{595}$/10 mg. of protein.

*Other assays.* Oxygen uptake by whole bacteria was measured by standard Warburg techniques.

Glucose content of the medium was determined with the glucose oxidase (Boehringer, Mannheim, Germany).

Nitrite content of the medium was assayed as described by Van't Riet, Stouthamer & Planta (1968).

Nitrate reductase activity was measured according to Lowe & Evans (1964); the assay mixtures were incubated for 15 min. at 37°.

Protein was estimated according to Lowry, Rosebrough, Farr & Randall (1951).

## RESULTS

### *Effect of anaerobiosis, induction of nitrate reductase and lysis*

In vigorously aerated cultures, no nitrate reductase could be demonstrated as long as the cell density was not too high. When a $NBYNO_3$ culture grown with vigorous aeration was shifted to anaerobic conditions, rapid lysis occurs (Fig. 1) and no nitrite was formed.

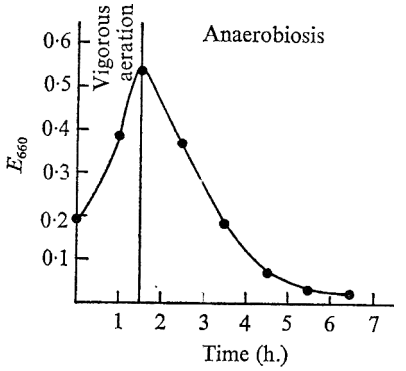


Fig. 1

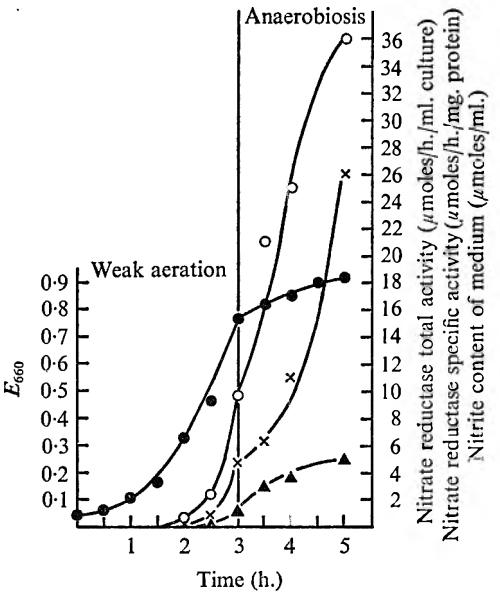


Fig. 2

Fig. 1. Lysis of *Bacillus licheniformis* s244 in $NBYNO_3$ medium after a shift from vigorous aeration to anaerobiosis.

Fig. 2. Growth, nitrite formation and nitrate reductase synthesis of *Bacillus licheniformis* s244 in $NBYNO_3$ medium during weak aeration, followed by a shift to anaerobiosis. ●, $E_{660}$; ○, nitrate reductase specific activity; ▲, nitrate reductase total activity; ×, nitrite content of medium.

When grown with weak aeration in $NBYNO_3$, the oxygen consumption by the bacteria soon exceeded the oxygen supply and the oxygen tension in the medium was reduced. Nitrate reductase was induced and nitrite was produced. During this period, oxygen and nitrate respiration were occurring simultaneously, and change to anaerobic conditions did not cause lysis until the nitrate was exhausted (Fig. 2).

In glucose-containing media, shifts from vigorous aeration to anaerobic conditions never caused lysis, but acid was produced immediately. In cultures without pH regulation, growth stopped when pH 5·7 was reached (Fig. 3*a*). The pH was regulated in all further experiments in glucose-containing media. Nitrate reductase was formed under anaerobic conditions in NBYG, in spite of the absence of nitrate (Fig. 3*a*).

Addition of nitrate caused a further 2·5-fold increase in specific activity (Fig. 3*b*). Specific activity in $NBYNO_3$ was considerably higher than in glucose-containing media: glucose repressed nitrate reductase. More nitrite was formed in $NBYNO_3$ than in $NBYGNO_3$, in spite of the greater total nitrate reductase activity in the latter medium. Apparently, in the presence of glucose the transfer of electrons to the nitrate reductase was less effective.

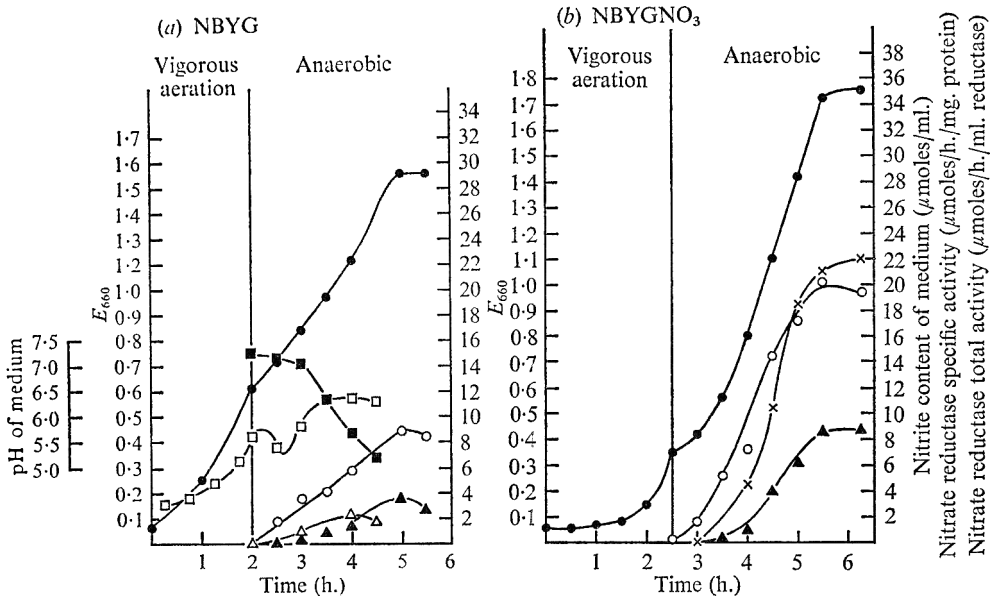


Fig. 3. Growth and nitrate reductase formation of *B. licheniformis* S244 in (*a*) NBYG and (*b*) $NBYGNO_3$ media after a shift from vigorous aeration to anaerobiosis, with and without pH control. With pH control: ●, $E_{660}$; ○, nitrate reductase specific activity; ▲, nitrate reductase total activity; ×, nitrite content of medium. Without pH control (applies to (*a*) only): □, $E_{660}$; △, nitrate reductase specific activity; ■, pH of the medium.

*Effect of aeration. Bacillus licheniformis* was grown in NBY, $NBYNO_3$, NBYG and $NBYGNO_3$ media with weak aeration, and the dissolved oxygen content, nitrate reductase and nitrite formation were followed. Nitrate reductase formation in glucose-free and glucose-containing media started at 20 and 0 % oxygen respectively (Fig. 4*a*, *b*). In NBY and $NBYNO_3$ nitrate reductase was produced at the same rate; the same applies to NBYG and $NBYGNO_3$. Also, in $NBYNO_3$ and $NBYGNO_3$, nitrite formation started at 20 and 0 % oxygen respectively (Fig. 4). When an oxygen concentration of 20 % was reached in NBY and $NBYNO_3$, the oxygen concentration did not decrease any further, and the growth rates dropped (Fig. 4*a*).

*Inactivation of nitrate reductase by aeration.* When an anaerobic $NBYGNO_3$ culture was aerated vigorously, the specific nitrate reductase activity dropped, but the total activity remained constant, and the nitrite content of the medium still increased. Therefore, in order to study inactivation of nitrate reductase by aeration, growth and oxygen consumption have to be decreased by suspending the bacteria in a buffer with chloramphenicol and without substrate (Fig. 5). The aerated suspension showed inactivation of nitrate reductase, whereas the nitrate reductase activity in the non-aerated suspension remained constant.

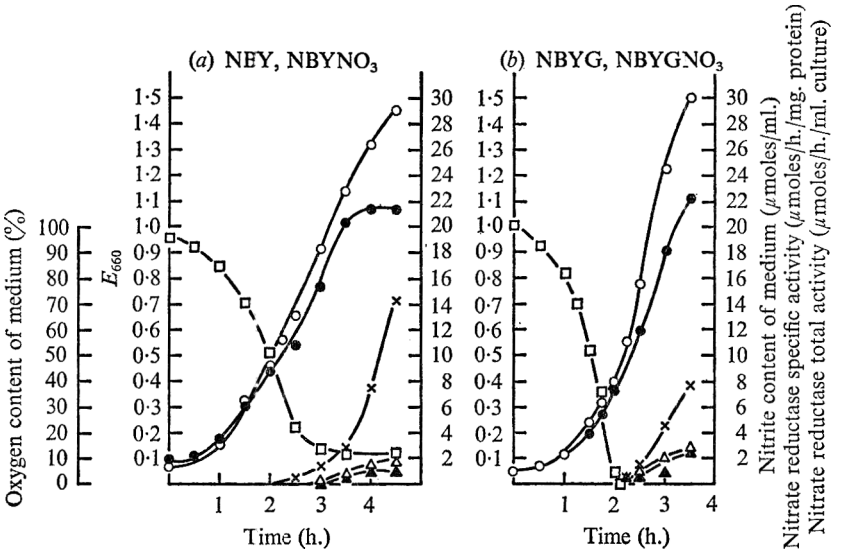


Fig. 4. Growth, oxygen consumption, nitrate reductase and nitrite formation in various media with weak aeration. Closed symbols: NBY, NBYG. Open symbols: $NBYNO_3$, $NBYGNO_3$. ○●, $E_{660}$; △▲, nitrate reductase; □, oxygen concentration (for all media); ×, nitrite content of medium ($NBYNO_3$ and $NBYGNO_3$ only).

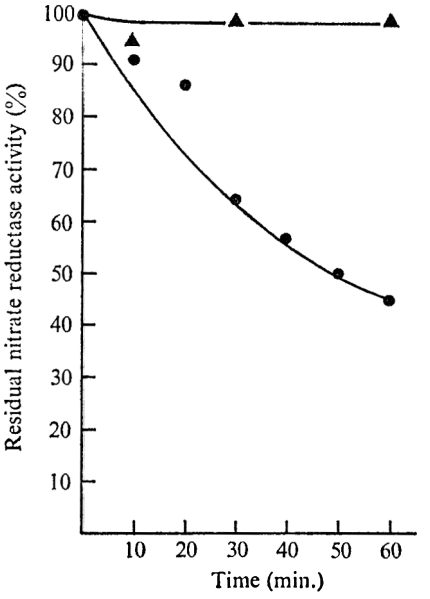


Fig. 5. Effect of aeration upon nitrate reductase in cells grown anaerobically in $NBYGNO_3$ to $E_{660} = 1{\cdot}5$. Cells were washed and resuspended in Mg-tris-KCl medium to $E_{660} = 1{\cdot}5$. 50 ml. was shaken in a 500 ml. flask at 37° in a water bath shaker at a speed of 250 strokes/min. (●). 50 ml. was left static at 37° in a 50 ml. flask (▲). At 5 min. intervals, 5 ml. samples were removed for determination of the residual nitrate reductase activity. During harvesting, washing and the inactivation experiment, 30 μg. of chloramphenicol/ml. was present.

*Changes in the respiratory system, concomitant with anaerobiosis and nitrate respiration.* Table 1 shows oxygen uptake by suspensions of washed bacteria, grown under various conditions. After anaerobic growth in $NBYNO_3$, respiration with all substrates was reduced to 30 to 40 % of the aerobic level, as was glucose respiration in glucose-containing media. The presence of glucose reduced respiration of all substrates except glucose and abolished that of acetate and citrate.

Table 1. *Effect of growth conditions on oxygen uptake*

Averages of two or three experiments are given. Correction has been made for endogenous oxygen uptake.

| | Oxygen uptake ($\mu$moles $O_2$/mg. protein/h.) | | | | | |
|---|---|---|---|---|---|---|
| | Aerobic | | | Anaerobic | | |
| Substrate | NBY or $NBYNO_3$ | $NBYGNO_3$ | NBYG | $NBYNO_3$ | $NBYGNO_3$ | NBYG |
| Glucose | 12·08 | 13·96 | 10·33 | 3·71 | 5·94 | 3·66 |
| Pyruvate | 10·52 | 5·22 | 3·42 | 3·25 | 2·13 | 2·53 |
| Acetate | 10·79 | 0 | 0 | 3·59 | 0 | 0 |
| Citrate | 2·16 | 0 | 0 | 0·91 | 0 | 0 |
| Succinate | 13·09 | 2·01 | 0·70 | 3·57 | 0·44 | 0 |

Table 2. *Effect of growth conditions on cytochrome content*

| | Cytochrome $c_1$ $E_{552}$/10 mg. protein | | | Cytochrome $a_1$ $E_{595}$/10 mg. protein | | |
|---|---|---|---|---|---|---|
| Medium | Vigorous aeration | Weak aeration | Anaerobic | Vigorous aeration | Weak aeration | Anaerobic |
| NBY | 0·210 | 0·187 | — | 0·058 | 0·051 | — |
| $NBYNO_3$ | 0·196 | 0·143 | 0·189 | 0·064 | 0·061 | 0 |
| NBYG | 0·046 | 0·059 | 0·056 | 0·081 | 0·011 | 0 |
| $NBYGNO_3$ | 0·042 | 0·047 | 0·110 | 0·083 | 0·015 | 0 |

Table 3. *Effect of nitrate in growth and test media upon rate of acid formation and glucose consumption*

| | Acid formation ($\mu$moles/mg. protein/h.) in | | Glucose consumption ($\mu$moles/mg. protein/h.) in | |
|---|---|---|---|---|
| Cultivation medium | NBYG | $NBYGNO_3$ | NBYG | $NBYGNO_3$ |
| NBYG | 12·70 | 8·4 | 5·04 | 5·60 |
| $NBYGNO_3$ | 11·78 | 7·3 | 5·70 | 5·30 |

The cytochrome contents of bacteria grown under different conditions are given in Table 2. Although some *b*-type cytochrome was present, this could not be determined quantitatively from our spectra. Glucose strongly repressed cytochrome $c_1$ formation; under anaerobic conditions, this repression was somewhat released by nitrate.

After growth with vigorous aeration with or without glucose, cytochrome $a_1$ was present. In bacteria grown anaerobically it was completely absent. The amount of cytochrome $a_1$ in bacteria grown without glucose was the same whether they had been vigorously or weakly aerated. In cultures grown in glucose-containing media with weak aeration, however, considerably less cytochrome $a_1$ was found. This could be

explained by the fact that in glucose-free media with weak aeration 20 % of the dissolved oxygen is left, and in glucose-containing media nothing.

*Effect of nitrate upon the rate of acid formation and glucose breakdown.* The rates of acid formation and glucose consumption were measured in NBY and NBYG$NO_3$, after cultivation in these media, as described under Methods. Nitrate decreased acid formation by 40 %, but did not influence the rate of glucose breakdown (Table 3).

## DISCUSSION

When aeration of cultures of *Bacillus subtilis* is interrupted, rapid lysis ('anaerobic lysis') occurs (Nomura, 1955). Similarly, lysis of *B. subtilis* during aerobic growth is observed after exhaustion of the energy source in the medium (Monod, 1942; Hadjipetrou & Stouthamer, 1963; Baillie & Holms, 1968). In this organism, lysis occurs when the generation of ATP is interrupted. *Bacillus licheniformis* behaves similarly in that anaerobic lysis can be prevented only by the presence of the nitrate-reducing system or a fermentable carbon source. *Bacillus subtilis* and *B. licheniformis* appear to be subject to autolysis in the absence of the continued synthesis of cell wall substance (Shockman *et al.* 1961), for which ATP is required. This autolytic activity interferes with the preparation of washed suspensions; 10 % $MgSO_4.7H_2O$ protected cells of *B. licheniformis*. In this medium the bacteria retained their normal rod-like shape, perhaps because the autolytic enzymes were inhibited by the high concentration of $Mg^{2+}$ ions.

The most striking feature of the regulation of nitrate reductase formation in *Bacillus licheniformis* is the induction by anaerobiosis, even in absence of nitrate. In *Escherichia coli* (Showe & De Moss, 1968), *Aerobacter aerogenes* (Van't Riet *et al.* 1968), *Proteus mirabilis* (De Groot & Stouthamer, 1970*a*) and *Haemophilus parainfluenzae* (Sinclair & White, 1970) nitrate reductase activity is low or absent during anaerobiosis in the absence of nitrate. Showe & De Moss (1968) have suggested that the formation of nitrate reductase in *E. coli* is regulated by two repressors, one of which is sensitive to nitrate, and a second which is sensitive to the intracellular redox potential. The presence of two repressors for the regulation of nitrate reductase formation has also been postulated for *H. parainfluenzae* (Sinclair & White, 1970) and for *P. mirabilis* (De Groot & Stouthamer, 1970*a*). In *B. licheniformis* the intracellular redox potential is of prime importance in the regulation of nitrate reductase formation, and there is no evidence for regulation by nitrate.

Azoulay, Puig & Couchaud-Beaumont (1969) have shown that redox enzymes in *Escherichia coli* chlorate-resistant mutants cannot be complexed in active membrane-bound form. Similarly, Azoulay, Puig & Martins Rosado de Sousa (1969) proposed that complexing of redox enzymes is also impossible during aerobic growth. De Groot & Stouthamer (1970*b*) have observed that in *Proteus mirabilis* the formation of redox enzymes is repressed when there is no electron flow to these enzymes, and suggested that such enzymes cannot be complexed in active membrane-bound form with other electron transport components without electron flow. Induction and glucose repression of nitrate reductase in *Bacillus licheniformis* can be explained in a similar way. With weak aeration, the oxygen supply is limiting and electrons can flow to nitrate reductase. In glucose-containing media there is hardly any electron flow to nitrate reductase from other substrates, because their oxidation is inhibited (Table 1). In

medium with glucose, active fermentation of glucose occurs and consequently the total electron flow to nitrate reductase is small. The possibility that glucose acts by catabolite repression cannot be ruled out.

