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# *Rhodotorula nitens* sp.nov. Isolated from the Atmosphere

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SUMMARY

A new species of the genus *Rhodotorula* isolated from the atmosphere in Edinburgh is described. It is characterized by the presence of a capsule and a predilection for low temperatures.

## INTRODUCTION

During a survey of the yeast flora associated with man (Mackenzie, 1961), a single colony of a new orange-coloured yeast developed on an atmospheric settling plate exposed in the bacteriology laboratory at the Astley Ainslie Hospital, Edinburgh. Its description according to the methods and criteria defined by Lodder & Kreger-van Rij (1952) is noted below.

## DESCRIPTION

*Rhodotorula nitens* nov. sp.

In musto maltato cellulae singulae rotundae aut subrotundae 3–8$\mu \times 3{\cdot}5$–9$\mu$. In agaro maltato formae et dimensiones cellularium eadem sunt quae in musto maltato. Gemmae singillatim formantur in basi subgrandi. Forma capsulae. Insulae et anulus formantur. Sedimentum grave. Cultura (post unum mensem, 17°) mollis, rasilis, mucosa, nitens, convexa, patula, colore aurantiosa, margines rasiles. Pseudomycelium nullum. Fermentatio nulla. In medio minerali cum glucoso galactoso saccharo maltoso et lactoso crescit. Nitras kalicus non assimilatur. In medio minerali cum alcohole aethylico non crescit. Arbutinum finditur. Non multum amidum producitur. Ex aere separata Edinburgi in Scotia.

Typus: Dept. Microbiol. Queen's Univ. Belfast, AA8.
CBS 4256
NCYC 607

*Growth in malt extract.* After 3 days at 17°, cells single, rounded or subspherical, occasionally short-oval, measuring 3–8$\mu \times 3{\cdot}5$–9$\mu$. Buds formed on a relatively broad base. Capsule present. After one month at 17°, conspicuous ring and islets; formation of a heavy flocculent sediment.

*Growth on malt agar.* After 6 days at 20° cells rounded or subspherical (Fig. 1). Buds formed at one point on the cell surface; prior to bud formation the cell is distinctly apiculate; subsequently, a prominent bud scar is present. Large prominent vacuole: numerous refractile cytoplasmic inclusions. Capsule present, ap-

proximately equal to the radius of the cell. After 1 month at 17°, the streak is soft, smooth, mucoid, glistening, convex, spreading, orange-coloured, tending to accumulate at the bottom of a vertical slant. Margins entire.

*Slide cultures.* Pseudomycelium absent. Very rarely, two or three elongated cells may be formed.

*Fermentation.* Absent.

*Sugar assimilation.* (Liquid medium) glucose +, galactose +, sucrose +, maltose +, lactose +.

*Assimilation of potassium nitrate.* Absent.

*Ethanol as sole source of carbon.* No growth.

*Splitting of arbutin.* Positive.

*Presence of starch.* Weak reaction.

Additional carbon sources tested for assimilation in liquid media as follows:

| | | | |
|---|---|---|---|
| adonitol | + | melezitose | + |
| aesculin | weak | melibiose | + |
| dl-arabinose | + | α-methylglycoside | + |
| cellobiose | + | raffinose | + |
| citric acid | – | rhamnose | – |
| dextrin | – | ribose | + |
| dulcitol | – | salicin | – |
| erythritol | – | sorbitol | + |
| glycerol | weak | l-sorbose | – |
| glycogen | – | starch | – |
| inulin | – | succinic acid | – |
| lactic acid | – | trehalose | weak |
| mannitol | + | d-xylose | + |

Mrs N. J. W. Kreger-van Rij (Delft) has also examined the biochemical characteristics of *Rhodotorula nitens*, and with the exception of soluble starch (+), ribose (–), adonitol (–), α-methylglycoside (–), results of assimilation tests are similar. In addition, Mrs Kreger-van Rij found that *R. nitens* assimilates L-arabinose but not D-arabinose.

### *Additional observations*

*Relation of growth to temperature.* In view of the apparent inability of the new yeast to grow at temperatures exceeding 26°, an investigation was made of the range of temperature supporting growth. Seven-day cultures on glucose nutrient agar slopes (glucose 1 % in Lemco nutrient agar) maintained at room temperature were washed off and the organisms suspended in sterile saline, the concentration being adjusted to about $2{\cdot}6 \times 10^6$ organisms/ml.

Twenty ml. quantities of glucose nutrient agar in 4 oz. (113·7 ml.) medicine bottles stoppered with cotton wool were inoculated with 0·7 ml. of the standardized suspension of the new yeast and allowed to stand for 1 hr. at room temperature. After removal of excess liquid the bottle was placed upright in a thermostatically controlled water bath. The low temperatures required were obtained by placing the water bath in a cold room at +4°. Incubations were made for 14 days each at 4°, 8°, 12°, 16°, 20°, 24° and 26°. Three bottles were inoculated and incubated at each temperature. After 14 days the yeast was washed off with sterile saline, filtered and the dry weight determined. Combined results of two trials are shown in Fig. 2.

The results show that *Rhodotorula nitens* is capable of growth between 4° and 20° inclusive, and that the optimum is about 14°. The new yeast is remarkably in-

tolerant of temperatures above 24°. Three-day cultures in 2 % malt broth are killed when placed at 37° for 8 to 10 hr. No growth occurs at 26°, but the organism remains viable at this temperature.

*Relation of growth to pH value.* Growth was studied over a range of values from pH 2·2 to pH 8, at intervals of 0·2 units. The pH values of samples of the basal medium [$(NH_4)_2SO_4$, 5 g.; $KH_2PO_4$, 1·0 g.; $MgSO_4.7H_2O$, 0·5 g.; $CaCl_2.6H_2O$, 0·1 g; NaCl, 0·1 g.; glucose, 5·0 g.; concentrated vitamin solution (Lodder & Kreger-van

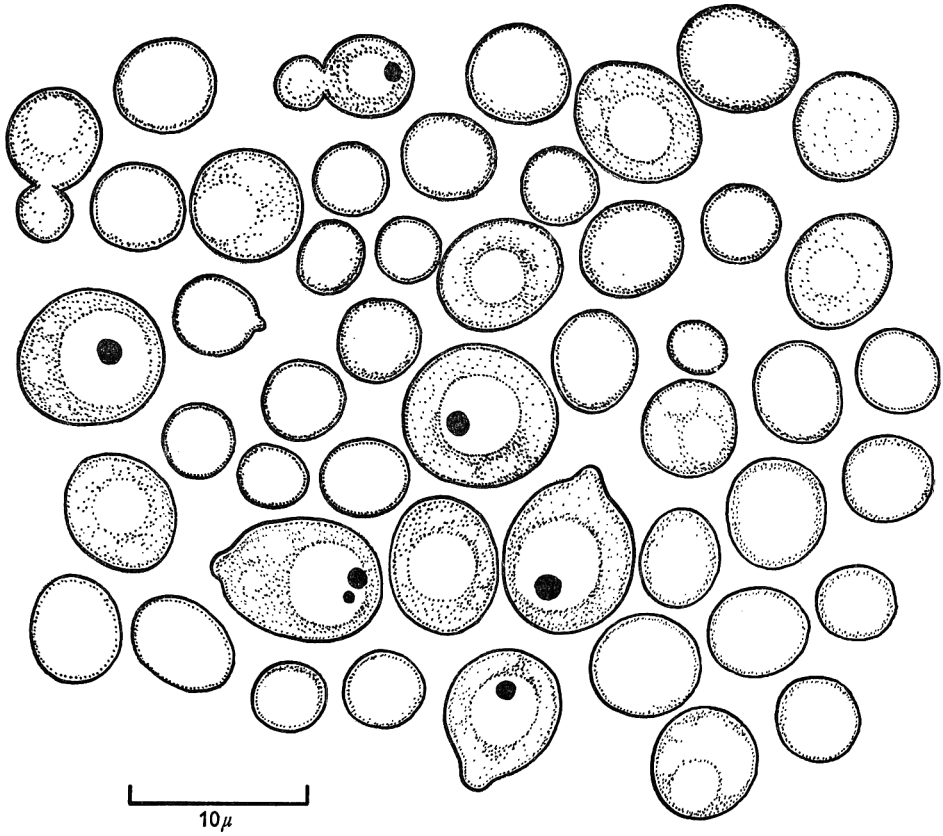


Fig. 1. *Rhodotorula nitens* nov.sp. Camera lucida drawing of cells from 3-day culture on 2·5 % malt agar.

Rij, 1952), 0·5 ml.; distilled water, 1000 ml.] were adjusted by 0·2 M–$Na_2HPO_4$ or 0·1 M-citric acid (McIlvaine, 1921). The presence of $SO_4''$, $Cl'$ and $PO_4'''$ ions in the nutrient solution appreciably altered the calculated pH values, particularly in the higher pH range, and pH values were accordingly adjusted by an electric pH meter. Two tubes were prepared for each pH value; the experiment was later duplicated. The pH stability was generally satisfactory over the 14-day period of incubation. Inoculated tubes showed a maximum drift of 0·25 pH units after incubation for 20 days. Potassium acid phthalate+sodium hydroxide, and potassium acid phthalate+hydrochloric acid buffer solutions (Clark & Lubs, 1915) were also used but were unsatisfactory because of toxicity and pH drift. The phosphate and citric

acid components of the buffer solution finally used in determination of growth at different pH values were non-toxic. No assimilation of either substance was noted in liquid assimilation tests.

The amount of growth, represented by the turbidity produced after 14 days of incubation at 20° was measured by an EEL (Evans Electroselenium Ltd.) absorptiometer. The results in Fig. 3 show that growth of *Rhodotorula nitens* at 20° occurs between pH 2·2 and pH 8 (opt. about pH 5). In further trials with 500 ml. quantities of unbuffered glucose nutrient broth (original pH 6·8), incubation for 4 weeks at room temperature resulted in a final pH value of 4·6.

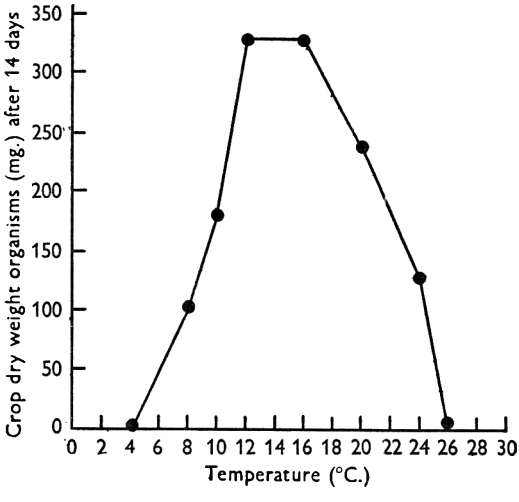


Fig. 2

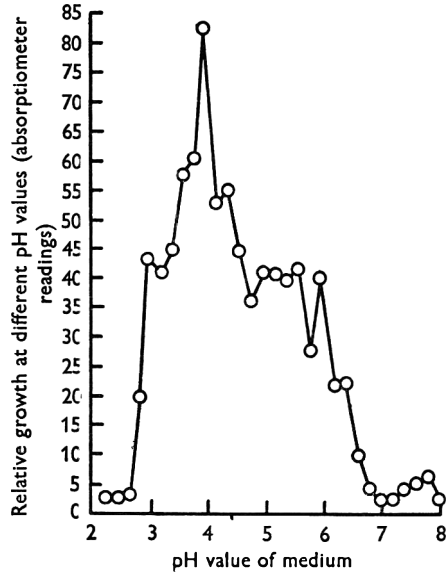


Fig. 3

Fig. 2. Effect of temperature on growth of *Rhodotorula nitens*, sp. nov.

Fig. 3. Effect of pH value of medium on growth of *Rhodotorula nitens*, sp.nov. Readings were made after 14 days of incubation at 20°. Relative amounts of growth shown as absorptiometer reading.

*Pigment production.* *Rhodotorula nitens* was incubated in glucose nutrient broth for 14 days at room temperature, centrifuged, washed with water and dried in air. The dried yeast was ground to a powder in a mortar and the pigments extracted by prolonged mechanical agitation in a mixture of 20 % methanolic KOH and benzene (Bonner, Sandoval, Tang & Zechmeister, 1946); after centrifugation the pigments were localized in the benzene layer. Separation of the pigments was achieved chromatographically on a small (1 × 10 cm.) column of a 4 + 1 mixture of calcium hydroxide and acid-washed alumina. Elution with light petroleum ether (b.p. 40–60°) containing 7·5 % (v/v) acetone, and finally with ethanol, gave three pigmented and three colourless fractions. Examination of the absorption spectra of each fraction, dissolved in light petroleum and subsequently in carbon disulphide, with a Unicam spectrophotometer and/or an Optica recording spectrophotometer, showed that none of the colourless fractions gave distinguishable peaks between 420 and 600 mμ. Details of absorption maxima are noted in Table 1.

One (yellow) fraction corresponded closely to figures obtained with authentic $\beta$-carotene; another (yellow) appeared to be $\gamma$-carotene. The composition (possibly complex) of fraction 6 was not determined.

The absorption maximum of a crude extract of pigment derived from cells grown in a potato+yeast medium (Hasegawa, Banno & Yamauchi, 1960) was 453 m$\mu$.

*Vitamins.* The vitamin requirements of *Rhodotorula nitens* were determined for eight vitamins in liquid media incubated for 13 days at room temperature. The basal medium was identical with that used in determining the pH range for growth. Individual vitamins were tested both singly and as the only deficiency in a solution containing the other seven. Each tube was triplicated. The results (Table 2) showed that *R. nitens* required thiamine; partial requirement was noted for Ca-pantothenate.

Table 1. *Absorption maxima (m$\mu$) of pigments isolated from* Rhodotorula nitens

| Solvent | Fraction 2 | Commercial $\beta$-carotene | Bonner *et al.* (1946) $\beta$-carotene | Fraction 4 | Fraction 6 |
|---|---|---|---|---|---|
| Light petroleum | 424 | 425 | | 435 | 450 |
| | 450 | 449 | 453 | 459 | 470 |
| | 476 | 478 | 480 | 489 | 503 |
| Carbon disulphide | 451 | 460 | 452 | 466 | 474 |
| | 482 | 490 | 484 | 494 | 508 |
| | 510 | 520 | 519 | 528 | 540 |

Table 2. *Vitamin requirements of* Rhodotorula nitens

| Vitamin | Amount ($\mu$g./10 ml. medium) | Growth* As sole vitamin | Growth* As sole deficiency in mixture of remaining vitamins |
|---|---|---|---|
| Biotin | 2 | ± | ++ |
| Ca-pantothenate | 400 | + | ++ |
| Inositol | 2000 | ± | ++ |
| Nicotinic acid | 400 | ± | ++ |
| p-Aminobenzoic acid | 200 | ± | ++ |
| Pyridoxine HCl | 400 | ± | ++ |
| Thiamine HCl | 400 | ++ | — |
| Riboflavin | 200 | − | ++ |
| Control 1. Vitamins absent | | − | . |
| Control 2. All vitamins present | | ++ | . |

* −, No growth; ±, trace of growth, faint turbidity; +, tube opalescent; ++, heavy growth, coalescent surface growth.

## DISCUSSION

The new yeast is placed in the genus *Rhodotorula* Harrison rather than the genus *Cryptococcus* Kützung emend. Vuillemin on the basis of its conspicuous carotenoid pigmentation and absence of a strongly positive starch reaction. It most closely resembles *Rhodotorula flava* (Saito) Lodder in sugar and nitrate assimilation patterns, inability to utilize ethanol and ability to split arbutin. Although the presence of a capsule is not generally associated with the red-pigmented yeasts, Hasegawa *et al.* (1960) record its existence in several species of Rhodotorula and observe that it is not unknown in *R. flava*. *Rhodotorula nitens* differs from *R. flava* in cell

morphology, appearance of the colony and in having an orange rather than a yellow colour. Characteristic features of the new yeast include the capsule and a marked intolerance of a temperature of 37°; there is a slight psychrophilic tendency, with optimum growth occurring between 12 and 16°.

The dependence on pigmentation for generic characterization of Rhodotorula has been criticized by several workers (Wickerham, 1952; Peterson, Bell, Etchells & Smart, 1954; Nakayama, Mackinney & Phaff, 1954). It has been shown that carotenoid pigments may occur in Cryptococcus (Nakayama *et al.* 1954) and because of this, Lodder & Kreger-van Rij (1955) have conceded that red pigmentation is not an entirely satisfactory criterion for the classification of Rhodotorula. Nakayama *et al.* (1954) suggest that separation of Cryptococcus and Rhodotorula is best achieved on the basis of starch formation, and although this may be of value in classifying doubtful cases, starch production (like red pigmentation) is not in itself an absolute character. There are marked inconsistencies in the characters (presence of capsules, starch and carotenoid pigments) used to distinguish the three genera *Cryptococcus*, *Rhodotorula* and *Torulopsis*, and these have yet to be resolved. The 'subgenus' *Flavotorula* created by Hasegawa *et al.* (1960) includes asporogenous, non-fermenting, capsulated, starch-forming, budding yeasts, producing yellow to pale orange colonies and having an absorption maximum of 450 m$\mu$ in light petroleum when grown in potato + yeast extract. In the latter respect *Rhodotorula nitens* resembles 'Flavotorula', apparently lacking the specific pigment or pigments giving the peak of 480 m$\mu$ which is characteristic of 'Rhodotorula'. In *Rhodotorula nitens* the absorption peak of the crude pigment extract is due to a high proportion of $\beta$-carotene. This was produced in abundance when the organism was grown in a potato + yeast extract medium, but the crude extract is a mixture of pigments, and changes in their relative proportions would alter the absorption maximum. If the qualitative aspects of carotenoid pigmentation are to be used as taxonomic criteria, it is essential that the organisms should be grown in a chemically defined medium.

The original isolate AA 8 is maintained at the Mycological Laboratory, Department of Microbiology, Queen's University of Belfast, Northern Ireland. Subcultures (isotypes) have been deposited at the Yeast Division of the Centraalbureau voor Schimmelcultures, Delft, Netherlands and the National Collection of Yeast Cultures, Brewing Industry Research Foundation, Nutfield, Surrey, England.

Acknowledgements are made of the assistance received from Mrs N. J. W. Kreger-van Rij and Dr Takezi Hasegawa in examining the new yeast, Professor M. J. Boyd for the Latin diagnosis, and the technical assistance of Miss Hilary Bell and Miss Lesley Rusk.

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# The Effect of Various Inactivating Agents on the Viral and Ribonucleic Acid Infectivities of Foot-and-Mouth Disease Virus and on its Attachment to Susceptible Cells

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SUMMARY

Foot-and-mouth disease (FMD) virus was partially inactivated by several methods: incubation with dilute formaldehyde or acetylethyleneimine, ultraviolet (U.V.) irradiation, heating, or by mixing with type specific antiserum, trypsin or extracts from cells susceptible to the virus. The serological properties of the treated virus preparations were studied by complement-fixation and agar diffusion tests and their content of infective ribonucleic acid (RNA) determined by phenol extraction. The ability of the treated preparations to attach to susceptible cultivated pig kidney cells was examined. The decrease in viral infectivity when FMD virus was treated with formaldehyde or acetylethyleneimine, U.V., or heat at 25° or 37° was proportional to the loss of infective RNA, with little impairment of its serological properties or its ability to attach to susceptible cells. In contrast, loss of viral infectivity on mixing with antiserum, trypsin or cell extracts was due to the failure of the virus to attach to susceptible cells; the viral RNA is still present in an infective form in these mixtures.

## INTRODUCTION

Now that several stages can be recognized in the multiplication of animal viruses the points at which this multiplication can be impeded or inhibited may be examined in more detail. The infectivity of many viruses can be considerably decreased by heating at 56° or by treatment with dilute formaldehyde or by mixing with the specific antiserum. It is not known, however, whether these processes result in the loss of infectivity of the nucleic acid core of the virus or whether the inactivation is due to some blocking action which prevents the virus from attaching to the susceptible cell or from releasing its nucleic acid when it does attach. This is fundamental to the study of virus multiplication and is also pertinent in considering whether a formalinized (inactivated) vaccine is in fact non-infective, i.e. whether its nucleic acid has been inactivated. To answer some of these questions for foot-and-mouth disease (FMD) virus the effect of several inactivating procedures on the viral and RNA infectivities and on the serological properties of the virus was studied, together with the ability of the inactivated virus to attach to cells able to support the growth of the virus.

## METHODS

*Virus and RNA preparations.* Strains 1 (type O) and 997 (type C) of FMD virus were used. The first is maintained by intradermal inoculation of the metatarsal pads of guinea pigs. The fluid collected from the local vesicles which develop is highly infectious for unweaned mice (about $10^{9.5}$ ID50/ml.). Strain 997 has been passaged more than 100 times in monolayers of pig kidney cells and the virus was routinely harvested when the cells had been stripped from the glass by its action. Infective RNA was prepared from the virus suspensions by the cold phenol method (Gierer & Schramm, 1956).

*Titration of virus and RNA.* Virus was titrated either by intraperitoneal inoculation of groups of 7 day mice (Skinner, 1951) or by the plaque method in monolayer cultures of pig kidney cells (Sellers, 1955) or occasionally by the metabolic inhibition test (Martin & Chapman, 1961). RNA from strain 1 was assayed by intracerebral inoculation of 7 day unweaned mice (Brown, Sellers & Stewart, 1958) and that from strain 997 in pig kidney cell monolayers (Brown & Stewart, 1959). Freedom of the RNA from residual virus was ensured by showing that the infectivity was destroyed by the addition of a trace of ribonuclease.

*Examination of serological properties of virus suspensions.* The serological properties were studied by complement-fixation and by diffusion in agar. The complement-fixing activity of FMD virus suspensions is distributed between the infective 25 m$\mu$ component and the accompanying non-infective 7 m$\mu$ component. This difference in size allows ready separation by ultracentrifugation so that an assessment of the proportion of each component in a suspension can be obtained by a combination of ultracentrifugation and complement-fixation. In these experiments the virus samples were spun for 1 hr. at 30,000 rev./min. (60,000 *g* mean) in the L 40 head of the Spinco Ultracentrifuge and the complement-fixing activity of the top 8 ml. and bottom 2 ml. compared with that of the initial suspension. Brooksby's (1952) method was used for the titration of the complement-fixing activity of the suspensions. In this method equal amounts of antigen and antiserum dilutions are allowed to react with different amounts of complement and the 50% haemolytic end-point calculated by the use of probits.

Similarly, an approximate estimation of the relative amounts of the two virus components can be made by the use of the agar gel diffusion test when the concentration of the two components is sufficiently high (Brown & Crick, 1958). The vesicular fluid from guinea pigs infected with virus (strain 1) can be used directly for agar diffusion but the tissue culture harvests (strain 997) used in this work required concentration (e.g. with polyethylene glycol) before they could be examined in this way.

*Adsorption of virus to cultivated pig kidney cells.* Suspensions were prepared by removing the pig kidney cells from primary monolayer cultures with a mixture of ethylenediaminetetra-acetic acid (EDTA) and trypsin (0·01% of each) in phosphate buffered saline free from $Ca^{2+}$ and $Mg^{2+}$. The cells were then washed several times in phosphate buffered saline. Virus was added in the proportion of one infective unit to one cell and the mixture incubated at 37°. With fully infective virus this results in the adsorption of more than 90% of the virus and the breakdown of the adsorbed 25 m$\mu$ infective component into its RNA and 7 m$\mu$ components (Brown,

Cartwright & Stewart, 1961). The events following addition of inactivated virus preparations were ascertained by: (*a*) estimating the infective RNA in the inocula, unadsorbed supernatant and cells; (*b*) examining the nature of the adsorbed virus antigen by complement-fixation and agar diffusion tests. Although mixing the virus with pig kidney cells results in the removal of about 90 % of the complement-fixing activity from the inoculum, the cells do not fix complement when mixed with the type specific antiserum. When the cells are extracted with ether, about 50 % of the adsorbed virus antigen can then be recovered.

## RESULTS

### *Action of formaldehyde and acetylethyleneimine*

Virus suspensions were inactivated by treating with 1/5000 formaldehyde ( = 1/2000 dilution of 40 % formalin solution) at pH 7·6 and 25° for various periods. The inactivation was interrupted at intervals by adding excess sodium bisulphite. Similar partially inactivated preparations were obtained by treatment with 0·05 % (v/v) acetylethyleneimine at 37° except that in this case sodium thiosulphate was used to stop the inactivation process. Each preparation was then dialysed before extraction of the infective RNA and examination of its serological properties.

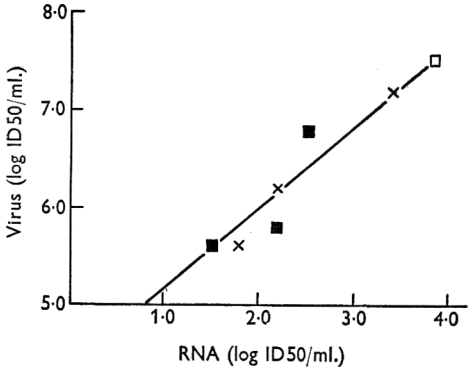


Fig. 1

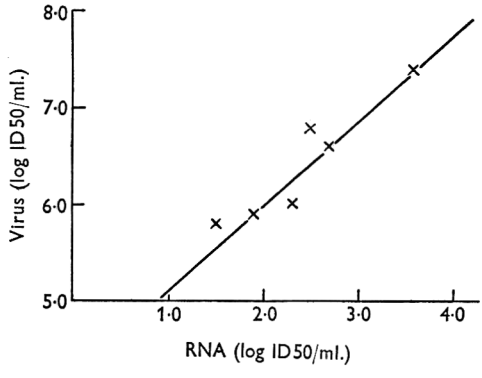


Fig. 2

Fig. 1. Effect of dilute formaldehyde (×) and acetylethyleneimine (■) on the infectivity of FMD virus and the RNA in the virus (initial virus (□)).

Fig. 2. Effect of ultraviolet irradiation on the infectivity of FMD virus and the RNA in the virus.

The yields of infective RNA obtained from the series of partially inactivated preparations were proportional to the residual virus infectivity (Fig. 1). Neither inactivation procedure led to any alteration in the total complement-fixing activity of the virus suspensions, even when carried on to complete inactivation. In addition, ultracentrifugation at 30,000 rev./min. still resulted in the deposition of about 70 % of the complement-fixing activity into the bottom 2 ml. of the tube. Similarly, two precipitin lines, corresponding to the 25 and 7 m$\mu$ components of the virus, were obtained in the agar diffusion test with the inactivated preparations as well as with the initial suspension.

*Action of ultraviolet radiation*

Preparations of the virus were U.V.-irradiated for various intervals with a Hanovia chromatolite; this emits radiation mainly at a wavelength of 2537 Å., which would be expected to inactivate the RNA rather than the protein part of the virus. Samples of irradiated material were removed at intervals and titrated for residual virus and the amount of residual infective RNA estimated by extraction with phenol. As with the formaldehyde and acetylethyleneimine inactivated preparations, the decrease in virus titre was accompanied by a corresponding decrease in the infective RNA (Fig. 2). Similarly, the inactivated preparations had serological properties which were indistinguishable from the starting material in the complement fixation and agar diffusion tests.

Table 1. *Effect of incubating FMD virus (strain 1) and viral RNA suspensions at 25° and 37° on their infectivity*

| | Virus titre (log. ID 50/ml.) | RNA titre (log. ID 50/ml.) |
|---|---|---|
| Initial suspension in 0·04 M-phosphate (pH 7·6) | 8·3 | 4·3 |
| Suspension at 25° for 24 hr. | 7·3 | 3·4 |
| Suspension at 25° for 24 hr. in 0·1 % sodium dodecyl-sulphate | 7·2 | 3·2 |
| Suspension at 37° for 8 hr. | 6·9 | 3·1 |
| Suspension at 37° for 8 hr. in 0·1 % sodium dodecyl-sulphate | 6·7 | 3·1 |
| Initial RNA | — | 4·3 |
| RNA at 25° for 24 hr. in 0·1 % sodium dodecyl-sulphate | — | 2·3 |
| RNA at 37° for 8 hr. in 0·1 % sodium dodecylsulphate | — | 2·5 |

*Action of heat*

The effects of prolonged heating at 25° and 37° were studied. Some of the results obtained when FMD virus is heated at 56° have already been reported (Brown & Cartwright, 1961). These will be referred to in the Discussion, to illustrate the marked differences in the results obtained at the different temperatures. About 90 % of the initial infectivity was lost in 24 hr. at 25° and the yield of infective RNA obtained from the heated virus was correspondingly lower (Table 1). Heating virus suspensions at 37° also caused a decrease in the virus titre and amount of infective RNA obtained from them. When the infective RNA was first extracted from the virus before heating at 25° or 37°, and protected from traces of residual ribonuclease by the addition of 0·1 % sodium dodecyl sulphate, its infectivity was lost rather more rapidly than that of the virus (Table 1). Virus suspensions held at 25° and 37° for periods of time which resulted in the loss of more than 99·9 % of the initial infectivity, showed the same pattern of two precipitin lines on diffusion in agar and possessed the same properties in the ultracentrifuge as the initial virus, thus indicating that the effect of heating was on the RNA core of the virus rather than on the protein coat.

*Action of trypsin, cell debris and viral antiserum*

Addition of crystalline trypsin to the tissue culture virus at a concentration of 1 mg./ml. resulted in a decrease in virus titre of about 2 logs. With smaller concentrations the decrease in titre was less but was about 1 log (90 %) even with 0·01 mg. trypsin/ml. Despite the decrease, as much infective RNA could be obtained from the virus + trypsin mixtures as from the initial virus suspension (Table 2).

Table 2. *Effect of mixing FMD virus (strain 997) with trypsin, viral antiserum and cell extracts on the viral and RNA infectivity*

| Addition to virus | Virus titre (log. ID50/ml.) | RNA titre (log. ID50/ml.) |
|---|---|---|
| Nil | 7·0 | 2·7 |
| Trypsin | 4·9 | 2·7 |
| Trypsin + EDTA | 4·8 | — |
| Trypsin + Arcton 113 | 4·9 | — |
| Antiserum | 1·5 | 2·5 |
| Antiserum + Arcton 113 | 7·1 | — |
| Cell extract | 5·1 | 2·6 |
| Cell extract + EDTA | 6·9 | — |

A similar loss in virus infectivity without loss of infective RNA occurred when the virus was added to cell extracts prepared by grinding cultivated pig kidney cells in a mortar (Brown, Cartwright & Stewart, 1962). In these experiments, however, the virus infectivity could be recovered by the addition of EDTA, which chelates $Ca^{2+}$ essential for the attachment of FMD virus to pig kidney cells. Similar treatment of the virus + trypsin mixtures with EDTA did not lead to recovery of virus infectivity. Treatment of the mixture with 'Arcton 113' (Trifluorotrichlorethane, I.C.I.) also failed to recover the virus infectivity.

The infectivity of FMD virus suspensions can be considerably decreased by mixing them with homotypic antiserum from guinea pigs or cattle. Here, too, as much infective RNA was obtained from the virus + antiserum mixtures as from the initial virus suspensions (Table 2). In these experiments, however, Arcton treatment led to full recovery of the virus infectivity (Brown & Cartwright, 1960).

*Fate of inactivated virus preparations added to susceptible cultivated pig kidney cells*

When infective tissue culture virus is added to cultivated pig kidney cells in the proportion of one infective unit per cell, the virus is rapidly adsorbed and engulfed by the cells; this is followed by disruption of the engulfed virus into its RNA and 7 m$\mu$ protein components (Brown *et al.* 1961). Virus suspensions inactivated with formaldehyde and acetylethyleneimine or by heating at 25° or 37° were adsorbed as efficiently as the untreated virus (Table 3). The cells did not fix complement when mixed with viral antiserum, although much of the complement-fixing activity of the inoculum had been adsorbed by the cells. These results suggest that the inactivated virus preparations were engulfed by the cells and were thus not available for reaction with antiserum.

As with cells which had been mixed with untreated virus preparations, complement-fixing antigen could be recovered by ether extraction of cells which had

adsorbed virus preparations inactivated by acetylethyleneimine and heat. Here too, the recovered complement-fixing antigen was not deposited by centrifugation at 30,000 rev./min. for 1 hr. in the L 40 head of the Spinco ultracentrifuge, indicating that these inactivated virus preparations had been disrupted by the cells (Table 3). In contrast to these observations, cells which had adsorbed virus inactivated by formaldehyde did not yield any complement-fixing antigen on extraction with ether, suggesting that such virus attaches to a different, presumably non-lipid, cellular constituent.

Virus partially inactivated by mixing with specific antiserum, cell extracts or trypsin was not adsorbed by the normally susceptible pig kidney cells. This was

Table 3. *Adsorption by cultivated pig kidney cells of FMD virus (strain 997) inactivated by formaldehyde, acetylethyleneimine or incubation at 37°*

| Treatment | Sample | Virus titre (log ID 50/ml.) | ml. 1/30 complement fixed by 1 ml. antigen | |
|---|---|---|---|---|
| | | | Total | Top 8 ml. |
| None | Initial virus | 7·0 | 0·80 | 0·25 |
| | Unadsorbed virus | 5·0 | 0·20 | — |
| | Cells | 4·5 | Nil | — |
| | Cells extracted with ether | 4·3 | 0·27 | 0·25 |
| Formaldehyde | Initial virus | Non-infective | 0·80 | 0·17 |
| | Unadsorbed virus | — | 0·17 | — |
| | Cells | — | Nil | — |
| | Cells extracted with ether | — | Nil | — |
| Acetylethyleneimine | Initial virus | Non-infective | 0·73 | 0·15 |
| | Unadsorbed virus | — | 0·17 | — |
| | Cells | — | Nil | — |
| | Cells extracted with ether | — | 0·28 | 0·28 |
| Incubation at 37° | Initial virus | Non-infective | 0·84 | 0·23 |
| | Unadsorbed virus | — | 0·16 | — |
| | Cells | — | Nil | — |
| | Cells extracted with ether | — | 0·39 | 0·30 |

Table 4. *Reaction with cultivated pig kidney cells of virus (strain 997) inactivated by mixing with antiserum, trypsin or pig kidney cell extracts*

| Treatment | Sample | Virus titre (log. ID 50/ml.) | RNA titre (log. ID 50/ml.) |
|---|---|---|---|
| None | Initial virus | 7·0 | 2·7 |
| | Unadsorbed virus | 5·0 | 0·5 |
| | Cells | 4·5 | 2·5 |
| Type specific antiserum | Virus-serum mixture | 1·5 | 2·5 |
| | Unadsorbed mixture | — | 2·5 |
| | Cells | — | None detected |
| Trypsin | Virus-trypsin mixture | 4·9 | 2·7 |
| | Unadsorbed mixture | 3·5 | 2·5 |
| | Cells | 3·0 | 0·5 |
| Cell extracts | Virus-cell extract mixture | 5·1 | 2·6 |
| | Unadsorbed mixture | 3·3 | 2·3 |
| | Cells | 2·9 | 0·7 |

ascertained by extracting samples of the inocula, unadsorbed supernatant fluids and cells with phenol after incubation of the mixtures with the pig kidney cells. The yields of infective RNA from each fraction (Table 4) indicate that the decrease of virus infectivity was due to non-attachment of virus to the cells.

It was considered possible that 1 mg. trypsin/ml. would affect the surface proteins of cultivated pig kidney cells, thus influencing their adsorptive properties. Suspensions of the cells were, therefore, held for 15 min. in trypsin solutions of this concentration, and then deposited in the centrifuge and mixed with virus. The treated cells adsorbed virus as efficiently as untreated cells and also produced as much virus when subsequently incubated at 37°.

The virus + trypsin complex was deposited as a pellet when centrifuged at 30,000 rev./min. for 150 min. in the L 40 head of the Spinco Ultracentrifuge. The pellet was readily suspended in phosphate buffered saline and yielded as much infective RNA as the initial virus when extracted with phenol. In some experiments the resuspended pellet was added to pig kidney cells; the RNA-containing material did not adsorb to these cells.

## DISCUSSION

Although FMD virus can be inactivated in several ways, the mechanisms of these inactivations have not been previously examined in any detail. Our experiments show that dilute formaldehyde or acetylethyleneimine or U.V.-radiation destroy the infectivity of the RNA core of the virus. The protein coat of the virus and the proportions of the 25 and 7 m$\mu$ components are unaffected by these treatments, as judged by complement-fixation and agar diffusion tests. More important is the fact that these inactivated preparations are taken up by susceptible cultivated pig kidney cells as efficiently as the untreated virus.

Virus inactivated by prolonged incubation at 25° or 37° also loses its RNA infectivity without any apparent change in the properties of the protein coat or alteration in the proportions of the 25 and 7 m$\mu$ components. This result is in striking contrast to the effect of heating the virus at 56°. At this temperature, the 25 m$\mu$ infective component is rapidly broken down into its infective RNA and 7 m$\mu$ protein sub-units (Bachrach, 1961; Brown & Cartwright, 1961) and as much infective RNA can be obtained by this treatment as by extracting the virus with phenol. The loss of RNA infectivity on prolonged incubation of the virus at 25° and 37° is probably due to heat denaturation rather than the action of environmental ribonuclease on the RNA core of the virus following rupture of the protein coat at some point, because the loss of RNA infectivity also occurs in the presence of sodium dodecyl sulphate, which inhibits the action of the enzyme. Nakamura (1961), working with mouse encephalomyelitis virus, also concluded that U.V. inactivation of the virus is due to destruction of the RNA whereas heat inactivation at 56° is due to destruction of the protein coat and not the RNA.

The loss of virus infectivity when FMD virus suspensions are mixed with cell extracts, trypsin or homotypic antiserum does not result in any loss of RNA infectivity. The loss is due to failure of the virus to attach to susceptible cells. In fact, virus can be recovered quantitatively from the mixtures with cell extracts and antiserum by treatment with EDTA and Arcton 113, respectively. So far it has not been possible to recover the inactivated virus from virus + trypsin mixtures,

suggesting that the site on the virus protein coat necessary for attachment to cells has been hydrolysed by the enzyme, thus preventing attachment to the susceptible cell. This is being further investigated.

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# Studies on the Deoxyribonucleic Acid of *Serratia marcescens*

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SUMMARY

A method for the quantitative isolation of pure highly polymerized DNA from *Serratia marcescens*, based on Kirby's phenol extraction procedure (Kirby, 1956, 1957), has been developed. No statistically significant differences were detected in the base composition or sequence between the DNA of a wild and a mutant strain of the organism. 6-Methylaminopurine was found in the DNA of *Serratia marcescens*. The properties of this base and its N-nitroso derivative were compared with those of a synthetic sample.

## INTRODUCTION

Deoxyribonucleic acids (DNA) are usually extracted from bacteria after they have been mechanically broken or lysed with lysozyme or sodium deoxycholate (e.g. McCarty & Avery, 1946; Zamenhof, Alexander & Leidy, 1953; Jones, 1953). The DNA thus obtained has usually been found to contain a much lower phosphorus content than the theoretical value, indicating the presence of non-phosphorus containing impurities (Overend, Stacey, Webb & Ungar, 1951; Dutta, Jones & Stacey, 1953; Burton, 1960). In the present work Kirby's method for the isolation of DNA from animal tissues (Kirby, 1956, 1957) was used to extract DNA from *Serratia marcescens* without the necessity for mechanical breakage of the cells, and the DNA so obtained was purified and analysed for purines and pyrimidines.

## METHODS

*Organism.* Two strains of *Serratia marcescens* were used: (i) a normal wild pigmented strain (NCTC 1377); (ii) a colourless mutant produced in this laboratory as follows. A culture of the pigmented strain was grown for 18 hr. at 25° on a medium containing NaCl (0·5%, w/v), Oxoid Lab Lemco (0·5%, w/v) and Oxoid peptone (1·0%, w/v) (10 ml.). The culture was centrifuged and the sediment of organisms washed three times with 0·85% (w/v) NaCl solution. The organisms were finally suspended in 0·85% NaCl (10 ml.) and a sample, in a 1 cm. quartz spectrophotometer cell, irradiated with ultraviolet light from a Hanovia Dectectolite unit (Hanovia Lamps Ltd. Slough, Bucks.) for 5 min. at a distance of 15 cm. The suspension was then plated on the medium described below and incubated at 25° for 24 hr. A colourless colony was picked off and propagated as usual. This strain gave colonies which were colourless when grown for 24 hr. but gave a very faint pink on prolonged incubation. When grown on a medium containing only inorganic salts and glucose this faint pink colour did not develop. The organism retained these properties after regular subculture for 6 years. The following characteristics were found to be

identical with those of the wild pigmented strain: motility +; indole reaction −; acid only produced from glucose, sucrose, inositol, mannose and maltose; no fermentation of lactose, dulcitol, arabinose, rhamnose and xylose.

*Medium.* The organism was grown on a nutrient agar containing NaCl (0·5%, w/v), Oxoid Lab Lemco (0·5%, w/v), Oxoid peptone (1·0%, w/v) and shred agar (0·2%, w/v): Astell Lab. Service Co., Ltd., London, S.E. 6).

*Conditions of growth.* The nutrient agar was poured into drying trays (Jencons Ltd., Hemel Hempstead, Herts.) 12 in. × 12 in., which were then covered with glass and allowed to cool. The inoculum was prepared from a 24 hr. nutrient broth culture, and was added (2 ml.) to each tray, which was then incubated at 26° for 48 hr.

*Harvesting.* The organisms were scraped off the surface of the agar, which was then washed with a little physiological saline. Organisms and washings were centrifuged at 4000 *g* for 20 min. and the deposit washed three times with saline solution.

*Nitrogen determination.* DNA samples were digested with the reagent described by Ma & Zuazaga (1942) containing selenium (0·05%, w/v) and the nitrogen determined by developing the indophenol blue colour by using the method of Holbrook & Jones (unpublished results). After digestion the solution was allowed to cool and saturated sodium bicarbonate solution (16 ml.) added, followed by sodium hypochlorite solution (1 ml., 1·0–1·4% (w/v) available chlorine, containing sodium nitroprusside 0·025% (w/v)). After 5 min., sodium phenate solution was added (1 ml., phenol 5·6% (w/v), sodium hydroxide 16·6% (w/v)) and the absorption of the solution read at 625 mμ after 20 min.

*Total phosphorus* was estimated by the method of Jones, Lee & Peacocke (1951).

*Inorganic phosphorus* was estimated by the method of Berenblum & Chain (1938).

*Pentose* was determined by the method of Euler & Hahn (1946) with a purified yeast nucleic acid as a standard.

*Base analysis.* Samples of DNA (5 mg. dried *in vacuo* at 110° for 3 hr.) were hydrolysed in formic acid (98–100% (w/v), 0·6 ml.) at 175° for 1 hr. Duplicate samples were hydrolysed and for each hydrolysate four chromatograms were run in propan-2-ol + 10N-hydrochloric acid + water (68 + 20·5 + 11·5, by vol.) on Whatman No. 1 paper.

## RESULTS

*Estimation of the DNA content of* Serratia marcescens. The method was a modification of Schmidt & Thannhauser's (1945) method introduced by Jones, Rizvi & Stacey (1958). The cells were harvested, weighed while wet, washed, freeze dried and reweighed, thus enabling a wet/dry weight ratio of the cells to be obtained so that the amount of DNA obtained in future isolations could be expressed as a percentage of the total amount present once the wet weight of cells harvested was known. Freeze-dried cells (70 mg.) were extracted with ether after refluxing with 95% methanol to remove the lipids, the acid soluble material was extracted with cold trichloroacetic acid (2 ml., 5%, w/v) and the residue dispersed in N-KOH (4 ml.) and incubated at 37° for 15 hr. The phosphorus content of the solution was measured before and after the precipitation of the DNA with perchloric acid (0·4 ml., 60%, w/v). It was found necessary to centrifuge the suspension at

173,000 *g* (Spinco ultracentrifuge model L). The amount of DNA obtained was 2·18 mg. and the DNA content of *Serratia marcescens* was calculated to be 3·1 %.

### *Isolation of the DNA*

All operations carried out at 0–4° unless otherwise stated. The organisms (7 g. wet weight, 1 g. dry weight from 6 trays) were added to a mixture of 6 % (w/v) sodium *p*-aminosalicylate (200 ml.) and phenol (Analar, 200 ml.) which had been saturated with salicylate solution, and the mixture shaken for 1 hr. The mixture was then centrifuged at 1000 *g* for 10 min. the water layer removed, and the phenol layer re-extracted with a salicylate solution (200 ml.) saturated with phenol until no more material precipitable with 3 volumes of ethanol was extracted from the cell debris; usually five extractions were necessary. Ethanol (3 volumes) was added to the combined aqueous layers and the crude DNA removed on a glass rod. The DNA was dissolved in M-NaCl (20 ml.) and left overnight. The solution was centrifuged at 25,000 *g* for 20 min. to remove cell debris and microsomal RNA, and brought to 20°. Cetyltrimethylammonium bromide (CTAB., British Drug Houses, Ltd.) was added to the supernatant liquid so that its final concentration was 1 % (w/v), and the concentration of the sodium chloride was decreased to 0·6 M by the addition of water (13·3 ml.). The precipitate of the cetyltrimethylammonium salt of DNA was removed on a glass rod, dissolved in M-NaCl, and the sodium deoxyribonucleate precipitated by adding ethanol (3 volumes).

The DNA so obtained was found by analysis to have a very low phosphorus content (about 7 % of the dry weight), which indicated the presence of impurity. The DNA was dissolved in water and shaken with chloroform to remove any traces of CTAB and protein, and the aqueous layer was then centrifuged at 80,000 *g*. The supernatant liquid was dialysed against frequent changes of distilled water and freeze dried (yield 30 mg., 97 % recovery). The precipitate, which in the case of DNA from the wild strain was coloured red, was very stable to acid-hydrolysis, but eventually gave a mixture of amino acids and sugars, indicating that the impurity probably contained cell-wall material.

### *Analysis of the DNA*

The DNA was isolated in three separate experiments from both the strains of *Serratia marcescens*. The analyses are shown in Table 1. The RNA and protein contents of all samples were less than 1 %. The base contents of the DNA samples were also determined. Adenine, guanine, cytosine and thymine were present in the amounts shown in Table 2.

### *Identification and properties of* 6-*methylaminopurine*

6-Methylaminopurine was identified chromatographically in the DNA of *Serratia marcescens* by using the method described by Dunn & Smith (1958), and by comparing its spectra in neutral, alkaline and acid solution with that given by a solution of the base which has been prepared from 6-methylmercaptopurine (Nutritional and Biochemical Corporation, Cleveland, Ohio) by the method of Elion, Burgi & Hitchings (1952). The $R_F$ values of the two compounds corresponded in the solvent systems butanol + ethanol + water (4 + 1 + 5, by vol.; $R_F$ 0·61), and propan-2-ol + 10 N-hydrochloric acid + water (68 + 20·5 + 11·5 by vol.; $R_F$ 0·50).

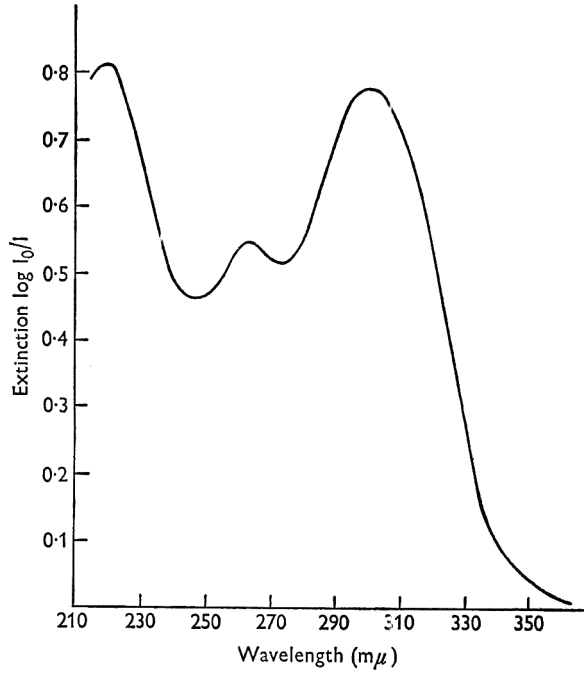


Fig. 1. Spectrum of N-nitroso derivative of 6-methylaminopurine (13·6 μg./ml.) in aqueous solution at pH 7·0.

Table 1. *Analysis of DNA samples isolated from* Serratia marcescens

Values are given for dry DNA. Nitrogen was determined by the method of Holbrook & Jones (unpublished results) and phosphorus by the method of Jones, Lee & Peacocke (1951). % DNA isolated from the cells calculated from the yield of wet cells obtained.

| | Wild strain | | | Colourless mutant | | |
|---|---|---|---|---|---|---|
| | S1 | S2 | S3 | S4 | S5 | S6 |
| Nitrogen (%) | 14·6 | 15·1 | 15·1 | 14·7 | 15·3 | 14·9 |
| Phosphorus (%) | 8·7 | 9·0 | 9·0 | 8·7 | 9·1 | 8·9 |
| $\epsilon_P$ | 7900 | 7800 | 8000 | 7800 | 8200 | 8200 |
| % isolated from cells | 97 | 100 | 97 | 100 | 100 | 100 |

S1–3, Separate samples of DNA isolated from the wild strain; S4–6, separate samples of DNA isolated from the colourless mutant; $\epsilon_P$, molar absorptivity, based on one gram-atom of phosphorus/litre.

The N-nitroso derivative of the synthetic 6-methylaminopurine was made by dissolving the base (100 mg.) in glacial acetic acid (2 ml.), adding a saturated solution of sodium nitrite (2 ml.) and allowing the solution to stand at room temperature overnight. Water (2 ml.) was added, the precipitate (80 mg.) filtered off and recrystallized from water to give orange needles (65 mg.; m.p. 242–6° (*d*). Found: C, 40·36; H, 3·76; N, 46·00%; calculated for $C_6H_6N_6O$: C, 40·44; H, 3·37; N, 46·12%). The spectrum given by this sample in neutral aqueous solution is given in Fig. 1; it does not agree with the spectrum given previously for this compound by Dunn & Smith (1958). This can be explained by the fact that they took their

Table 2. *Base composition of the DNA of* Serratia marcescens

The amounts were determined from the ultraviolet absorption of the eluate from paper chromatograms after hydrolysis of the DNA in formic acid. The number of samples hydrolysed is shown in parentheses and the mean amounts ± standard deviation are given in moles of base/100 g. atoms of P.

| Source | Adenine | 6-MAP* | Thymine | Guanine | Cytosine | Nitrogen materi recover (%) |
|---|---|---|---|---|---|---|
| Wild strain | 19·61 ± 0·21 (6) | 0·69 (2) | 20·16 ± 0·14 (6) | 29·70 ± 0·50 (6) | 29·84 ± 0·27 (6) | 97·7 |
| Colourless mutant | 19·59 ± 0·41 (6) | 0·73 (2) | 19·76 ± 0·14 (6) | 29·98 ± 0·59 (6) | 29·94 ± 0·39 (6) | 97·9 |

* 6-Methylaminopurine.

Table 3. *Ratio of the inorganic phosphorus liberated during a Burton* (1960) *degradation to the total phosphorus content of* Serratia marcescens *DNA*

DNA (5 mg.) was taken in each case and allowed to stand for 16 hr. at 30° C. in diphenylamine (2 %, w/v) in formic acid (30 ml., 68 %, w/v). Inorganic phosphate was measured by Berenblum & Chain's (1938) method and total phosphorus by Jones, Lee & Peacocke's (1951) method.

| | Wild strain | | | | | | Colourless mutant | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | S1 | | S2 | | S3 | | S4 | | S5 | | S6 | |
| Phosphorus | | | | | | | | | | | | |
| Inorganic (*I*) μg.P | 3·45 | 4·15 | 4·38 | 4·15 | 4·14 | 4·91 | 3·81 | 5·05 | 4·17 | 4·50 | 5·00 | 5·3 |
| Total (*T*) μg.P | 14·2 | 19·0 | 19·0 | 18·0 | 17·5 | 22·0 | 14·9 | 22·2 | 17·7 | 19·2 | 19·7 | 21·0 |
| *I*/*T* | 24·2 | 21·8 | 23·0 | 23·0 | 23·6 | 22·3 | 25·5 | 22·7 | 23·5 | 23·4 | 25·3 | 25·2 |
| Average *I*/*T* | | | 23·0 ± 0·7 | | | | | | 24·2 ± 1·1 | | | |

S1–3, Separate samples of DNA isolated from the wild strain; S4–6, separate samples of DNA isolated from the colourless mutant.

spectra of the compound after they had eluted it from a chromatogram either at pH 1 or at pH 13. Under these conditions we found that the N-nitroso derivative breaks down to yield the parent base.

The base (17 $\mu$g. eluted from paper chromatograms in 0·1 N-HCl, 3·4 ml.) isolated from the DNA of *Serratia marcescens* gave when treated with nitrous acid a compound with the same $R_F$ values as N-nitrosomethylaminopurine, but when an attempt was made to obtain the spectrum of this compound by eluting it from chromatography paper, the blank reading was so high, that it was impossible to obtain an accurate spectrum, but the peak was in the region of 300 m$\mu$.

From the value obtained for the molar extinction coefficient of the pure synthetic 6-methylaminopurine ($15{\cdot}1 \times 10^{-3}$ at 267 m$\mu$; pH 1·0), it was found to be present to the extent of 3·4% of the adenine content of the wild strain DNA, and 3·6% of the adenine in the colourless strain DNA.

No 5-methylcytosine was found in the DNA preparations of *Serratia marcescens* examined.