Inactivation of nitrate reductase has been observed in *Proteus mirabilis* only when the electron flow is withdrawn from the enzyme (De Groot & Stouthamer, 1970*a*). In *Bacillus licheniformis* nitrate reductase is less easily inactivated and occurs only with vigorous aeration in the absence of a substrate that can be respired (Fig. 5). In a complete medium there is repression, no inactivation. It seems that inactivation requires complete withdrawal of the electron flow, whereas for repression a partial withdrawal is sufficient.

The effect of glucose inhibiting oxidation of other substrates by *Bacillus licheniformis* has also been shown in *Staphylococcus aureus* (Strasters & Winkler, 1963). This is surprising, as the effect of glucose upon the citric acid cycle enzymes in the closely related species *B. subtilis* and even in another strain of *B. licheniformis* is completely different. In these organisms the enzymes of the first part of the citric acid cycle are partially repressed by glucose and almost completely repressed by a mixture of glucose and glutamate. The enzymes of the second part of the citric acid cycle are not affected (Flechtner & Hanson, 1969). Repression of cytochrome *c* by glucose is also known from *S. aureus* (Strasters & Winkler, 1963), and from *Salmonella typhimurium* (Richmond & Maaløe, 1962).

The influence of anaerobiosis upon respiration has recently been reviewed by Wimpenny (1969). Aeration of cells grown anaerobically generally leads to a three- or four fold increase of overall respiratory activity. Thus *Bacillus licheniformis* fits well into the general pattern.

Increase of cytochrome-*c* content and complete disappearance of *a*-type cytochromes during nitrate respiration has been reported for *Micrococcus denitrificans* (Scholes & Smith, 1968) and for *Bacillus stearothermophilus* (Downey & Kiszkiss, 1969). Downey & Kiszkiss (1969) suppose that disappearance of *a*-type cytochromes is a common feature of denitrifying bacteria, and that it is caused by active nitrate respiration. When *B. licheniformis* is cultivated anaerobically in NBYG medium, however, nitrate reductase is formed, but no active nitrate respiration occurs because of the absence of nitrate. So we conclude that in *B. licheniformis* disappearance of *a*-type cytochrome is merely caused by anaerobiosis.

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# The Electrophoretic Properties and Some Surface Components of Penicillium Conidia

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SUMMARY

Conidia of *Penicillium expansum* are covered with a surface layer of polyphosphate when grown on a high phosphate medium. The composition of this polyphosphate layer, which appears 2 days after conidial initiation, is dependent on the phosphate content of the growth medium; the layer is absent from conidia grown on a low phosphate medium. The rodlet layer which lies beneath the polyphosphate is free of cutin and does not consist of a unique protein. The amino acid composition of the surface protein is, however, different from that of the total wall protein. The rodlet layer appears to be an integral part of the spore wall.

The pH–mobility curves of Penicillium conidia are constant and species-specific when the fungi are grown on defined media.

## INTRODUCTION

The chemical composition of hyphal cell walls has been studied in some detail (Bartnicki-Garcia, 1968) but less attention has been paid to the physical and chemical properties of the spore wall. The composition of sporangiospore walls of *Mucor rouxii* (Bartnicki-Garcia & Reyes, 1964) and conidial walls of *Aspergillus oryzae* (Horikoshi & Iida, 1964) have been investigated, while Rizza & Kornfeld (1969) have compared the carbohydrate and amino acid composition of conidial and hyphal walls of *Penicillium chrysogenum*.

The surface ornamentation of fungal spores may be examined by the replica technique (Bigelow & Rowley, 1968) or by freeze-etching (Laseter *et al.* 1968; Hess & Stocks, 1969). The freeze-etching technique has shown that the surface of Penicillium conidia is covered with a distinctive pattern of rodlets (Hess, Sassen & Remsen, 1968).

Ionizable surface groups on fungal spores can be detected by particle electrophoresis in conjunction with chemical and enzymic treatments; the pH–mobility curves of fungal spores may be species specific. Conidia of *Penicillium expansum* are covered with a phosphate layer easily removed by washing to reveal an underlying amino–carboxyl surface (Fisher & Richmond, 1969).

The purpose of the present paper is to describe further the surface components of *Penicillium expansum* conidia and to determine whether closely related species of Penicillium can be differentiated by their pH–mobility curves.

## METHODS

*Fungal material.* Conidia from 7 day cultures of *Penicillium expansum* Link ex Thom, *P. digitatum* Sacc., *P. roquefortii* Thom, *P. thomii* Maire, kindly supplied by Mr R. C. Codner of the University of Bath, and *P. notatum* Westling (CMI 17969), from the Commonwealth Mycological Institute, were grown on malt agar and harvested as previously described (Richmond & Somers, 1963). Cultures of *P. expansum* were also grown on Fries medium (Richmond & Somers, 1962) containing 0·3, 3·0 and 6·0 g. $KH_2PO_4$/l. The pH of this medium was adjusted to 6·0. Spore walls were obtained by shaking dense spore suspensions with an equal volume of ballotini (no. 12) in a Mickle disintegrator at 4° for 15 min. (Somers & Fisher, 1967). The centrifuged walls were washed ten times with 10% (w/v) sucrose, five times with 0·9% (w/v) NaCl and five times with water following the technique of Dyke (1964). The final washing was free of u.v. absorbing material.

*Examination of water-soluble wall components.* Soluble surface material was removed from conidia by repeated washing with water or by using the Dyke (1964) technique (described above). Polyphosphates and sugars were examined in the combined water washings after concentration to 5 ml. in a rotary evaporator at 40°.

Polyphosphates were identified by thin-layer chromatography on starch (Canic, Turčic, Petrovic & Petrovic, 1965). The solvent system was trichloroacetic acid + *iso*-propanol + distilled water containing 2·5 mM-EDTA and 2·5 mM-$NH_4OH$ (5 g. + 80 ml. + 40 ml.). Polyphosphates were detected by spraying the plate with molybdate-perchloric acid (Hanes & Isherwood, 1949). For the determination of metachromatic activity, polyphosphates were precipitated from the concentrated spore washings by a saturated solution of barium acetate. The precipitate was washed, resuspended in 5 ml. water and shaken with 200 mg. Amberlite resin I R-120 ($H^+$ form). The barium-free solution was examined for metachromatic activity with 0·006% (w/v) aqueous toluidine blue (Nassery, 1969). The values of $E_{530}/E_{630}$ were then calculated. Total phosphorus was determined by the method of Hanson (1950) after digestion with $HNO_3$ followed by $HClO_4$.

Sugars in the soluble surface material were examined after hydrolysis in 0·5 M-$H_2SO_4$ for 12 h. at 105° in a sealed tube. Excess sulphate was removed by precipitation with barium hydroxide and the supernatant solution was passed down columns of Amberlite IR-120 ($H^+$ form) and 400 (acetate form). Total reducing sugars were determined by the arsenomolybdate method (Chan & Cain, 1966). Individual monosaccharides were identified by paper chromatography. The solvent system was *sec*-butanol + acetic acid + water (70 + 2 + 28, v/v). Sugars were detected by spraying the paper with *p*-anisidine (Mukherjee & Srivastava, 1952).

*Examination of surface protein.* Surface protein was extracted from spores by incubating with urea (7 M, pH 2·8) containing 10% (w/v) 2-mercaptoethanol for 1 h. at 37°. The suspension was cooled and centrifuged, and the spores were washed four times with water then incubated with 0·1 M-NaOH for 15 min. at 4°. The alkaline extract was dialysed against running water overnight (Gould, Stubbs & King, 1970).

Amino acids in the extracted non-dialysable wall material were determined on a Technicon TSM.1 amino acid analyser after hydrolysis with 6 M-HCl at 100° in an atmosphere of $N_2$ for 21 h.

*Total protein.* Total protein was determined by the method of Lowry, Rosebrough,

Farr & Randall (1951) after treatment of the walls with 2 M-NaOH at 100° for 30 min. (Shah & Knight, 1968).

*Detection of cutin acids.* Washed spore walls were refluxed with 1 % ethanolic KOH for 3 h. The fatty acids were extracted with ether, methylated with diazomethane and examined by thin-layer chromatography on Kieselguhr HR using chloroform + ethyl acetate (7 + 3) as solvent or by gas–liquid chromatography using a Hewlett-Packard 5750 gas chromatograph (Baker & Holloway, 1970).

*Freeze-etching.* Conidia were suspended in 15 % glycerol for 1 h., centrifuged into a pellet, frozen in liquid Freon 12 at −150° and then treated as described by Moor (1966). Replicas were prepared in a Balzers freeze-etching plant BA 360 M and viewed in an AEI EM6B microscope.

*Electrophoretic measurements.* The electrophoretic mobilities of conidia were measured in a laterally mounted rectangular cell enclosed in a water bath maintained at 25·0 ± 0·2° (Fisher & Richmond, 1969). Measurements were made on conidia which had been washed once with the appropriate buffer before suspension in HCl + NaCl or barbiturate + acetate buffer (*I*: 0·05) of the required pH (Gittens & James, 1963). Movement was timed over 180 $\mu$m. in both directions (current reversal). Each mobility was the mean of at least 20 observations; the standard error of the mean was less than 4 %. Mobilities are expressed as $10^{-8}$ m.$^2$ V$^{-1}$ s.$^{-1}$ (equivalent to $\mu$m. cm. V$^{-1}$ s.$^{-1}$ in c.g.s. units).

## RESULTS

*Electrophoretic properties of Penicillium conidia.* The pH–mobility curves of conidia from five species of Penicillium grown on malt agar were all different and characteristic (Fig. 1). The pH–mobility curves were affected neither by repeated reculturing of the fungi nor by storage of conidia in water for up to 2 days. There was no evidence to suggest that suspension of conidia in the acid or alkaline buffers caused any surface denaturation; after suspension in pH 7·0 buffer, mobilities were the same as those of normal control conidia. A single washing in buffer before mobilities were determined was necessary to ensure complete removal of water from the conidia, but this washing had no effect on mobility; conidia shaken dry from culture plates had the same mobility as conidia harvested by the normal procedure.

*Effect of age on pH–mobility curve of conidia of Penicillium expansum.* The pH–mobility curve of 1 day conidia grown on malt agar had a typical amino–carboxyl shape with an iso-potential point at pH 3·5 (Fig. 2). After 2 days the pH–mobility curve (Fig. 2) showed an acid surface with an iso-potential point of 2·0 closely resembling that from 7 day conidia (Fig. 1). As the shape of the pH–mobility curve showed little change after 2 days, all further tests were carried out on 7 day cultures.

*Influence of growth medium on pH–mobility curve.* The pH–mobility curve of conidia grown on Fries medium (3·0 g./l. $KH_2PO_4$) was quite different from that of conidia grown on malt agar and indicated an amino–carboxyl surface (Fig. 3). When the phosphate content of the Fries medium was reduced to 0·03 g./l. $KH_2PO_4$ the pH–mobility curve had a similar shape although mobilities were higher above pH 7·0. When the phosphate content of the medium was increased to 6·0 g./l. $KH_2PO_4$ an entirely acid surface was formed (Fig. 3).

*Effect of washing on pH–mobility curve.* The surface of conidia grown on malt agar became progressively less acid as the conidia were washed, until a typical amino–

carboxyl surface was revealed (Table 1). The rapid increase in iso-potential point from 2·0 to 3·4 after only five washings shows that most surface phosphate was easily removable. Further washings produced only a small increase in iso-potential point, but the large increase in positive mobility at pH 2·0 and the similar increase in negative at pH 5·0 suggests that prolonged washing removed non-ionogenic material. When conidia grown on Fries medium with the highest phosphate content were washed the

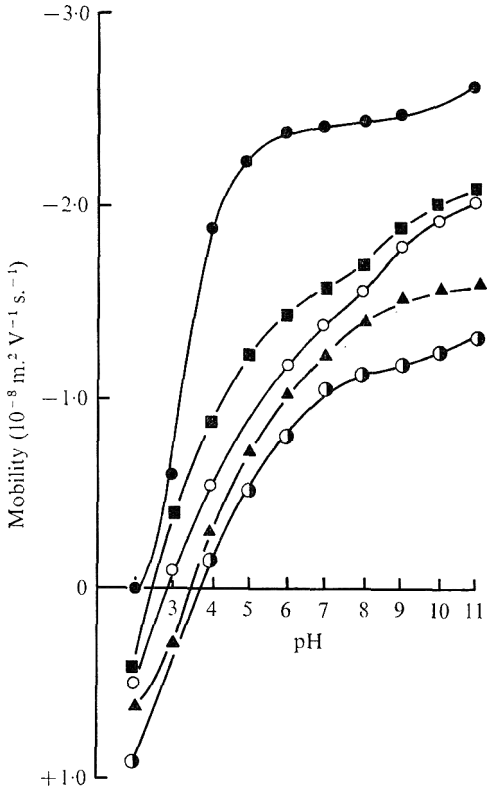


Fig. 1

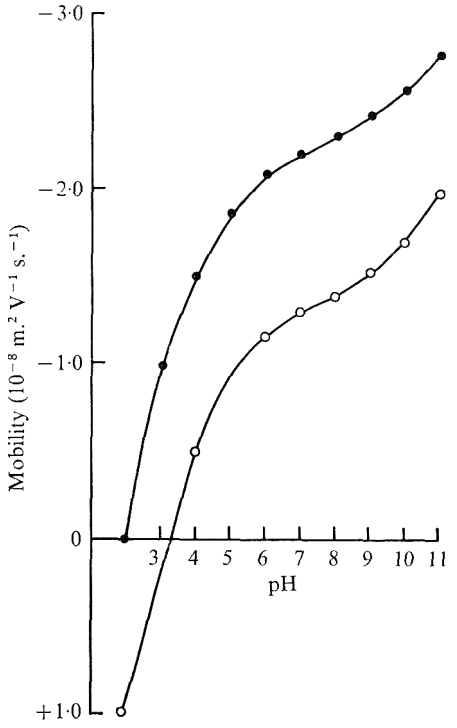


Fig. 2

Fig. 1. pH–mobility curves of *Penicillium expansum*, ●——●; *P. thomii*, ■——■; *P. roquefortii*, ○——○; *P. digitatum*, ▲——▲; and *P. notatum*, ◑——◑, 7 day conidia from malt agar.

Fig. 2. pH–mobility curves of *Penicillium expansum*, 1 day conidia, ○——○; 2 day conidia, ●——●, from malt agar.

Table 1. *Mobilities of washed conidia of Penicillium expansum grown on malt agar*

| Washing procedure | Iso-potential point (pH) | Mobilities ($10^{-8}$ m.$^2$ V$^{-1}$ s.$^{-1}$) pH 2·0 | pH 3·0 | pH 4·0 | pH 5·0 |
|---|---|---|---|---|---|
| Control unwashed | 2·0 | 0·00 | −0·60 | −1·87 | −2·23 |
| Water (×5) | 3·4 | +0·25 | +0·08 | −0·13 | −0·36 |
| Water (×10) | 3·5 | +0·55 | +0·24 | −0·21 | −0·42 |
| Water (×15) | 3·6 | +0·63 | +0·37 | −0·33 | −0·71 |
| 10 % Sucrose (×10), 0·9 % NaCl (×5), Water (×5) | 3·7 | +1·08 | +0·77 | −0·55 | −1·10 |

acid surface was only partially removed even after prolonged washing (Fig. 4). The acid groups appeared to have become an integral part of the surface possibly because the polyphosphate was present in a higher polymeric form.

*Composition of spore washings.* The amount of phosphorus compounds washed from *Penicillium expansum* conidia varied widely with the nature and phosphorus content of the medium (Table 2). The small amount of phosphorus removed from conidia

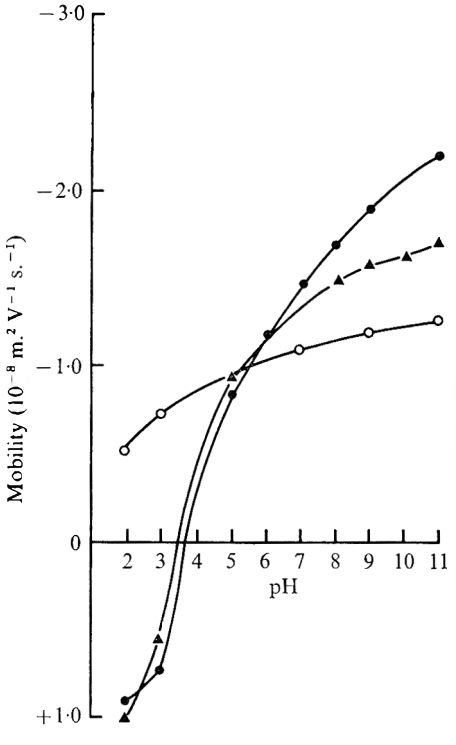


Fig. 3

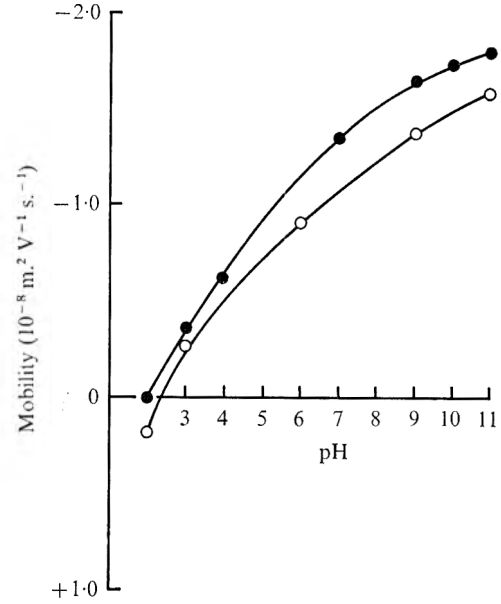


Fig. 4

Fig. 3. pH–mobility curves of *Pencillium expansum* grown on Fries medium, containing 0·03 g./l. $KH_2PO_4$, ●——●; 0·30 g./l. $KH_2PO_4$, ▲——▲; 6·0 g./l. $KH_2PO_4$, ○——○.