Table 4. *Amount of thymidine liberated from the* Serratia marcescens *DNA during the Burton* (1960) *degradation*

The amounts were determined from the ultraviolet absorption of the eluates from paper chromatograms (Burton & Petersen, 1960). The mean amounts ± standard deviation are given in moles of thymidine/100 g. atoms of P on the chromatograms.

| | Wild strain | | | Colourless mutant | | |
|---|---|---|---|---|---|---|
| Thymidine liberated | S1 | S2 | S3 | S4 | S5 | S6 |
| | 5·62 | 5·44 | 5·37 | 5·38 | 5·47 | 5·58 |
| Average | 5·48 ± 0·11 | | | 5·48 ± 0·08 | | |

S1–3, Separate samples of DNA isolated from the wild strain; S4–6, separate samples of DNA isolated from the colourless mutant.

*Base sequence of the DNA*

To detect any difference in the base sequences of the DNA of the two strains of *Serratia marcescens* especially in the thymine distribution (since only this base occurred in what might be significantly different amounts in the DNA of the two strains; Table 2), the DNA was subjected to the degradation procedure described by Burton & Petersen (1957, 1960). The same preparations of the DNA were used as were taken for the base analysis (S 1-6); the results are given in Table 3. The products of the degradation were separated by removal of the terminal phosphate groups, followed by a chromatographic separation of the oligonucleotides (Burton, 1960). The amount of thymidine thus liberated from the DNA of the two strains was then determined and is shown in Table 4.

## DISCUSSION

When the phenol extraction method (Kirby, 1956) for isolating DNA is applied to micro-organisms, it is usually necessary to break the cells mechanically. In the present case with *Serratia marcescens* on the addition of phenol and water, the DNA at once passed into the aqueous layer. By using this method and with a suitable

salt concentration for the aqueous layer, the ribosomal RNA was left with the cell debris, enzyme activity was completely inhibited and the protein was completely removed from the DNA. When this method was combined with the method of Dutta *et al.* (1953) for separating DNA from RNA by the use of cetyltrimethylammonium bromide, it was possible to isolate quantitatively the DNA from *S. marcescens*, and to obtain it in an undegraded form, free from protein (microsomal and transfer), RNA and polysaccharide. The DNA as initially isolated contained an impurity, probably cell-wall material, which was removed from the solution by centrifugation at 80,000 *g* for 1 hr. Samples of DNA which have been isolated from bacterial sources have often had low phosphorus contents (Burton, 1960); perhaps these too were contaminated by cell-wall material, although in one case the contaminant was shown to be a polysaccharide (Overend *et al.* 1951).

For comparison, DNA was isolated from the two strains of *Serratia marcescens*, and care was taken to ensure that identical growth, harvesting, isolation and purification procedures were used in each case; even so no statistically significant difference was found in the base contents of the DNA preparations from the two strains. No difference in the distribution of purines and pyrimidines in the DNA's from the two strains could be detected by analysing the results of the percentage inorganic phosphorus liberated during a Burton degradation. Nor was any difference detected in the amounts of thymidine liberated during the degradation. However, because no difference was found in the base composition or sequence between the DNA preparations from the two strains, this does not mean that they are identical, but rather that any difference was too small to be detected by the methods used.

The amount of 6-methylaminopurine found in the DNA is the highest so far known to occur naturally and is only exceeded by the amount found in the DNA from *Escherichia coli* 15T$^-$ grown under conditions of thymine deficiency (Dunn & Smith, 1958). If it be assumed that 6-methylaminopurine has similar base-pairing properties to adenine, then it can be seen that in this Serratia DNA there are typical Watson & Crick (1953) base pairs, i.e. adenine + 6-methylaminopurine = thymine; guanine = cytosine.

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# The $B_{12}$ Vitamins and Growth of the Flagellate *Prymnesium parvum*

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SUMMARY

Vitamin $B_{12}$ is an essential nutrient for the flagellate *Prymnesium parvum* when growing in axenic culture and apparently can be replaced only by a few related 'incomplete' analogues, i.e. only those devoid of one or both benzimidazole-methyl groups; and, of the substituted analogues, some (e.g. the mono-acid, the dicarboxylic acid and the ethylamide of $B_{12}$) can replace vitamin $B_{12}$, but some others of this group, e.g. pseudovitamin $B_{12}$, 2-methylmercaptoadenine cobamide, the ethylamide mono-acid, methylamide and anilide of the vitamin $B_{12}$, act as competitive inhibitors of the growth of *P. parvum*.

## INTRODUCTION

Micro-organisms which respond to vitamin $B_{12}$ as a nutrient may be divided into three groups in accordance with their ability to accept some other metabolite in place of vitamin $B_{12}$ (Lascelles & Cross, 1955). In the present paper vitamin $B_{12}$ means $\alpha$-(5,6-dimethyl benzimidazolyl)-cobamide cyanide. The first group comprises a mutant of *Escherichia coli* for which methionine may replace vitamin $B_{12}$ (Davis & Mingioli, 1950). The second group, comprising several lactobacilli, can have vitamin $B_{12}$ replaced by certain deoxyribosides and purines (Kitay, McNutt & Snell, 1950). To the third group belong micro-organisms such as *Ochromonas malhamensis*, whose vitamin $B_{12}$ requirement can only be satisfied by the vitamin itself or by some of its analogues (Ford, 1958). These micro-organisms may alternatively be divided into three groups, each with its own specific requirements in relation to the vitamin $B_{12}$ group (Kon & Pawelkiewicz, 1958). To the first group belongs *Escherichia coli* 113-3, which can satisfy its vitamin $B_{12}$ requirements not only by the benzimidazole and purine analogues but also by 'incomplete' analogues devoid of the nucleotide. The second group is represented by *Lactobacillus leichmanii* and *Euglena gracilis*; members of this group utilize vitamin $B_{12}$ and its benzimidazole or purine analogues. The third group includes *Ochromonas malhamensis*, which can utilize only the benzimidazole analogues.

*Prymnesium parvum* (Chrysomonadina) has been shown to require vitamin $B_{12}$ for its growth in axenic culture (Droop, 1954*a*; Rotberg, 1958); McLaughlin (1958) tested the specificity of its requirement for some of the $B_{12}$ analogues. In the present paper, an attempt has been made to determine the position of *P. parvum* within the aforementioned scheme by studying its specific vitamin $B_{12}$ requirements.

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Some of the $B_{12}$ analogues are known to inhibit the growth of various micro-organisms (Ford, 1958, 1959; Baker, Frank, Pasher, Hutner & Sobotka, 1960); these analogues were also tested for their effect on the growth of *P. parvum*.

## METHODS

Of the two strains of *Prymnesium parvum* isolated, respectively, by Droop (1954*b*) and by Reich & Kahn (1954), the latter (Reich) strain has been used in this study. Cultures of *P. parvum* were grown in a simplified version of a culture medium for marine algae (Provasoli, McLaughlin & Droop, 1957), the composition of which is shown in Table 1.

Stock cultures of *Prymnesium parvum* were maintained on basal medium fortified

Table 1. *Composition of basal culture medium*

Glass-distilled water was used. The medium was adjusted to pH 8·2 and sterilized by autoclaving at 120° for 15 min.

| | | | |
|---|---|---|---|
| NaCl | 10·00 mg./ml. | $FeCl_3.6H_2O$ | 1 μg./ml. |
| $MgSO_4.7H_2O$ | 3·00 mg./ml. | $NaMoC_4.2H_2O$ | 1 μg./ml. |
| KCl | 0·80 mg./ml. | $ZnSO_4.7H_2O$ | 0·15 μg./ml. |
| $CaCl_2.2H_2O$ | 0·10 mg./ml. | $CoCl_2.6H_2O$ | 0·003 μg./ml. |
| $H_3BO_3$ | 0·01 mg./ml. | Casamino acids* | 1 mg./ml. |
| $NaNO_3$ | 0·2 mg./ml. | Tris† buffer | 1 mg./ml. |
| $Na_2HPO_4$ | 0·05 mg./ml. | Thiamine hydrochloride | 10 mμg./ml. |
| $MnCl_2.4H_2O$ | 5 μg./ml. | | |

* Casamino acids = Bacto vitamin-free casamino acids (Difco).
† Tris = 2-amino-2-hydroxy methyl propane-1,3-diol.

Table 2. *Compounds of the vitamin $B_{12}$ group used in the present work*

In this paper 'vitamin $B_{12}$' stands for α-(5,6-dimethylbenzimidazolyl) cobamide cyanide. Other analogues are presented by specific name.

| | Abbreviations used | The analogues |
|---|---|---|
| 1. | $B_{12}$ | α-(5,6-dimethylbenzimidazolyl) cobamide cyanide |
| 2. | 5MBIA Cob | α-(5-methylbenzimidazolyl) cobamide cyanide |
| 3. | BIA Cob | α-(benzimidazolyl) cobamide cyanide |
| 4. | $FIII_m$ | α-(5-methoxybenzimidazolyl) cobamide cyanide |
| 5. | FIII | α-(5-hydroxybenzimidazolyl) cobamide cyanide |
| 6. | $PVB_{12}$ | α-(adenyl) cobamide cyanide |
| 7. | FA | α-(2-methyladenyl) cobamide cyanide |
| 8. | 2MMA Cob | α-(2-methylmercaptoadenyl) cobamide cyanide |
| 9. | FB | cobinamide cyanide |
| 10. | FIb | cobinamide phosphoribose |
| 11. | DMBIA | 5,6-dimethyl benzimidazole |
| 12. | MO | $B_{12}$ monoacid |
| 13. | DA | $B_{12}$ dicarboxylic acid |
| 14. | EA | $B_{12}$ ethylamide |
| 15. | EAM | $B_{12}$ ethylamidemono acid |
| 16. | MA | $B_{12}$ methylamide |
| 17. | An | $B_{12}$ anilide |

Vitamin $B_{12}$ commercial, Nutritional Biochemicals Co., U.S.A. Analogues 2–10 received from K. Bernhauer, Technische Hochschule, Stuttgart, Germany. Analogue 11 received from Merck, Sharp and Dohme, U.S.A. Analogues 12–17 received from E. Lester Smith, Glaxo Laboratories, England.

with suboptimal quantities (10 $\mu\mu$g./ml.) of vitamin $B_{12}$. In some of the experimental cultures, however, vitamin $B_{12}$ was intentionally omitted. All cultures were grown in 16 × 160 mm. test tubes fitted with aluminium caps to prevent possible organic contamination. Inoculation was accomplished by transferring 0·1 ml. of a 10- to 12-day culture into 5 ml. of basal medium containing the substances to be tested. The cultures were kept in a thermostat under constant illumination at 20 ± 2°. Growth measured as optical density (O.D.) was determined after incubation for 12 days with a Junior Coleman Spectrophotometer at 480 m$\mu$. Control cultures of *P. parvum*, grown in basal medium + 100 $\mu\mu$g. vitamin $B_{12}$/ml., attained a count of about $5 \times 10^6$ organisms/ml.

The various compounds of the vitamin $B_{12}$ group to be tested (Table 2) were kept sterile in solution in sealed glass ampoules, and were added to the medium before autoclaving.

## RESULTS

The nutrient requirement of *Prymnesium parvum* satisfied by vitamin $B_{12}$ could not be replaced by various amounts of DNA, RNA, thymidine, thymine, deoxyadenosine, adenosine, cytidine, guanosine or uridine. Attempts to stimulate the growth of *Prymnesium parvum* in the absence of vitamin $B_{12}$ by addition of various amino acids, such as alanine, glycine, homocysteine, homocystine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, serine, threonine, tryptophan or valine, were unsuccessful; negative results were obtained also with betaine or

Table 3. *Growth of* Prymnesium parvum *in the presence of incomplete vitamin* $B_{12}$ *analogues*

| Analogue* added | | Optical density reading (O.D.) × 1000 | |
|---|---|---|---|
| | | In presence of 100 $\mu\mu$g./ml. vitamin $B_{12}$ | In absence of vitamin $B_{12}$ |
| FB | 1 m$\mu$g./ml. | 337 | 27 |
| FIb | 1 m$\mu$g./ml. | 367 | 22 |
| DMBIA | 1 m$\mu$g./ml. | 409 | 27 |
| FB + DMBIA | 1 m$\mu$g./ml. each | — | 27 |
| FIb + DMBIA | 1 m$\mu$g./ml. each | — | 18 |
| BIA Cob | 100 $\mu\mu$g./ml. | — | 208 |
| 5MBIA Cob | 100 $\mu\mu$g./ml. | — | 229 |
| $B_{12}$ | 100 $\mu\mu$g./ml. | — | 328 |

* For code, see Table 2.

choline. These findings can be compared to results with *Ochromonas malhamensis* (another chrysomonad) where methionine can spare vitamin $B_{12}$ and enable some growth even in the absence of the latter (Hutner, Provasoli & Filfus, 1953; Johnson, Holdsworth, Porter & Kon, 1957). Table 3 shows growth of *P. parvum* in media containing 'incomplete' analogues of vitamin $B_{12}$ in which some part of the molecule is missing.

As seen from Table 4, the remaining vitamin $B_{12}$ analogues fell into two groups with respect to their influence on the growth of *P. parvum*. Members of the one

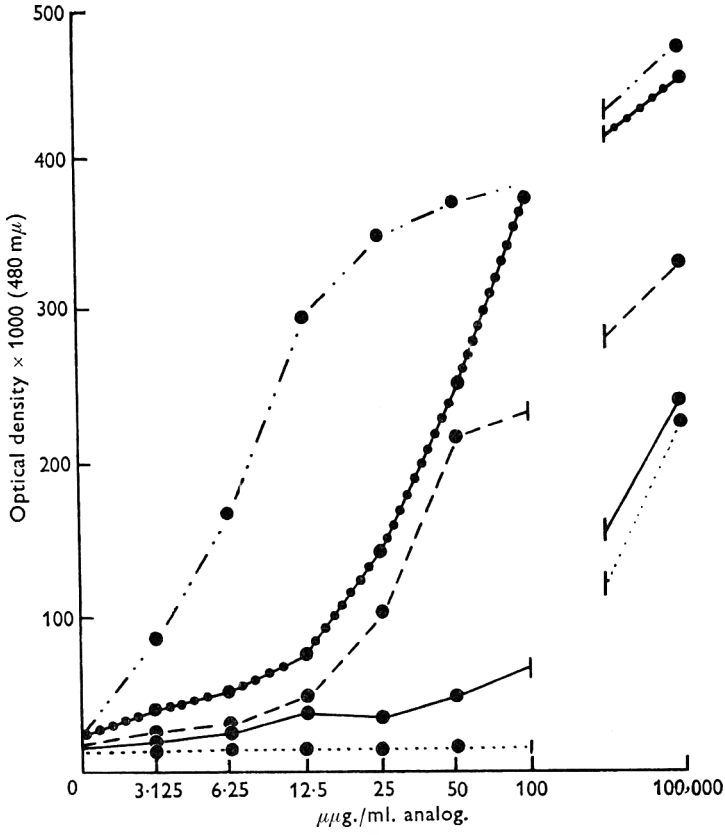


Fig. 1. Growth of *Prymnesium parvum* in media containing growth-enhancing analogues of vitamin $B_{12}$. —·—, Vitamin $B_{12}$; —•—•—, $FIII_m$*; – – –, MO; ——, EA; · · · · ·, DA.

* For code, see Table 2.

Table 4. *Growth of* Prymnesium parvum *in media containing substituted vitamin $B_{12}$ analogues; in the presence or absence of vitamin* $B_{12}$

| Analogue added* (1 μg./ml.) | Optical density reading (o.d.) × 1000: In presence of vitamin $B_{12}$ 100 μμg./ml. | Optical density reading (o.d.) × 1000: In absence of vitamin $B_{12}$ |
|---|---|---|
| FA | 22 | 9 |
| $PVB_{12}$ | 22 | 9 |
| 2MMA Cob | 22 | 13 |
| FIII | 22 | 9 |
| $FIII_m$ | 328 | 444 |
| MO | 523 | 337 |
| DA | 456 | 244 |
| EA | 509 | 237 |
| EAM | 31 | 22 |
| MA | 22 | 22 |
| An | 36 | 18 |
| No analogue added | 469 | 20 |

* For code, see Table 2.

group, namely $FIII_m$, MO, DA and EA, replaced the vitamin quantitatively; however, each of these analogues elicited a different growth response (Fig. 1).

The second group of analogues included FA, $PVB_{12}$, 2MMA Cob, FIII, EAM, MA and An; these had an inhibitory effect on the growth of *Prymnesium parvum* even in the presence of vitamin $B_{12}$. Since it had been claimed by Droop, McLaughlin, Pintner & Provasoli (1959) that FIII replaces vitamin $B_{12}$ for *P. parvum*, an attempt was made to compare the growth of the 'Droop' and the 'Reich' strains in the presence of this analogue. Such comparison showed that both strains reacted in similar fashion to compound FIII, the growth of both being inhibited by this

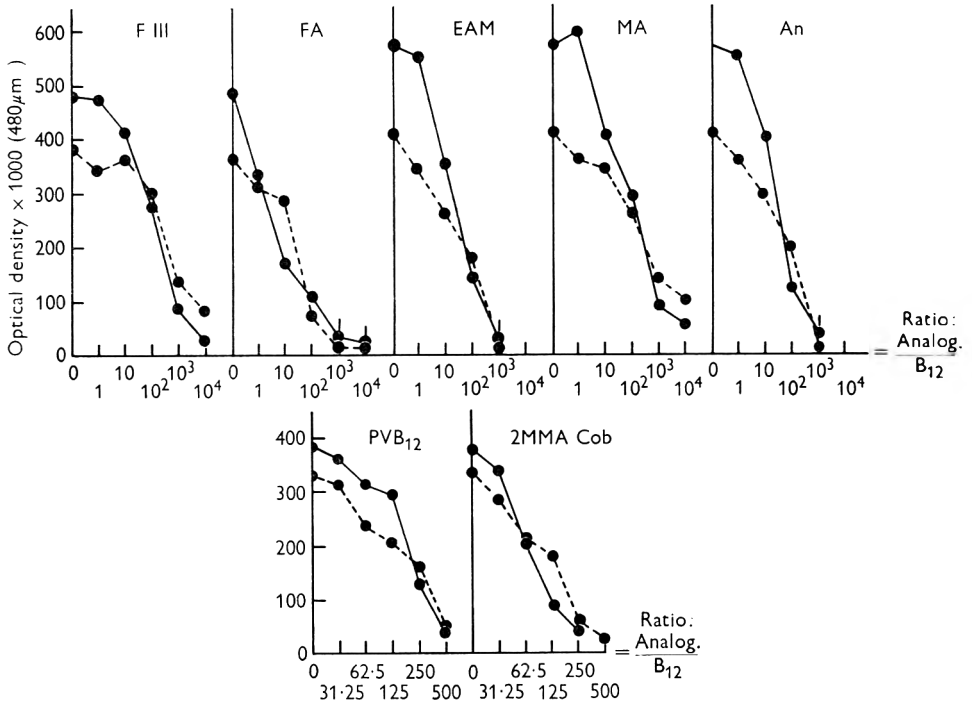


Fig. 2. Growth of *Prymnesium parvum* in media containing the inhibitory analogues and vitamin $B_{12}$ at various ratios. ——, Vitamin $B_{12}$ constant (100 μμg./ml.), analogues according to ratio. – – – –, Analogues constant (1 μg./ml. (except $PVB_{12}$ and 2MMA Cob, 50 mμg./ml.)), vitamin $B_{12}$ according to ratio.

analogue. The source and amount of compound FIII used in the work of Droop *et al.* was not indicated by these authors. This apparent discrepancy of results remains.

The inhibitory analogues were further tested quantitatively for their inhibitory effect. For this, two parallel series of media were used: the first series contained 100 μμg. vitamin $B_{12}$/ml., while the amount of the analogue tested varied successively from a ratio of analogue/$B_{12}$ of 1:1 to 10,000:1 (w/w), except for $PVB_{12}$ and 2MMA Cob, for which the ratios were from 31·25:1 to 500:1 (unbroken line in Fig. 2). Media of the second series contained 1 μg. analogue/ml., with the exception of $PVB_{12}$ and 2MMA Cob, of which 50 mμg./ml. were given, while the amount of vitamin $B_{12}$ varied according to the desired ratio (broken line in Fig. 2).

From Fig. 2 it is clear that, in a medium containing both vitamin $B_{12}$ and the inhibitory analogues, the growth of *P. parvum* was dependent on the ratio of analogue to vitamin $B_{12}$ and not on the absolute amount of inhibitor. From the above figures, the inhibition indices of the various analogues may be roughly estimated; their approximate values are given in Table 5.

Table 5. *Inhibition indices of various vitamin* $B_{12}$ *analogues in growth of* Prymnesium parvum

| Analogue* | Inhibition index |
|---|---|
| FIII | 500 |
| MA | 500 |
| $PVB_{12}$ | 175 |
| 2MMA Cob | 100 |
| An | 80 |
| EAM | 80 |
| FA | 10 |

* For code, see Table 2.

## DISCUSSION

The nutrient requirement of *Prymnesium parvum* for vitamin $B_{12}$ is very exacting. Vitamin $B_{12}$ was not replaced by any of the various unrelated metabolites tested, nor could the 'incomplete' analogues substitute for it. These facts at once place *P. parvum* among the most exacting of the micro-organisms which have a nutrient requirement satisfied by vitamin $B_{12}$. Furthermore, *P. parvum* is apparently incapable of synthesizing vitamin $B_{12}$ even when some of the preformed parts of this vitamin are supplied. On the other hand, analogues which lack one or both of the benzimidazole methyl groups can substitute for vitamin $B_{12}$. This could be explained by assuming either that *P. parvum* is capable of adding the missing methyl groups to analogues such as benzimidazolyl cobamide cyanide, or that the function of vitamin $B_{12}$ is not affected by the absence of these groups. The former assumption seems the more plausible, since compound $FIII_m$, which has a methoxybenzimidazole group, replaced vitamin $B_{12}$, whereas compound F III, with an hydroxybenzimidazole group at the same site, inhibited growth. Analogues in which a purine replaces the benzimidazole of the vitamin $B_{12}$ molecule, such as compounds FA, 2MMA Cob and $PVB_{12}$, all inhibited growth. This indicates that the benzimidazole part of the molecule is essential for the normal function of vitamin $B_{12}$ in *P. parvum*.

There are conflicting reports in the literature about the influence on the growth of micro-organisms of analogues substituted in the propionamide groups of the corrinoid part of the vitamin $B_{12}$ molecule. Thus, analogue MO ($B_{12}$ monoacid), which can replace vitamin $B_{12}$ for *Prymnesium parvum*, was reported by Baker *et al.* (1960) to do the same for *Ochromonas malhamensis*. Ford (1959), however, found it inhibitory for the growth of *O. malhamensis*. Such discrepancy in the data for *O. malhamensis* is found also with regard to analogue MA (vitamin $B_{12}$ methylamide), which inhibited the growth of *P. parvum*. Ford (1959) found that *O. malhamensis* adapts to the utilization of the inhibitory MA, or that vigorously growing cultures can probably transform this analogue to vitamin $B_{12}$. Analogues

DA ($B_{12}$ dicarboxylic acid) and EA ($B_{12}$ ethylamide), which are inhibitory for *O. malhamensis* (Ford, 1959; Baker *et al.* 1960), were in the present study able to substitute for vitamin $B_{12}$. Such contradictory results may be attributable either to differences in the experimental procedures used and their effect on the analogues, or to differences in ability of the given micro-organism to take up the analogues and change them to the functional forms. As the coenzyme forms of $B_{12}$ are known to contain different analogues (Barker, Weissbach & Smyth, 1958; Barker *et al.* 1960*a*; Barker *et al.* 1960*b*), it follows that a micro-organism's requirements for a given coenzyme form can determine the respective influence of the analogues on its growth. Further work on the particular function of each analogue is therefore desirable.

The inhibitory effect of analogues of vitamin $B_{12}$ on the growth of *Prymnesium parvum* has, in the present work, been shown to depend on the ratio of analogue to vitamin $B_{12}$ in the culture medium and not on the absolute amount of analogue. This indicates a form of competitive inhibition, with analogue and vitamin competing for a particular enzyme site in *P. parvum*. However, as pointed out by Woolley (1952), 'the inhibition index as found in a living organism may represent the resultant of several forces rather than the relative affinity of the two compounds for a specific protein'.

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# The $B_{12}$ Vitamins and Methionine in the Metabolism of *Prymnesium parvum*

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(*Received* 31 *August* 1962)

SUMMARY

Methionine or ethionine, although capable of serving as sole nitrogen source for *Prymnesium parvum*, cannot replace or spare the vitamin $B_{12}$ nutrient requirement for the growth of this flagellate. In the presence of vitamin $B_{12}$ methionine counteracted the inhibition of growth exerted by some vitamin $B_{12}$ analogues which are substituted at the benzimidazole part of the molecule. No such effect was observed against the inhibition by certain other analogues. Analogue F III ($\alpha$-(5-hydroxybenzimidazolyl)-cobamide cyanide) replaced vitamin $B_{12}$ in the presence of methionine, and to a lesser degree also in the presence of other methyl donors such as methylmethionine sulphonium iodide, dimethylpropiothetin, choline chloride and betaine. The conclusion drawn is that for *P. parvum* analogue F III is capable of replacing vitamin $B_{12}$ in all the metabolic pathways other than methyl-group synthesis. For the latter process, the benzimidazole methyl groups present in vitamin $B_{12}$ and analogue $F III_m$ ($\alpha$-(5-methoxy-benzimidazolyl)-cobamide cyanide) are indispensable.

## INTRODUCTION

Vitamin $B_{12}$ participates in many biochemical reactions (see Heinrich, 1957, 1962). Davis & Mingioli (1950) showed that vitamin $B_{12}$ is essential for the growth of a mutant of *Escherichia coli* and that methionine can replace the vitamin for this mutant. Helleiner, Kisliuk & Woods (1957) and Guest, Helleiner, Cross & Woods (1960) showed that vitamin $B_{12}$ is necessary for methionine synthesis by cell-free extracts of *E. coli*. A dependence on vitamin $B_{12}$ is also known for *Ochromonas malhamensis*, an allied flagellate of *Prymnesium parvum*, the growth of which is enhanced by methionine in the presence of vitamin $B_{12}$ (Hutner, Provasoli & Filfus, 1953; Johnson, Holdsworth, Porter & Kon, 1957; Ford, 1958). The growth of *O. malhamensis* is inhibited by ethionine, but this inhibition can be annulled by methionine. High concentrations of methionine, however, are also inhibitory (Johnson *et al.* 1957).

Vitamin $B_{12}$ is an essential nutrient for *Prymnesium parvum* (Droop, 1954; Rotberg, 1958), and may be replaced by some of its analogues, while other analogues inhibit the growth of *P. parvum* even in the presence of vitamin $B_{12}$ (Rahat & Reich, 1962). To date, no compound has been found which can replace vitamin $B_{12}$ for *P. parvum*, except the few related compounds just mentioned. Though some amino acids may serve as sole nitrogen source for *P. parvum*, vitamin $B_{12}$ is still an

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essential nutrient (Rahat & Reich, 1963). It is the purpose of the present paper to discuss the function of vitamin $B_{12}$ and some of its analogues in the metabolism of *P. parvum*, and the possible role of this vitamin in the synthesis of methionine and free methyl groups by this flagellate.

## METHODS

The Reich strain of *Prymnesium parvum* was used in the present work. Data on medium composition and methods of culture are given elsewhere (Rahat & Reich, 1963). The amount of growth of *P. parvum* was determined at the end of 12 days by measuring the optical density of a culture with a Junior Coleman Spectrophotometer at 480 m$\mu$. A list of vitamin $B_{12}$ analogues used in the present work is given in Table 1.

Table 1. *Compounds of the vitamin* $B_{12}$ *group used in the present work*

In this paper vitamin $B_{12}$ means $\alpha$-(5, 6-dimethylbenzimidazolyl) cobamide cyanide. Other analogues are presented by name and code.

| | Abbreviation used | The analogues |
|---|---|---|
| 1. | $B_{12}$ | $\alpha$-(5,6-dimethylbenzimidazolyl) cobamide cyanide |
| 2. | FIII | $\alpha$-(5-hydroxybenzimidazolyl) cobamide cyanide |
| 3. | $FIII_m$ | $\alpha$-(5-methoxybenzimidazolyl) cobamide cyanide |
| 4. | $PVB_{12}$ | $\alpha$-(adenyl) cobamide cyanide |
| 5. | FA | $\alpha$-(2-methyladenyl) cobamide cyanide |
| 6. | 2MMA Cob | $\alpha$-(2-methylmercaptoadenyl) cobamide cyanide |
| 7. | EAM | $B_{12}$ ethylamidmonoacid |
| 8. | MA | $B_{12}$ methylamide |
| 9. | An | $B_{12}$ anilide |

Vitamin $B_{12}$ commercial, Nutritional Biochemical Co., U.S.A.; analogues 2–6 received from K. Bernhauer, Technische Hochschule, Stuttgart, Germany; analogues 7–9 received from E. Lester Smith, Glaxo Laboratories, England.

## RESULTS

Experiment showed that methionine and ethionine could be used by *Prymnesium parvum* as sole nitrogen sources. No growth inhibition occurred with either of these amino acids even at high concentrations (Fig. 1). In a second series of experiments, almost identical growth curves were obtained when the above-mentioned amino acids were added at concentrations of 0·25 mg./ml. (Fig. 2) and 2·0 mg./ml. Neither methionine nor ethionine show such sparing of vitamin $B_{12}$ for *P. parvum* as is known for *Escherichia coli* (Davis & Mingioli, 1950) and *Ochromonas malhamensis* (Johnson *et al.* 1957). Even though no sparing of vitamin $B_{12}$ was detected, nevertheless methionine, when added to media containing vitamin $B_{12}$ and growth-inhibitory analogues, was found to counteract the inhibition caused by some of these analogues (Fig. 4). This annulment of inhibition by methionine was clearly evident when the compounds FIII, $PVB_{12}$, 2MMA Cob and FA were the inhibitors; these are substituted in the nucleotide part of the molecule. However, with inhibitory analogues which were substituted in the propionamide of the corrinoid part of the molecule (e.g. compounds MA, EAM, An), only a slight annulment of inhibition was observed, which may have been due merely to the addition of another organic metabolite.

Table 2 shows the growth of *Prymnesium parvum* in media devoid of vitamin $B_{12}$, in the presence of the inhibitory analogues and with or without methionine. It can be seen at once that of all the analogues tested only FIII replaced vitamin $B_{12}$ in the presence of methionine. As pointed out earlier only analogues substituted in the

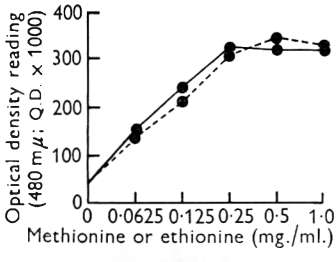


Fig. 1

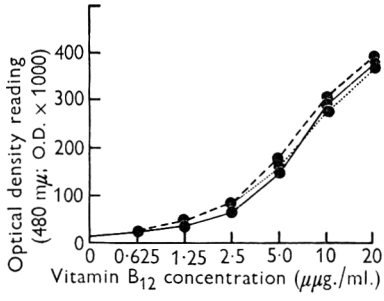


Fig. 2

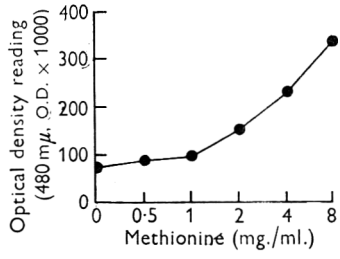


Fig. 3

Fig. 1. Growth of *Prymnesium parvum* in inorganic medium with methionine or ethionine as sole nitrogen source. In these experiments Casamino acids were omitted from the basal medium (see Rahat & Reich, 1962). ——, Methionine; – – –, ethionine.

Fig. 2. Growth of *Prymnesium parvum* at various suboptimal concentrations of vitamin $B_{12}$ in the presence of methionine or ethionine (0·25 mg./ml.). – – –, Vitamin $B_{12}$; ——, vitamin $B_{12}$ + methionine; ....., vitamin $B_{12}$ + ethionine.

Fig. 3. Growth of *Prymnesium parvum* at various concentrations of methionine, in the presence of compound FIII (10 mμg./ml.) with vitamin $B_{12}$ omitted.

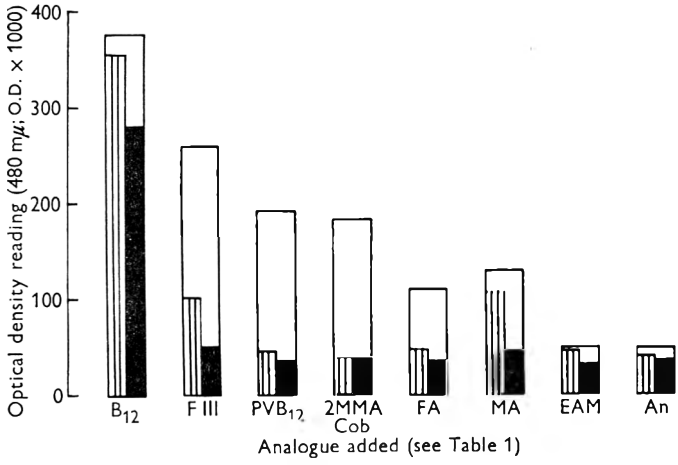


Fig. 4. Effect of methionine or ethionine (4 mg./ml.) on the growth of *Prymnesium parvum* in the presence of vitamin $B_{12}$ and growth inhibitory analogues. Vitamin $B_{12}$ was added at 100 μμg./ml. and the respective analogues at 100 mμg./ml. Broad open column = with methionine; right half (solid black) = with ethionine; left half (vertical rules) = no addition.

nucleotide part of the molecule had their growth-inhibitory effect annulled by methionine. Of these analogues, only FIII has the benzimidazole group unchanged, whereas in compounds FA, $PVB_{12}$ and 2MMA Cob (see Table 1), this group is replaced by adenine or its derivative.

In the absence of vitamin $B_{12}$ but with 10 m$\mu$g. FIII/ml. +8 mg. methionine/ml., growth of *Prymnesium parvum* was equal to that obtained with vitamin $B_{12}$ alone (Fig. 3). Higher concentrations of compound FIII gave no additional growth. Noteworthy, however, is the fact that, whereas growth of *Ochromonas malhamensis* is inhibited by 0·8 mg. methionine/ml., for *P. parvum* maximal growth was attained in the presence of 8 mg. methionine/ml. An attempt was made to replace methionine, in the presence of compound FIII, by other potential methyl donors with homocysteine added as a methyl acceptor. As can be seen from Table 3, these methyl donors exerted an effect qualitatively similar to that of methionine.

Table 2. *Growth of* Prymnesium parvum *in the absence of vitamin* $B_{12}$ *but ± methionine and various analogues*

| Analogues (at 2 m$\mu$g./ml.) | Optical density reading (o.d. × 1000) With methionine (4 mg./ml.) | Without methionine |
|---|---|---|
| FA | 13 | 8 |
| FIII | 268 | 50 |
| 2MMA Cob | 18 | 13 |
| $PVB_{12}$ | 13 | 8 |
| EAM | 22 | 18 |
| MA | 31 | 22 |
| An | 22 | 18 |

Table 3. *Growth of* Prymnesium parvum *in the presence of compound FIII* (α-[5-*hydroxybenzimidazolyl*]-*cobamide cyanide;* 10 m$\mu$g./ml.) *and potential methyl-donors with homocysteine as methyl acceptor*

| Methyl-donor added | Optical density reading (o.d. × 1000) |
|---|---|
| MMT | 65 |
| MMT + HCSTE | 114 |
| Thet | 60 |
| Thet + HCSTE | 108 |
| Ch | 91 |
| Ch + HCSTE | 114 |
| Bt | 75 |
| Bt + HCSTE | 119 |
| HCSTE | 65 |
| No addition | 46 |

MMT = Methylmethionine sulphonium iodide (100 $\mu$g./ml.); Thet = Dimethyl propiothetin HCl (100 $\mu$g./ml.); Ch = Choline chloride (1·0 mg./ml.); Bt = Betaine (1·0 mg./ml.); HCSTE = Homocysteine (100 $\mu$g./ml.).

It is known that vitamin $B_{12}$ participates in nucleic acid metabolism (Dinning & Young, 1960; Manson, 1960; Downing & Schweigert, 1956*a*, *b*). On the assumption that compound FIII in presence of methionine might be replaced by a nucleotide, various nucleotides were added to basal media free from any members of the vitamin $B_{12}$ group. The nucleotides tested, at 0·1 mg./ml., were: adenine, adenosine, deoxyadenosine, adenylic acid, cytidine, cytosine, cytidylic acid, guanosine, thymine, thymidine, uridine, RNA, DNA. No growth occurred in any culture tested.

## DISCUSSION

Methionine and ethionine can both be used by *Prymnesium parvum* as sole nitrogen source. Even at high concentrations, neither of these two amino acids exerts on *P. parvum* the growth inhibitory effect they can exert on *Ochromonas malhamensis* (Johnson *et al.* 1957). This may possibly be attributable to a low uptake by *P. parvum* of methionine and ethionine into sites where they are required in unchanged form, whereas at sites where they function as catabolic substrates these amino acids may be utilized even at high concentrations. Since with *P. parvum* there is no antagonistic interaction between methionine and ethionine, it must be assumed that the metabolic system of this organism is either so specific as to prevent ethionine from interfering with the action of methionine, or is such as to utilize both amino acids equally well, enabling ethionine to be incorporated into cell protein, as is the case with Tetrahymena (Gross & Tarver, 1955).

The addition of methionine, but not of ethionine, to media containing vitamin $B_{12}$ and one of the growth-inhibitory benzimidazole-substituted analogues annulled the inhibitory effect of the latter. There was no such annulment in cultures in which the inhibitory analogues were ones substituted in the corrinoid part of the molecule. This shows that the various inhibitory analogues exert their inhibitory effects at different sites. The benzimidazole-substituted analogues apparently inhibit methionine or methyl-group synthesis, and therefore their inhibition is annulled when methionine is added. The propionamide-substituted analogues seem to inhibit some other biosynthetic pathway. To obtain the above-mentioned annulment, a relatively high concentration of methionine was required. This also might be explained by assuming a low uptake of methionine as such, at the sites where it is needed unchanged. Perhaps it is at these sites that methionine is synthesized when vitamin $B_{12}$ is not subject to competitive inhibition of an inhibitory analogue. Methionine, while obviously synthesized only in the presence of vitamin $B_{12}$, shows no sparing effect for this vitamin. It seems likely therefore that only a small part of the vitamin $B_{12}$ is utilized in methyl metabolism, the rest participating in other metabolic processes.

As mentioned before, the growth of *Prymnesium parvum* is inhibited by benzimidazole-substituted analogues (e.g. compounds FA, $PVB_{12}$ and 2MMA Cob, Table 1) even in the presence of vitamin $B_{12}$; however, the addition of methionine enables growth. This suggests that, of the functions of vitamin $B_{12}$ in the organism, only that of methionine synthesis is inhibited by these analogues. Hence the addition of methionine enables growth of *P. parvum* even in the presence of such inhibitory analogues. For *Ochromonas malhamensis* the inhibition was shown to be exerted on the uptake of the vitamin (Ford, 1958).

Such a mechanism of action of $B_{12}$ antagonists cannot be excluded in *P. parvum*. A final conclusion whether Ochromonas and Prymnesium are different in this respect will have to await further experiments on both organisms.

Compound $FIII_m$ (-5-methoxy) replaces vitamin $B_{12}$ for *Prymnesium parvum* while compound FIII (-5-hydroxy) inhibits growth, even in the presence of vitamin $B_{12}$ (Rahat & Reich, 1963). However, compound FIII in the presence of methionine replaces vitamin $B_{12}$ for *P. parvum*. No precursors of methionine or allied amino acids tested had such effect with compound FIII. This emphasizes the importance

of the methyl groups in methionine, compound $FIII_m$ and vitamin $B_{12}$. There is, however, the possibility that compound FIII in the presence of labile methyl groups of methionine may be converted to $FIII_m$ or even to vitamin $B_{12}$ proper. If this be so, then the reverse transfer of methyl groups should be possible, i.e. transformation of a methionine precursor into methionine as a result of transmethylation of the vitamin $B_{12}$ benzimidazole methyl groups. In *P. parvum* cultures containing compound FIII instead of vitamin $B_{12}$, the addition of various methyl donors (methylmethionine sulphonium iodide, dimethylpropiothetin, choline, betaine) in the presence or absence of homocysteine as methyl acceptor gave enhancement of growth, qualitatively like that attained with vitamin $B_{12}$. This again emphasizes the importance of free methyl groups for the growth of *P. parvum* in the absence of vitamin $B_{12}$, which obviously participates in the synthesis of these groups. Compound FIII has a benzimidazole moiety in its molecule, and can replace vitamin $B_{12}$ in the presence of methionine. In the other nucleotide-substituted analogues tested the benzimidazole moiety was replaced by a purine group. It would seem, therefore, that compound FIII can assume all the functions of vitamin $B_{12}$ except that of methionine or methyl group synthesis, for which the methyl groups of the benzimidazole moiety are indispensable. The latter syntheses are actually inhibited by the presence of hydroxybenzimidazole moiety in the molecule of FIII. Thus, the provision of methionine can relieve vitamin $B_{12}$ from one of its functions, methionine synthesis, and compound FIII can assume the remaining functions of the vitamin. Attempts to find a compound that can replace FIII in the presence of methionine have thus far been unsuccessful.

The authors are indebted to Professor K. Bernhauer, Technische Hochschule, Stuttgart, Germany, and to Dr E. Lester Smith, Glaxo Laboratories, England, for the generous donation of the analogues of vitamin $B_{12}$ used in the present work.

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# Transducing Phages for *Bacillus subtilis*

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SUMMARY

Transducing phage PBS1 and a clear-plaque type mutant PBS2 derived from PBS1 show similar growth characteristics with a relatively small burst size (23 to 24), and are related serologically. They are morphologically indistinguishable and both have a dense head of about 100 m$\mu$ in diameter and a tail about 250 m$\mu$ in length. PBS1 is more efficient in transduction and its plaque-forming activity is more sensitive to ultraviolet (u.v.) irradiation than that of PBS2. The host range of the phages is limited to strains of *Bacillus subtilis*. All Marburg strains tested were sensitive to the lytic action of the phages. Prophages of PBS1 and PBS2 were not u.v.-inducible and the irradiation of lysogenic cultures with a relatively high dose of u.v. radiation resulted in considerable decrease of the free phage titre. However, transducing activities of lysates treated with a high dose of u.v. radiation were not affected significantly. Organisms lysogenic either for PBS1 or to PBS2 were not stable and reverted to the sensitive state.

## INTRODUCTION

It has been shown that the temperate phage PBS1 is capable of transducing a number of genetic traits in *Bacillus subtilis* (Takahashi, 1961). A clear-plaque type mutant (PBS2) derived from PBS1 is also able to carry out general transduction, although the frequency of transduction is always lower than that of PBS1. Thorne (1961) also isolated a temperate phage from soil and demonstrated transduction in the same organism. However, the phages reported here differ in host range and other properties from the temperate phages for *B. subtilis* reported hitherto (Romig & Brodetsky, 1961; Thorne, 1962). This paper describes the general characteristics of transducing phages PBS1 and PBS2.

## METHODS

The bacterial strains used in this work are listed in Table 1. Organisms grown overnight on Difco Tryptose Blood Agar Base (TBB agar) were used as inoculum. Soft TBB agar was made up to contain the same ingredients as the prepared product with the exception that the agar content was 1 %. As the *Bacillus subtilis* strains used sporulated poorly on TBB agar, inocula were essentially all vegetative forms. Liquid cultures were obtained by growing the organisms in Penassay Broth (Difco) with shaking for 4 hr. The sporulation medium of Schaeffer (1961) was used to obtain spores. Medium Y (Cook & Lochhead, 1959) was used as diluent in the assay of plaque-forming particles. All incubations were at 37° unless otherwise stated.

Phages were isolated from soil samples collected at various places in Ottawa by a modification of the method described by Ivanovics & Lantos (1958). Samples of cultivated soil (about 50 g.) were suspended in 50 ml. tap water containing a small number (about $10^7$) of streptomycin-resistant indicator bacteria *Bacillus subtilis* SB19. After 2 days at room temperature the soil suspensions were shaken vigorously and allowed to settle for 30 min. A sample of supernatant liquid was mixed with an equal volume of medium Y and shaken for 3 hr. To a 10 ml. portion of the mixture were added 5 ml. of indicator culture and streptomycin sulphate (Mann Research Laboratories, Inc.) to a final concentration of 500 $\mu$g./ml., and incubation continued for a further 6 hr. with shaking. The number of plaque-forming particles in the culture was determined, after a brief centrifugation, on soft agar containing streptomycin sulphate 500 $\mu$g./ml.

Since the soft agar-layer method of Adams (1959) did not give satisfactory results in the assay of PBS1, the following modification of the pre-adsorption technique of Potter & Nelson (1952) was used. Three ml. samples of lysate diluted in medium Y were mixed in sterile test tubes (16 × 125 mm.) with 1·5 ml. medium Y containing about $5 \times 10^7$ indicator bacteria. After 5 min. at room temperature 4·5 ml. of soft TBB agar (previously melted and kept in a 55° water bath) were added to the tubes with a 10 ml. graduated pipette (large tip opening) and mixed thoroughly. Fresh TBB agar plates were overlaid with 3 ml. samples of this phage + bacteria mixture. By this modified technique the number of plaques that developed on each plate represented the number of plaque-forming particles/ml. lysate tested. The assay plates were made in duplicate. The soft agar plates were incubated overnight at 28°. The omission from medium Y of yeast extract and ferric chloride decreased the plaque counts by about 80 %. Under the same condition the adsorption of phage to host bacteria was also decreased to the same extent. Thus the low plaque counts observed were probably due to poor adsorption of phage to host.

Phage lysates were made by the following technique. Phage-sensitive cultures in Penassay Broth in the logarithmic growth phase were infected with phage at a multiplicity of infection of about 1·0 and shaken for one additional hour. The infected cultures were further incubated overnight without shaking. After a brief centrifugation at low speed, the lysates were filtered through Millipore filter membranes (0·45 $\mu$ pore size). Lysates were also made by growing lysogenic cultures in Penassay Broth for 4 hr. with shaking, followed by overnight incubation without shaking. Plaque counts of lysates made by either methods were about $5 \times 10^9$/ml. for PBS1 and $5 \times 10^9$ to $1 \times 10^{10}$ for PBS2.

Ultraviolet irradiations of lysogenic cultures and phages were carried out at a distance of 20 cm. from a 'Mineralight' source (Ultraviolet Products Inc., South Pasadena, California) equipped with a 2537 Å filter.

Procedures for the transduction experiments were as previously described (Takahashi, 1961). In initial experiments, recipient organisms were grown in minimal media supplemented with growth factors. Higher frequencies of transduction, however, were obtained when the organisms were grown in a complex medium such as Penassay Broth.

## RESULTS

*Phage isolates.* By using the method described it was possible to isolate, from 12 of 15 soil samples, phages active on *Bacillus subtilis* at a titre of $10^2$ to $10^4$ particles/ml. Among the phage isolates, temperate phage PBS1 was able to transduce prototrophy, resistance to antibiotics (streptomycin, erythromycin, neomycin) and sporogenesis in strains of *B. subtilis*.

When a large number of particles of phage PBS1 were plated on soft agar with *Bacillus subtilis* SB19 as indicator, the presence of clear plaques which were distinct from those of PBS1 was noted. This clear-plaque type mutant (PBS2) was also temperate and able to carry out general transduction in *B. subtilis*, although the frequency of transduction was lower than that with phage PBS1.

*Plaque morphology.* Plate 1, figs. 1, 2, show the plaque morphology of phages PBS1 and PBS2. PBS1 forms turbid plaques of about 1 mm. in diameter with peripheral rings. PBS2 forms clear plaques of about 1 mm. in diameter which become slightly turbid after prolonged incubation.

*Morphology of the phage particles.* Electron micrographs were prepared by the pseudo-replica technique described by Wyckoff (1951). Phages PBS1 and PBS2 were almost indistinguishable in their morphology (Pl. 1, figs. 3, 4). They were spermlike with a dense head (about 100 m$\mu$ diameter) and tail (about 250 m$\mu$ length). These dimensions are comparable to those of T4 phage which is one of the largest phages described (Luria, 1962).

*Serology.* Rabbit antisera were made and their velocity constants (K) of neutralization were measured according to the procedures of Adams (1959). An antiserum made against phage PBS1 having a K value of 1830 had a K value of 810 when measured against phage PBS2. It was found that the lysates of PBS1 and PBS2 contained, with different proportions, four density species determined in the CsCl density gradient; one was purely transducing and had no plaque-forming activities (unpublished results). This suggests that phage particles in the lysates of PBS1 and PBS2 are not homogeneous. Therefore, the difference in the rate of inactivation observed here may not be significant, since the K values were calculated only by the decrease in the number of plaque-forming particles. Nevertheless, the results obtained indicate that the two phages are closely related serologically.

*Host range.* The host range of the phages was determined by spotting a drop of cell-free lysate on a soft agar layer seeded with test organisms. Table 1 shows the results of host range determinations made with several aerobic spore-forming bacteria belonging to different groups. The Marburg strain of *Bacillus subtilis* and all its derivatives were susceptible to the lytic action of phages PBS1 and PBS2, and general transduction could be performed between these strains. Although other *B. subtilis* strains isolated from various sources were sensitive to the phages, attempts to transduce a streptomycin marker to these strains with *B. subtilis* SB19 as donor did not succeed.

*Growth characteristics.* Single-step growth experiments were made according to the procedures described by Adams (1959). The results obtained with *Bacillus subtilis* SB19 as host are summarized in Table 2. Phages PBS1 and PBS2 both have a relatively small burst size and their growth characteristics are very similar. In the same experiments, from viable counts made on infected and uninfected

bacteria, it was found that only 15 % of bacteria which were killed by phage PBS1 gave rise to plaques. The proportion of plaque formers among bacteria infected by phage PBS2 was higher (35 %). This observation suggests the presence in the lysates of defective particles which kill the host bacteria but do not initiate phage multiplication. These resemble 'killer particles' found in defective lysogenic transductants of *Shigella dysenteriae* (Luria, Adams & Ting, 1960).

Table 1. *Susceptibility of species of Bacillus to phages PBS1 and PBS2*

| *Bacillus* | | Source of culture | Lysis by phage* PBS1 | PBS2 |
|---|---|---|---|---|
| *B. subtilis* | | NCTC 3329 | − | − |
| | | NCTC 2586 | + | + |
| | | ATCC 6633 | + | + |
| | Marburg | ATCC 6051 | + | + |
| | SB19 | Dr E. W. Nester (Stanford University) | + | + |
| | Wild type | Dr J. Spizizen (University of Minnesota) | + | + |
| | 168 | Dr J. Spizizen (University of Minnesota) | + | + |
| *B. subtilis* var. *niger*† | | | + | + |
| *B. megaterium* | | MRI‡ 151 | − | − |
| *B. brevis* | | MRI 515 | − | − |
| *B. circulans* | | MRI 602 | − | − |
| *B. sphaericus* | | MRI 566 | − | − |
| *B. cereus* | | MRI 247 | − | − |
| *B. cereus* | | MRI 818 | − | − |
| *B. thuringiensis* var. *thuringiensis* | | Dr D. Kushner (NRC)§ | − | − |
| *B. thuringiensis* var. *alesti* | | Dr D. Kushner (NRC) | − | − |
| *B. polymyxa* | | ATCC 7070 | − | − |

*+ = lysis; − = lack of lysis; † determined by Dr J. Marmur, Brandeis University; ‡ MRI: Microbiology Research Institute, Ottawa; NRC: § National Research Council of Canada, Ottawa.

Table 2. *Growth characteristics of phages PBS1 and PBS2**

Host: *Bacillus subtilis* SB19

| Phage | Latent period (min.) | Rise period (min.) | Burst size |
|---|---|---|---|
| PBS1 | 35 | 15 | 23 |
| PBS2 | 37 | 13 | 24 |

* Averages of 4 experiments for each phage.

*Stability of the phages.* The phages PBS1 and PBS2 appear to be fairly stable when stored at 5°. Plaque counts decreased only about 50 % when lysates of phages PBS1 or PBS2 were stored at 5° for 3 months. No measurable decrease in phage titre was noted when lysates were treated with chloroform. The transducing activity in lysates, on the other hand, declined at a much faster rate under the same conditions. In a typical experiment, the number of *indole*$^+$ colonies developing from *Bacillus subtilis* 168 (*indole*$^-$) when treated with 0·1 ml. of lysates made from *B. subtilis* SB19 decreased from 3500 to 510 after 3 months at 5°, while the plaque count of phage PBS1 decreased from $6{\cdot}8 \times 10^8$/ml. to $3{\cdot}8 \times 10^8$/ml.

*Stability of lysogenic cultures.* It was reported that stably lysogenic bacteria could be isolated from turbid plaques formed by phage PBS1 (Takahashi, 1961). The

lysogenic cultures retained their prophage through repeated subcultures on sporulation agar and heating at 85° for 15 min. Control experiments showed that the same heat treatment decreased the phage titre of a lysate from $10^7$/ml. to < 1/ml. It was found, however, that the individual lysogenic bacteria were not quite as stable as previously thought. When about 100 colonies derived from heated spores of *Bacillus subtilis* SB19 lysogenic for phage PBS1 were examined, 50 % of them were found to be in the typical lysogenic state while the remainder were immune to phage infection but had lost their ability to produce free phage. This at first suggested that the immune state was intermediate between sensitivity and lysogeny. However, in further experiments when a colony of lysogenic *B. subtilis* SB19 was grown in Penassay Broth containing phage antiserum at 1/500 (K value of the serum estimated at a dilution of 1/2000 being 1830) for 4 hr. and plated on TBB agar, 30 % of the colonies were fully lysogenic and the remainder were sensitive, there being no detectable colonies of the immune type. The necessity for postulating the intermediate state was thus eliminated since, during the initial growth of lysogenic *B. subtilis* SB19 in the absence of phage antiserum, the presence of a large number of free phages could have served to eliminate sensitive bacteria and to select resistant (immune) bacteria derived from them. Bacteria lysogenic for phage PBS2 were also unstable and eventually reverted to the sensitive state. Because of this instability of the lysogenic state, it was not possible to establish any exact relationship between transduction and lysogenization.