Fig. 4. pH–mobility curves of *Penicillium expansum* grown on Fries medium containing 6·0 g./l. $KH_2PO_4$, conidia washed ten times with 10 % (w/v) sucrose, five times with 0·9 % (w/v) NaCl and five times with water, ●——●; conidia washed twice by above procedure, ○——○.

grown on Fries medium with the highest phosphate content is in agreement with the electrophoretic results which showed that even prolonged washing had little effect on the acid surface (Fig. 4). The metachromatic activity of the washings established that polyphosphates were present on all conidial surfaces. The highest polyphosphate content occurred on conidia grown on malt agar but small amounts were present on conidia grown on Fries medium. Conidial production was greater on malt agar than on Fries medium, even when the phosphorus contents of the two media were similar. Thin-layer chromatography of the concentrated water washings from conidia grown on malt agar confirmed the presence of polyphosphates and showed them to contain less than ten phosphorus atoms. The extracted material was free of nucleic acid, having

no absorption at 260 nm., and imidophosphate linkages were absent as no infrared absorption peak occurred at 7·15 $\mu$m. (Correll, 1966).

As prolonged washing of conidia grown on malt agar seemed to remove non-ionogenic material (Table 1) the water washings were examined for carbohydrates. The total reducing sugars (as glucose) from 20 successive water washes was 7·1 $\mu$g./g. dry wt of spores before hydrolysis and 12·6 $\mu$g./g. after. Glucose and xylose were present before hydrolysis and, in addition, arabinose after hydrolysis.

Table 2. *The effect of growth medium on the phosphorus content of conidia and conidial washings from Penicillium expansum*

Conidia were grown, harvested and washed as described in the text.

| Medium | Phosphorus (g./l.) | Dry wt of conidia* (mg.) | Dry wt of conidia (mg./g.) | | Phosphorus removed by washing (%) | Meta-chromatic activity of washings† |
|---|---|---|---|---|---|---|
| | | | Phosphorus in conidia | Phosphorus in conidial washings | | |
| Malt agar | 1·33 | 810 | 10·7 | 0·15 | 1·4 | 0·17 |
| Fries | 0·07 | 74 | 9·8 | —‡ | —‡ | 0·05 |
| | 0·70 | 584 | 9·3 | 0·07 | 0·8 | 0·04 |
| | 1·40 | 561 | 9·3 | 0·04 | 0·4 | 0·03 |

* From 50 plates. † $E_{530}/E_{630}$ nm. − blank. ‡ = Not determined.

*Nature of the rodlet layer.* Hess *et al.* (1968) suggested that the outer rodlet layer on Penicillium conidia might consist of cutin or a similar material, since they found the layer to be removed by treatment with aqueous or ethanolic KOH. None of the hydroxy fatty acids characteristic of cutin (Baker & Holloway, 1970) could be detected in ethanolic KOH wall extracts either by thin-layer or by gas–liquid chromatography. The fatty acids extracted from the cell walls after methylation and analysis by gas–liquid chromatography consisted mainly of palmitic (46·5 % of total peak area), oleic (19·9 %) and stearic (19·5 %) acids, although five other acids were present in small amounts. Although many studies have been made on the lipid composition of fungi (Shaw, 1966) less work has been done on isolated cell walls. Laseter, Weete & Weber (1968) have, however, found that palmitic and oleic acids were the most abundant fatty acids in the surface wax from chlamydospores of *Ustilago maydis*.

The rodlets on freeze-etched Pencillium conidia closely resemble the patterns seen on some bacterial spores (Holt & Leadbetter, 1969). The patterned surface layer on *Bacillus coagulans* spores consists of an alkali-soluble protein which can be removed from the spores by treatments which rupture disulphide bonds (Gould *et al.* 1970). Examination of freeze-etched replicas of *Penicillium expansum* conidia showed that the surface rodlets were not removed by incubation with mercaptoethanol in urea followed by treatment with 0·1 M-NaOH. The rodlets were, however, less distinct after alkali treatment, suggesting that some protein material may have been removed. The electrophorectic mobility of water-washed conidia fell, after alkali treatment, from $-2{\cdot}0$ to $-1{\cdot}16\times10^{-8}$ m.$^2$ V$^{-1}$ s.$^{-1}$ at pH 10·0 and from $+1{\cdot}1$ to $+0{\cdot}64\times10^{-8}$ m.$^2$ V$^{-1}$ s.$^{-1}$ at pH 2·0. The iso-potential point was the same in control and treated spores. The decreases in mobility confirm that the treatment may have partially removed some protein material. Analysis of the non-dialysable alkali-soluble material showed it to contain 11·2 % protein. The remainder of the extracted material probably consisted

of polymeric polysaccharides (Grisaro, Sharon & Barkai-Golan, 1968). The extracted protein which represents only a small proportion of the total conidial wall protein (Table 3) had a high tyrosine and methionine content, proline was absent, and threonine, leucine, isoleucine, histidine, valine and cyst(e)ine were low as compared with conidial wall protein (Table 4). The distinctive amino acid composition of the extracted protein confirmed the partial removal of a definite surface layer. The total conidial wall protein was similar in composition to the cytoplasmic protein. Mercaptoethanol pretreatment had little effect on the removal of surface protein and the amino acid composition of the alkaline extract was the same whether mercaptoethanol was used or not.

Table 3. *Some components of conidial walls of Penicillium expansum grown on malt agar*

| | % |
|---|---|
| Protein | 7·67 |
| Phosphorus | 0·23 |
| Nitrogen (by Kjeldahl) | 3·32 |
| Alkali-soluble surface protein | 0·37 |
| Other alkali-soluble material | 2·92 |

Table 4. *Amino acid components of Penicillium expansum conidia*

| | Mole ratio | | |
|---|---|---|---|
| Amino acid | Alkaline extract from conidia | Conidial wall | Cytoplasm |
| Aspartic acid | 1·38 | 1·25 | 1 24 |
| Glutamic acid | 1·48 | 1·13 | 1·11 |
| Threonine | 0·50 | 1·07 | 0 84 |
| Serine | 1·00 | 1·10 | 1 14 |
| Alanine | 1·00 | 1·00 | 1 00 |
| Glycine | 1·13 | 1·02 | 0 85 |
| Leucine | 0·49 | 0·76 | 0 74 |
| Phenylalanine | 0·40 | 0·31 | 0 40 |
| Lysine | 0·26 | 0·31 | 0 20 |
| Isoleucine | 0·18 | 0·39 | 0 23 |
| Arginine | 0·43 | 0·50 | 0 48 |
| Tyrosine | 1·09 | 0·31 | 0·36 |
| Proline | n.d. | 0·77 | 0·51 |
| Histidine | 0·30 | 0·61 | 0·53 |
| Valine | 0·34 | 0·72 | 0·48 |
| Half cystine | 0·23 | 1·30 | 1·78 |
| Methionine | 0·86 | 0·13 | 0·13 |

n.d. = Not detected.

## DISCUSSION

It is at first sight surprising that closely related species should have such widely different mobility curves; the explanation probably lies in the presence of a polyphosphate layer which is not an integral part of the conidial surface.

The presence of surface phosphate on conidia of *Penicillium expansum* was previously demonstrated by treatment with acid phosphatase and confirmed by the decrease in mobility at pH 7·0 which occurred in the presence of $Ca^{2+}$. Removal of phosphate by enzyme action or prolonged washing revealed an underlying amino–carboxyl surface

(Fisher & Richmond, 1969). The curves of the other Penicillium species (Fig. 1) are indicative of amino–carboxyl surfaces containing varying amounts of phosphate. The curve of *P. notatum* is remarkably similar to that of *P. expansum* after complete phosphate removal (Fisher & Richmond, 1969).

No imidophosphate linkages or nucleic acids were detected in extracts from *Penicillium expansum* conidia and phospholipids are absent as the surface is lipid-free (Fisher & Richmond, 1969). Although surface phosphate groups have been detected on yeast cells (Eddy & Rudin, 1958) and *Neurospora crassa* conidia (Somers & Fisher, 1967) as well as on *P. expansum* conidia (Fisher & Richmond, 1969), the exact nature of the phosphate was not specified. Rothstein & Meier (1951) have, however, suggested that uranyl ions may react with polyphosphate-like groups on the yeast surface, and Harold (1962) has shown that cytoplasmic polyphosphate can bind to hyphal walls of *N. crassa*.

The Penicillium conidium has a three-layered wall covered with an outer patterning of rodlets (Sassen, Remsen & Hess, 1967). Carbon replica studies suggest that the rodlets are themselves covered with an additional very thin film (Hess *et al.* 1968). This film may constitute the polyphosphate layer present on unwashed conidia. Our results show that the polyphosphate layer is not an integral part of the conidial surface. Eddy & Rudin (1958) also found that phosphate groups were absent from the surface of cells grown in phosphate-deficient media.

No specific function can, at present, be suggested for the surface polyphosphate which may act simply as an inorganic phosphate reserve (Harold, 1966). Alternatively, polyphosphate may be implicated in phosphorylation reactions involved in the transport of glucose into the cell on germination (Rothstein & Meier, 1951).

The presence of free xylose in the carbohydrate layer of conidia is of interest. Xylose has been found in hyphal walls of *Penicillium chrysogenum* (Hamilton & Knight, 1962), *P. digitatum* and *P. italicum* (Grisaro *et al.* 1968). Rizza & Kornfeld (1969) were, however, unable to detect xylose in either hyphal or conidial walls of *P. chrysogenum*. Arabinose which was found in the carbohydrate layer after hydrolysis has also been found in small amounts in hyphal walls of *Aspergillus niger* (Johnston, 1965).

The rodlet layer appears to be an integral part of the wall structure since it is not easily separated from the rest of the wall by mechanical disruption. This layer is free of cutin and is not composed of a unique protein. The surface layer does, however, contain protein of a different amino acid composition from that present in the whole wall. The amino acid composition of the whole conidial wall is similar to that of the hyphal wall of *Penicillium notatum* (Applegarth, 1967) except for the presence of valine. Conidial walls of *P. chrysogenum* are, however, quite distinct, since tyrosine, phenylalanine, methionine and histidine are absent (Rizza & Kornfeld, 1969). Fungal cell walls have frequently been reported to contain a full complement of amino acids (Crook & Johnston, 1962; Shah & Knight, 1968; Aronson & Fuller, 1969).

The high tyrosine and methionine content of the surface protein may be significant. Tyrosine is a precursor of melanin, which can protect fungi from enzymic lysis (Kuo & Alexander, 1967; Bull, 1970), while methionine as *S*-adenosylmethionine, an important methyl donor in plants (Meister, 1965), may detoxify injurious substances. Although the rodlet layer superficially resembles the surface layer of *Bacillus coagulans* spores (Gould *et al.* 1970), the two layers are different in structure and composition.

Water repellency can probably be attributed to the rodlet layer. The polyphosphate

layer does not contribute materially to the water-repellent properties of the spores since conidia grown on low-phosphate media lack polyphosphate and are still water-repellent.

The Penicillium spore surface has previously been shown to be lipid-free (Fisher & Richmond, 1969); the present work has not found any other substances which could be responsible for water-repellency. The physical conformation of the surface may itself be sufficient to prevent wetting.

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# Effect of Light on β-Carotene Accumulation in *Blakeslea trispora*

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SUMMARY

Cultures of a plus and a minus strain of *Blakeslea trispora*, grown either separately or together in light, accumulated only 40% of the β-carotene of corresponding cultures grown in darkness. The trisporic acids which accumulated in culture media of plus and minus strains grown together in light differed in their extinction spectra from the trisporic acids in the medium of cultures grown in darkness. Exposure of a trisporic acid extract from dark-grown cultures to light altered its extinction spectrum and decreased its carotenogenic activity. Only the ultraviolet portion of the spectrum of the lamps employed brought about this change in the extinction spectrum of the trisporic acid extract. The visible portion of the spectrum, however, was able to depress β-carotene accumulation in cultures without bringing about a change in the extinction spectrum of the trisporic acids produced. It seems likely that the formation of trisporic acids with altered extinction spectra is not responsible for the decrease in the accumulation of β-carotene.

## INTRODUCTION

*Blakeslea trispora* Thaxter, a zygomycete, order Mucorales, is heterothallic. Plus (+) and minus (−) strains grown separately synthesize small amounts of β-carotene. Plus and minus strains grown together (+/−) reproduce sexually, synthesize more β-carotene than separate + and − strains (Ciegler, 1965; Plempel, 1965; Sutter & Rafelson, 1968) and produce a family of acidic compounds termed β-factor or trisporic acids (Prieto, Spalla, Bianchi & Biffi, 1964; Sebek & Jager, 1964; Caglioti *et al.* 1966). Trisporic acids enhance β-carotene production when added to cultures of the − strain (Prieto *et al.* 1964; Sebek & Jager, 1964; Thomas, Harris, Kirk & Goodwin, 1967; Sutter & Rafelson, 1968; van den Ende, 1968) and are postulated to be sex hormones in *B. trispora* (van den Ende, 1968; Austin, Bu'Lock & Gooday, 1969; Reschke, 1969; van den Ende, Wiechmann, Reyngoud & Hendricks, 1970). This report is on the effect of light on β-carotene accumulation in +, −, and +/− cultures and on the trisporic acids produced by +/− cultures.

## METHODS

*Strains.* Plus and minus strains of *Blakeslea trispora* (USDA, Northern Regional Research Laboratories, no. 9216 and 9159, respectively) were obtained from Dr George Scherr of Colab Laboratories, Chicago Heights, Illinois.

*Medium and culture methods.* Maintenance of cultures and culture techniques have been described elsewhere (Sutter & Rafelson, 1968). Cultures were grown in 500 ml.

Erlenmeyer flasks containing 150 ml. of medium composed of (%, w/v): potato extract, 1·25; glucose, 2·0; thiamine hydrochloride, 0·0002. Cotton plugs were used on culture flasks. Incubation was at 250 rev./min. on an inch stroke Gyrotory-Incubator Shaker Model G-25 (New Brunswick Scientific Co. Inc., New Jersey) at 28°. Cultures grown in darkness were in flasks wrapped with three layers of aluminium foil, which was also placed around the tops of flasks of cultures grown in light.

*Light sources and illumination procedures.* Cool white, white, red and blue fluorescent (F15T8-CW, F40W, F40R, F40B, respectively) and red incandescent (40 W) lamps were made by Sylvania (Salem, Massachusetts) and black fluorescent (F40T12-BLB) and sun (275 W) lamps by General Electric (Schenectady, New York). Light sources were switched on 5 min. before experiments. The spectral distribution of radiation between 380 and 1100 nm. from light sources was measured with an ISCO (Instrumentation Specialties Company, Lincoln, Nebraska) Spectroradiometer Model SR. The instrument was calibrated with an ISCO Spectroradiometer Calibrator Model SRC with a lamp calibrated against a National Bureau of Standards lamp. The direct readout of the instrument was converted to intensity ($\mu$W/cm.$^2$) by the appropriate correction factor. Intensity was plotted against wavelength to give a curve of spectral intensity. The intensity of radiation between given wavelengths was obtained by comparing the area under the corresponding portion of the spectral distribution curve with the total area beneath the curve.

Experiments on the effect of light on trisporic acid extracts were performed at 28° in 18 mm. × 150 mm. test-tubes which were capped with parafilm and contained 5 ml. of extract. Dark controls were wrapped in five layers of aluminium foil. In experiments with filters, a fan circulated air between the light source and filter to prevent destruction of the filter.

*Extraction and analytical methods.* The extraction and analysis of $\beta$-carotene and trisporic acids have been described elsewhere (Sutter & Rafelson, 1968). In preparing tris buffer extracts of trisporic acids, the chloroform extracts of culture filtrates adjusted to pH 2 were flash evaporated at 28°. The residue, which contained trisporic acids, was dissolved in 0·1 M-tris–$H_2SO_4$ (pH 7·5) and stored at −20°. The relative concentration of trisporic acids in tris buffer extracts was expressed as the total extinction at 328 nm. (i.e. the $E_{328}$ reading times the dilution factor). The relative concentration of trisporic acids in +/− culture filtrates was expressed as the net extinction at 325 nm. (i.e. the $E_{325}$ of the +/− culture filtrates less the $E_{325}$ of the − culture filtrates). Extinction spectra were measured with a Hitachi Double Beam Spectrophotometer Model 124 and Recorder Model 165 (Coleman Instruments, Maywood, Illinois). All operations involving potentially light-labile compounds were performed under red fluorescent lamps. All experiments were performed at least three times.

## RESULTS

*Effect of light on $\beta$-carotene accumulation.* The effect of continuous illumination by white fluorescent lamps upon growth, $\beta$-carotene accumulation and trisporic acids in *Blakeslea trispora* is shown in Table 1. The dry weights of +, −, and +/− cultures grown in light differed little from comparable cultures grown in darkness. However, +, −, and +/− cultures grown in light accumulated only 40% of the $\beta$-carotene of comparable cultures grown in darkness. This observation suggests a similar mechanism

might be responsible for decreased β-carotene accumulation in both single and combined cultures. *Choanephora cucurbitarum* (Chu & Lilly, 1960) and *Blastocladiella emersoni* (Cantino & Horenstein, 1956) also accumulate less carotene when grown in light than in darkness. In contrast, most carotenoid-producing micro-organisms such as *Fusarium aquaeductuum* (Rau, Feuser & Rau-Hund, 1967), *Mycobacterium* species (Howes & Batra, 1970), *Neurospora crassa* (Harding, Huang & Mitchell, 1969), and *Phycomyces blakesleeanus* (Lilly, Barnett & Krause, 1957) accumulate more carotenoids when grown in light than in darkness.

Table 1. *Effect of light on growth, β-carotene and trisporic acid production by cultures of Blakeslea trispora*

| | | | | Trisporic acids | |
|---|---|---|---|---|---|
| Conditions | Strains | Growth† | β-Carotene‡ | Culture filtrates§ | Tris buffer extracts‖ |
| Darkness | + | 753 | 381 | — | — |
| | − | 821 | 303 | — | — |
| | +/− | 795 | 2550 | 0·71 | 2·62 |
| Light* | + | 851 | 161 (42 %) | — | — |
| | − | 916 | 107 (35 %) | — | — |
| | +/− | 783 | 1000 (39 %) | 0·41 | 1·14 |

* Continuous illumination by cool-white fluorescent lamps. Intensity, 413 μW/cm.²; wavelengths, 380 to 725 nm.

† Dry weight of mycelium (mg.) after 5·2 days growth.

‡ μg. β-Carotene/g. dried mycelium. Yields in light are also expressed as a percentage of corresponding yields in darkness.

§ Net extinction at 325 nm. of culture filtrate diluted 20-fold with tris buffer.

‖ Extinction at 328 nm./extinction at 258 nm.

*Effect of light on trisporic acids accumulation.* Plus/minus cultures grown in darkness appeared to accumulate twice as much trisporic acid as cultures grown under fluorescent lamps, as judged by the extinction at 325 nm. of their culture filtrates. However, when the spectra of tris buffer extracts of trisporic acids were examined, it was found that the trisporic acids from cultures grown under white fluorescent lamps had a spectrum different from that of the trisporic acids from cultures grown in darkness (Fig. 1). The ratio of extinction at 328 nm. to extinction at 258 nm. ($E_{328}/E_{258}$) of trisporic acid extracts from cultures grown in light was 1·14 instead of 2·62, the value with extracts from dark-grown cultures. The extinction at 328 nm. was decreased, the 328 nm. peak was shifted to 303 nm., and the extinction at 258 and 234 nm. was increased.

*Ultraviolet irradiation of extracted trisporic acids.* Light has been shown to alter the extinction spectrum of extracts of trisporic acids (Caglioti *et al.* 1966; van den Ende, 1967). Van den Ende (1967) observed that the alteration was accompanied by decreased sex hormone activity. To test if light also decreased carotenogenic activity, 8 ml. samples of filter-sterilized tris buffer extracts of trisporic acid ($E_{328}$ = 438) were placed 36 in. from a sun lamp (which emits mainly u.v. radiation) for various times (0 to 300 min.) to obtain extracts with a series of different $E_{328}/E_{258}$ ratios from 2·74 to 1·30. Three ml. of each extract were added to each of two 26 h. −cultures in 70 ml. medium in 250 ml. flasks. Cultures were incubated for 4 days and the amounts of β-carotene determined. The production of β-carotene decreased linearly with the $E_{328}/E_{258}$ ratio

of added trisporic acid (Fig. 2). In another experiment with trisporic acid extracts with $E_{328}/E_{285}$ ratios ranging from 2·5 to 0·8, $\beta$-carotene production also decreased linearly. Thus loss of carotenogenic activity accompanied the changes in the spectrum of irradiated trisporic acids. It was also shown that the rate at which the extinction spectrum was altered was dependent upon the concentration of trisporic acids, light intensity and the type of light-source employed.

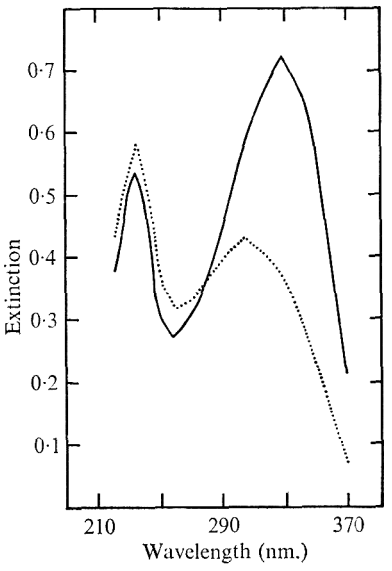


Fig. 1

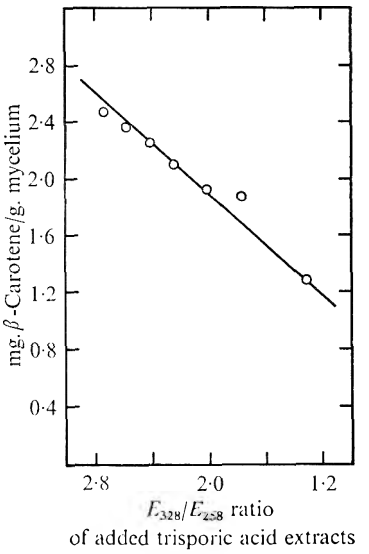


Fig. 2

Fig. 1. Extinction spectra of tris buffer extracts of culture filtrates from 5·2 day +/− cultures grown in the presence (dotted) and absence (solid line) of cool-white fluorescent light (intensity, 413 $\mu$W/cm.$^2$; wavelength, 380 to 725 nm.). Portions of tris buffer extract (7 ml.) were diluted 200-fold for measurements.

Fig. 2. Effect of near u.v. radiation upon carotenogenic properties of trisporic acids. Trisporic acid extracts ($E_{328}$ = 438) were exposed to a sun lamp for 0 to 300 min. to obtain acids with different $E_{328}/E_{258}$ ratios prior to their addition to − cultures.

*Effect of visible radiation on trisporic acids and carotene.* Since trisporic acids absorb only in the u.v. region of the spectrum, lamps not emitting u.v. radiation should not affect the extinction spectrum of trisporic acids. This was confirmed as no detectable changes occurred in the spectrum of extracts ($E_{328}$ = 1·2) exposed to red fluorescent (107 $\mu$W/cm.$^2$; 575 to 850 nm.) or red incandescent (533 $\mu$W/cm.$^2$; 575 to 1100 nm.) lamps for 48 h. although extracts exposed to black fluorescent or sun lamps (both of which emit large amounts of u.v. radiation) exhibited rapid changes in extinction spectra. To test whether u.v. radiation alone was responsible for altering the spectrum of extracts, samples were exposed for 4 h. to light from one blue and two white fluorescent lamps which had been passed through filters to eliminate u.v. radiation. No detectable changes occurred in the spectrum of extracts when filters excluded radiation below either 475 or 400 nm. although extensive alteration occurred in controls not protected by filters (Table 2). Separate experiments showed that slight changes occurred in the spectrum of extracts exposed for 24 h. to light from which u.v. radiation

was excluded by a filter of Mylar W (DuPont, Delaware). This can be accounted for as Mylar W transmits and trisporic acids absorb slight amounts of radiation at 400 nm.

To determine if the decreased accumulation of β-carotene in +/− cultures grown under white fluorescent lamps resulted from alteration of trisporic acids or whether the two events occurred independently, +, −, and +/− cultures were grown in darkness and in light from which u.v. radiation was excluded by Mylar W. All the illuminated cultures accumulated less than half the β-carotene of comparable cultures in darkness (Table 3), a result similar to that obtained in experiments in which cultures were grown in light from which u.v. radiation was not excluded (Table 1). The trisporic acid extracts from both light-grown and dark-grown +/− cultures, however, exhibited

Table 2. *Effect upon trisporic acids of light of various wavelengths and intensities*

| | Illumination† | | Trisporic acids§ $E_{328}/E_{258}$ | |
|---|---|---|---|---|
| Filters | Intensity‡ | Wavelength | Light | Darkness |
| None | 150 | 380 to 725 nm. | 1·25 | 2·61 |
| Kapton 100H* | 75 | 475 to 725 nm. | 2·61 | 2·61 |
| Mylar W* | 148 | 400 to 725 nm. | 2·61 | 2·60 |

* Names are registered trademarks of DuPont. Two sheets of filter were placed between two sheets of window glass over a black box containing the samples of trisporic acid.

† Two white and one blue fluorescent lights. Exposure time 4 h.

‡ Intensity in μW/cm.².

§ $E_{328} = 1·2$.

Table 3. *Effects upon Blakeslea trispora of light from which u.v. radiation was excluded by Mylar W*

| | | | | Trisporic acids | |
|---|---|---|---|---|---|
| Conditions | Strains | Growth† | β-Carotene‡ | Culture filtrates§ | Tris buffer extracts‖ |
| Darkness | + | 823 | 329 | — | — |
| | − | 860 | 321 | — | — |
| | +/− | 774 | 3095 | 0·62 | 2·65 |
| Light* | + | 843 | 237 | — | — |
| | − | 975 | 114 | — | — |
| | +/− | 816 | 1350 | 0·61 | 2·35 |

* Continuous illumination by cool-white fluorescent lamps. Intensity, 290 μW/cm.²; wavelengths, 400 to 725 nm.

† Dry weight of mycelium (mg.) after 5·3 days growth.

‡ μg. β-Carotene/g. dried mycelium.

§ Net extinction at 325 nm. of culture filtrate diluted 20-fold with tris buffer.

‖ Extinction at 328 nm./extinction at 258 nm.

similar extinction spectra. Thus less β-carotene accumulated in +/− cultures grown in light from which u.v. radiation was excluded even though the nature and amounts of trisporic acids was unchanged by illumination. Therefore, the accumulation of less β-carotene and the alteration of the extinction spectrum of trisporic acids in +/− cultures grown under white fluorescent lamps are independent events even though the change in extinction spectrum of extracted trisporic acids is accompanied by a reduced carotenogenic activity.

*Possible relationship between trisporic acids and β-carotene accumulation.* Trisporic acids are synthesized by − cultures (Sutter, 1970; van den Ende *et al.* 1970) and can stimulate carotenogenesis in − cultures. Since the latter reaction is inhibited by actidione (Thomas *et al.* 1967) and since labelled trisporic acids are not incorporated into β-carotene (Sebek & Jager, 1964), trisporic acids presumably act by inducing *de novo* synthesis of rate-limiting enzymes in the carotenoid pathway. It seems likely that in +/− cultures grown in light the trisporic acids synthesized by minus cultures induce carotenogenesis before being released into the medium where their extinction spectra are altered with a concomitant loss of biological activity. A remote possibility does exist that trisporic acids in the mycelium might be acted upon by visible light via an endogenous photosensitizer such as riboflavin prior to the induction step, thereby reducing carotene synthesis in +/− cultures. However, the percentage decrease in accumulation of β-carotene in all light-grown as compared with dark-grown cultures (+, −, +/−) is very similar. This strongly suggests that the same mechanism, such as increased destruction of β-carotene, is operative in all light-grown cultures.

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# Induction of DNA Breakdown and Inhibition of Cell Division by Colicin E2. Nature of Some Early Steps in the Process and Properties of the E2-specific Nuclease System

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(*Accepted for publication 15 October 1970*)

SUMMARY

DNA breakdown was detected 3 to 4 min. after addition of colicin E2 to sensitive cells; inhibition of cell division followed 5 to 10 min. later, but inhibition of DNA synthesis was observed only after several more minutes. Adsorption of E2, which takes place even at 4°, led to the formation of a specific surface complex (I). Complex I did not promote DNA breakdown. We suggest that the transition from this complex to a surface complex (II) which promoted DNA breakdown depended upon several factors which include temperature, concentration of E2, specific membrane proteins and, under certain conditions, high concentrations of extracellular $KH_2PO_4$. The formation of complex II did not depend on concomitant DNA or protein synthesis. The continued promotion of DNA breakdown by complex II and its associated nuclease was blocked by inhibition of energy metabolism. In addition, the removal of E2 from the cell surface by trypsin treatment during the early stages of the process greatly decreased the rate of DNA breakdown. E2-induced DNA breakdown, which appears to commence from a limited number of chromosomal sites, proceeded normally in $UVr^-$, $RecB^-$, $RecC^-$, $Hsr^-$, $Hss^-$, $PolA^-$ and in several tsDNA replication mutants.

## INTRODUCTION

Colicins appear to act by inducing physicochemical changes in the membrane, which promote specific intracellular changes in the sensitive cell. Evidence has been presented that: (1) Colicin E2 adsorbs to, but does not penetrate, the surface layer of sensitive bacteria (Maeda & Nomura, 1966); (2) E2 does not induce detectable changes in the permeability of the membrane (Nomura, 1964); (3) bacteria may be rescued from the lethal effects of E2 for several minutes after adsorption, by digestion of the extracellular colicin with trypsin (Reynolds & Reeves, 1963; Maeda & Nomura, 1966); (4) mutants which still adsorb E2 but remain insensitive to its presence appear to have altered membranes (Holland *et al.* 1970; Samson & Holland, 1970).

To explain the mode of action of colicin, Nomura (1964) proposed that specific transmission systems must exist in the membrane which connect the extracellular colicin to its intracellular target. Such a transmission system or pathway should therefore have specific and identifiable intermediates between the initial colicin-surface complex and the final modified cell component. Since a primary effect of E2 action is rapid degradation of DNA (Nomura, 1963; Holland, 1968), we assumed for the

purposes of this investigation that a final product in the E2 pathway is a specific DNA–nuclease complex which promotes rapid degradation of DNA. We further assumed that the release of soluble breakdown products of DNA provides a reasonable measure of formation of this complex.

In this study, attempts have been made to establish more clearly the presence of intermediate steps in colicin E2 action and to determine the nature of the processes which affect their formation. The mechanism of DNA breakdown itself appears to proceed via a specific mechanism unrelated to degradative systems involved in known repair or recombination processes.

## METHODS

*Strains. Salmonella typhimurium* LT2 strain 906, a colicinogenic strain carrying the E2 (P9) factor, was the source of colicin E2 (Holland, 1968). *Escherichia coli* K12 laboratory strain 206 (Hfr *thy*$^-$ *his*$^-$ $\lambda^-$) was used as the colicin-sensitive strain. The bacteria were normally grown in Difco nutrient broth supplemented with appropriate amounts of thymine.

*Colicin E2.* Colicin was used as a crude sterile lysate prepared from a mitomycin C induced culture of strain 906 as described previously (Hill & Holland, 1967). This material contained no antibacterial activity in addition to E2 (Hill & Holland, 1967) and behaved in all respects like highly purified preparations of E2 (Holland, 1968). For the assay of colicin, serial dilutions of the E2 preparations were spotted on nutrient broth agar plates previously seeded with strain 206. After 14 h. at 37° the highest dilution giving the minimal inhibition of growth was determined and the reciprocal of this dilution taken as the colicin titre. Preparations usually contained $2 \times 10^5$ arbitrary units of colicin E2/ml. and were stable for several months at 4°. Concentrations of 0·2 to 0·4 units of E2/ml./$10^8$ bacteria normally killed 50% at 37°.

*Bacterial counts.* For total bacterial counts, 0·05 ml. culture samples were diluted into non-pyrogenic N-saline (Polyfusor, Boots Pure Drug Co. Ltd, Nottingham) and counted in duplicate in a model F Coulter counter at densities around $2 \times 10^4$ bacteria/ml.

*Determination of DNA breakdown in treated cultures.* Strain 206 was aerated in nutrient broth + 2·5 $\mu$g. thymine/ml., until it reached $5 \times 10^7$ bacteria/ml. The culture was diluted tenfold into the same medium containing 40 $\mu$Ci methyl-[$^3$H]thymine (21·8 Ci/mmole)/ml.; incubation was continued for four to five generations to about $2 \times 10^8$ bacteria/ml. The culture was washed twice in nutrient broth + 25 or 100 $\mu$g. cold thymine/ml. and finally suspended in this medium at about $2 \times 10^7$ bacteria/ml. The labelled culture was then incubated at 37° for 10 min., colicin E2 was added and samples (0·2 ml.) were removed at intervals, mixed with 0·2 ml. ice-cold 10% trichloroacetic acid. After standing 60 min. on ice, the samples were analysed for cold and hot acid-soluble radioactivity as described by Howard-Flanders & Theriot (1966). Radioactive samples were counted with a Packard Tri-Carb scintillation counter.

*Measurement of DNA synthesis.* Incorporation of [$^3$H]thymine into acid-precipitable material was used as a measure of DNA synthesis in treated cultures as described previously (Holland, 1968).

*Materials.* Mitomycin C, trypsin (type III, twice crystallized) and trypsin inhibitor (Soybean, type I-S, twice crystallized) were obtained from the Sigma Chemical Company. Nalidixic acid was a gift from Bayer Products, Surbiton-upon-Thames, Surrey.

## RESULTS

### *Kinetics of DNA breakdown and inhibition of cell division induced by colicin E2*

Previous studies indicated that colicin E2 causes inhibition of cell division in addition to induction of rapid DNA breakdown (Holland, 1968). To determine which was the primary effect, the kinetics of the appearance of these two consequences of colicin action were measured in cultures of *Escherichia coli* strain 206 prelabelled with [$^3$H]thymine and exposed to different concentrations of colicin E2. In other experiments the effect of E2 on division and DNA synthesis were measured simultaneously. Fig. 1 presents a composite figure of some typical results. The colicin

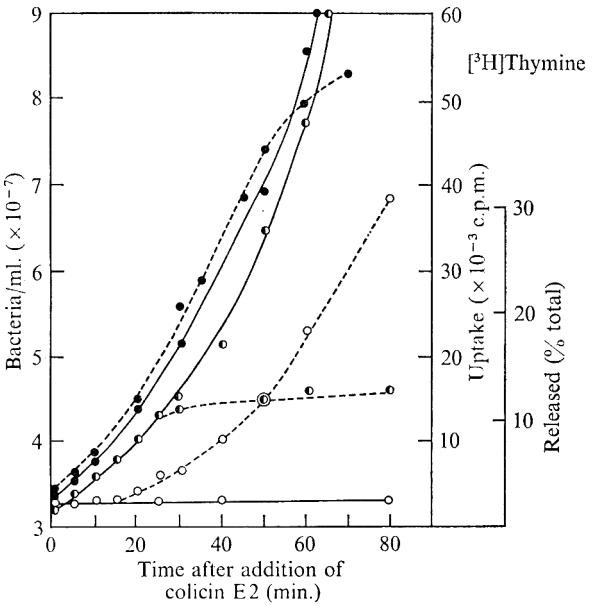


Fig. 1

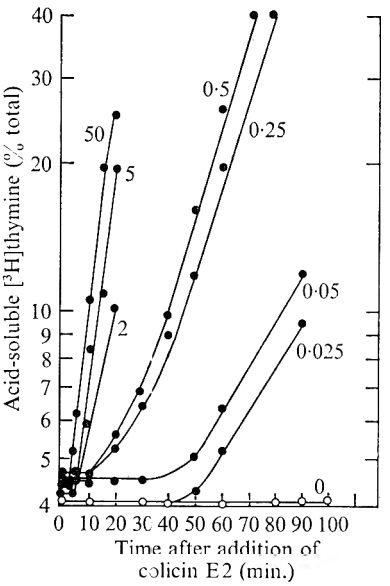


Fig. 2

Fig. 1. Kinetics of colicin E2-induced DNA breakdown and inhibition of bacterial division and DNA synthesis. [$^3$H]Thymine-labelled bacteria of *Escherichia coli* strain 206 were suspended in broth + 100 $\mu$g. cold thymine/ml. at $3 \times 10^7$ bacteria/ml. and treated with colicin E2 (0·5 units/$10^7$ bacteria) at time zero. In a parallel unlabelled culture [$^3$H]thymine (0·2 $\mu$Ci/$\mu$g. thymine) and E2 were added at zero time and samples were removed at intervals. Solid lines denote untreated controls, broken lines denote colicin-treated cultures; ○, cold acid-soluble [$^3$H]thymine; ◑, cell count; ●, cold acid-precipitable [$^3$H] incorporation.