*Induction by u.v. irradiation.* Attempts to induce phage development by u.v. irradiation of lysogenic bacteria were unsuccessful. The bacteria were irradiated in physiological saline with a 30–95 % killing dose of u.v. radiation and transferred into Penassay Broth. At a relatively low dose of u.v. radiation in no case was there an appreciable increase in phage titre when compared with unirradiated cultures. Both irradiated and unirradiated lysogenic cultures gave a titre of about $10^9$/ml. after they were incubated overnight without shaking. Relatively high doses of u.v. radiation (95 % killing) decreased the free phage titre by about 90 %.

Table 3. *Effect of u.v. irradiation on plaque count and transducing activity of phage PBS1*

Recipient: *Bacillus subtilis* 168 (*ind*$^-$). *B. subtilis* W-*ery* (prototrophic and resistant to 1 μg./ml. of erythromycin) was used as donor.

| Dose of u.v. irradiation (sec.) | Plaque count/ml. | Fraction surviving | Prototrophs* (recipient:168) | Erythromycin resistant colonies* (recipient:168) |
|---|---|---|---|---|
| 0 | $3{\cdot}6 \times 10^9$ | 1·0 | 850 | 1400 |
| 20 | $5{\cdot}4 \times 10^8$ | 0·15 | 630 | 1750 |
| 40 | $7{\cdot}5 \times 10^7$ | 0·021 | 870 | 1570 |
| 80 | $9{\cdot}5 \times 10^6$ | 0·0026 | 1020 | 1640 |
| 120 | $2{\cdot}1 \times 10^6$ | 0·00058 | 1570 | 1470 |

* Number of transductants developed from 0·1 ml. of lysates and 0·9 ml. of recipient culture.

*Effect of u.v. irradiation on the phages.* Cell-free lysates were treated with various doses of u.v. radiation in very thin layers in Petri dishes which were agitated continuously. The plaque counts and transducing activities of the irradiated lysates are shown in Tables 3 and 4. Exponential decreases in the plaque count of phages

PBS1 and PBS2 were obtained up to 40 sec. exposure; thereafter, the inactivation proceeded at a somewhat lower rate. The lytic activity of phage PBS2 was more resistant to u.v. irradiation than that of phage PBS1. On the other hand, the transducing activity was apparently unaffected; it doubled for the indole marker even though the plaque count was decreased from $10^9$/ml. to $10^5$/ml.

Table 4. *Effect of u.v. irradiation on plaque count and transducing activity of phage PBS2*

Recipients: *Bacillus subtilis* 168 (*ind*$^-$); *B. subtilis* S10. *B. subtilis* SB19 (prot, str-r) was used as donor. S10 was derived from the wild type strain of Spizizen (see Table 1) by u.v. irradiation and was glutamic acid$^-$.

| Dose of u.v. radiation (sec.) | Plaque count/ml. | Fraction surviving | Prototrophs* (recipient:168) | Prototrophs* (recipient:S10) |
|---|---|---|---|---|
| 0 | $2{\cdot}7 \times 10^9$ | 1·0 | 390 | 260 |
| 20 | $5{\cdot}3 \times 10^8$ | 0·19 | 380 | 230 |
| 40 | $1{\cdot}4 \times 10^8$ | 0·051 | 550 | 190 |
| 80 | $2{\cdot}7 \times 10^7$ | 0·010 | 630 | 230 |
| 120 | $6{\cdot}1 \times 10^6$ | 0·0022 | 840 | 260 |

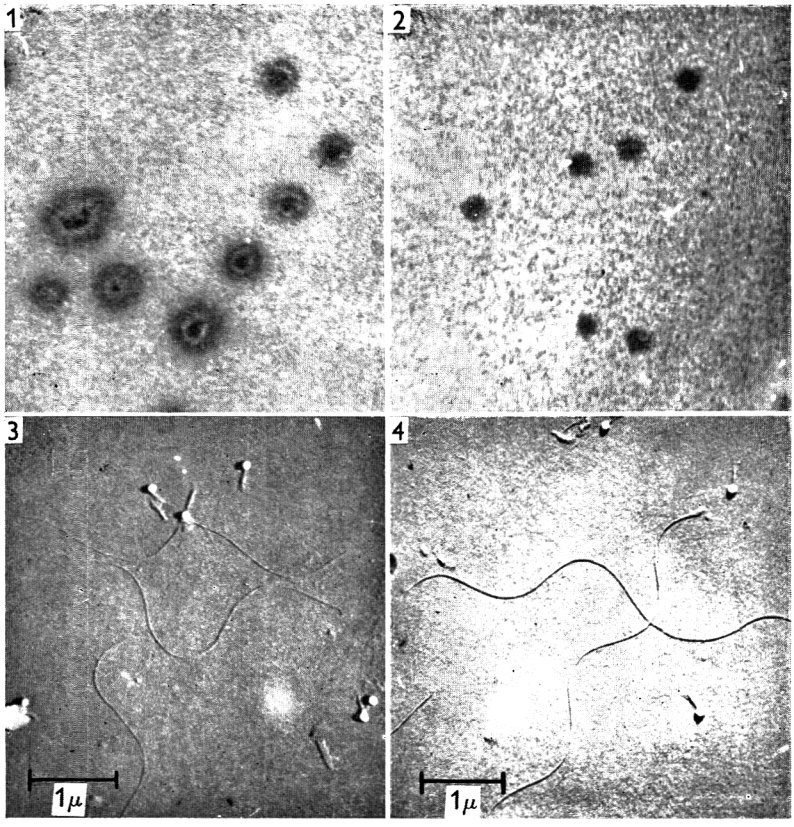
* Number of transductants developed from 0·1 ml. of lysates and 0·9 ml. of recipient culture.

## DISCUSSION

The host ranges of phages PBS1 and PBS2 seem to be restricted to certain strains of *Bacillus subtilis*, while the temperate phage SP 13 for *B. subtilis* isolated by Romig & Brodetsky (1961) is active on several *Bacillus* species, including *B. megaterium* and *B. brevis*. Thorne (1962) reported that his transducing phage SP10 was active on *B. subtilis* W23 and its derivatives but had no lytic activity on *B. subtilis* 168, although this strain can be used as recipient in transduction. As shown in Table 1, phages PBS1 and PBS2 form plaques on *B. subtilis* 168 as well as on other derivatives of the Marburg strain. This difference in host range may be explained by the fact that phage SP10 was isolated with *B. subtilis* W23 as indicator and, in the present report, *B. subtilis* SB19 was used for the isolation of phage PBS1. *B. subtilis* SB19 is a prototrophic derivative of *B. subtilis* 168 obtained by transformation with deoxyribonucleic acid (DNA) prepared from *B. subtilis* 23 (Nester & Lederberg, 1961) and presumably still possesses the genetic background of *B. subtilis* 168. Moreover, buoyant densities of the DNA prepared from phages PBS1 and SP10 are quite different from each other (Dr J. Marmur, personal communication). It appears, therefore, that phage PBS1 belongs to a phage race which is distinct from phages SP10 or SP13, although they are all temperate.

Unexpectedly, during the single-step growth experiments, it was found that only a small portion of bacteria killed by the phage infection were able to form plaques. Lysates containing phage PBS1, therefore, seem to contain defective particles as well as plaque-forming particles. At present the nature of these killing particles and their relations to the transducing particles are not known. Investigations are being undertaken to clarify this situation.

Garen & Zinder (1955) reported that the transducing activity of phage P22 for *Salmonella typhimurium* was not significantly depressed by a high dose of u.v. radiation; similar observations have been made with phages PBS1 and PBS2.






(*Facing p.* 217)

Presumably the transducing activity of these phages also involves only a small portion of u.v.-sensitive material (DNA) of the phages.

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## EXPLANATION OF PLATE 1

Fig. 1. Plaques formed on *Bacillus subtilis* SB19 by phage PBS1. × 5.
Fig. 2. Plaques formed on *B. subtilis* SB19 by phage PBS2. × 5.
Fig. 3. Electron photomicrograph of phage PBS1. × 11,000.
Fig. 4. Electron photomicrograph of phage PBS2. × 11,000.

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# Lysogeny in *Proteus rettgeri* and the Host-Range of *P. rettgeri* and *P. hauseri* Bacteriophages

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SUMMARY

Twenty-two different phage-types of *Proteus rettgeri* were investigated for lysogeny by growing strains singly, in mixtures and by ultraviolet induction. Five of the strains were lysogenic for other members of the group. The phages differed from one another in host-ranges. Eighteen different lytic phages were isolated from sewage with action on one or more of the strains. The host-range of all 23 phages was tested against a number of *P. hauseri*, *P. morganii*, Providence and other intestinal organisms. Twelve of the phages had productive cycles of infection on 24 strains of *P. hauseri*; 21 of the phages attacked 19 Providence strains. Two of the phages lysed the same 2 out of 40 strains of *Escherichia coli*. The efficiencies of plating varied from $10^{-4}$ to unity. No action was demonstrated on any of the *P. morganii* or other strains tested. The host-range of 23 *P. hauseri* phages previously isolated from sewage and from lysogenic *P. hauseri* was tested against *P. rettgeri*, *P. morganii* and Providence strains. Three Providence and 2 *P. morganii* strains proved susceptible to productive infection by a number of the *P. hauseri* phages; 15 *P. rettgeri* strains were lysed by one or more of 18 *P. hauseri* phages. Some of the *P. hauseri* phages also caused abortive infection associated with cell death in some *P. morganii* and *P. rettgeri* organisms. The results indicate close ties between the *P. hauseri* and *P. rettgeri* groups, and between *P. rettgeri* and the Providence strains.

## INTRODUCTION

Differences of opinion exist whether organisms of the *Proteus rettgeri* and *P. morganii* groups should be included with *P. hauseri* in the genus *Proteus*. Kauffmann (1951) suggested combining *P. rettgeri* and *P. morganii* strains in one group. *Topley & Wilson's Principles* (1955) included *P. morganii* strains in the Proteus group but considered *P. rettgeri* strains to be more closely related to paracolon organisms and proposed the name 'Bacterium rettgeri' for them. On the basis of the guanidine + cytosine content of their deoxyribonucleic acids Falkow, Ryman & Washington (1962) showed that *P. morganii* strains were distinct from *P. hauseri*, *P. rettgeri* and Providence strains. The latter three organisms possess very similar base compositions. The taxonomic relationships of the Providence group is also uncertain. Kauffmann (1951) described Providence strains as intermediate between Shigella and Proteus. The latter author as well as Ewing, Tanner & Dennard (1954) stressed the biochemical relationships between Providence, *P. rettgeri* and *P. morganii*. *Topley & Wilson's Principles* (1955) relegated Providence strains to a subgroup of paracolon organisms. *P. hauseri* is certainly a well-defined group; it differs markedly

in its biochemical reactions from other members of the Enterobacteriaceae (Topley & Wilson, 1955) and only isolated instances of serological cross-reactions with other members of this family have been recorded (Kauffmann, 1951; Namioka & Sakazaki, 1959). Bacteriophage reactions have confirmed the above findings. Phages of *P. hauseri* were tested by Brandis & Schwarzrock (1956), Coetzee (1958) and Vieu (1958) for action on many strains of *Escherichia coli*, Salmonella, Shigella, Pseudomonas, Staphylococcus, and paracolons. Vieu also tested *P. hauseri* phages against a number of *P. morganii*, *P. rettgeri* and Providence strains. Apart from the finding by Vieu that an unspecified number of *P. hauseri* phages produce 'incomplete lysis or some isolated plaques' on 2 of 18 *P. rettgeri* strains no other positive results have been recorded. In examining the haemagglutinating activity of *P. rettgeri* and *P. morganii* strains (Coetzee, Pernet & Theron, 1962) it was found that while *P. rettgeri* strains differed from *P. hauseri* and *P. morganii* in the range of species of vertebrate red cells agglutinated the haemagglutinins of all three groups shared the property of not being inhibited by mannose (Shedden, 1962). This finding and the observation by Vieu (1958) led us to investigate the bacteriophage relationships of *P. hauseri*, *P. rettgeri*, Providence and *P. morganii* organisms.

## METHODS

*Organisms.* Twenty-eight strains of *Proteus hauseri* were used. Nine were 'vulgaris' varieties and the remainder 'mirabilis' strains. They were isolated from human faeces or urine and all but four (nos. 108, 121, 171, 192) are hosts for a series of phages (Coetzee, 1958; Coetzee & Sacks, 1960*a*). Identifying numbers are given in Table 3. All the cultures were in the A morphological phase (Coetzee & Sacks, 1960*b*) and when the swarms were matched on nutrient agar plates they all showed a narrow line of demarcation. This is taken to mean that the strains differ in some respect (Dienes, 1946; Krikler, 1953; Story, 1954). Fourteen locally isolated strains of *P. rettgeri* and 8 *P. rettgeri* cultures obtained from the National Collection of Type Cultures (NCTC; Colindale, London) were used. Identifying numbers are given in Table 1. The *P. morganii* strains used comprised 6 NCTC strains and 11 locally isolated cultures. The identifying numbers are NCTC 1707, 2815, 2818, 5845, 7381, 10041, and local no. M2, M3, M21, M47, M49, M71, M86, M92, M232, M235, M336. Twenty-four different O serological groups of Providence strains were used; they were supplied by Miss Shona Wright (Bacteriology Department, University of Edinburgh). They are all NCTC cultures and their identifying numbers are given in Table 4. Five different phage-types of *Salmonella typhosa*, 23 other Salmonella serological varieties, 40 different isolates of *Escherichia coli*, 8 different isolates of paracolon organisms, 8 Shigella strains and 7 strains of *Pseudomonas aeruginosa* were used in host-range experiments. Cultures were stored on agar slopes at 4°.

*Phages.* The 23 *Proteus hauseri* phages used are listed in Table 2. Twenty-one were isolated from sewage (Coetzee, 1958), the remaining two are virulent clear plaque mutants of temperate phages 34/13 and 12/57 (Coetzee & Sacks, 1960*a*, *c*). Twenty-three *P. rettgeri* phages were isolated during the present work from sewage and from lysogenic strains. The sewage isolations were by the enrichment technique of Adams (1959). Lysogeny was tested for by examining the supernatants of

10-day-old broth cultures of single organisms and mixtures of organisms for phage ultraviolet irradiation of cultures was also used. These three methods of induction have been described (Coetzee & Sacks, 1959, 1960*a*). All the *P. rettgeri* strains were used as possible indicators. Phages isolated from sewage are identified by the number of the original indicator organism. Temperate phages are identified by fractions: the number of the lysogenic strain is the nominator and that of the indicator strain the denominator. The *P. rettgeri* phages are listed in Table 1.

The nutrient agar and general methods used were those of Adams (1959); the broth was as described by Coetzee & Sacks (1960*b*). Phages were purified by three successive single-plaque propagations on the host organism. Broth lysates were shaken with 0·1 vol. chloroform to kill bacteria, decanted to remove excess chloroform and aerated for about 30 min. at 45° to remove residual chloroform. Lysates were stored at 4° in sterile containers. The host ranges of the phages were determined by spotting drops of phage suspensions on agar plates which had been layered with soft agar seeded with enough test organism to give a lawn. The phage suspensions contained about $10^8$ plaque-forming particles/ml. Plates were incubated at 37° overnight before reading. Phages which showed reactions were then titrated simultaneously on the original host organism and on the new strains and the relative efficiency of plating (e.o.p.) calculated. Abortive infections were investigated by mixing phage and organism at a multiplicity of infection of about 1 at 37°. After an adsorption period of 5 min. an aliquot was treated with chloroform and titrated for unadsorbed phage on the homologous host. Simultaneously another aliquot was titrated for viable bacteria and for total infective centres on both the organisms involved (Coetzee, De Klerk & Sacks, 1960). Phages which had a low e.o.p. on heterologous hosts were investigated according to the above method using the homologous and heterologous organisms in separate experiments. These experiments were then repeated with the phage prepared on the heterologous host. In these experiments total infective centres were also assayed at the end of the latent periods (Bertani & Weigle, 1953). In attempts to increase the e.o.p. or to convert abortive to productive infections the top-layer agar was enriched with 10 g. vitamin-free pancreatic digest of casein/l. (Nutritional Biochemicals Corp., Cleveland, Ohio), 0·2 M-sodium chloride and ammonium phosphate, 0·01 M-calcium chloride and 0·01 M-magnesium sulphate individually or together.

## RESULTS

*Demonstration of lysogeny in* Proteus rettgeri. Five of the *P. rettgeri* strains were lysogenic for one or more of the 22 strains of this group; phages were obtained by all three methods used. The intra-group host-range of these phages (Table 1) shows that they can be differentiated on their host-ranges. Taubeneck (1962) did not find lysogeny in 4 *P. rettgeri* strains he examined.

*Isolation of* Proteus rettgeri *phages from sewage.* Phages were isolated from sewage against 18 of the *P. rettgeri* strains. The intra-group host-range (Table 1) shows that these phages can be distinguished on the basis of host-range and that the 22 hosts differ from one another in susceptibility to these phages. The e.o.p. of all the *P. rettgeri* phages on heterologous *P. rettgeri* hosts was unity.

*Intra-group host-range of* Proteus hauseri *phages.* The phages isolated on *Proteus*

Table 1. *Intra-group host-range of* Proteus rettgeri *phages*

Plaque-forming titres were determined on homologous and heterologous hosts and the relative efficiencies of plating calculated.

| | *P. rettgeri strains* | | | | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | NCTC no. | | | | | | | | Local organisms no. | | | | | | | | | | | | | | |
| *P. rettgeri* phage | 8893 | 7481 | 7480 | 7479 | 7478 | 7477 | 7476 | 7475 | R400 | R327 | R326 | R325 | R322 | R300 | R83 | R67 | R65 | R51 | R34 | R13 | R12 | R6 |
| 8893 | 1 | 1 | 1 | 1 | . | 1 | . | 1 | . | 1 | 1 | 1 | 1 | 1 | . | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| 7481 | 1 | 1 | . | . | . | 1 | . | . | 1 | . | 1 | 1 | 1 | . | . | . | 1 | . | 1 | 1 | 1 | 1 |
| 7480 | . | 1 | 1 | 1 | . | 1 | . | . | . | . | . | . | . | 1 | . | . | . | . | . | . | . | . |
| 7479 | 1 | . | 1 | 1 | . | 1 | . | . | . | 1 | . | 1 | 1 | 1 | . | . | . | . | . | . | . | . |
| 7477 | 1 | 1 | 1 | . | . | 1 | . | 1 | 1 | . | 1 | 1 | 1 | 1 | 1 | . | 1 | 1 | 1 | 1 | 1 | 1 |
| 7475 | 1 | 1 | 1 | 1 | . | . | . | 1 | 1 | . | . | 1 | 1 | 1 | . | . | . | . | . | . | . | . |
| R400 | . | 1 | 1 | 1 | . | 1 | . | . | 1 | . | . | . | . | 1 | . | . | . | . | . | . | . | 1 |
| R326 | 1 | . | 1 | 1 | . | 1 | . | 1 | . | . | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| R325 | 1 | 1 | . | . | . | . | . | . | 1 | 1 | 1 | 1 | 1 | . | . | . | . | . | . | . | . | . |
| R322 | . | 1 | . | . | . | 1 | . | . | 1 | . | . | 1 | . | . | . | . | . | . | . | . | . | . |
| R300 | 1 | 1 | . | . | . | . | . | . | 1 | . | . | 1 | 1 | 1 | 1 | . | 1 | 1 | 1 | 1 | 1 | 1 |
| R83 | . | . | 1 | . | . | 1 | . | 1 | . | . | 1 | . | . | . | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| R67 | 1 | . | 1 | . | . | 1 | . | 1 | . | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| R65 | 1 | . | . | 1 | . | 1 | 1 | . | . | . | . | 1 | 1 | . | 1 | 1 | 1 | 1 | 1 | . | 1 | 1 |
| R51 | 1 | 1 | 1 | . | . | . | . | 1 | 1 | 1 | 1 | 1 | . | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| R13 | 1 | . | 1 | 1 | . | 1 | 1 | 1 | 1 | . | . | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| R12 | 1 | 1 | 1 | . | . | 1 | . | 1 | . | 1 | 1 | 1 | . | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| R6 | 1 | . | . | 1 | . | 1 | 1 | 1 | . | . | 1 | 1 | 1 | . | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| 7478/325 | . | . | . | 1 | . | . | . | . | . | . | . | 1 | . | 1 | . | . | 1 | . | . | . | 1 | . |
| 7476/322 | 1 | 1 | . | . | 1 | 1 | . | . | 1 | . | . | 1 | 1 | . | 1 | . | 1 | . | . | . | . | . |
| 400/300 | . | . | . | 1 | 1 | . | . | . | . | . | . | 1 | . | 1 | . | . | 1 | . | . | . | . | 1 |
| R51/R67 | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | 1 | . | . | . | . | 1 | . |
| R6/322 | 1 | . | . | . | . | 1 | . | . | . | . | . | 1 | . | . | . | 1 | 1 | 1 | . | 1 | . | . |

Numbers = relative efficiency of plating. Points = no action.

Table 2. *Intra-group host-range of* Proteus hauseri *phages*

Plaque-forming titres were determined on homologous and heterologous hosts and the relative efficiency of plating calculated. Only phage-susceptible strains are listed.

| | *P. hauseri* strains | | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 'mirabilis' | | | | | | | | | | | | | | 'vulgaris' | | | | | | |
| *P. hauseri* phage | 6 | 12 | 13 | 14 | 19 | 20 | 23 | 24 | 25 | 34 | 57 | 58 | 63 | 65 | 74 | 9 | 48 | 49 | 51 | 54 | 61 | 78 |
| 6 | 1 | 1 | 1 | 1 | . | 1 | . | 1 | 1 | 1 | . | 1 | . | . | . | 1 | 1 | . | . | . | 1 | . |
| 12 | . | 1 | . | . | . | . | . | 1 | . | 1 | . | 1 | . | . | . | 1 | 1 | . | . | . | 1 | . |
| 13 | 1 | 1 | 1 | . | . | 1 | 1 | . | 1 | 1 | . | . | . | . | . | . | 1 | . | 1 | . | . | . |
| 14 | 1 | 1 | 1 | 1 | . | 1 | 1 | 1 | 1 | 1 | . | 1 | . | . | . | 1 | 1 | . | . | . | . | . |
| 19 | . | . | . | . | 1 | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . |
| 20 | . | . | . | . | . | 1 | 1 | 1 | 1 | 1 | . | 1 | . | . | . | . | 1 | . | . | . | 1 | . |
| 23 | 1 | 1 | . | 1 | . | 1 | 1 | . | 1 | 1 | . | 1 | . | . | . | . | . | . | . | . | . | . |
| 24 | . | . | . | 1 | . | 1 | . | 1 | . | . | . | 1 | . | . | . | 1 | . | . | 1 | . | 1 | . |
| 25 | . | . | . | . | . | . | . | . | 1 | . | . | 1 | . | . | . | . | . | . | . | . | . | . |
| 34 | 1 | . | . | . | . | 1 | 1 | 1 | 1 | 1 | . | . | . | . | . | . | 1 | 1 | . | . | 1 | . |
| 58 | 1 | . | . | 1 | . | 1 | . | 1 | 1 | 1 | . | 1 | . | . | . | . | . | . | . | . | . | . |
| 63 | . | 1 | 1 | 1 | . | . | . | . | . | . | . | . | 1 | . | . | 1 | . | 1 | . | . | 1 | 1 |
| 65 | . | . | 1 | . | . | . | . | . | . | . | . | . | . | 1 | . | . | . | . | . | . | . | . |
| 74 | . | 1 | . | . | . | . | . | . | . | . | . | . | . | . | 1 | . | . | . | . | . | . | . |
| 34/13 vir | . | . | 1 | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . |
| 12/57 vir | . | . | . | . | . | . | . | . | . | . | 1 | . | . | . | . | . | . | . | . | . | . | . |
| 9 | . | . | . | . | . | . | . | 1 | . | . | . | . | . | . | . | 1 | . | 1 | . | 1 | . | . |
| 48 | 1 | 1 | 1 | 1 | . | 1 | 1 | 1 | 1 | 1 | . | 1 | . | . | . | 1 | 1 | . | . | . | 1 | . |
| 49 | . | . | . | . | . | . | . | 1 | . | . | . | 1 | . | . | 1 | 1 | 1 | 1 | 1 | 1 | . | . |
| 51 | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | 1 | . | . | . |
| 54 | . | 1 | . | . | . | . | . | . | . | . | . | . | . | . | . | 1 | . | . | . | 1 | 1 | . |
| 61 | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | 1 | . | 1 | 1 | . | 1 | 1 |
| 78 | 1 | . | . | . | . | . | . | . | . | . | 1 | . | . | . | 1 | . | . | . | . | . | . | 1 |

Numbers = relative efficiency of plating. Points = no action.

## Table 3. *Action of* Proteus rettgeri *phage on strains of* P. hauseri

Plaque-forming titres were determined on homologous and heterologous hosts and the relative efficiency of plating calculated. Only phages with one or more positive reactions are listed.

| | *P. hauseri* strains | | | | | | | | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 'mirabilis' | | | | | | | | | | | | | | | | | | 'vulgaris' | | | | | | | | |
| phage *P. rettgeri* | 6 | 12 | 13 | 14 | 19 | 20 | 23 | 24 | 25 | 34 | 57 | 58 | 63 | 65 | 74 | 108 | 121 | 171 | 192 | 9 | 15 | 48 | 49 | 51 | 54 | 61 | 67 | 78 |
| 7481 | . | . | 0 | . | . | . | . | . | . | . | . | . | . | . | . | . | . | 0 | . | . | . | . | . | . | . | . | . | . |
| 7477 | . | . | . | . | . | . | . | − 1 | . | . | . | . | . | . | 0 | . | . | . | − 2 | . | . | . | − 1 | . | . | . | . | . |
| 7475 | . | 0 | . | . | . | . | . | . | . | . | . | . | . | . | . | . | 0 | . | . | . | . | . | . | . | . | . | . | . |
| 400 | 0 | . | 0 | . | . | . | . | − 1 | − 1 | . | . | . | . | . | − 1 | . | . | . | . | . | . | . | . | . | . | . | . | 0 |
| 325 | . | 0 | . | . | . | . | . | 0 | . | . | . | . | . | . | . | − 1 | . | . | . | 0 | . | . | . | . | . | . | . | 0 |
| 322 | . | 0 | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | − 2 | − 2 | . | . | . | . | . | . |
| 300 | . | . | . | . | . | . | . | . | . | − 1 | . | . | . | . | . | . | − 1 | . | . | . | . | . | − 1 | . | . | . | . | . |
| R51 | . | . | . | . | 0 | 0 | . | . | . | . | . | 0 | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . |
| R13 | 0 | . | . | . | . | . | . | . | . | − 1 | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | − 1 | . | . |
| 7478/325 | . | . | . | . | . | . | . | . | . | . | 0 | . | . | . | . | . | . | . | . | . | . | . | . | 0 | . | . | . | . |
| 7476/322 | . | . | . | . | . | . | . | . | . | . | . | − 2 | − 2 | . | . | . | . | . | . | . | . | . | . | . | − 1 | . | . | . |
| 400/300 | . | . | . | . | . | . | . | . | − 1 | . | . | . | . | . | . | . | . | . | − 2 | . | . | . | . | . | . | . | − 2 | − 2 |

Numbers = $\log_{10}$ of relative efficiency of plating. Points = no action.