Fig. 2. Effect of colicin E2 concentration on rate of DNA breakdown in growing cultures. Exponential cultures ($3 \times 10^7$ bacteria/ml.) of *Escherichia coli* strain 206 in nutrient broth previously labelled with [$^3$H]thymine were treated with different concentrations of E2 at time zero. The release of soluble [$^3$H]thymine was then determined at intervals. Figures against the curves are the E2 concentrations/$10^7$ bacteria; ○, untreated control.

concentration used in Fig. 1 (0·5 units/$10^7$ bacteria/ml.) was normally sufficient to kill 99 % of the bacteria; DNA breakdown was first detected at 20 min. whilst the increase in bacterial number continued at the normal rate until minute 30. Thereafter total biomass (turbidity) increased at the normal rate for at least 25 min. and the bacteria became filamentous. DNA synthesis also continued at the normal rate for at least

50 min. after adding E2. Similar experiments were carried out with several different concentrations of E2 and, although the time of onset of DNA breakdown varied (see also Fig. 2), the result was always the same: DNA degradation began 5 to 10 min. before inhibition of division was observed. Thus inhibition of bacterial division did not appear to be a primary effect of colicin E2 action nor did it result from inhibition of DNA synthesis. The effect of E2 upon the division machinery therefore remains obscure.

*Factors affecting initiation of DNA breakdown by E2*

*Effect of E2 concentration on DNA breakdown in growing bacteria.* A striking effect of colicin E2 action observed in these studies was that the time of commencement of DNA breakdown in exponentially growing cultures depended on the amount of E2 added. A similar effect was observed by Nomura (1964). Labelled cultures of

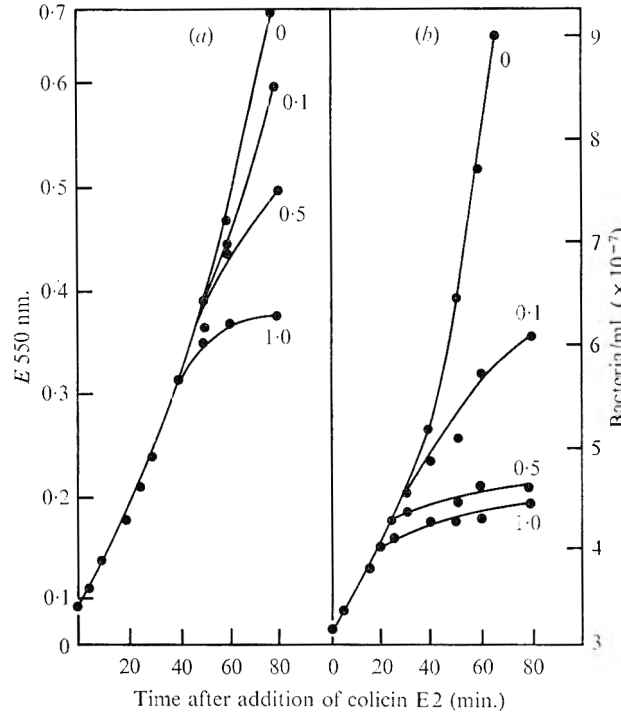


Fig. 3. Effect of colicin E2 on growth and division of exponentially growing cultures of *Escherichia coli* strain 206 in nutrient broth treated with E2 at time zero. Figures against the curves are the colicin concentrations added/$10^7$ bacteria; (*a*) extinction (*E*) rate changes, (*b*) total bacterial counts.

*Escherichia coli* strain 206 growing exponentially in nutrient broth were treated with various concentrations of E2 and the kinetics of DNA breakdown measured. Fig. 2 shows that, over a 100-fold range of E2 concentration, DNA breakdown was detected as early as 4 min. or as late as 50 min. (equivalent to 1·5 generations). Similar results were obtained even when adsorption of E2 was done at 4° and the bacteria washed to remove excess E2 before raising the temperature to 37°. Measurement of the survival

kinetics of bacteria treated with various E2 concentrations also showed that the delayed induction of DNA breakdown by small amounts of E2 was not due to slow adsorption of the colicin. When the effect of E2 upon bacterial division was measured at different colicin concentrations, similar results to those on the induction of DNA breakdown were obtained. Addition of E2 was followed by normal increases in bacterial number until, abruptly, the rate of increase changed at a time determined in some way by the amount of E2 present (Fig. 3).

Table 1. *Effect of colicin E2 multiplicity on induction of DNA breakdown*

Exponential broth cultures of *Escherichia coli* strain 206 ($3 \times 10^7$ bacteria/ml.) treated at time zero with colicin E2 at 37° as in Fig. 2. Rates of DNA breakdown calculated from the slopes of the curves shown in Fig. 2.

| E2 concentration | | | DNA breakdown | |
|---|---|---|---|---|
| Units/$10^7$ bacteria | Estimated no. molecules/ bacterium | Bacterial survival (after 60 min.) | Time of onset (min.) | Rate % sol. [$^3$H]thymine released/min. |
| 0·025 | 25 | 53 % | 45 | 0·16 |
| 0·05 | 50 | 23 % | 40 | 0·21 |
| 0·1 | 100 | 16 % | 20 | 0·37 |
| 0·25 | 250 | 0·4 % | 10 | 0·42 |
| 0·5 | 500 | 0·1 % | 10 | 0·62 |
| 5 | 5,000 | 0·01 % | 5 | 0·86 |
| 50 | 50,000 | 0·001 % | 4 | 1·23 |

This effect of concentration of colicin E2 on the pattern of DNA breakdown in sensitive bacteria was investigated in many experiments, and the relationship between E2 concentration, the timing of DNA breakdown and the subsequent rate of this process is examined in Table 1, which also shows the bactericidal effect of E2 and presents each colicin concentration as the number of E2 molecules/cell. This can be estimated since the molecular weight of E2 is known (Herschman & Helinski, 1967) and the specific activity of purified E2 (1000 units/$\mu$g. protein) was determined previously (Holland, 1968). On the basis of these calculations, the maximum number of E2 molecules adsorbed/organism was 2000, which is in good agreement with the figure of 2000 to 3000 E2 receptors calculated by Maeda & Nomura (1966). The results in Table 1 show that when sufficient E2 was added to saturate all receptors there was a minimum delay of 4 min. before breakdown was detected. At E2 concentrations sufficient to kill only 47% of the bacteria, less than 5% of the receptors should be occupied, and in this case breakdown was only detected at 40 min. These results indicate that, with exponentially growing bacteria, very few of the initial receptor colicin complexes promoted a lethal interaction between colicin and membrane. At high E2 concentrations, at least one lethal hit was quickly attained but at subsaturation concentrations growth and division appeared to be needed before a lethal hit, as expressed by the induction of DNA breakdown, took place. The data in Table 1 also show that, in contrast to its effect upon the timing of breakdown, increasing the concentration of E2 did not greatly influence the rate of DNA breakdown. Although E2, when added in excess, stimulated higher rates of breakdown, at subsaturation concentrations the rate of degradation was virtually independent of the E2 concentration, particularly when the fraction of bacteria killed by the colicin is taken into account.

These results do not rule out the possibility that DNA breakdown was initiated by the co-operative effect, upon the membrane, of many colicin molecules, i.e. a multihit mechanism. Since, however, there is evidence that the killing action of colicin is a single-hit process (Jacob, Siminovitch & Wollman, 1952; Nomura, 1963; Shannon & Hedges, 1967), we favour the alternative interpretation that DNA degradation is initiated by an all or none process, and that in growing cultures the main effect of increasing the E2 concentration was to increase the probability of an early single hit which then initiated DNA breakdown (or inhibition of division) at the maximum rate.

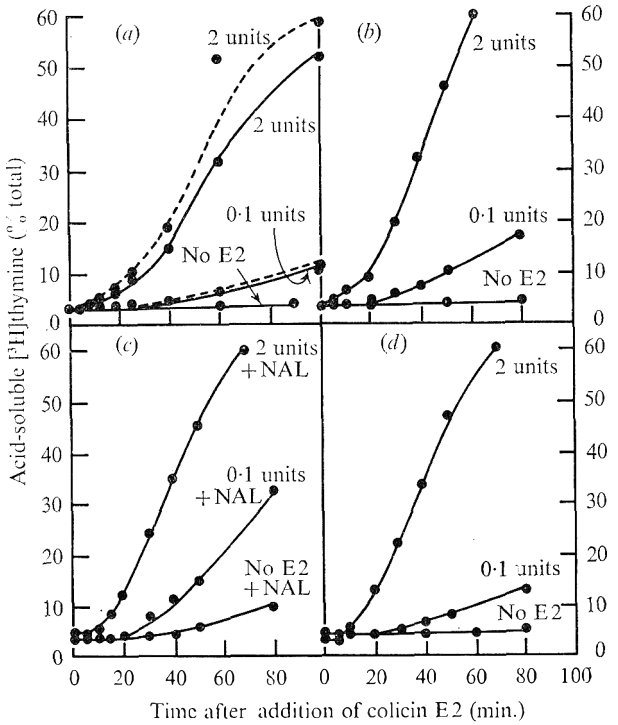


Fig. 4. Effect of chloramphenicol and nalidixic acid on colicin E2 action. Cultures of *Escherichia coli* strain 206 growing exponentially in nutrient broth were labelled with [³H]thymine. (*a*) Chloramphenicol (200 μg./ml.) was added to cultures (broken lines) at −10 min.; colicin E2 was added to all cultures at time zero and release of cold acid-soluble [³H]thymine determined at intervals. Colicin E2 concentration/$10^7$ bacteria is indicated for each curve. (*c*) Nalidixic acid (NAL) (20 μg./ml.) was added to cultures at −2 min. followed by E2 at time zero. (*d*) Cultures first treated with NAL (20 μg./ml.) for 30 min.; NAL was then removed by centrifugation, cultures resuspended in nutrient broth and E2, 2 and 0·1 units/$10^7$ bacteria, added at time zero. The control received no colicin. (*b*) Cultures treated with E2 alone (controls for the NAL treatments).

*Effect of inhibition of DNA and protein synthesis upon E2-induced DNA breakdown.* Energy is required for the conversion of the initial colicin E2–cell complex into a state from which viable bacteria can no longer be recovered by trypsin action (Reynolds & Reeves, 1969). Nomura & Maeda (1965) have shown that no DNA breakdown takes place in bacteria treated with E2 in the presence of 2,4-dinitrophenol. In addition, the results described in the previous section indicate that, particularly at low concentrations of E2, some growth was required to initiate DNA breakdown. The question whether

protein synthesis was needed for the formation of, for example, a specific colicin-surface complex or for the synthesis *de novo* of a specific DNase was therefore studied. Cultures prelabelled with [$^3$H]thymine were exposed to different concentrations of colicin E2 with chloramphenicol (200 $\mu$g./ml.). As shown in Fig. 4, neither the length of the prebreakdown lag nor the subsequent rate of breakdown were affected. In a few experiments chloramphenicol did produce some decrease in rate of breakdown, particularly with low E2 concentrations, but this effect was not reproducible and may have been due to disturbance in the growth of the bacterial surface induced by chloramphenicol (see Rothfield & Pearlman-Kathencz, 1969). Amino acid starvation before and during treatment with colicin E2 was also without effect upon the DNA breakdown pattern. These results clearly showed that the promotion of DNA breakdown by E2 did not require the synthesis *de novo* of a DNase. In addition, these results showed that the lag period before the initiation of DNA breakdown by low E2 concentrations was apparently not associated with the synthesis of any cellular protein component.

The effect of inhibition of DNA synthesis upon colicin E2 action is also shown in Fig. 4. Labelled cultures of *Escherichia coli* strain 206 were exposed to E2 after treatment in various ways with nalidixic acid (NAL). NAL did not inhibit DNA breakdown induced by high or low E2 concentrations. Furthermore, experiments discussed below showed that E2-induced DNA breakdown proceeded normally at 42° in mutants defective in ability to replicate DNA at high temperature. Promotion of DNA breakdown by E2 could therefore be initiated at any point in the DNA replication cycle and degradation did not depend upon continued synthesis. Fig. 4*d* also shows that pretreatment with NAL under conditions which promoted more chromosomal replication forks per bacterium (see Pritchard, Barth & Collins, 1969) did not stimulate the rate of DNA breakdown on subsequent addition of colicin E2. This result suggests that DNA breakdown in E2-treated bacteria is not specifically triggered from the replication point on the chromosome.

*Induction of DNA breakdown by E2 in non-growing bacteria.* The results described above indicated that, particularly at low E2 concentrations, initiation of DNA breakdown depended on growth or accumulation of some cellular component. This interpretation predicted that, in non-growing bacteria (e.g. in buffer suspensions), high E2 concentrations should initiate breakdown more or less normally, but small amounts of E2 should initiate breakdown only poorly. Contrary to expectation, as shown in Fig. 5, extensive breakdown of DNA was observed with both high and low E2 concentrations. In the latter case the delay in initiation of breakdown observed in growing bacteria was not found, and soluble [$^3$H]thymine was detected in all cultures within a few minutes of adding E2. DNA breakdown in buffered suspensions quickly became linear in contrast to the exponential rates found with growing bacteria. Nevertheless, initial rates were usually at least half those for broth suspensions treated with comparable E2 concentrations. Again, as found with growing bacteria, the rate of DNA breakdown in buffer suspension was not very dependent upon the colicin concentration: over at least a 100-fold range of E2 concentration the rate of DNA breakdown increased at most sixfold.

The long delays in initiation of DNA breakdown observed in broth cultures were thus due to some effect unique to growing bacteria and were not essential for E2 action. Possible explanations for the effect of E2 multiplicity upon the timing of initiation of DNA breakdown will be discussed below.

*Effect of phosphate on initiation of DNA breakdown in buffer suspensions.* Bacteria in tris+HCl buffer showed very little DNA breakdown in the presence of colicin E2 although adsorption and killing by E2 were normal. Addition of $KH_2PO_4$ to treated bacteria in tris+HCl buffer stimulated DNA breakdown, and rates comparable to those in phosphate buffer were obtained with $10^{-2}$ M-phosphate. As shown in Fig. 6, $KH_2AsO_4$ was quite effective in stimulating breakdown in tris buffer, in contrast to NaCl, KCl or lower concentrations of $KH_2PO_4$. The stimulation produced by added phosphate was usually six- to eightfold although in a few experiments DNA breakdown in tris buffer alone was more extensive, and the effective stimulation by added phosphate was only two- to threefold.

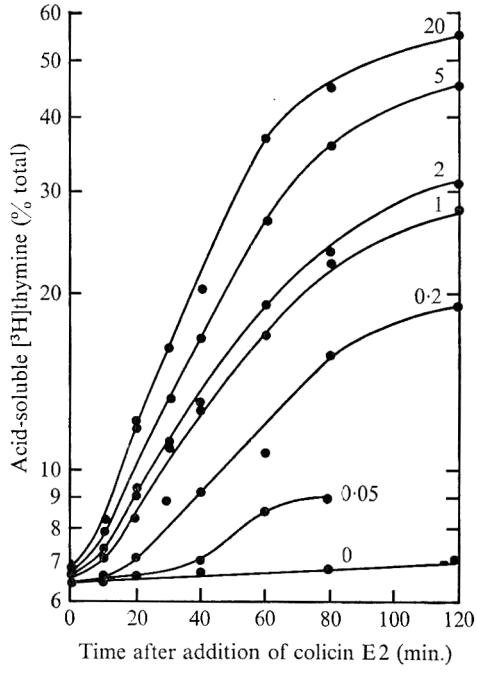


Fig. 5

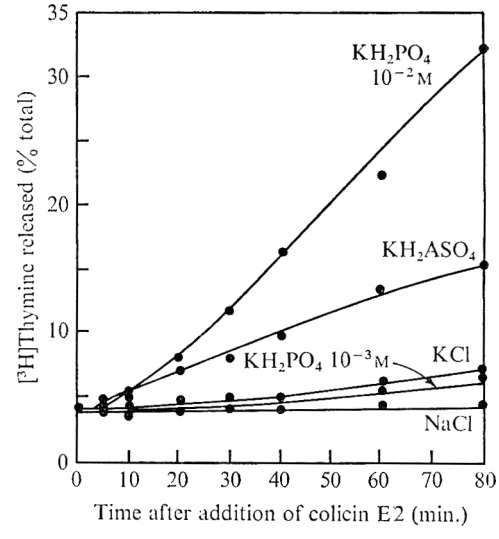


Fig. 6

Fig. 5. Induction of DNA breakdown by colicin E2 in resting bacteria. Growing cultures of *Escherichia coli* strain 206 were labelled in the usual way, harvested, washed three times in 0·1 M-tris buffer (pH 7·2) containing $10^{-2}$ M-$KH_2PO_4$, $10^{-3}$ M-$MgSO_4$ and $10^{-4}$ M-$CaCl_2$ and the bacteria finally resuspended to $2 \times 10^7$/ml. in the same buffer+25 $\mu$g. cold thymine/ml. After incubation for 10 min. at 37°, colicin E2, diluted in buffer, was added at time zero. Incubation was continued at 37° and release of labelled thymine determined at intervals. The figures by the curves are the colicin concentrations/$10^7$ bacteria.

Fig. 6. Effect of anions on induction of DNA breakdown by colicin E2. Isotopically labelled cultures of *Escherichia coli* strain 206 were washed three times in 0·1 M-tris buffer (pH 7·2) and resuspended in the same buffer containing 25 $\mu$g. thymine/ml., $10^{-3}$ M-$MgSO_4$, $10^{-4}$ M-$CaCl_2$. After shaking cultures at 37° for 10 min., different samples received NaCl, KCl, $KH_2AsO_4$ (all $10^{-2}$ M) or $KH_2PO_4$ ($10^{-2}$, $10^{-3}$ M). Colicin E2 added to 5 units/$10^7$ bacteria at time zero.