Table 4. *Action of* Proteus rettgeri *phage on Providence strains*

Plaque-forming titres were determined on homologous and heterologous hosts and the relative efficiencies of plating determined. Only phages with one or more positive reactions are listed.

| *P. rettgeri* phage | Providence strains (NCTC no.) | | | | | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 9000 | 9190 | 9211 | 9213 | 9215 | 9219 | 9224 | 9246 | 9248 | 9250 | 9260 | 9262 | 9265 | 9268 | 9271 | 9276 | 9278 | 9279 | 9282 | 9283 | 9290 | 9295 | 9298 | 9402 |
| 8893 | −1 | . | . | 0 | −1 | −1 | . | −2 | . | −1 | . | . | −2 | −1 | . | . | . | . | . | −2 | −1 | . | . | 0 |
| 7481 | −1 | . | . | −3 | . | . | . | −1 | −4 | −1 | −1 | . | 0 | . | . | 0 | . | 0 | . | −3 | . | . | . | 0 |
| 7480 | −1 | . | . | . | −2 | −1 | −4 | −2 | . | −2 | . | . | −2 | −2 | . | . | . | . | . | . | −2 | . | . | −2 |
| 7479 | 0 | 0 | −4 | 0 | −1 | 0 | . | . | . | 0 | . | . | 0 | 0 | . | . | . | . | . | 0 | 0 | . | . | 0 |
| 7477 | −1 | . | . | −1 | 0 | −1 | . | −1 | −1 | −1 | −1 | . | 0 | −1 | . | 0 | . | 0 | . | −1 | −1 | . | . | −1 |
| 7475 | −3 | −4 | . | −1 | −1 | −2 | . | −1 | . | −3 | . | . | −1 | −4 | . | −1 | . | −1 | . | . | −2 | . | . | −2 |
| R400 | −2 | −2 | . | −1 | −1 | −1 | . | −1 | . | −1 | −1 | . | 0 | −1 | . | 0 | . | 0 | . | −1 | −1 | . | . | −2 |
| R326 | −1 | 0 | . | 0 | −2 | −2 | . | . | −2 | −2 | −1 | . | 0 | −2 | . | −1 | . | −1 | . | −1 | −1 | −2 | . | 0 |
| R325 | −3 | . | . | 0 | . | . | . | . | . | . | . | . | −1 | . | . | −1 | . | −3 | . | . | . | . | . | −1 |
| R322 | −1 | . | . | −2 | −2 | . | . | . | . | −3 | . | . | −2 | . | . | −1 | . | −1 | . | . | . | . | . | −1 |
| R300 | −3 | −1 | . | −1 | −1 | −1 | . | . | . | . | . | . | −1 | −1 | . | . | . | −4 | . | . | . | . | . | −4 |
| R83 | . | 0 | −1 | −3 | −3 | . | −3 | . | −3 | . | −3 | . | . | . | . | . | . | . | . | . | . | −2 | . | −2 |
| R67 | . | . | . | 0 | −1 | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | 0 | −1 | . | 0 |
| R65 | . | . | . | 0 | 0 | −2 | −2 | . | . | . | . | . | −2 | . | . | . | . | . | . | −1 | −1 | −1 | . | . |
| R51 | −1 | . | −4 | −3 | −3 | . | . | . | . | . | . | . | −3 | . | . | −3 | . | . | . | . | . | . | . | −3 |
| R13 | −2 | 0 | −1 | 0 | 0 | −2 | 0 | . | −2 | . | 0 | . | −1 | −2 | . | −2 | . | . | . | 0 | 0 | . | . | 0 |
| R12 | −4 | 0 | −1 | −4 | −1 | . | . | . | . | . | −1 | . | −4 | . | . | . | . | . | . | . | . | −1 | . | −1 |
| R6 | . | . | . | 0 | −2 | −1 | . | . | −2 | −2 | −1 | . | −2 | −2 | . | . | . | . | . | −2 | 0 | . | . | 0 |
| R6/322 | . | . | . | . | −2 | . | . | . | . | −2 | . | . | . | −1 | . | −1 | . | . | . | . | −1 | . | . | . |
| 7478/325 | . | . | . | . | −3 | −2 | . | −2 | . | −2 | . | . | . | . | . | . | . | . | . | . | −1 | . | . | . |

Numbers = $\log_{10}$ of relative efficiency of plating. Points = no action.

*hauseri* strains (Table 2) differed from one another in host-range and, as shown (Coetzee, 1958), many have the same e.o.p. on *P. mirabilis* and *vulgaris* varieties of *P. hauseri*.

*Action of* Proteus rettgeri *phages on* P. hauseri *organisms*. Table 3 shows that 12 of the *P. rettgeri* phages have productive cycles on several *P. hauseri* hosts. The e.o.p. was often unity. A few of the systems with low e.o.p. were further examined. A typical example is the action of *P. rettgeri* phage 7477 on *P. mirabilis* strain 192. This phage has an e.o.p. of slightly less than $10^{-2}$ on the 'mirabilis' strain, adsorbs equally well on both the organisms but kills few of the 'mirabilis' organisms. Phage prepared on strain 192 plates with equal efficiency on the two strains. However, a single cycle of growth in *P. rettgeri* 7477 is sufficient for it to revert to the original state with an e.o.p. of about $10^{-2}$ on *P. mirabilis* 192. The efficiency of this transformation makes an explanation on grounds of host-range mutants untenable (Bertani & Weigle, 1953) and suggests a phenotypic modification of the phage by the *P. rettgeri* strain (Luria, 1953). Addition of casein digest or the various salts to the top-layer agar did not affect the e.o.p. The phenomenon was not further investigated.

Table 5. *Action of* Proteus hauseri *phage on* P. rettgeri *strains*

Plaque-forming titres determined on homologous and heterologous hosts and relative efficiencies of plating calculated. Only systems showing one or more positive reactions are presented.

| | *P. rettgeri* strains | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *P. hauseri* phage | NCTC 7481 | NCTC 7479 | R400 | R327 | R326 | R325 | R322 | R309 | R83 | R67 | R51 | R34 | R13 | R12 | R6 |
| 6 | −1 | . | . | . | −1 | . | . | . | . | . | . | . | . | . | 0 |
| 12 | −1 | . | . | . | −1 | . | A | . | −3 | . | . | A | A | . | . |
| 13 | −1 | . | . | . | −1 | . | . | . | . | . | −1 | . | −1 | . | . |
| 14 | −1 | . | . | . | −1 | −3 | . | . | −3 | . | . | . | . | A | . |
| 19 | −1 | . | . | . | −1 | . | . | −2 | . | . | . | . | . | . | . |
| 20 | −1 | . | −3 | . | −1 | A | . | . | . | −2 | . | . | . | . | . |
| 23 | −1 | . | . | . | −1 | . | A | . | . | . | . | . | −2 | . | . |
| 24 | −1 | . | . | −2 | −1 | . | . | . | −2 | . | . | −3 | . | A | . |
| 25 | −1 | . | . | . | −1 | . | −3 | −3 | A | . | . | . | . | . | −1 |
| 34 | −1 | . | . | . | −1 | . | . | . | . | . | . | −1 | . | . | . |
| 58 | −1 | . | . | . | −1 | A | . | . | . | . | . | . | . | A | . |
| 63 | −1 | . | . | −3 | −1 | . | . | . | . | . | −1 | . | −3 | . | . |
| 74 | −1 | . | . | . | −1 | . | 0 | . | −2 | . | . | . | . | . | −1 |
| 34/13 vir | −1 | −1 | . | . | . | A | . | . | . | . | . | . | . | . | . |
| 9 | −1 | . | . | . | −1 | . | . | . | . | −1 | . | . | . | −2 | . |
| 48 | −1 | . | . | . | −1 | . | . | . | . | . | A | . | . | . | . |
| 54 | −1 | . | −2 | . | −1 | . | 0 | . | . | . | . | . | . | . | . |
| 61 | −1 | . | . | . | −1 | . | . | . | . | . | A | . | . | A | . |

Numbers = $\log_{10}$ of relative efficiency of plating. A = abortive infection. Points = no action.

*Action of* Proteus rettgeri *phage on* P. morganii *organisms*. No action of any kind was observed for *P. rettgeri* phages on the 17 *P. morganii* strains used.

*Action of* Proteus rettgeri *phage on other members of the family Enterobacteriaceae*. The *P. rettgeri* phages did not have any action on any of the Salmonella, Shigella, paracolon or Pseudomonas strains tested. Phages isolated on *P. rettgeri* strains R65 and NCTC 7477 plated with efficiencies of $10^{-2}$ on two strains of *Escherichia coli*

(one strain locally isolated from faeces, the other a colicine F producing strain no. CA.42 supplied by Professor P. Fredericq, Liége, Belgium). The action of *P. rettgeri* phages on 24 different Providence strains is recorded in Table 4; 20 of the phages acted on one or more of these 19 strains. The e.o.p. varied between $10^{-4}$ and unity. A few systems with low e.o.p. were further examined. Enrichment of the top-layer agar with casein digest or with the electrolytes used before did not affect the e.o.p. Phage which had passed through a Providence strain plated with equal efficiency on this strain and the particular *P. rettgeri* host. One cycle of growth in this latter strain re-established the original plating efficiency. The cases examined appeared to be examples of phenotypic modifications of the particular phages.

Table 6. *Action of* Proteus hauseri *phage on* P. morganii *strains*

Plaque-forming titres were determined on homologous and heterologous hosts and relative efficiencies of plating calculated. Only systems showing one or more positive results are presented.

| | *P. morganii* | | | | | |
|---|---|---|---|---|---|---|
| *P. hauseri* phages | NCTC 10041 | NCTC 2815 | M235 | M232 | M47 | M21 |
| 6 | −3 | . | . | . | . | . |
| 12 | −3 | A | . | A | A | . |
| 14 | −3 | −3 | . | . | . | A |
| 19 | −3 | . | . | . | . | . |
| 24 | −3 | A | . | . | . | A |
| 34 | −3 | . | . | . | A | A |
| 48 | . | . | A | . | . | . |
| 63 | −3 | A | . | . | . | . |

Numbers = $\log_{10}$ of relative efficiency of plating. A = abortive infection. Points = no action.

*Action of* Proteus hauseri *phages on* P. rettgeri *organisms.* The e.o.p. of *P. hauseri* phages on strains of *P. rettgeri* is given in Table 5. The original observation by Vieu (1958) that some *P. hauseri* phages have an action on *P. rettgeri* strains was confirmed and extended. Eighteen of the *P. hauseri* phages caused productive infection in one or more of 15 *P. rettgeri* strains. The e.o.p. of most of the phages on the new hosts varied between $10^{-1}$ and $10^{-3}$, but some plated with an efficiency of unity. Enrichment of the top-layer agar with amino acids or salts as before had no effect on the e.o.p. A few systems with low e.o.p. were further examined. A single passage through the heterologous host increased the e.o.p. to unity. The phenomenon was not further examined, it may be a phenotypic change like the cases previously mentioned. Discrepancies were sometimes observed in the host range of *P. hauseri* phages as determined by the spotting and titration techniques on *P. rettgeri* hosts. In these instances an area of bacterial inhibition in the form of complete or partial clearing was present where the drop of phage was placed but the plaque-forming titre of the phage on the particular organism was zero. These cases were all due to abortive infection with killing of the adsorbing organism. No phage production was ever found (Coetzee *et al.* 1960; Amati, 1962). As with efforts to increase the e.o.p. the top-layer agar was enriched with the electrolytes and amino acids in attempts to convert abortive infections to productive ones but no difference in the course of adsorption could be demonstrated.

*Action of* Proteus hauseri *phage on* P. morganii *strains.* Only organisms and phage which showed positive results are presented in Table 6. *P. hauseri* phage is decidedly less active on *P. morganii* strains than on *P. rettgeri* organisms. Only two *P. morganii* organisms (NCTC no. 10041, 2815) supported the productive cycle of one or more *P. hauseri* phages. The e.o.p. was $10^{-3}$ in all cases. The remaining positive reactions were all of the abortive infection variety associated with host killing. Vieu (1960) could not find any action by 33 temperate *P. morganii* phages on four strains of *P. hauseri.* Taubeneck (1962), with a series of temperate *P. hauseri* and *P. morganii* phages, did not find any action of *P. morganii* phage on *P. hauseri* strains; his *P. hauseri* phages also did not act on any *P. morganii* strains.

*Action of* Proteus hauseri *phage on Providence strains.* Only three strains showed evidence of phage action: strain NCTC 9219 supported productive infection by phages 19 and 74, strain NCTC 9224 was similarly attacked by phages 12 and 48, and strain NCTC 9246 was productively lysed by phages 12/57 vir, 6, 9, 12, 13, 14, 23, 24, 48, 51, 58, 67. The e.c.p. varied between $10^{-2}$ and unity.

## DISCUSSION

Organisms able to support the growth of a particular phage are considered to be closely related (Stocker, 1955) and the many examples of productive infection caused by *Proteus hauseri* phages on *P. rettgeri* strains, and vice versa, should serve to strengthen the position of *P. rettgeri* within the Proteus group and confirms the findings of Falkow *et al.* (1962). The finding of Falkow *et al.* of very similar guanidine +cytosine contents in the deoxyribonucleic acids of Providence, *P. rettgeri* and *P. hauseri* strains may also reflect the finding that many *P. rettgeri* phages productively infect Providence organisms. The relationship between Providence and *P. hauseri* strains, on the other hand, is possibly less close as judged by the small number of Providence strains susceptible to *P. hauseri* phages.

The productive infection of a few *Proteus morganii* hosts by *P. hauseri* phage may also be regarded as evidence of a relation between these groups. That these interactions are not as frequent as those between *P. hauseri* and *P. rettgeri* strains and the fact that no *P. rettgeri* phage was found to attack *P. morganii* strains may support the finding of Falkow *et al.* (1962) that *P. morganii* strains are not as closely related to *P. hauseri* as are *P. rettgeri* organisms. The finding of two *P. rettgeri* phages which caused productive infection in 2 of 40 strains of *Escherichia coli* was interesting. At present these reactions appear to be rare; perhaps more weight should be given to the other reactions encountered.

The abortive infections encountered mean at the very least that the strains have common phage receptors. No abortive infections were encountered in the non-Proteus organisms tested and this adds to the significance of the finding.

The e.o.p. of phages on the heterologous hosts was often low but in all cases the phage progeny from the latter plated with the same efficiency on both the organisms involved. This investigation has shown close relationships between *P. hauseri*, *P. rettgeri*, Providence and to a lesser extent *P. morganii* strains which will have to be taken into account in any future taxonomic juggling with these organisms.

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# The Identity of Streptococcal Group D Antigen with Teichoic Acid

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SUMMARY

The 'intracellular' teichoic acids from two strains of group D streptococci (strains 8191, 39) were purified and their serological and chemical properties examined. Both compounds reacted serologically with group D streptococcal antiserum. Chemical analysis showed them to be polymers of glycerol phosphate containing a high proportion of glucose and bearing alanine ester residues on the sugar; it is likely that the glucose comprises di- and tri-saccharide residues attached to most or all of the glycerol units. Analysis of group antigen preparations from three other strains of group D streptococci previously studied indicated that they are serologically identical with and chemically similar to the teichoic acid from strain 39. The difference in serological activity between the intracellular teichoic acid from strain 8191 and that from the other four strains is probably associated with small differences in chemical composition, particularly with respect to glucose content.

## INTRODUCTION

Most streptococci of intestinal origin fall into a single serological group, Lancefield's group D. Whereas in group A streptococci cell-wall polysaccharides have been shown to be the group antigens (Salton, 1953; McCarty, 1952, 1960), in group D streptococci wall material confers type- rather than group-serological specificity (Elliott, 1959). In a recent communication (Elliott, 1962) it was suggested that the group D antigen is probably a polymer of glucosylglycerol phosphate. Polymers of either ribitol- or glycerol phosphate, often containing sugars in glycosidic linkage with the polyol moieties, have been isolated from a wide range of Gram-positive bacteria; such compounds have been called teichoic acids (Armstrong *et al.* 1958, 1959). An immunologically active polymer of glycerol phosphate has been isolated from group A streptococci by McCarty (1959). Ribitol teichoic acids in the walls of some staphylococci are responsible for the immunological group specificity in these organisms (Haukenes, Ellwood, Baddiley & Oeding, 1961), and a relationship between serological behaviour and teichoic acids in the walls of lactobacilli has been proposed (Baddiley & Davison, 1961). The present communication confirms the identity of streptococcal group D antigen with intracellular teichoic acid. Moreover, it is likely that slight differences in the serological activity of the intracellular teichoic acids isolated from different strains are reflected by, and may be due to, differences in chemical composition.

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## METHODS

*Streptococci.* *Streptococcus faecalis* strains 39, C1 and D76, and *S. durans* strain C3 were characterized by physiological criteria (*Topley & Wilson's Principles*, 1955). Strain 8191 was examined by Dr E. M. Barnes (Low Temperature Research Station, Cambridge); she reported that it is intermediate between *S. faecium* and *S. durans*, but does not resemble *S. faecalis* (Barnes, 1956). Serologically, strain 8191 belongs to cell-wall type 39 of Sharpe & Fewins (1960). Strain 6681 belongs to group N. Strains C1, D76 and C3 were obtained from Dr R. C. Lancefield and strain 39 from Professor E. F. Gale, F.R.S.; strains 8191 and 6681 were obtained from the National Collection of Industrial Bacteria (NCIB).

*Conditions for growth and extraction of antigens.* The conditions for growth, extraction and study of composition of group D antigenic material from strains C1, D76 and C3 have already been described (Elliott, 1960, 1962).

*Antisera.* Group D streptococcal antisera were provided by Dr R. C. Lancefield and prepared by a method previously described (Elliott, 1960).

*Precipitin tests.* These were carried out by using the capillary techniques (Swift, Wilson & Lancefield, 1943) and the agar gel diffusion method of Ouchterlony.

*Conditions for growth of strains* 8191 *and* 39. Organisms were grown under the direction of Mr A. L. Davison in batch culture (15 l.) at 37° for 16 hr. in a liquid medium of the following composition: Oxoid Tryptone, 20 g.; Oxoid yeast extract, 10 g.; sodium acetate, 10 g., glucose, 20 g.; potassium dihydrogen phosphate, 4·5 g.; sodium hydroxide, 1·04 g.; inorganic salts (Barton-Wright, 1946), 5 ml.; distilled water, 1000 ml.

*Extraction of teichoic acids from strains* 8191 *and* 39. Cocci (about 60 g. wet weight in 15 l. culture) were harvested in a refrigerated Sharples centrifuge and washed with 0·95 % sodium chloride solution at 0°. The washed cocci were resuspended in cold distilled water (25 g. wet weight in 100 ml. water) and disrupted with Ballotini beads in a centrifuge shaker head (Shockman, Kolb & Toennies, 1957) at 1400 rev./min. for 30 min. After removal of the Ballotini beads by filtration through a no. 1 sintered glass funnel, the suspension was centrifuged at 3500 rev./min. for 30 min. The cloudy supernatant fluid was separated from the lower layers of particulate material, the latter comprising whole cocci, cell walls and a fairly mobile layer of disintegrated cell walls and other material. Further centrifugation of the supernatant fluid at 29,000 rev./min. for 60 min. in a Spinco Model L preparative ultracentrifuge gave a clear gel (equiv. 2–3 g. dry wt. from 15 l. culture). Examination, by acid hydrolysis and paper chromatography, of freeze-dried samples of the various fractions showed that most of the glycerol teichoic acid was present in the gel fraction. The supernatant fluid from the 29,000 rev./min. centrifugation contained a small amount of glycerol teichoic acid as well as a little ribitol phosphate polymer that is normally found in the walls of these organisms (J. J. Armstrong, J. Baddiley & A. J. Wicken; unpublished observations). The mobile layer of disintegrated walls also contained small quantities of a similar admixture of the two types of polymer.

The freeze-dried precipitated gel fractions contained, in addition to the glycerol teichoic acids, protein and nucleic acid, the latter accounting for about 98 % of the material. Removal of protein and much of the nucleic acid was effected by stirring

a suspension of the dry gel in 10% (v/v) trichloroacetic acid solution (1 g. gel/40 ml. trichloroacetic acid solution) for 3 days at 0–5°. After centrifugation in the cold, the clear supernatant fluid was removed and treated with 5 vol. cold 96% ethanol. Crude teichoic acid was precipitated during 24 hr. in the cold, collected by centrifugation, washed with ethanol and ether, and then dried *in vacuo* over $P_2O_5$. A further extraction of the gel for 4 days in a similar manner yielded an additional small quantity of crude teichoic acid (total yield, about 180 mg. from 1 g. dry gel). Examination of this material chromatographically, before and after acid hydrolysis, showed that protein had been completely removed but that considerable quantities of material which absorbed ultraviolet (u.v.) radiation were still present, much of the latter being small oligonucleotides arising from degradation of nucleic acid by the prolonged action of trichloroacetic acid.

Considerable concentration of the teichoic acid was achieved by trituration of crude material for 1–2 min. in ice-cold 10% trichloroacetic acid (50–100 mg. in 1 ml. trichloroacetic acid solution), followed by centrifugation to remove undissolved degraded nucleic acid. The teichoic acid was recovered from the supernatant fluid as described earlier. Two such triturations gave material (30–40 mg. from 1 g. gel) which was composed of approximately equal amounts of teichoic acid and material which absorbed light at 260 m$\mu$.

*Purification of teichoic acid from strain* 8191. Final purification of the teichoic acid was achieved by chromatography on Sephadex G. 75 (Pharmacia, Sweden). Crude teichoic acid (274 mg.) in a little water was neutralized with dilute ammonia to pH 6·8 and applied to a column (60 cm. × 2·5 cm.) of Sephadex G. 75 (previously allowed to swell in water and washed with 0·05 M-sodium chloride solution and then with water until free from chloride ions). Elution with water at an initial rate of 0·8 ml./min. was carried out while fractions (2·5 ml.) were collected with a Towers Automatic Fraction Collector (Model A). The extinction at 260 m$\mu$ and phosphorus content of each fraction were determined.

Typically, two peaks corresponding to material absorbing ultraviolet radiation were observed from a fully developed chromatogram; a sharp peak corresponding to components with high molecular weight immediately followed the solvent front and a very broad peak corresponding to nucleotides with lower molecular weight was observed after 150 ml. water had passed through the column. Teichoic acid was eluted between these two fractions and was contaminated with the slower moving nucleotides. Repeated chromatography of the teichoic acid fraction on Sephadex G. 75 as above gave further partial separation from nucleotides. The purest samples (10–15 mg. from 1 g. gel) of teichoic acid obtained in this way contained 0·3% of material which absorbed u.v. radiation.

Chromatography of the crude teichoic acid from strain 39 gave a similar elution pattern, except that the extent of contamination of the teichoic acid with nucleotides was slightly greater than in the case of strain 8191; this supports the view that the teichoic acid from strain 39 has a lower molecular weight than that from strain 8191. Thus, yields of the purest fractions were lower and contained 2% of material which absorbed u.v. radiation.

*Chemical examination of teichoic acids.* Acid and alkali hydrolysis and paper chromatographic identification of the products were carried out essentially as described previously for other teichoic acids (cf. Archibald, Baddiley & Buchanan,

1961; Armstrong *et al.* 1958). Organic phosphate was determined by the method of Chen, Toribara & Warner (1956) and glucose by the method of Dubois *et al.* (1956). Alanine was determined in neutralized acid hydrolysates by the method of Rosen (1957). The presence of alanine ester residues was demonstrated by reaction with ammonia or hydroxylamine (Armstrong *et al.* 1958). D-Glucose was demonstrated chromatographically with the Glucostat reagent (Worthington Biochemical Corp., Freehold, N.J., U.S.A.) as a spray reagent (Salton,1960).

## RESULTS

### *Serological reactivity of streptococcal group D antigen preparations and purified teichoic acids*

*Precipitin reactions in capillary tubes.* Table 1 shows the results of precipitin tests with a representative group D antiserum (R986) prepared against strain D76 (*Streptococcus faecalis*). Similar results were obtained with three additional antisera made with different strains of *S. faecalis*. It was confirmed that the group D antiserum reacted only with material extracted from group D streptococci; no reaction occurred with material from group N streptococci or from staphylococci. It was

Table 1. *Precipitin reactions between group D streptococcus antiserum and glycerol teichoic acids from streptococci and staphylococci*

R986 antiserum prepared with strain D76 (*Streptococcus faecalis*) was used throughout.

| | Antigen or teichoic acid concentration (mg./ml.) | | | | |
|---|---|---|---|---|---|
| | 0·1 | 0·05 | 0·025 | 0·01 | 0·005 |
| Source of teichoic acids | Precipitin reaction | | | | |
| Streptococcus | | | | | |
| Group D. Strain 8191* | + | ± | ± | − | − |
| Group D. Strain 39* (*S. faecalis*) | ++ | ++ | ++ | + | ± |
| Group D. Strain D76† (*S. faecalis*) | ++ | ++ | ++ | + | + |
| Group D. Strain C1† (*S. faecalis*) | ++ | ++ | ++ | + | ± |
| Group D. Strain C3† (*S. durans*) | ++ | ++ | ++ | + | ± |
| Group N. Strain 6681‡ | − | − | − | − | − |
| Group A. Strain 'Richards' (type 3)§ | − | − | − | − | − |
| Staphylococcus | | | | | |
| *S. epidermis*‡ | − | − | − | − | − |
| *S. citreus*‡ | − | − | − | − | − |

* Purified teichoic acid. † Antigen preparation previously described (Elliott, 1960, 1962). ‡ Unpurified intracellular glycerol teichoic acid prepared by Mr A. L. Davison. § Purified polyglycerophosphate prepared by Dr M. McCarty (1959).

shown previously (Elliott, 1962) that no reaction occurred between the group D antiserum and the polyglycerophosphate antigen from group A streptococci (McCarty, 1959). With all the *S. faecalis* antisera used, teichoic acid from strain 8191 had about 10% of the reactivity of that isolated from the other group D strains examined. No group antiserum prepared with strain 8191 was available for comparison.