In an attempt to determine which particular step in the E2 pathway was affected, the kinetics of inactivation of bacteria treated with E2 in tris buffer were examined and found to be similar whether phosphate was present or not. Phosphate was not

therefore required for irreversible binding of colicin E2. Similarly, phosphate was not required for the degradative process itself since removal of phosphate from the suspending medium after the onset of breakdown did not affect further degradation (Table 2). Several experiments, however, indicated that *exogenous* phosphate was required for formation of the specific colicin-envelope complex which rapidly initiated phosphate-independent DNA breakdown. Table 2 also shows: (1) suspension of bacteria in phosphate buffer did not stimulate DNA breakdown when exogenous phosphate was removed before addition of E2; (2) phosphate starvation of bacteria for at least 30 min. before addition of E2 did not affect subsequent phosphate stimulation of DNA breakdown; (3) after preadsorption of E2, bacteria showed increased DNA breakdown immediately upon addition of phosphate; treatment of organisms with E2 in the presence of phosphate at 4° did not stimulate subsequent DNA breakdown when phosphate was removed before incubation at 37°.

Table 2. *Effect of phosphate on initiation of DNA breakdown by colicin E2*

Exponentially growing cultures of *Escherichia coli* strain 206 were labelled with [$^3$H]thymine and resuspended in 0·1 M-tris buffer as for Fig. 5. Colicin E2 (5 units/$10^7$ bacteria) was added and adsorption completed by 10 min. incubation at 37° or for 30 min. at 4°. Cultures were then centrifuged, deposit resuspended in the same buffer and DNA breakdown determined at intervals. Potassium phosphate buffer ($10^{-2}$ M final concentration) was present during, before or after adsorption of colicin as indicated.

| | Adsorption temperature | Presence of phosphate | DNA breakdown* % total DNA released |
|---|---|---|---|
| Expt. 1 | 37° | Throughout | 28 |
| | | During adsorption and for further 10 min. | 25 |
| | | After adsorption only | 25 |
| | | Ten min. before addition of E2 only | 8 |
| Expt. 2 | 4° | Throughout | 28 |
| | | After adsorption only | 26 |
| | | During adsorption only | 6 |

* The amount of breakdown shown is that obtained after 90 min. postadsorption incubation at 37°. When phosphate was present throughout, the amount of breakdown obtained was 25 to 40 % over several experiments; without phosphate, between 3 and 8 % of DNA was usually broken down in 90 min. at 37°.

In summary, the above results indicate that, in bacteria suspended in tris buffer, the initial colicin–cell complex was only poorly active in initiating DNA breakdown. In the presence of exogenous phosphate, a temperature-dependent process then converted the colicin–surface complex into an active form which promoted DNA breakdown.

*Mechanism of DNA breakdown in bacteria treated with colicin E2*

*The degradative process requires energy.* Since earlier studies showed that energy was required to promote colicin E2 action, the possibility was examined that the DNA breakdown process itself might be energy-dependent. As shown in Fig. 7, 2,4-dinitrophenol (DNP) immediately and dramatically decreased the rate of breakdown by treated bacteria. The effect was reversible and upon removal of DNP, breakdown immediately resumed at the original rate (Fig. 7*b*). Fig. 7*a* shows that DNP was equally effective in suppressing breakdown when added at late times. The breakdown

process itself is therefore inhibited by DNP. Energy metabolism in E2-treated bacteria was also inhibited at various times by cyanide ($2 \times 10^{-3}$ M) or colicin K (an uncoupler of oxidative phosphorylation; see Levinthal & Levinthal cited by Luria, 1964) and in each case the rate of breakdown was greatly decreased, confirming that energy is required to maintain the colicin–membrane–DNA complex in a state propitious for breakdown, or alternatively that the E2-specific nuclease itself is ATP-dependent.

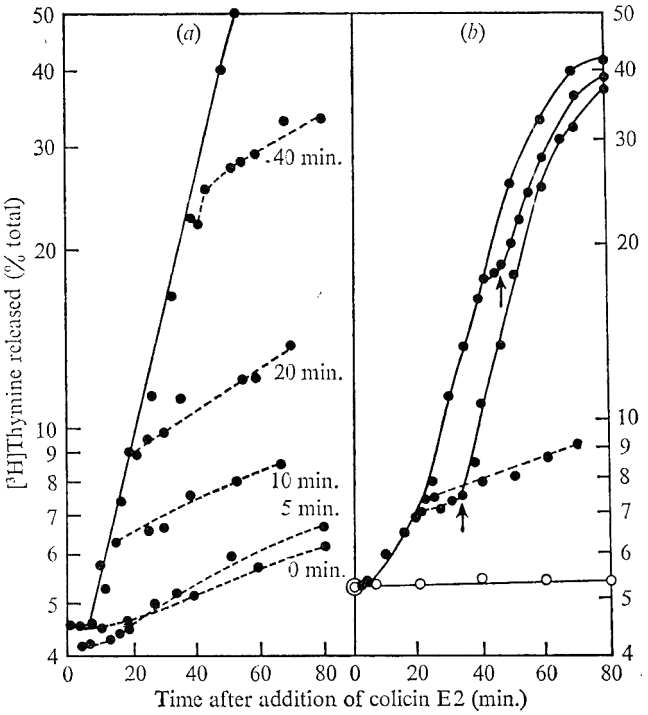


Fig. 7. Effect of inhibition of energy metabolism upon colicin E2-induced DNA breakdown. (*a*) Exponential cultures ($2 \times 10^7$ bacteria/ml.) of *Escherichia coli* strain 206 in nutrient broth at 37° were treated with colicin E2 (2 units/$10^7$ bacteria) at time zero, ●——●. At the intervals indicated by the curves ●----●, 2,4-dinitrophenol (DNP) (final concentration $2 \times 10^{-3}$ M) was added. Release of acid-soluble [³H]thymine was then measured as described in Methods. (*b*) Strain 206 in nutrient broth treated with E2 (2 units/$10^7$ bacteria/ml.). Separate fractions were treated with DNP at 20 min. and at 40 min.; duration of DNP treatment is indicated by broken lines. At the times indicated by arrows, DNP was removed by centrifugation and incubation of bacteria in nutrient broth resumed. The control culture (○) was treated with DNP only.

*Effect of trypsin on E2-induced DNA breakdown.* Nomura & Nakamura (1962) previously showed that macromolecule synthesis, completely suppressed by colicin K, was quickly resumed after addition of trypsin. The membrane alteration induced by colicin K is therefore only maintained in its presence and reverts to normal when it is removed. Since a primary consequence of colicin E2 action is the promotion of DNA breakdown, we determined whether nuclease activity also depended completely upon the continued presence of E2. In the presence of small amounts of E2 (Fig. 8*a*), addition of trypsin to *Escherichia coli* strain 206 greatly inhibited further breakdown even when added at 30 min.; during the early lag period it completely

suppressed DNA breakdown. This latter effect provides further confirmation that the formation of an active surface–E2 complex, which promotes DNA breakdown, is delayed at low E2 concentrations. At saturating concentrations of E2, trypsin was most effective when added up to 10 min. after initiation of breakdown and, as shown in Fig. 8*b*, was progressively less effective when added at later times. Phase-contrast

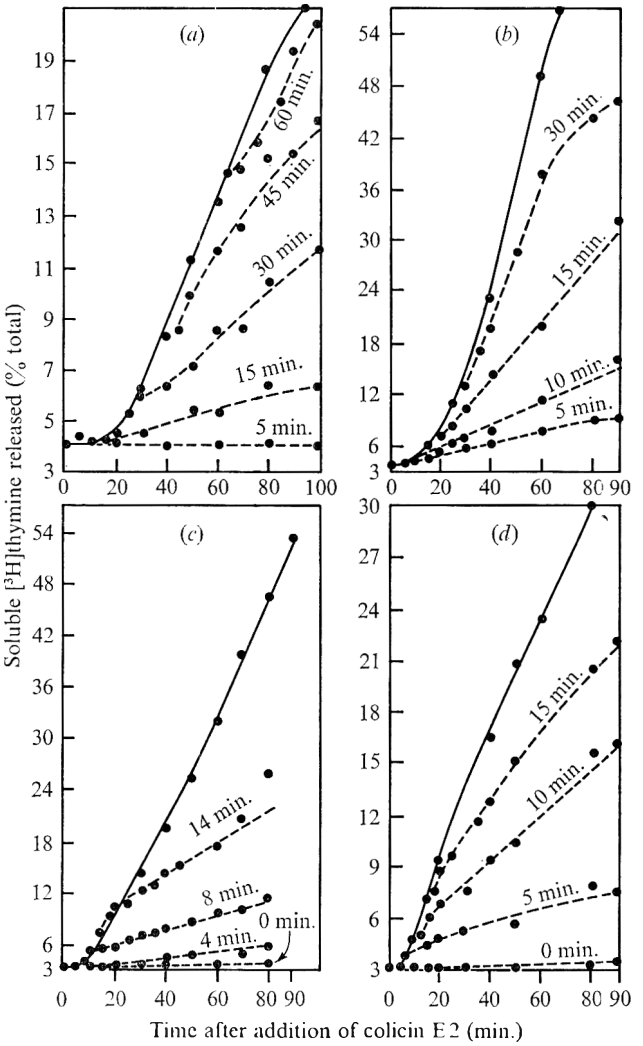


Fig. 8. Effect of trypsin on colicin E2-induced DNA breakdown. Cultures of *Escherichia coli* strain 206 were labelled in the usual way, washed and suspended in nutrient broth, pH 7·8 (*a*, *b*), or in tris buffer, pH 7·8 (*c*, *d*), containing $10^{-2}$ M-$KH_2PO_4$, $10^{-3}$ M-$MgSO_4$ and $10^{-4}$ M-$CaCl_2$. Solid lines represent the primary cultures treated with colicin E2, broken lines denote samples removed from primary culture and treated with trypsin (1 mg./ml.). (*a*) Nutrient broth culture treated with colicin E2 (0·2 units/$10^7$ bacteria) at zero time and trypsin treatment done at the times indicated. (*b*) Nutrient broth cultures treated with E2 (2 units/$10^7$ bacteria). (*c*) Bacteria suspended in tris phosphate buffer and treated with E2 (8 units/$10^7$ bacteria). (*d*) Preadsorption of E2 (10 units/$10^7$ bacteria) for 45 min. at 4°, cultures then centrifuged and deposit suspended in tris phosphate buffer before trypsin treatment.

microscopy of such cultures showed that trypsin was also most effective in preventing filament formation when added early.

Three possible explanations of these features of trypsin inhibition are: (1) initiation of DNA breakdown is not synchronized in all of the population, and trypsin only affects those bacteria in which E2-induction of DNA breakdown is not yet established; (2) colicin E2 becomes, with time, increasingly inaccessible to the trypsin molecule; (3) the specificity of the degradative process is progressively lost as breakdown becomes extensive.

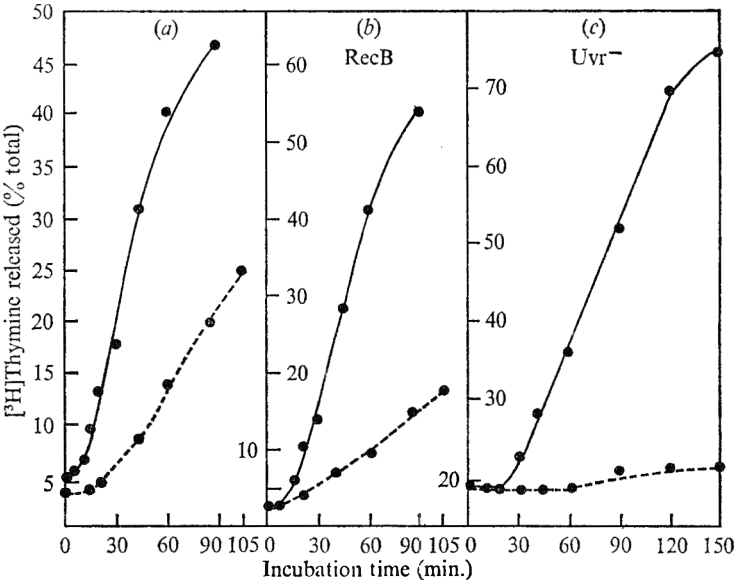


Fig. 9. Mitomycin C and colicin E2-induced DNA breakdown in Rec and Uvr mutants of *Escherichia coli*. Bacterial strains labelled with [$^3$H]thymine in the usual way and colicin E2 (5 units/$10^7$ bacteria) or mitomycin C (40 $\mu$g./ml.) added at time zero. Solid lines denote E2-treated, broken lines mitomycin C-treated cultures. (*a*) Wild-type strain 206; (*b*) Strain JC4457, RecB$^-$; (*c*) Strain KMBL90, UvrB$^-$.

In an attempt to test the first possibility, the effect of trypsin on E2 action in non-growing bacteria was examined in the hope that any asynchrony arising from some aspect of growth would be minimal. The effect of trypsin upon DNA breakdown was, however, very similar to that obtained with growing cultures (Fig. 8*c*). Early addition of trypsin completely abolished subsequent breakdown, and the trypsin effect was progressively lost as breakdown proceeded. Nevertheless, even when breakdown was well under way, addition of trypsin still produced a significant, although sometimes delayed, inhibitory effect. Similar results were obtained when adsorption of E2 was completed at 4° and non-adsorbed E2 removed by centrifugation before shifting the treated bacteria to 37°, conditions under which colicin is irreversibly adsorbed although at a slower rate. It therefore seems unlikely that trypsin affects only those bacteria in which DNA degradation has not yet been initiated.

Several relevant control experiments were also made: (*a*) under the cultural conditions normally used, trypsin treatment had no effect upon mitomycin C-induced DNA breakdown; (*b*) with E2-treated bacteria, subsequent removal of trypsin did not lead

to a resumption of the normal rate of DNA breakdown, and when equimolar amounts of trypsin and trypsin inhibitor were added breakdown was not inhibited; (*c*) incubation of bacteria with trypsin followed by removal of the enzyme and addition of colicin E2 had no inhibitory effect upon subsequent DNA degradation.

The observed effects of trypsin upon colicin E2 action appear therefore to be the result of the proteolytic digestion of the surface-bound colicin and not to any non-specific effects. On the basis of all these results it seems reasonable to conclude that, at least at early times, removal of E2 by trypsin treatment can have a direct inhibitory effect upon the E2-specific nuclease.

*Effect of E2 on Escherichia coli K12 mutants defective in nuclease functions.* A number of strains of *Escherichia coli* mutants possibly lacking nuclease functions (see Table 3) have been studied. All of them were sensitive to colicin E2 and showed normal E2-induced DNA breakdown at 37° (or at 42° in the case of temperature-sensitive DNA-replication mutants) including strains which lack an ATP-dependent nuclease (Buttin

Table 3. *Escherichia coli mutants tested for colicin E2-sensitivity and E2-induced DNA breakdown*

The tsDNA mutants, E279 and E613, which show immediate cessation of DNA synthesis on shift to 42° were kindly provided by Dr J. Wechsler. The tsDNA mutant 16 was kindly provided by Mr H. G. Nandadasa, this strain and all the other ts mutants tested show delayed inhibition of DNA synthesis on shift to high temperature.

| *E. coli* mutant strain | Genotype or phenotype | Linked to chromosomal locus | Reference |
|---|---|---|---|
| JG64 | *pol*A⁻ | *met*E | Gross & Gross (1969) |
| 4K | *hss*⁻ | *ser*B | Glover (1970) |
| 5K | *hsr*⁻ | *ser*B | Hubacek & Glover (1970) |
| KMBL90 | *uvr*B | *gal* | Van de Putte, Van Sluis, Van Dillewijn & Rörsch (1965) |
| JC4457 | *rec*B | *arg*A | Clark (1967) |
| KMBL243 | *rec*C | *arg*A | Van de Putte, Zwenk & Rörsch (1966) |
| E279 | tsDNA | | From Dr J. Wechsler |
| E613 | tsDNA | | From Dr J. Wechsler |
| FA21 | tsDNA | *str* | Fangman & Novick (1968) |
| 16 | tsDNA | *ilv* | From Mr H. S. Nandadasa |
| T83 | tsDNA | *ilv* | Hirota, Ryter & Jacob (1968) |
| T42 | tsDNA | *mal*B | Hirota, Ryter & Jacob (1968) |
| T46 | tsDNA | *ilv* | Hirota, Ryter & Jacob (1968) |

& Wright, 1968) and restriction-deficient strains, which probably lack an ATP-dependent endonuclease (Meselson & Yuan, 1968). As found previously, colicin E2 also promoted normal breakdown in a Uvr⁻ mutant (Holland, 1967) although mitomycin C-induced DNA breakdown was greatly decreased compared to wild-type strains (Fig. 9). Uvr⁻ mutants appear to lack the capacity to excise thymine dimers (Howard-Flanders & Theriot, 1966) and to repair mitomycin C-induced damage (Boyce & Howard-Flanders, 1964), and since such mutants show normal sensitivity to colicin E2 it appears most unlikely that E2-directed DNA breakdown involves any of the normal repair nucleases. Several mutants that were temperature-sensitive for DNA replication were also examined, including mutants possibly defective in either replication or the initiation of replication (Table 3), but all showed normal E2-induced DNA breakdown at high temperature.

## DISCUSSION

### *Factors affecting initiation of DNA breakdown by colicin E2*

On the basis of our studies and those of other workers, we envisage the following scheme for the pathway of colicin E2 action:

bacteria + E2 → Surface complex I → Surface complex II (promotes DNA breakdown).

The initial adsorption step is a two-stage process (Reynolds & Reeves, 1969) leading to an irreversible state, complex I. This appears to require magnesium (Reynolds & Reeves, 1969) but not energy and is completely reversible by trypsin treatment. The specificity for complex I is shared by colicin E2 and E3 (Maeda & Nomura, 1966), although the two colicins have quite different intracellular effects. The chemical nature of the primary receptor is not known but it may well be analogous to a phage receptor. Some intermediate stages, presumably involving interaction between E2 and specific membrane proteins, may then be involved before the pathway culminates in the formation of complex II, a conformational state of the membrane which promotes rapid degradation of DNA and inhibition of cell division.

The formation of complex II is a complicated process, the efficiency and timing of which varies in relation to the colicin concentration and to the physiological conditions of the organism. Nevertheless, the initiation of DNA breakdown always appears to be an all-or-none process, with increasing concentrations of colicin E2 hastening initiation of breakdown rather than its ultimate rate. Although this multiplicity effect of E2 is not understood, it is clear that synthesis of neither protein nor DNA is required for the development of the final surface complex which triggers DNA breakdown. Differential growth or extension of various surface layers in the presence of small concentrations of E2 may, however, be important in determining the timing of complex II formation, but it cannot be an absolute requirement since in non-growing bacteria low doses of E2 still trigger DNA breakdown without appreciable delay.