*Precipitin reactions in agar gel.* The difference in serological reactivity of teichoic acids isolated from strain 8191 and from the other group D streptococci was further

examined by carrying out the precipitin tests in agar gel (Ouchterlony's method). Drawings of the results with serum R 986 are shown in Fig. 1; identical patterns were produced with the other group D antisera. A continuous 'line of identity' was formed by the zone of precipitation resulting from the interaction of group D antiserum with the group D antigen preparations from strains C1 and D76 (*Streptococcus faecalis*); strain C3 (*S. durans*) and the purified teichoic acid from strain 39

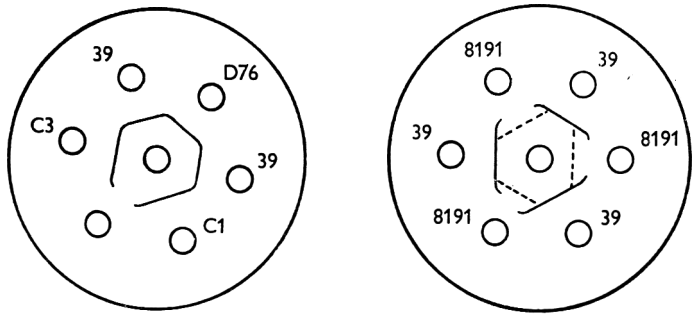


Fig. 1. Agar gel precipitin reactions of group D streptococcal antiserum (R986) with teichoic acids from different group D streptococci. (*a*) Antiserum in centre well. Teichoic acids in peripheral wells from different strains as indicated; 0·025 mg./ml. (*b*) Antiserum in centre well. Teichoic acid from strains 39 and 8191 as indicated. Strain 39, 0·025 mg./ml.; strain 8191, 2·0 mg./ml.

Table 2. *Products obtained by hydrolysis of teichoic acids from streptococcal strains* 39 *and* 8191

Samples were heated at 100° for 3 hr. in 2N-HCl or N-NaOH. The products were identified by paper chromatography with solvent mixtures described previously.

| | Acid hydrolysis | Alkali hydrolysis* |
|---|---|---|
| Glycerol | + | − |
| Glycerol monophosphates | + | Trace |
| Glycerol diphosphates | + | Trace |
| Glucose | + | − |
| Alanine | + | + |
| Lysine† | + | + |
| Inorganic phosphate | + | − |

* Most of the material after treatment with alkali was unhydrolysed and could be eluted from the base-line of the chromatogram.

† Strain 8191 only.

(*S. faecalis*). This indicates probable identity of the serologically reactive determinants in these preparations. It should be mentioned that all strains had serologically distinct cell-wall carbohydrates. On the other hand, intracellular teichoic acid from strain 8191 formed with the antiserum a weak zone of precipitation which was not continuous with that produced by teichoic acid from strain 39. The two lines approached one another at an angle but did not cross, although the strain 39 precipitate continued in the form of a spur into the surrounding agar. Teichoic

acid from strain 8191 did not form a line of precipitation when used at concentrations of less than 1·0 mg./ml.

*Chemistry of intracellular teichoic acids from streptococcal strains* 39 *and* 8191

Table 2 shows qualitatively the products of acid and alkali hydrolysis of the teichoic acids, identified by paper chromatography with reference to known standards. Table 3 gives the molar ratios of glucose, alanine and phosphorus in the purified polymers.

Table 3. *Analysis of teichoic acids from streptococcal strains* 39 *and* 8191

Results are expressed as molar ratios

| Strain from which teichoic acid was prepared | Glucose | Alanine | Phosphorus† |
|---|---|---|---|
| 8191 | 2·79 | 0·85 | 1·00 |
| 39 | 1·45 | 0·41 | 1·00 |
| *D 76 | 1·42 | — | 1·00 |
| *C 1 | 1·01 | — | 1·00 |
| *C 3 | 2·01 | — | 1·00 |

* Glucose/phosphorus ratios calculated from published analyses of extracted group antigens (Elliott, 1962).

† Corrected for nucleic acid phosphorus where applicable.

## DISCUSSION

Acid hydrolysis of a glycerol phosphate polymer occurs randomly on either side of the phosphodiester linkages, giving a mixture of glycerol, its mono- and diphosphates, and inorganic phosphate; substituents such as glucose and alanine would appear as free glucose and amino acid, respectively. These products were found after acid hydrolysis of the purified teichoic acids from streptococcal strains 8191 and 39 (Table 2). On the other hand, alkali hydrolysis can only proceed through intermediate formation of cyclic phosphates involving free polyol hydroxyl groups (cf. Kelemen & Baddiley, 1961; and others). As glycosidic linkages are relatively stable towards alkali, a glycerol phosphate polymer bearing sugar on each glycerol would not be hydrolysed with alkali. It was found that these two teichoic acids were largely or entirely unaffected by alkali, suggesting that most of the glycerol moieties were substituted with glucose; this is also supported by the high glucose: phosphorus ratio in these preparations (Table 3). It is interesting that strain 8191 contains a small amount of L-lysine, in addition to D-alanine. Evidence to be presented elsewhere indicates that the lysine is in ester linkage in the teichoic acid. Amino acid ester linkages other than those involving alanine have not been observed in teichoic acids before.

In all teichoic acids previously examined D-alanine is in ester linkage with polyol hydroxyl groups. From the above considerations it would appear that in the two teichoic acids under discussion few or no glycerol hydroxyl groups would be available for substitution with alanine; thus these polymers do not conform to the general pattern. Presumably, amino acid residues in these polymers must be in ester linkage with glucose hydroxyl groups.

The products of acid hydrolysis of the group D antigens extracted from steptococcal

strains C1, C3, and D76 have already been reported (Elliott, 1962), and indicate the presence in these extracts of teichoic acids similar to those extracted and purified from strains 8191 and 39. Their alkali stability and high glucose content resembled the teichoic acids from the other strains. Alanine ester residues were not demonstrated in the earlier extracts, but aminoacyl esters are exceptionally labile, especially above pH 7, and it is probable that they were hydrolysed during extraction (Elliott, 1960) In teichoic acids from staphylococci, the presence or absence of alanyl ester residues did not affect their immunological specificity (Haukenes *et al.* 1961), and from the present results it would also appear that alanine is not involved in the immunological properties of the polymers from group D streptococci.

Immunological identity between preparations of the group D antigen from strains C1, C3, and D76, and purified teichoic acid from strain 39, was shown by the capillary precipitin technique (Table 1) and more particularly by the Ouchterlony plate method (Fig. 1) where a continuous 'line of identity' was formed by the zone of precipitation. Thus, it is concluded that the intracellular glycerol teichoic acids in these strains are responsible for the group serological activity.

The lower reactivity of the teichoic acid from strain 8191 with *Streptococcus faecalis* antisera, and the differences in the Ouchterlony plate pattern as compared with the teichoic acids from the other strains, was interesting. It is possible that the preparations have common determinants, but that strain 8191 lacks additional determinants present in the teichoic acid of the other strains. Chemically there is a striking difference in the glucose:phosphorus ratio of the teichoic acid from strain 8191 as compared with the ratios in other strains. The high glucose content of all these preparations suggested that the polymers might be substituted with di- or tri-saccharides of D-glucose. Experiments on partial hydrolysis with acid indicate the presence of kojitriose residues in the teichoic acid from strain 8191 and kojibiose residues in strain 39 (Wicken & Baddiley, 1963).

It is likely that the immunological specificity of these antigens resides mainly in the carbohydrate portion of the molecule. Thus, no reaction occurs between group D antisera and the polyglycerophosphate antigen from group A streptococci (Elliott, 1962); this antigen appears to be a glycerol phosphate polymer with little or no sugar residues (McCarty, 1959). On the other hand, high concentrations of group D antigen precipitate with group A antisera, suggesting that the polyol phosphate chain of the polymer has a role as an immunological determinant; both purified teichoic acids were homogeneous on Sephadex chromatography, paper electrophoresis and DEAE-cellulose (Wicken & Baddiley, 1963), so it is unlikely that the preparations were mixtures containing small amounts of the group A glycerol phosphate polymer.

A carbohydrate with type antigenic properties has been isolated from group D streptococcal walls (Elliott, 1960). Chemical studies indicate that the walls of strain 8191 contain a ribitol teichoic acid and a soluble polysaccharide which have so far resisted all attempts at complete separation (J. J. Armstrong, A. J. Wicken and J. Baddiley, unpublished). Further work on the purification of this ribitol teichoic acid should assist in the chemical characterization of the type antigen.

The exact location of the group D antigen is of some interest. Although it was known not to be in the wall, unequivocal evidence for its presence exclusively

within the cell was lacking (Jones & Shattock, 1960). Recent work (Hay, Wicken & Baddiley, 1963) with protoplasts obtained by the action of lysozyme on strain 8191, and similar experiments with *Bacillus megaterium*, indicate that 'intracellular' teichoic acid is located between the wall and the protoplast membrane. Studies on differential extraction of wall and intracellular teichoic acids (Critchley, Archibald & Baddiley, 1962), and their distribution after disruption of washed bacteria precludes their occurrence on the outer surface of the wall.

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# Mutants of *Aerobacter aerogenes* Blocked in the Accumulation of Inorganic Polyphosphate

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SUMMARY

Mutants of *Aerobacter aerogenes* blocked in the accumulation of inorganic polyphosphate were isolated by the following method. Organisms previously subjected to ultraviolet (u.v.) irradiation were induced to accumulate polyphosphate in medium containing $^{32}P$. Upon subsequent growth in medium devoid of phosphate, RNA and DNA synthesis took place at the expense of polyphosphate. The organisms containing radioactive DNA were inactivated by decay of the $^{32}P$ during prolonged cold storage. From the survivors, mutants unable to form volutin granules were selected by microscopic examination. The mutants fell into two classes. The majority did not accumulate polyphosphate when phosphate was added to a phosphate-starved culture, but did so upon prolonged sulphur starvation. The remainder of the mutants did not accumulate polyphosphate under either condition. Both kinds of mutant grew well in a defined medium and showed no obvious physiological disabilities.

## INTRODUCTION

Bacteria from cultures whose growth has ceased as a result of certain kinds of nutrient imbalance often contain prominent intracellular granules characterized by metachromatic staining with basic dyes and by their opacity in the electron microscope. These 'volutin granules' have been identified as deposits of inorganic polyphosphate (reviewed by Wilkinson & Duguid, 1960; Kuhl, 1960). The nature of volutin granules is controversial. While most investigators regard them as distinct entities, some continue to identify them with nuclei or with mitochondrial equivalents. Their composition has been claimed to include ribonucleic acid (RNA), protein, lipids and various metal ions (Widra, 1959) in addition to polyphosphate. The function of polyphosphate granules likewise remains to be clarified. They have been considered to be storage deposits of phosphorus (Wiame, 1949), high-energy phosphate (Hoffmann-Ostenhof & Weigert, 1952) or divalent cations (Katchman & Fetty, 1955). Inorganic polyphosphate has also been assigned a specific role in cell division (Sall, Mudd & Takagi, 1958; Scherbaum, 1960) and in the control of energy metabolism through ATP dissipation (Harold, 1962).

Mutants genetically blocked in various phases of polyphosphate metabolism should provide incisive tools for the resolution of these controversies. It is the purpose of the present paper to describe a method for the isolation of such mutants and to report preliminary investigations of their physiology. The principle used was the inactivation of bacteria by the decay of $^{32}P$ incorporated into their deoxy-

ribonucleic acid (DNA) (Fuerst & Stent, 1956). Ultraviolet-irradiated *Aerobacter aerogenes* organisms were induced to accumulate polyphosphate in medium containing radioactive phosphate. Upon subsequent transfer to medium devoid of phosphate, RNA and DNA synthesis took place at the expense of the accumulated radioactive polyphosphate. The organisms were then collected and stored in solid $CO_2$ for several weeks. Decay of the $^{32}P$ incorporated into DNA inactivated most of the organisms. Any mutants unable to accumulate polyphosphate would not have incorporated $^{32}P$ into their DNA and would thus be expected to survive. The survivors did in fact include up to 5% of mutants blocked in the accumulation of polyphosphate.

## METHODS

*Organism.* *Aerobacter aerogenes* strain A3(0) was supplied by Dr J. F. Wilkinson. A clone of organisms showing good polyphosphate accumulation was picked and used in all subsequent experiments.

*Growth experiments.* Two types of growth medium were employed: (*a*) W medium designates the medium of Smith, Wilkinson & Duguid (1954), containing 2300 $\mu$g. Pi/ml. as phosphorus source and buffer. (*b*) $T_{subscript}$ media are a modification of this medium containing tris buffer (10 g./l., pH 7·6); the subscript indicates the phosphorus content in $\mu$g. phosphorus/ml.

All cultures were grown at 37° on a rotary shaker. Viable counts were performed by plating on W medium. The optical density of suspensions of organisms was estimated turbidimetrically at 600 m$\mu$. An optical density value at 600 m$\mu$ (OD 600) of 1·0 corresponds to $7 \times 10^8$ organisms/ml.

Polyphosphate accumulation was induced by two distinct procedures described by Smith *et al.* (1954): (i) Bacteria were grown overnight on W medium, centrifuged, washed with phosphate or tris buffer, resuspended in medium devoid of sulphate, and incubated for 6 hr. (ii) An overnight culture was prepared in $T_{1·8}$ medium, growth being limited by the available phosphate. Next morning glucose and phosphate were added and the culture incubated for 1 hr. Alternatively, organisms from an overnight culture in W medium were centrifuged, washed and incubated overnight in $T_{0·4}$ medium. Addition of glucose and phosphate then induced excellent accumulation of polyphosphate within 1 hr.

*Analytical methods.* Samples (20 ml.) were withdrawn and the organisms centrifuged down. Fractionation of the organisms and estimation of inorganic polyphosphate was carried out by methods described earlier (Harold, 1960). Briefly, the organisms were extracted in succession with cold perchloric acid (PCA; 0·5 N; 15 min.), ethanol, ethanol + ether and hot PCA (0·5 N; 15 min. at 70°). Most of the polyphosphate was found in the hot PCA extract ('insoluble polyphosphate'); it was estimated as acid-labile phosphate after removal of nucleic acid by adsorption on Norit A. Organisms subjected to procedure (ii) also contained soluble polyphosphate in the cold PCA extract. This was determined as acid-labile phosphate after removal of nucleotides with Norit A.

*Chemicals.* Carrier-free $^{32}P$ was purchased from Volk Radiochemical Co., Skokie, Ill., U.S.A.

*Isolation of mutants.* An overnight culture of *Aerobacter aerogenes* A3(0) grown in W medium was harvested and the organisms resuspended in tris-buffer to OD 600 of 0·10. Ten ml. of this suspension were u.v.-irradiated with a Mineralight V-41

lamp for a period of time sufficient to decrease the viable count by 99·9 %. The irradiated organisms were then diluted tenfold in W medium or in nutrient broth and incubated for 10–18 hr. These and all subsequent operations were carried out under aseptic conditions. The organisms were then harvested by centrifugation, washed once with tris buffer and resuspended in $T_{0·4}$ medium at an OD600 value of 0·1–0·3. After incubation for 18 hr. the organisms were collected and resuspended in $T_0$ medium at OD600 about 0·8. A sample (0·20 ml.) of this suspension was pipetted into a dry tube containing 3 $\mu$g. phosphorus and 300 $\mu$c. $^{32}P$ (as $KH_2PO_4$). The tube was then incubated for 1 hr. at 37° to permit accumulation of radioactive polyphosphate by the wild-type organisms. At the end of this period the organisms were centrifuged down, washed twice with $T_0$ medium and resuspended in 0·40 ml. of $T_0$ medium. They were then incubated for 4 hr. at 37° to permit growth and nucleic acid synthesis by the wild-type organisms at the expense of accumulated $^{32}P$-polyphosphate. Finally the culture was diluted tenfold with $T_0$ medium, and glycerol added to a final concentration of 10%. Samples (1 ml.) were dispensed into screw-cap test tubes. These were frozen in solid $CO_2$ + methylcellosolve and stored in solid $CO_2$.

At intervals these tubes were thawed at 37° and appropriate dilutions plated on W medium. As shown in Fig. 1 the viable count decreased exponentially, levelling out after about 1 week with about 0·01 % of the organisms surviving. Most of the survivors were identical with the parent strain with respect to polyphosphate accumulation; the mutants, which constituted 2–5 % of the population, were picked out by microscopic screening as follows. Colonies to be screened were replated on W medium. Simultaneously, part of each colony was transferred to 2·5 ml. of $T_{0·4}$ medium and incubated with shaking overnight. Next morning glucose and phosphate (100 $\mu$g./ml.) were added to each tube. After 1 hr. at 37° the organisms were sedimented, a sample smeared on a microscope slide, heat fixed and stained for volution granules by Laybourn's procedure (1924). Clones which did not show volutin granules were thus detected; in general they turned out to be polyphosphateless mutants on subsequent investigation. Such potential polyphosphateless mutants were repurified and tested for polyphosphate accumulation. The extent of polyphosphate accumulation was determined chemically or by the use of $^{32}P$.

## RESULTS

By the techniques described in the previous section, some 25 clones were isolated from three separate u.v.-irradiations. All were incapable of accumulating 'polyphosphate when radioactive phosphate was added to a culture previously subjected to phosphorus starvation. Two clones from each u.v. irradiation were compared with the parent strain with respect to typical reactions of *Aerobacter aerogenes* (Wilkinson, Duguid & Edmonds, 1954). The Gram reaction and motility, fermentation of sucrose, lactose, mannitol, glycerol and inositol, colony appearance on eosine methylene blue agar, citrate utilization and production of indole and acetylmethylcarbinol, were all like the reactions given by *Aerobacter aerogenes* A3(0). The mutants, like *A. aerogenes* A3(0), produced neither capsule nor loose slime.

The patterns of phosphorus metabolism in mutant and wild-type organisms were compared in growth experiments. Cultures whose growth was limited by phosphorus deficiency were prepared by overnight growth in $T_{1·8}$ medium. These organisms were then harvested and resuspended in $T_{230}$ medium. Under these

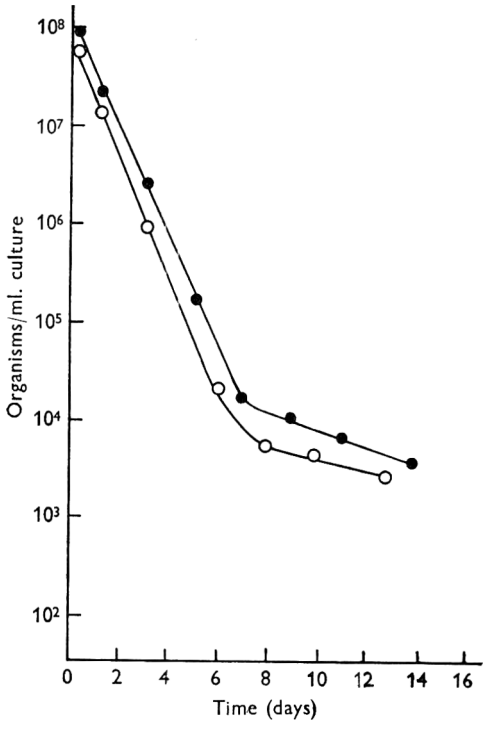


Fig. 1

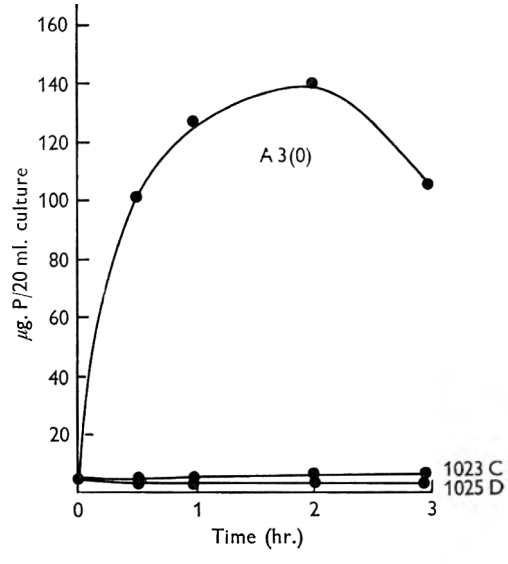


Fig. 2

Fig. 1. Inactivation of *Aerobacter aerogenes* containing $^{32}P$- labelled nucleic acids upon storage in solid $CO_2$. For details of experiments see text. The open and solid circles represent separate experiments.

Fig. 2. Accumulation of inorganic polyphosphate in wild-type *Aerobacter aerogenes* A 3(0) and mutants 1023B and 1025D. Overnight cultures were prepared in $T_{1\cdot8}$ medium. Phosphate and glucose were added at 0 hr.

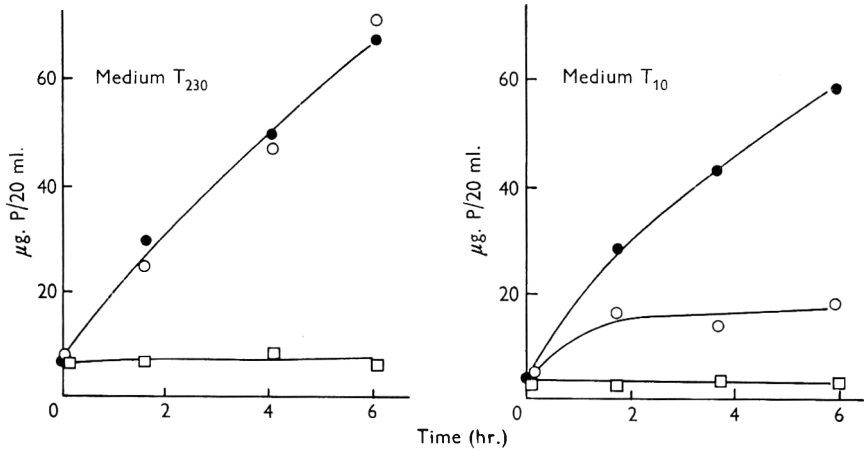


Fig. 3. Accumulation of inorganic polyphosphate in wild-type *Aerobacter aerogenes* A 3(0) and mutants 1023B and 1025D when sulphur starved. Organisms from overnight cultures in $T_{230}$ medium were resuspended in sulphur-deficient media containing 230 or 10 μg./ml. P at 0 hr. ●—● = Strain A 3(0); ○—○ = mutant 1025D; □—□, = mutant 1023B.

conditions there was rapid accumulation of polyphosphate in strain A 3(0) but none in any of the mutants (Fig. 2). This sharp difference in pattern was independent of the amount of phosphorus added to the starved cultures and of the duration of starvation.

In other experiments, the response of the wild-type and mutant organisms was compared in a sulphur deficient medium. Cultures were grown overnight on $T_{230}$ medium; the organisms were collected, washed and resuspended in T media devoid of sulphur. Under these conditions wild-type organisms exhibited a marked accumulation of polyphosphate, regardless of the concentration of phosphorus in the medium. The mutants fell into two classes: two clones (which might have been progeny of a single mutational event), 1023B and 1023C, accumulated no polyphosphate upon sulphur starvation in any medium tried. There was, however, a small variable increase in the concentration of orthophosphate and of acid-soluble organic phosphate. The remainder of the mutants were capable of accumulating polyphosphate upon sulphur starvation, but only in media of relatively high phosphorus content. Accumulation was poor on medium containing only 10 $\mu$g. radioactive phosphate (Fig. 3).

Thus far, only preliminary attempts have been made to determine whether the polyphosphateless mutants suffer any physiological disabilities as a result of their inability to accumulate polyphosphate. All of the mutants tested were identical with the parent strain with respect to growth rate and growth yield in W and $T_{230}$ media. Also there was no difference in the length of the lag period which preceded resumption of growth of sulphur- or phosphorus-starved cultures when the missing nutrient was supplied.

## DISCUSSION

The principle underlying our method for the selection of mutants with defective polyphosphate metabolism is the decay of $^{32}P$ incorporated into DNA; there is no rigorous proof that the inactivation of the wild-type population was indeed due to this process. However, the proposed mechanism is supported by the exponential inactivation curve (Fig. 1) and by preliminary experiments which showed the transfer of phosphorus from polyphosphate to both DNA and RNA under the conditions used. While our method has undoubtedly permitted the isolation of mutants blocked in the accumulation of polyphosphate, a measure of selection may well be inherent in it. If a genetic block in polyphosphate metabolism is such as to be lethal to the organism, or to prevent its multiplication in the medium used, the mutant would be lost. Consequently the only mutants recovered will be those which exhibit non-lethal modifications of polyphosphate metabolism. It would thus be not too surprising if all the mutants so isolated grew well in minimal medium, despite their inability to accumulate polyphosphate. In the absence of information about the enzymic capacities of our mutants, failure to accumulate polyphosphate need not indicate a block in polyphosphate synthesis, since a high turnover rate might prevent net accumulation. The information available so far is therefore not incompatible with a vital role for polyphosphate in cellular economy, provided that its function can be discharged by trace amounts of it. For instance, a catalytic concentration of polyphosphate would suffice for the dissipation of ATP via the polyphosphate cycle (Harold, 1962). However, the very existence of mutants such as 1023B, which appears to be incapable of accumulating

polyphosphate under any conditions, argues that volutin granules and the capacity to form a large polyphosphate pool are dispensable functions. (The fact that volutin granules were not observed in our mutants supports their identification as deposits of inorganic polyphosphate.) We have here evidence against the obligatory participation of a large polyphosphate pool in a vital process, such as cell division (Scherbaum, 1960). The nature of the enzymic defects which prevent polyphosphate accumulation in the mutants is under investigation. However, we may anticipate two distinct blocks: one which prevents polyphosphate accumulation altogether, while the other permits the slow accumulation seen in sulphur starvation but which prevents the dramatic burst normally observed when radioactive phosphate is restored to a phosphorus-starved culture. The induction of polyphosphate accumulation by these two procedures thus appears to reflect distinct and separable metabolic processes.

It is a pleasure to thank Professor G. S. Stent for suggestions about the selection technique described. We are grateful to Miss Norma Layher for skilful assistance. This investigation was supported in part by a research grant (E-3568) from the National Institutes of Health, Public Health Service, U.S.A.

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*Note added in proof:* Isolation of mutants which failed to accumulate polyphosphate following phosphorus starvation has now been repeated, using a methionine-requiring mutant of *Aerobacter aerogenes* A3 (0).

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# The Uptake of Some Sulphur-containing Amino Acids by a Brewer's Yeast

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SUMMARY

A study of the accumulation by a brewer's yeast of six sulphur-containing amino acids labelled with sulphur-35 showed several distinct patterns of uptake. L-Methionine was accumulated rapidly and completely from the medium, L-cysteine was taken up slowly, and L-cystine not at all. DL-Ethionine accumulation was incomplete. *S*-Methyl-L-cysteine and *S*-ethyl-L-cysteine were taken up rapidly for a short period, and part of the sulphur of these compounds was then released into the medium. Accumulation of the sulphur amino acids was inhibited by certain other amino acids with a close structural relationship to them and when present in high concentration. Cysteine uptake was enhanced by the presence of reduced glutathione. These effects on accumulation were shown to be primarily changes in the transport of the sulphur amino acids into the yeast, although subsequent incorporation into protein may also be affected.