It is still not clear whether complex II formation is energy-dependent. Promotion of E2 action is temperature-dependent and is inhibited by 2,4-dinitrophenol, but the finding that the DNA breakdown process itself requires energy makes it difficult to establish whether any of the intermediate steps are also energy-requiring.

Genetic analyses have previously shown that mutants refractory to colicin E2 may be blocked at several postadsorption steps (Hill & Holland, 1967; Nomura & Witten, 1967; Nagel de Zwaig & Luria, 1967) probably by alteration of specific proteins (Nomura & Witten, 1967; Holland, 1968; Nagel de Zwaig & Luria, 1969). Recent biochemical studies have shown that refractory mutants may lack or contain altered membrane proteins (Samson & Holland, 1970; C. Schnaitman, personal communication), suggesting that membrane or surface proteins may participate in complex II formation. Colicin E2 may therefore not interact directly with a membrane-bound nuclease but may act indirectly through the intermediacy of specific membrane proteins, possibly as suggested by Changeux & Thiery (1967) via conformational changes of repeating membrane protomers, leading to a final specific protein or membrane configuration which then promotes nuclease action. The finding that resting bacteria in tris buffer require added phosphate for efficient initiation of DNA breakdown but not for adsorption of colicin, provides further evidence for the presence of intermediate steps in E2 action.

We have suggested above that the final formation of complex II simply involves a series of physical changes in the cell membrane in response to the fixation of colicin. Several lines of evidence suggest that the ultimate biochemical changes induced by different colicins, including E2, derive from the altered properties of membrane-bound proteins or enzymes *in situ* and not from the activities of, for example, enzymes displaced from the membrane and acting through the cytoplasm. Thus the effects of colicin K upon energy metabolism and macromolecular synthesis are completely reversed upon subsequent removal of the colicin from the cell surface by trypsin treatment (Nomura & Nakamura, 1962). Moreover, although modification of 30S ribosomal subunits is induced by colicin E3 fixation to sensitive bacteria, extracts from such bacteria are incapable of modifying normal ribosomes *in vitro* (Konisky & Nomura, 1967). DNA breakdown induced by colicin E2 can, at least in the early stages, be halted or decreased by digestion of the adsorbed colicin with trypsin. Thus it appears that the membrane conformation induced by colicin E2 may return, as in the case of colicins E1 and K, to its original state when colicin is removed. The failure of trypsin to inhibit DNA breakdown effectively when added at later times is unexplained; asynchronous initiation of DNA breakdown within the population appears to be excluded as a major factor. More likely, the specificity of the degradative process is soon lost or the colicin becomes inaccessible to trypsin as DNA breakdown proceeds and division is halted.

*Specificity of E2-induced DNA breakdown*

With the formation of complex II the altered membrane induces rapid degradation of DNA and, as a consequence or as an independent effect, the inhibition of division. The degradative process appears to be highly specific to the *Escherichia coli* chromosome; in T4-infected bacteria colicin E2 does not promote phage DNA breakdown (Nomura, 1963), and invading $\lambda$ DNA is not degraded in E2-treated bacteria and appears to replicate normally (unpublished results). Also, since DNA replication may continue in E2-treated bacteria without loss of newly synthesized strands until at least 10% of the chromosome is degraded (Fig. 1), it appears probable that breakdown is initiated at a few strictly localized sites, possibly at the replication fork or at the chromosomal origin, both of which have been shown to be membrane-bound (Sueoka & Quinn, 1968). The kinetics of breakdown of pulse-labelled DNA in colicin E2-treated bacteria (unpublished results), however, indicate that breakdown does not begin at the replication fork, unless recently copied parental strands only are affected. The possibility is now being examined that breakdown is initiated at the chromosomal origin.

Enzymes concerned in thymine dimer excision and in host specificity mechanisms are not activated by colicin E2, nor is the ATP-dependent nuclease which is determined by the RecB and RecC genes (Buttin & Wright, 1968; Barbour & Clark, 1970). We found no decrease in colicin E2-directed DNA breakdown in a variety of *Escherichia coli* mutants which are temperature-sensitive for DNA replication. Obinata & Mizuno (1970) have shown that at least in the early stages of E2-induced DNA breakdown, a specific endonuclease (but not endonuclease I) is involved. Further screening among DNA repair and replication mutants may well yield strains which lack one or more of the E2-specific nucleases.

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## ADDENDUM

After preparation of this paper, Ringrose (*Biochimica et biophysica acta*, 1970, vol. 213, pp. 320–334) reported that DNA breakdown induced by E2 proceeds initially via single-strand breaks and that these can be repaired and their further formation halted if the bacteria are treated at an early stage with trypsin. This report further supports the hypothesis proposed here that membrane changes induced by E2 are reversible and that these changes directly affect the activity of a membrane-bound nuclease.

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# SHORT COMMUNICATIONS

## Vitamin, Purine and Pyrimidine Requirements of Oral Fusiforms

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(*Accepted for publication 2 October 1970*)

Fusiforms isolated from saliva and gingival debris were allocated on morphological, cultural and biochemical grounds to *Fusobacterium nucleatum*, *F. polymorphum*, *Leptotrichia buccalis* A and *L. buccalis* B (Hadi & Russell, 1968*a*). The viable count of such fusiforms increases significantly in both saliva (Hadi & Russell, 1968*b*) and gingival material (Hadi & Russell, 1969) in cases of acute ulcerative gingivitis (A.U.G.).

Few investigations have been made of the nutritional requirements of fusobacteria (Omata, 1953, 1959; Coles, 1968) and no work done on leptotrichiae. The present study was undertaken to determine the vitamin, purine and pyrimidine requirements of oral fusobacteria and leptotrichiae.

### METHODS

*Test organisms and their maintenance.* Three strains of each of the following were employed: *Fusobacterium nucleatum*, *F. polymorphum*, *Leptotrichia buccalis* A and *L. buccalis* B. These organisms were originally isolated from saliva and gingival material from healthy individuals, and from cases of A.U.G.

The test strains were maintained in Reinforced Clostridial Medium (R.C.M.) (Oxoid) containing 8 % $CaCO_3$. The cultures were stored at 4° and transferred to a freshly prepared medium every two weeks.

*Basal nutritional test medium.* The composition of the basal nutritional test medium (pH 7·2) was as follows (g./l.): vitamin-free casitone (Difco), 10·0; glucose, 5·0; $KH_2PO_4$, 1·2; $K_2HPO_4$, 1·2; DL-tryptophan, 0·2; sodium thioglycollate, 0·5; $FeSO_4.7H_2O$, $2{\cdot}5 \times 10^{-3}$; $MnSO_4.4H_2O$, $2{\cdot}5 \times 10^{-3}$; $MgSO_4.7H_2O$, 0·025; thiamine HCl, riboflavin, nicotinic acid, pyridoxine HCl, *p*-aminobenzoic acid, each 0·001; folic acid, $10^{-5}$; biotin, $5 \times 10^{-6}$; calcium pantothenate, 0·002; choline chloride, 0·1; inositol, 0·025; adenine sulphate.$H_2O$, guanine HCl.$H_2O$, xanthine, uracil, each 0·012.

All glassware was immersed in chromic-sulphuric acid for 8 h., washed with tap-water and then with distilled water. Culture tubes were further filled with distilled water and autoclaved at 121° for 30 min. Stock solutions of vitamins (apart from biotin) were prepared in 50 % ethanol. Biotin solution was made in 0·001 N-HCl, such a solution being stable for 18 months (Gallant & Toennies, 1949). Adenine, xanthine, guanine and uracil were dissolved in 0·5 N-NaOH. Stock solutions were

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stored at −20°. Test media made from the stock solutions were distributed in 10 ml. amounts into optically matched tubes (15 × 125 mm.) plugged with cotton-wool and autoclaved at 116° for 10 min.

*Inoculum and incubation conditions.* The organisms were grown in 20 ml. aliquots of R.C.M. (adjusted to pH 7·2 to 7·4) in McCartney bottles. The inoculated medium was incubated at 37° for 48 h. After incubation, the cultures were centrifuged at 1040 g. for 15 min. and washed three times in 0·067 M-phosphate buffer (pH 7·2) containing 0·05 % sodium thioglycollate. Nitrogen gas (passed through glycerol and sterile cotton-wool) was bubbled into the cell suspension during the washing procedure in order to remove any traces of oxygen.

Appropriate volumes of cell suspensions were transferred to optically matched tubes and brought to suitable volumes, in 0·067 M-phosphate buffer (pH 7·2) containing 0·05 % sodium thioglycollate, to give a turbidity reading of 80 on an E.E.L. (Evans Electroselenium Ltd, Halstead, Essex) nephelometer. An amount of 0·1 ml. of this suspension was then inoculated into tubes of test media. All cultures were incubated at 37° for 4 to 5 days (as found to be optimal for maximum growth) in B.T.L. (Baird & Tatlock Ltd, Chadwell Heath, Essex) anaerobic jars filled with a mixture of 95 % $H_2$ and 5 % $CO_2$. The growth was homogenized by means of a 'Rotamixer' (Hook and Tucker Ltd, London S.W.9) and estimated nephelometrically. In all experiments the organisms were inoculated in duplicate tubes and each nephelometer reading stated was an average of two. 'Essentiality' of a factor was indicated by a reduction of growth in its absence to one-quarter of the control; a fall to about one-half showed stimulation.

## RESULTS

In preliminary work none of the organisms grew in semi-synthetic medium containing vitamin-free Casamino acids (Difco) as source of amino acids, and only two strains

Table 1. *Growth of fusiforms in deficient media*

| Substance deleted | Nephelometer reading | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | *Fusobacterium nucleatum* | | *Leptotrichia buccalis* A | | | *L. buccalis* B | | |
| | A2 | A3 | C1 | C2 | C3 | D1 | D2 | D3 |
| None | 66·0 | 29·0 | 27·5 | 31·5 | 31·0 | 29·0 | 29·0 | 29·0 |
| Thiamine HCl | 61·0 | 30·5 | 25·0 | 29·5 | 30·5 | 30·0 | 29·5 | 27·5 |
| Riboflavin | 62·0 | 28·0 | 28·5 | 30·5 | 28·5 | 26·5 | 26·0 | 27·0 |
| Ca-pantothenate | 7·5 | 0 | (12·0) | (14·0) | (14·0) | (11·0) | (11·0) | (10·0) |
| Inositol | 62·5 | 28·0 | 21·0 | 28·0 | 25·0 | 28·0 | 23·5 | 24·0 |
| Choline chloride | 70·0 | 27·0 | 20·5 | 27·5 | 25·5 | 27·0 | 27·0 | 26·5 |
| Biotin | 66·0 | 20·5 | 4·5 | 4·5 | 1·0 | 2·0 | 3·5 | 2·5 |
| Nicotinic acid | (24·0) | (11·0) | 24·5 | 32·0 | 32·0 | 24·5 | 25·5 | 27·0 |
| PABA | 53·5 | 28·5 | 2·5 | 3·0 | 2·5 | 3·5 | 3·0 | 1·0 |
| Pyridoxine.HCl | 51·0 | 22·0 | 0 | 1·5 | 0 | 2·0 | 0 | 0 |
| Folic acid | 61·5 | 21·0 | 24·5 | 30·5 | 30·5 | 28·0 | 29·5 | 25·5 |
| Adenine (A) | 12·0 | 7·5 | 29·5 | 23·5 | 23·5 | 24·0 | 23·5 | 22·0 |
| Guanine (G) | 59·5 | 28·5 | 29·0 | 24·0 | 27·5 | 26·5 | 24·5 | 24·0 |
| Xanthine (X) | 55·0 | 27·0 | 25·0 | 30·0 | 31·0 | 25·0 | 25·0 | 24·5 |
| Uracil (U) | 60·5 | 27·5 | 24·5 | 24·5 | 28·5 | 26·0 | 29·0 | 29·5 |
| A, G, U, X | 14·5 | 7·0 | 27·5 | 25·0 | 30·0 | 29·0 | 27·5 | 27·0 |

Essentiality indicated by underlined figures and stimulation by figures in parentheses.

of *Fusobacterium nucleatum* and three strains each of *Leptotrichia buccalis* A and *L. buccalis* B grew in the basal medium with casitone. In experiments (Table 1) on the single deletion of vitamins from the basal medium, *F. nucleatum* A 2 and A 3 required calcium pantothenate and were stimulated by nicotinic acid. *Leptotrichia buccalis* (A and B) differed considerably from *F. nucleatum* and required biotin, pyridoxine hydrochloride and *p*-aminobenzoic acid. Furthermore, calcium pantothenate – essential for *F. nucleatum* – was found stimulatory for *L. buccalis* and nicotinic acid was not required.

Since the omission of *p*-aminobenzoic acid or folic acid did not affect the growth of *Fusobacterium nucleatum* (see Table 1), a medium deficient in both factors was tested. The two strains of *F. nucleatum* (A 2 and A 3) grew as well in the doubly deficient as in the complete medium, indicating that the lack of a requirement for either vitamin was not due to a mutual replacement effect.

The lack of requirement for biotin by isolates of *Fusobacterium nucleatum* could have been due to the presence of $CO_2$ in the atmosphere and the presence of asparagine in the vitamin-free casitone. A medium deficient in biotin was inoculated with the test organisms and incubated in an atmosphere of pure hydrogen. The control medium was that without biotin but incubated in a mixture of 95 % $H_2$ and 5 % $CO_2$. The complete medium, incubated in pure hydrogen, was also included as an additional control. It was observed that *F. nucleatum* A 2 and A 3 grew equally well in the above three media. This suggests that there was no requirement for biotin either in the presence or absence of $CO_2$.

The purine and pyrimidine requirements were first tested in media deficient in a single factor. The results are given in Table 1. It is clear that *Fusobacterium nucleatum* showed an absolute requirement for adenine. A subsidiary experiment showed that complete growth was obtained in the presence of adenine alone and that guanine, uracil and xanthine (singly or in combination) did not substitute for adenine: in contrast, deletion of adenine, guanine, uracil and xanthine as a group from the basal medium still did not affect the growth of *Leptotrichia buccalis*.

Media containing the compounds found essential and stimulatory for the organisms were prepared. *Leptotrichia buccalis* (A and B) grew nearly as well in the medium with only biotin, *p*-aminobenzoic acid, pyridoxine and calcium pantothenate as in the complete medium (with ten vitamins). On the contrary, *Fusobacterium nucleatum* A 2 and A 3 failed to grow when only calcium pantothenate, nicotinic acid and adenine were added. An experiment was then conducted in which single vitamins were added to the minimal medium used for *F. nucleatum*; in the case of folic acid there was a very slight response. The further addition of pyridoxine improved the growth of this organism to nearly one-half that in the complete medium.

## DISCUSSION

The lack of growth of *Fusobacterium nucleatum* A 1 and the three strains of *F. polymorphum* in medium containing vitamin-free casitone or Casamino acids made it impossible to study the nutrition of these organisms in such a medium. However, Omata (1959) reported that *F. nucleatum* did grow in a medium essentially similar to the basal medium used in this study (with Casamino acids). Similarly, Coles (1968) found that *F. polymorphum* responded to Omata's (1959) medium when glucose was

heat-sterilized with the medium and the adenine concentration reduced to 0·5 μmole/ml., i.e. 185 mg./l. from the 5 μmoles/ml. employed by Omata (1959); he also stated that adenine was inhibitory at concentrations higher than 0·5 μmole/ml. In fact, Omata sterilized all the components of his medium together by autoclaving at 116°, and reported that *F. nucleatum* gave maximum response to adenine at a concentration of 5 μmoles/ml. (1·85 g./l.) and no growth at 0·3 μmole/ml. (111 mg./l.). It should be noted that in the present work, even when the concentration of adenine was matched to those used by Omata (1959) and Coles (1968) respectively, one strain of *F. nucleatum* and three strains of *F. polymorphum* still failed to grow.

The requirement of *Fusobacterium nucleatum* for pantothenate is in agreement with the findings of Omata (1959), who found that this vitamin is a nutrilite for six strains. In the present study, however, the growth of the two strains of *F. nucleatum* studied was stimulated by nicotinic acid, although Omata did not indicate any stimulation by this vitamin. In addition, this organism requires adenine for active growth – a requirement also shown by Omata (1959).

It is strange to find that the highly fastidious organism *Fusobacterium nucleatum* apparently requires only one vitamin (pantothenic acid) and a purine (adenine). The use of casitone which had been treated with activated charcoal led to a reduction in growth, even though all the vitamins listed in Table 1 were present. It is clear there are unknown growth factors in the 'vitamin-free' casitone. However, the present work showed that pantothenic acid and adenine (plus nicotinic acid, being stimulatory) were not in fact the minimal vitamin requirements in the medium used. The further addition of both folic acid and pyridoxine resulted in moderate growth (approximately one-half the control).

It has been shown that the growth of *Fusobacterium nucleatum* in the experimental medium deficient in both folic acid and *p*-aminobenzoic acid is as good as that in the complete medium. It was not possible to eliminate components such as methionine and adenine from the medium; the former is presumably present in casitone, whereas the latter is essential for growth. This non-requirement for folic acid or *p*-aminobenzoic acid must imply the synthesis of these compounds by *F. nucleatum*. The failure to demonstrate a biotin requirement either in the presence or in the absence of $CO_2$ and, it may be added, in the absence of unsaturated fatty acids may be related to the fact that asparagine was not removed from the medium.

In the case of *Leptotrichia buccalis*, biotin, pyridoxine, *p*-aminobenzoic acid and calcium pantothenate could meet the minimal vitamin requirements. Similar observations were made on *Bacteroides ruminicola* by Bryant, Small, Bouma & Chu (1958). These workers found that this organism also grew well in the absence of purines and pyrimidines, and that medium containing vitamin-free enzymic digest of casein with biotin and *p*-aminobenzoic acid supported as good growth as medium with nine vitamins.

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# Flocculation in *Schizosaccharomyces pombe*

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Spontaneous formation of macroscopic aggregates of yeast cells is termed flocculation. Many papers have been written on the subject (for reviews see Comrie, 1952, Gilliland, 1957; Jansen, 1958; Rainbow, 1966; Windisch, 1968), but because of the importance of the phenomenon to the brewer most of the studies have been confined to brewer's yeast. An analogous phenomenon in a fission yeast is described here.

*Schizosaccharomyces pombe* NCYC 132 was grown in 125 ml. Erlenmeyer flasks each containing 10 ml. malt-extract broth (Oxoid, 2%, w/v). The inoculum consisted of $5 \times 10^6$ stationary-phase yeast cells from a 24 h. static culture in the same medium in a tightly capped McCartney bottle. Cultures were shaken on a rotatory shaker at 150 rev./min. at 32°; the generation time was 120 min.

## RESULTS AND DISCUSSION

Flocculation, operationally defined as the formation of aggregates consisting of more than 10 organisms, occurred only during the stationary growth phase. Observed with a microscope, the first flocs appeared 2 to 3 h. after the end of logarithmic growth. Maximum flocculation was attained within 5 to 10 h., when flocs contained 25 to 35% of the population. The largest floc contained up to $10^5$ organisms. Static cultures, such as those used for inocula, did not flocculate even when incubated for weeks, provided they remained largely anaerobic. Organisms grown in a medium containing (per l.) 5 g. yeast extract (Difco) + 30 g. glucose, static or shaken, never flocculated.

Formation of flocs was not due to failure of daughter organisms to separate after fission. An hour or two before flocculation, aggregates consisting of more than 10 organisms were never observed. Moreover, separation of pairs and larger aggregates by ultrasonic treatment at the end of logarithmic growth did not prevent subsequent flocculation.

Flocs were stable to dilution in deionized water and could be separated from free (non-flocculated) organisms by differential sedimentation. A flocculated culture was transferred to a 15 ml. tapered centrifuge tube and allowed to stand undisturbed for 5 min. at room temperature. The flocs settled to the bottom of the tube, whereas free organisms remained in the supernatant fluid which could then be decanted from the flocs or sampled *in situ* for counting with a haemocytometer. Flocs were purified by repeated sedimentation in deionized water. Free organisms separated from flocs during the time of maximum flocculation could not be induced to flocculate among themselves when shaken in their own medium and thus were considered to be non-induced or incompetent.

That flocs were stable in deionized water indicates that no special component of spent medium sustained floc formation. Addition of uninoculated medium, or of

spent medium separated from logarithmically growing cultures, to purified flocs did not cause deflocculation. This observation indicates that the failure of growing organisms to flocculate was not due to the presence of a deflocculating agent in the medium.

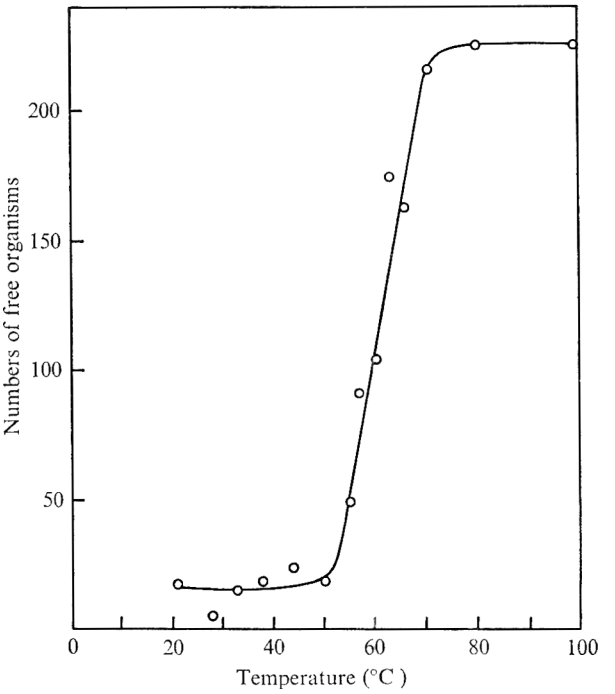


Fig. 1. Effect of temperature on the stability of purified flocs of *Schizosaccharomyces pombe* NCYC 132. The extent of deflocculation is expressed as the number of free organisms in the suspensions observed microscopically.

The effect of temperature on the stability of flocs in deionized water is shown in Fig. 1. A 10 ml. suspension of purified flocs in a tapered centrifuge tube was incubated in a water bath, and the temperature of the bath was raised 1 ° every 5 min. Counts of free organisms were made as follows. The tube was withdrawn, inverted five times, and returned to the bath to remain undisturbed until sampled. At the end of 5 min., the supernatant fluid was sampled for counting, 10 mm. below the liquid surface. Only organisms in groups of 10 or less were counted. Purified flocs were stable to 50 °; higher temperatures caused deflocculation, which was complete at 80 ° and above. The temperature at which half the floc population deflocculated was 61 °. A roughly similar value has been reported for brewer's yeast (Mill, 1964).

Deflocculation by heat was reversible. When a tube of heat-deflocculated organisms was allowed to stand undisturbed at room temperature, organisms slowly reflocculated. The time taken for half the population to reflocculate ($R_t$) was 10 to 15 h. Continuous gentle agitation shortened $R_t$ values to an hour or so. Addition of 0·1 M salt, such as NaCl or $CaCl_2$, decreased the $R_t$ value to a few minutes.

Calcium ions have been directly implicated in the flocculation of brewer's yeast (Mill, 1964) and in other cell-aggregating systems (for a review, see Curtis, 1967). I

found no evidence that $Ca^{2+}$ were directly involved in flocculation of this fission yeast *Schizosaccharomyces pombe*. The stability of purified flocs in deionized water and the ineffectiveness of EDTA (pH 8) as a deflocculating agent are evidence against the involvement of $Ca^{2+}$. Moreover, repeated washing of deflocculated organisms with deionized water did not inhibit subsequent reflocculation. Furthermore, the use of salts to increase the rate of reflocculation could be obviated by packing the organisms by centrifugation. Then, upon resuspension, the organisms were seen to have reflocculated. Incompetent organisms sedimented in a centrifuge did not flocculate.

Because the population of free organisms could be determined from a sample of the supernatant fluid, and the total population estimated after deflocculation, it was possible to calculate the number of flocculated organisms in a culture without isolating the flocs. A culture whose population of free organisms had been sampled was deflocculated at 90°. After cooling to room temperature, it was sampled for a total count. The number of organisms in flocs was then equivalent to the total population determined after deflocculation minus the free organism population determined after standing for 5 min. and before deflocculation.

Incubation of purified flocs in 3 M-guanidinium chloride or in 5 M-urea caused complete deflocculation, an observation that has been made with brewer's yeast (Mill, 1964). Removal of either reagent led to instantaneous reflocculation. Sodium dodecyl sulphate also caused reversible, but very slow, deflocculation. In contrast, deflocculation by trypsin or papain was irreversible, in the same sense that the effect of neither reagent could be removed by washing. The use of proteolytic enzymes as disaggregating agents for brewer's yeast (Eddy, 1958) and other cell-aggregating systems (for a review, see Curtis, 1967) is well known. Sugars, reported to cause deflocculation of brewer's yeast (Eddy, 1955; Mill, 1964) were ineffective even at concentrations as high as 50% (w/v). High concentrations of NaCl were likewise ineffective.

Since heat-killed organisms reflocculated, incompetent organisms and organisms in flocs were separately ruptured by means of a Braun cell homogenizer. The envelopes were separated from cellular debris and whole organisms by differential centrifugation and washed repeatedly with deionized water. Envelopes derived from organisms in flocs flocculated; those from incompetent organisms did not. Thus whatever forces were involved in floc formation must have been associated with the envelopes.

Reflocculation of organisms reversibly deflocculated by any of the ways described above showed significant hysteresis. The highest reflocculation value observed was 95%.

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# A Rapid Dilution Technique

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Viable counts by ordinary dilution procedures and plating is a routine practice in many fields of microbiology, Many attempts have been made to simplify these procedures in terms of both time and material consumed. There are fields of research where sampling at close intervals is essential. In such cases storing of samples, although undesirable because of possible deleterious effects on viability of the bacteria, may become necessary.

Faced with this problem in our growth experiments where very frequent sampling was required, we adopted the technique described below which eliminates the requirement for sterile pipettes and tubes of diluent, thus reducing time spent on preparations of materials and in manipulations. All the apparatus required for this technique is shown in Plate 1.

The stock diluent is conveniently contained in a Petri dish and is dispensed with a graduated 1 ml. syringe. The syringe is inserted into the Petri dish through a hole in the plastic lid, and a diluent volume of 0·9 ml. is transferred into a glass dish previously sterilized by burning alcohol. No change of syringe has been found necessary during platings over 7 to 8 h. The inoculum is measured by means of a pipette delivering 50 drops/ml. of the diluent used. Five drops of inoculum mixed with the diluent give a 1/10 dilution. Alternate use of two pipettes saves waiting time for cooling. The main feature is that the diluent and inoculum are placed in a small glass dish $4 \times 4 \times 1{\cdot}5$ cm. with a hemispheric cavity 1·5 cm. wide and 1·2 cm. deep (Baird and Tatlock) and mixed by means of a magnetic stirrer 6 mm. long and about 0·5 mm. thick sealed in a piece of capillary tubing (Voss Instruments, Faraday Works, Maldon, Essex). In the meantime the *inoculating* pipette is rinsed in alcohol and ignited and the diluent for the next dilution is placed in another sterile glass dish.

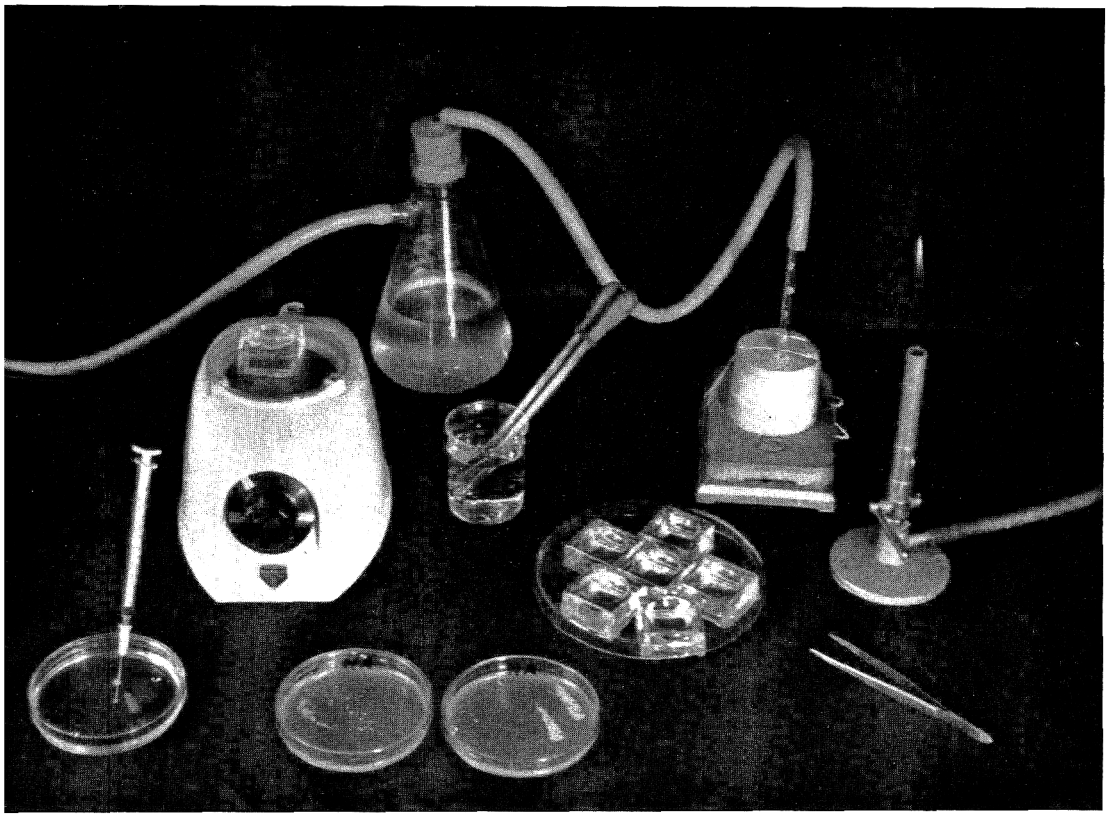
After plating the appropriate dilutions of culture, all glass dishes are vacuum-emptied into a conical flask connected to a vaccum line. Plugging the nozzle into a rubber stopper is a quick method of sealing the vacuum line. The dishes are then wetted with a piece of cotton wool soaked in alcohol and the residual alcohol ignited. The magnets, similarly sterilized, are replaced in the dishes which can then be stored under a large Petri dish lid. The whole procedure of making a dilution up to $10^{-6}$, plating, cleaning and sterilization takes no longer than 6 min.

No special contamination problems have been experienced over a period of several months. No aerosol could be detected with agar plates placed over the glass dishes during the magnetic mixing, but application of this technique to pathogenic organisms would require additional precautions, e.g. use of a fan cabinet and covers for the glasses. We find that this technique is considerably faster and more convenient than other

dilution procedures and makes possible sampling and plating at intervals as close as 6 to 8 min. It is also economical in terms of materials, technical assistance and effort.

EXPLANATION OF PLATE

An illustration of equipment for the dilution technique.



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# Genetic Transfer in *Mycobacterium phlei*

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Transfer of genetic information in the genus *Mycobacterium* has been reported (Juhasz, 1960; Redmond, Ward & Wright, 1967) but the role of phage in such transfers could not be established. We would like to report on genetic transfer in *M. phlei* in which transducing phage Bo 2 plays a major role.

The donor strain, *Mycobacterium phlei* SN 109, obtained from the Borstel Culture Collection, Borstel, West Germany, could grow on D-xylose (*xyl*$^+$) when this sugar was present as the sole carbon source, and was sensitive to 1·0 μg./ml. streptomycin (*str-s*). The recipient strain, a mutant of *M. phlei* F89 resistant to 100 μg./ml. streptomycin (*str-r*), could not utilize D-xylose (*xyl*$^-$) as a carbon source. Original *M. phlei* F89 was obtained from S. Froman, Olive View Hospital, Olive View, California. The phage employed was mycobacteriophage phlei Bo 2, originally designated B 2, isolated from dung and propagated on *M. phlei* F89 (Juhasz & Bönicke, 1965). The selective medium chosen for these experiments was a minimal medium (Gordon & Mihm, 1959) containing 1 % D-xylose (Pfanstiehl), to which 50 μg./ml. $FeCl_3$ and 50 μg./ml. streptomycin sulphate (Sigma) were added. Several controls were employed. These consisted of bacteria treated with (1) heat-killed phage (autoclaved at 15 lb. pressure for 15 min.); (ii) phage and DNase (Calbiochem); (iii) heat-killed (autoclaved) phage and DNase; and (iv) sterile heart infusion broth (Difco) in place of phage.

In our experiments phage Bo 2 was propagated on the donor strain *Mycobacterium phlei* SN 109 (*xyl*$^+$, *str-s*), harvested, filtered and tested for bacterial sterility. Sterile phage preparations were then used to infect the recipient *M. phlei* F89 (*xyl*$^-$, *str-r*). Various multiplicities of infection were tried. Positive results were obtained with multiplicities of infection of 1·0 and 2·0, the results being proportional to the phage concentrations employed. However, if the phage concentration was too high, background bacterial growth, stemming from nutrients present in the phage preparation itself, made isolation of *xyl*$^+$ transductants difficult. The phage–bacterium mixture was incubated at 37° for 48 h. and subsequently inoculated on to the selective medium. Growth was recorded after 4 weeks. In order to insure that mutants could in fact utilize xylose and that bacterial growth had not been due to nutrients in the phage inoculum, randomly selected colonies from each experiment were washed twice in physiological saline and reinoculated on to the selective medium. All the colonies thus tested were able to grow on xylose as sole carbon source. Phage conversion did not seem to account for the emergence of the *xyl*$^+$ character since infection with phage Bo 2, which has been propagated previously on F89 (*xyl*$^-$, *str-r*) did not convert F89 (*xyl*$^-$, *str-r*) to *xyl*$^+$.

Transfer of the *xyl*$^+$ marker by *transduction* is firmly supported by the data from four transfer experiments summarized in Table 1. The larger number of prototrophic colonies obtained with live as opposed to heat-killed phage, the proportionality of the number of such colonies to the number of phage employed, and, finally, the transfer activity retained by DNase-treated phage are major characteristics of the known transduction systems. The peculiarity of the transfer system presented in this paper was,

Table 1. *Transfer of the xyl*$^+$ *character to Mycobacterium phlei F89 (xyl*$^-$, *str-r)*

| Experiment* | A | B | C | D |
|---|---|---|---|---|
| Phage input/ml. (plaque-forming units) | $1{\cdot}2 \times 10^8$ | $3 \times 10^8$ | $8 \times 10^8$ | $4 \times 10^8$ |
| Multiplicity of infection | 1·2 | 1·C | 2·0 | 1·0 |
| 1. Bacteria and phage mixture | 217 | 481 | 1031 | 451 |
| 2. Bacteria and phage and DNase mixture | — | 411 | — | 367 |
| 3. Bacteria and heat-killed phage mixture | — | 84 | 343 | 108 |
| 4. Bacteria and heat-killed phage and DNase mixture | — | — | 56 | — |
| 5. Bacteria and sterile heart infusion broth | 13 | — | 38 | — |

* Numbers in columns refer to the number of colonies obtained on 20 plates containing 0·1 ml. amounts of the incubation mixture/plate (in the presence of xylose as sole carbon source).

however, that heat-killed phage preparations retained part of the original transfer activity. Upon treatment with DNase, this residual activity of heat-killed phage disappeared and the number of spontaneous revertants was comparable with that found when heart infusion broth replaced phage. Since the heat-killed phage appeared to retain the transfer activity lost when the phage preparation was treated with DNase (sum of lines two and three of Table 1 compared with line one) one can hardly escape the conclusion that the observed residual activity was due to transformation by bacterial DNA. Direct evidence for transformation to *xyl*$^+$ of *Mycobacterium phlei* F89 will be obtained only upon isolation of biologically active DNA from either donor SN 109 or from phage Bo 2 which has been propagated previously on SN 109.

## ADDENDUM

After this paper was submitted for publication an article appeared in *Nature, London*, on 'Transduction in *Mycobacterium smegmatis*'. (C. V. Sundar Raj & T. Ramakrishnan, 1970, *Nature London* **228**, 280.)

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