## INTRODUCTION

Previous studies (Maw, 1960, 1961*a*, *b*) on the ability of various compounds to act as sources of sulphur for the growth of a strain of *Saccharomyces cerevisiae* indicated marked differences between the sulphur-containing amino acids. Whereas L-methionine was almost as effective a sulphur source as inorganic sulphate, *S*-methyl-L-cysteine was poorly so, and L-cysteine, *S*-ethyl-L-cysteine and L-ethionine were inhibitory of growth. In view of these findings the mode of uptake of these compounds and the extent of utilization of their sulphur by the yeast was examined. The present paper deals with a comparison of the rates of uptake by yeast suspensions of sulphate and six sulphur-containing amino acids, the compounds used being labelled with sulphur-35. Striking differences in rate and pattern of uptake were found which have a bearing on some of the findings of the growth experiments reported earlier; for example, the differences in effectiveness between methionine, cysteine and *S*-methylcysteine when used as sulphur sources. An examination of the effects on the uptake of the labelled compounds of several related compounds was also made, to provide information on the specificity of the uptake mechanisms. In several instances changes in uptake of the sulphur amino acids were observed, although in general a high concentration of added compound was necessary to produce any marked effect. Trichloroacetic acid fractionation of materials containing the labelled sulphur which entered the yeast was then carried out to determine whether the changes which occurred were due entirely to effects on the amino acid transport mechanism, or whether other factors, such as intracellular incorporation, were involved. A preliminary account of some aspects of this work has already been reported (Maw, 1961*c*).

## METHODS

*Media for growth and uptake experiments.* For growth of the yeast a medium of the following composition was used (the amounts given are for 1 l. final medium): glucose, 50 g.; $NH_4Cl$, 1·52 g.; KCl, 0·42 g.; $KH_2PO_4$, 0·55 g.; potassium citrate monohydrate, 5 g.; citric acid, 1·125 g.; $CaCl_2.6H_2O$, 0·2 g.; $MgCl_2.6H_2O$, 0·1 g.; $MnCl_2.4H_2O$, 5 mg.; $FeCl_3$, 4·5 mg.; $CuCl_2.2H_2O$, 0·36 mg.; $Na_2MoO_4.2H_2O$, 0·34 mg.; $H_3BO_3$, 0·6 mg.; $(CH_3.CO_2)_2Zn.2H_2O$, 6·8 mg.; inositol, 25 mg.; thiamine chloride, 0·5 mg.; pyridoxine, 0·5 mg.; calcium pantothenate, 10 mg.; biotin, 0·1 mg. Sodium sulphate was added to give a final concentration of 0·312 mM (10 mg. sulphate-S/l.). This medium without the sulphate was used in the uptake experiments and is referred to as the sulphate-free medium.

*Yeast used.* A Guinness strain of *Saccharomyces cerevisiae* was used. The yeast was grown from a small inoculum in the sulphate-containing medium for 2 days at 30° with shaking. The yeast was harvested by filtration, washed free from sulphate by three resuspensions in 0·85% sodium chloride solution and partly dried by suction on a Buchner funnel for 15 min. The yield from 400 ml. medium was 7–8 g. wet weight pressed yeast. Suspensions (20% wet weight pressed yeast/vol. were prepared in water. Yeast dry weight contents were obtained by centrifuging off the yeast and drying to constant weight at 104°.

*Compounds used.* Sodium [$^{35}$S]sulphate (carrier-free) and DL-[$^{35}$S]ethionine (1·14 mC./m-mole) were purchased from the Radiochemical Centre, Amersham, Buckinghamshire.

L-[$^{35}$S]Cystine and L-[$^{35}$S]methionine were obtained biosynthetically by growing the yeast in the sulphate medium described above, supplemented with carrier-free $Na_2{}^{35}SO_4$ (2 mC./l. medium). The methods for the isolation of the labelled compounds followed closely those of Williams & Dawson (1952). In their preparations these workers used a medium containing about 60 mg. sulphate-S/l. In the present work, more efficient incorporation of sulphur-35 by the yeast was ensured by keeping the sulphate content of the medium in the region of 10 mg. S/l. which is just adequate for optimum growth (Maw, 1960).

*S*-Methyl-L-[$^{35}$S]cysteine and *S*-ethyl-L-[$^{35}$S]cysteine were prepared from L-[$^{35}$S]cystine by reduction with sodium in anhydrous ammonia followed by alkylation with the appropriate alkyl iodide (du Vigneaud, Loring & Craft, 1934). The alkylcysteines were extracted with 95% (v/v) ethanol in water and recrystallized once from this solvent. Both compounds gave negative tests for halide ($AgNO_3$ test) and for cystine (nitroprusside test after reduction with NaCN), the only likely contaminants. Each gave a single ninhydrin spot and a single radioactive peak coincident with the ninhydrin spot on one-dimensional chromatograms run in three solvent systems.

L-[$^{35}$S]Cysteine hydrochloride was prepared by the reduction of labelled L-cystine with sodium in anhydrous ammonia (du Vigneaud, Audrieth & Loring, 1930) Ammonia was removed from the product by adding cold dilute NaOH and bubbling nitrogen gas through the solution. After neutralization and evaporation to dryness under reduced pressure, the hydrochloride was obtained free from NaCl by extraction with hot *n*-butanol.

*Procedure for uptake experiments.* Each radioactive compound was diluted with

the appropriate carrier to a specific activity of about 50 $\mu$C/m-mole and made up in the medium to a final strength of 0·156 mM (equivalent to 5 mg. S/l.) unless otherwise stated. At this concentration the compounds when assayed by the methods described below gave counting rates of about 6000 counts/min./10 ml medium.

Measurements of the uptake of the various sulphur compounds were carried out in 25 ml. conical flasks containing a total fluid volume of 10 ml., consisting of 5 ml. sulphate-free medium of twice the required strength (Maw, 1960), 1 ml. yeast suspension (20% wet weight pressed yeast/vol.) and the labelled compound under study, together with other additions if any, in 4 ml. The flasks were loosely capped and shaken at 100 oscillations/min. in a thermostat at 30°. At suitable times pairs of flasks were removed from the bath, cooled in ice, and the contents of each centrifuged for 1 min. in pre-cooled tubes in a Camlab bench angle centrifuge at 1200 *g*. The supernatant fluids were decanted into a second pair of pre-cooled tubes and again centrifuged for 1 min. The temperature of the contents of the tubes did not rise above 4° during these stages. The final supernatant fluids, free from yeast, were removed and 5 ml. of each used for the assay of sulphur-35. When determinations were being carried out on the yeast itself, the organisms were washed by two resuspensions in ice-cold saline and centrifugation in pre-cooled tubes as before.

*Extraction of yeast with trichloroacetic acid.* The yeast pellet obtained by centrifugation from each flask in uptake experiments was resuspended in 10 ml. ice-cold 5% (w/v) trichloroacetic acid (TCA) in a tube placed in ice. The suspension was stirred at intervals, and after 2 hr. it was centrifuged and the precipitate washed by two resuspensions in ice-cold water and centrifugation in pre-cooled tubes.

*Assay of* $^{35}$S-*sulphate in the medium.* In studies on the uptake of labelled sulphate, 5 ml. of the centrifuged medium were pipetted into a tube containing 5 ml. of $Na_2SO_4$ solution (0·45 g./l.). The contents of the tube were adjusted to pH 2·8, 2 ml. of aqueous 1·6% (w/v) benzidine hydrochloride added, followed 2 min. later by 4 ml. of 95% (v/v) ethanol in water. After standing for 10 min. the precipitate of benzidine sulphate was collected by filtration as a 16 mm. disk on an 18 mm. diameter Whatman No. 1 filter paper. The paper and disk were mounted by means of Perspex cement in an aluminium planchet, and dried in a desiccator for 1 hr. The activity of the sample was determined with the aid of a thin window (1·5 mg./cm.$^2$) Geiger–Muller tube. The paper and benzidine sulphate disk were then removed from the planchet, and the weight of benzidine sulphate present determined by titration with 0·01 N-NaOH. The radioactivity counts were corrected for the resolving time of the equipment, for self-absorption by the disk and for background activity.

Since the incubation medium used contained only a low concentration of sulphate (0·312 mM), the addition of sodium sulphate during the assay was necessary to give an amount of benzidine sulphate (about 5 mg.) sufficient to ensure complete precipitation of the labelled sulphate under the conditions described and to provide a disk of uniform thickness. To obtain benzidine sulphate disks of a suitably fine and uniform texture which would spread evenly during filtration and which would not curl or fritter on drying, it was also found necessary to carry out the precipitation with a minimum of acid present, namely by using the minimum amount of HCl to prepare the benzidine hydrochloride.

*Assay of organic-*$^{35}$S. Labelled organic-S was first converted to inorganic sulphate by a modification of Benedict's procedure (Benedict, 1909), and then precipitated as benzidine sulphate as described above. Samples (5 ml.) of medium were evaporated to dryness in silica crucibles with the copper nitrate reagent, heated in a furnace at 460° overnight, and finally transferred with 5 ml. water to tubes containing 5 ml. $Na_2SO_4$ solution (0·45 g./l.). Samples of yeast and of the TCA-insoluble fraction of yeast were assayed in a similar manner after being transferred to crucibles with 8 ml. water together with 2·5 ml. of the sulphate-free medium. The original Benedict method gives incomplete conversion to sulphate of several sulphur compounds, including methionine, ethionine and the alkylcysteines, although cysteine, cystine and glutathione are quantitatively oxidized. It was found in the present experiments that the presence of the incubation medium in the samples prevented spattering and increased the extent of oxidation of the more intractable compounds from 70–80 to 90–100%. Whenever analyses of pure compounds were carried out, 2·5 ml. of the medium were routinely added to aid the oxidation.

## RESULTS

### *Uptake of sulphur amino acids*

Accumulation by the yeast of the various compounds under study was assessed from the percentage loss of sulphur-35 from the incubation medium. This was generally preferable to determination of the direct appearance of sulphur-35 in the yeast, since the latter requires careful washing of the yeast to free it from residual radioactivity of the medium. Comparisons were made under various conditions of the sulphur-35 which appeared in the yeast with that which disappeared from the medium. The close agreement between the two with all the labelled compounds tested justified the use of determinations made on the medium. Determinations on the yeast were, however, carried out in the trichloroacetic acid fractionation experiments described later.

Time curves were obtained for the uptake of inorganic sulphate and six sulphur-containing amino acids at several concentrations in the medium over a 4 hr. period at 30° Fig. 1 shows the results obtained at 0·312 mM (equivalent to 10 mg. S/l.). Four distinct patterns of accumulation were observed: (i) The uptakes of sulphate and L-methionine were similar in that they increased steadily with time, resulting ultimately in virtually complete removal of each compound from the medium. Methionine accumulation was considerably more rapid than that of sulphate, and was also greater than that of the other sulphur compounds so far tested. (ii) L-Cystine was not taken up by the yeast to any significant extent, and L-cysteine was taken up only at a very slow rate. (iii) DL-Ethionine accumulation took place for a period of about 1·5 hr., during which never more than 40% of the amino acid was removed from the medium. The labelled sulphur taken up was retained in the yeast during the remainder of the 4 hr. experimental period. (iv) The uptakes of *S*-methyl-L-cysteine and *S*-ethyl-L-cysteine showed characteristic features. The sulphur of these compounds was taken up rapidly for an initial period of about 1 hr. and there was then a rapid release of sulphur-35 back into the medium, amounting to 75% of the amount initially taken up in the case of *S*-ethylcysteine (0·312 mM) and 48% in the case of *S*-methylcysteine (0·312 mM). With lower concentrations

of *S*-ethylcysteine, more than 90% of the sulphur taken up was subsequently released.

The uptakes of all the compounds examined were unaffected by the presence of sodium fluoride (0·5 mM) but were markedly decreased by sodium azide (0·5 mM) or 2,4-dinitrophenol (0·5 mM) and were negligible at 0° (see Table 1). Yeast growth during the 4 hr. experimental period was small. No detectable growth was observed for the first 2 hr. and after 4 hr. it amounted to an increase on a dry weight basis of never more than 18–20%.

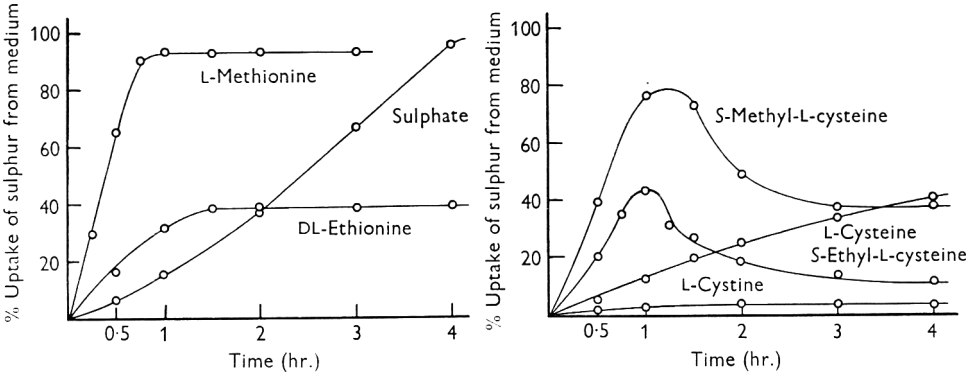


Fig. 1. Uptake of sulphur compounds by a brewer's yeast as a function of time. Compounds in medium 0·312 mM. Yeast 2 % (wet weight pressed yeast/vol.). Temperature 30°.

Table 1. *Effect of metabolic inhibitors on the uptake of sulphur amino acids by a brewer's yeast*

L-[$^{35}$S]Cysteine 0·312 mM, other [$^{35}$S]amino acids 0·156 mM. Yeast 2 % (wet wt. pressed yeast/vol.). Temperature 30°. Inhibitors 0·5 mM.

| Sulphur amino acid | Period of uptake (hr.) | % Decrease in uptake in the presence of 2,4-Dinitrophenol | Sodium azide | Sodium fluoride |
|---|---|---|---|---|
| L-Methionine | 0·33 | 92·1 | 94·3 | 4·8 |
| DL-Ethionine | 1 | 67·4 | 56·3 | 2·1 |
| L-Cysteine | 2 | 89·8 | 88·2 | 7·8 |
| *S*-Methyl-L-cysteine | 1 | 97·6 | 90·7 | 0 |
| *S*-Ethyl-L-cysteine | 0·75 | 89·7 | 90·8 | — |

*Relation between amino acid uptake and concentration in the medium.* Fig. 2 illustrates the effects on the accumulation of methionine, ethionine, cysteine and *S*-ethylcysteine of different concentrations of each compound in the medium, from 0·156 to 3·12 mM (equivalent to 5–100 mg. S/l.). Data for inorganic sulphate is also given for comparison. The lengths of the uptake periods were chosen to allow for substantial but not maximum uptake. Over this concentration range the uptake of sulphate was increased only slightly (40%) and that of methionine was about doubled. On the other hand, there was a much greater accumulation of the other amino acids, amounting to as much as a 23-fold increase in the case of cysteine. It will be noted that above 0·312 mM the uptake/concentration relationships for methionine, ethionine and cysteine were roughly linear, while *S*-ethylcysteine accumulation became maximal at about 1·56 mM.

*Concentration of amino acid-sulphur by yeast*

To obtain comparative data for the accumulation of the various sulphur amino acids, uptake values were recalculated as the ratio of the internal concentration of amino acid-$^{35}S$ to that in the medium. Approximate values for the internal 'free space' were obtained from the wet weight of pressed yeast and dry weight determinations. Intercellular water and water occupying the cell wall space were taken

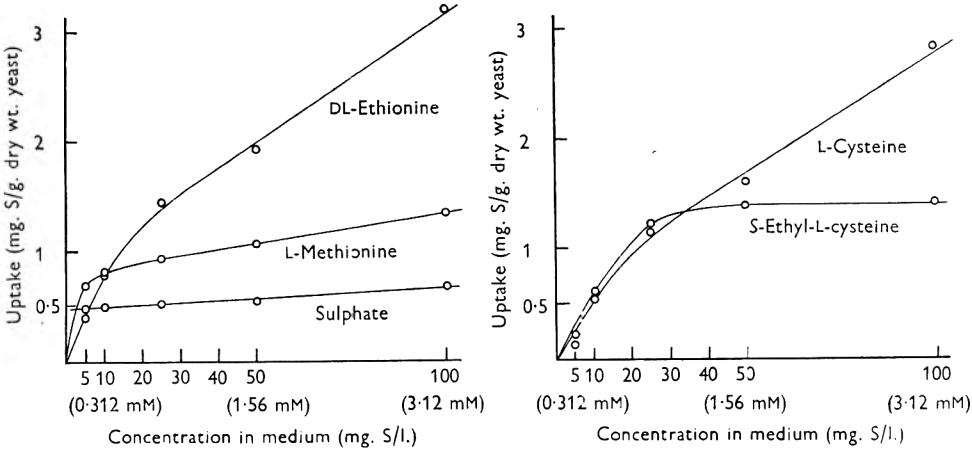


**Fig. 2.** Uptake of sulphur compounds by a brewer's yeast as a function of concentration. Yeast 2% (wet weight pressed yeast/vol.). Temperature 30°. Uptake periods: $Na_2SO_4$ and ethionine, 1 hr.; methionine, 20 min.; *S*-ethylcysteine, 0·75 hr.; cysteine, 2 hr.

Table 2. *Ability of a brewer's yeast to concentrate sulphur amino acids*

[$^{35}S$]Sulphur amino acids and $Na_2{}^{35}SO_4$ 0·312 mM. Yeast 2% (wet wt. pressed yeast/vol.). Temperature 30°. Uptake period 0·5 hr.

| Compound | Ratio of internal concentration of $^{35}S$ in yeast to concentration in medium |
|---|---|
| L-Methionine | 56·2 |
| DL-Ethionine | 11·5 |
| L-Cysteine | 17·2 |
| *S*-Methyl-L-cysteine | 30·5 |
| *S*-Ethyl-L-cysteine | 21·1 |
| $Na_2SO_4$ | 4·9 |

as amounting to 10% of the total water in the yeast when prepared in the manner described earlier. No allowance as made for any metabolism of the amino acids which might have taken place. Table 2 shows the extent to which the yeast concentrated the sulphur of the labelled compounds after a period of 0·5 hr. when they were present in the medium at 0·312 mM. Methionine was clearly the most readily accumulated amino acid at the external concentration chosen, but at 3·12 mM, owing to the greater sensitivity of uptake of the other amino acids to increase in

concentration, the difference was not so marked and at the higher concentration methionine accumulation after 1 hr. was only twice that of ethionine.

*Competition studies*

The effects of several compounds on the uptake of the sulphur amino acids was examined. The compounds used were chosen for their structural or metabolic relationships with the amino acid in question, and were added to the medium at the same concentration as the sulphur amino acid and at twice, five times and ten times this concentration. The labelled amino acids were added to 0·156 mM except for cysteine, which on account of its low rate of uptake, was added to 0·312 mM. The periods of uptake were chosen to provide substantial but not maximum accumulation. The results are summarized in Tables 3–7. For each of the five sulphur amino acids studied only a few compounds produced any significant effects on their accumulation, and then only when the molar ratio of added compound to sulphur amino acid was high (five to ten times greater).

Table 3. *Effect of various compounds on the uptake of* L-[$^{35}$S]*methionine by a brewer's yeast*

L-[$^{35}$S]Methionine 0·156 mM. Yeast 2% (wet weight pressed yeast/vol.). Temperature 30°. Uptake period 20 min. Effects of compounds expressed as the percentage decrease in methionine uptake compared with controls in the absence of added compounds.

| | Molar ratio compound/methionine | | | |
|---|---|---|---|---|
| | 1 | 2 | 5 | 10 |
| Compound added | % Decrease in methionine uptake | | | |
| $Na_2SO_4$ | 1·1 | 1·7 | 1·6 | 1·8 |
| L-Cysteine.HCl | 2·3 | 5·5 | 9·0 | 13·2 |
| DL-Homocysteine | 8·5 | 14·9 | 27·0 | 37·7 |
| *S*-Methyl-L-cysteine | 6·6 | 9·6 | 13·3 | 16·6 |
| *S*-Ethyl-L-cysteine | — | — | 15·2 | 21·4 |
| DL-Methionine sulphoxide | 11·8 | 20·9 | 36·3 | 46·5 |
| DL-Methionine sulphone | 16·1 | 26·4 | 40·4 | 45·9 |
| L-Ethionine | 12·5 | 20·9 | 32·5 | 46·0 |
| Glutathione (reduced) | 5·7 | 5·6 | 7·6 | 2·5 |
| DL-Norleucine | — | — | 16·9 | 23·3 |
| DL-α-Aminobutyric acid | — | — | — | 8·9 |
| L-α-Alanine | — | — | — | 8·9 |
| Amino acid mixture *A** | 7·5 | — | — | — |
| Amino acid mixtures *B*† | 7·9 | — | — | — |
| Amino acid mixture *A*+*B* | 14·1 | — | — | — |

* Mixture *A* contained the following amino acids, each 0·156 mM: L-α-alanine, L-arginine.HCl, L-aspartic acid, L-citrulline, L-glutamic acid, glycine, L-proline, L-serine, L-threonine.

† Mixture *B* contained the following amino acids, each 0·156 mM: L-cystine.HCl, L-histidine.HCl, DL-isoleucine, L-leucine, L-lysine.HCl, L-phenylalanine, DL-tryptophan, L-tyrosine, DL-valine.

Methionine sulphoxide, methionine sulphone and ethionine were about equally effective at a ten-fold molar concentration in decreasing methionine uptake by half (Table 3); homocysteine also decreased uptake slightly, but *S*-methylcysteine and α-aminobutyric acid were ineffective. A mixture of eighteen commonly occurring α-amino acids, each equimolar with respect to methionine, was also without any

marked effect. Methionine was the only effective inhibitor of ethionine accumulation, and then only when present at a five or tenfold concentration (Table 4).

In examining related thiols which might be expected to inhibit cysteine uptake, it was found that whereas homocysteine was without effect, reduced glutathione produced a distinct and reproducible increase in uptake (Table 5). In a series of six experiments increases in cysteine uptake ranging from 64·4 to 80·7% were

Table 4. *Effect of various compounds on the uptake of* DL-[$^{35}$S]*ethionine by a brewer's yeast*

DL-[$^{35}$S]Ethionine 0·156 mM. Uptake period 1 hr. Other details as in Table 3.

| | Molar ratio compound/ethionine | | | |
|---|---|---|---|---|
| | 1 | 2 | 5 | 10 |
| Compound added | % Decrease in ethionine uptake | | | |
| $Na_2SO_4$ | 0 | 0 | 0 | 0 |
| L-Methionine | 4·7 | 25·5 | 38·2 | 59·6 |
| *S*-Methyl-L-cysteine | 0 | 3·6 | 6·5 | 9·9 |
| *S*-Ethyl-L-cysteine | 2·0 | 4·6 | 11·0 | 17·7 |
| L-Cysteine.HCl | 0 | 0 | 0 | 6·4 |
| Glutathione (reduced) | 3·7 | 0 | 0 | 0 |

Table 5. *Effect of various compounds on the uptake of* L-[$^{35}$S]*cysteine by a brewer's yeast*

L-[$^{35}$S]Cysteine 0·312 mM. Uptake period 2 hr. Other details as in Table 3.

| | Molar ratio compound/cysteine | | | |
|---|---|---|---|---|
| | 1 | 2 | 5 | 10 |
| Compound added | % Decrease in cysteine uptake | | | |
| $Na_2SO_4$ | — | — | 0 | 0 |
| DL-Homocysteine | — | — | 1·4 | 4·9 |
| Glutathione (reduced) | −30·0 | −41·1 | −52·9 | −65·0 |
| L-Methionine | — | — | 21·3 | 23·3 |
| *S*-Methyl-L-cysteine | — | — | 12·1 | 31·9 |
| L-Serine | — | — | 4·0 | 6·0 |

Table 6. *Effect of various compounds on the uptake of* S-*methyl*-L-[$^{35}$S]*cysteine by a brewer's yeast*

*S*-Methyl-L-[$^{35}$S]cysteine 0·156 mM. Uptake period 1 hr. Other details as in Table 3.

| | Molar ratio compound/methylcysteine | | | |
|---|---|---|---|---|
| | 1 | 2 | 5 | 10 |
| Compound added | % Decrease in methylcysteine uptake | | | |
| $Na_2SO_4$ | 0 | 0 | 0 | 0 |
| *S*-Ethyl-L-cysteine | 2·5 | 10·9 | 42·9 | 59·9 |
| L-Cysteine.HCl | 0 | 0 | 0 | 0 |
| L-Methionine | 0 | 0 | 2·2 | 12·0 |
| L-Ethionine | 0 | 0 | 3·5 | 10·8 |

produced by 3·12 mM reduced glutathione. *S*-Methylcysteine and *S*-ethylcysteine were effective in decreasing the uptake of each other (Table 6 and 7) while neither methionine nor ethionine influenced *S*-methylcysteine uptake. The effects on the uptake of *S*-ethylcysteine of several compounds tested were variable but not very large. In experiments reported earlier (Maw, 1961*c*) the effect of *S*-methylcysteine was less marked than that shown in Table 7, and methionine was found to be without effect.

Table 7. *Effect of various compounds on the uptake of S-ethyl-*L-[$^{35}$S]*cysteine by a brewer's yeast*

*S*-Ethyl-L-[$^{35}$S]cysteine 0·156 mM. Uptake period 0·75 hr. Other details as in Table 3.

| | Molar ratio compound/ethylcysteine | | | |
|---|---|---|---|---|
| | 1 | 2 | 5 | 10 |
| | % Decrease in ethylcysteine uptake | | | |
| $Na_2SO_4$ | 0 | 0 | 0 | 0 |
| *S*-Methyl-L-cysteine | 8·7 | 17·1 | 50·3 | 64·5 |
| L-Methionine | 0 | 6·6 | 22·4 | 34·7 |
| L-Ethionine | 0 | 0 | 14·1 | 24·1 |
| L-Cysteine.HCl | 0 | −8·4 | −14·9 | −17·3 |
| Glutathione (reduced) | — | — | −13·2 | −23·2 |
| DL-Homocysteine | — | — | −17·8 | −11·6 |

*Intracellular distribution of accumulated sulphur*

The distribution, between the cold trichloroacetic acid (TCA)-soluble and TCA-insoluble fractions of yeast, of the sulphur of methionine, ethionine and cysteine was determined in the presence and absence of compounds which influenced their uptake. The added compounds were present at ten times the concentration of the labelled sulphur amino acid. The data from a series of duplicated experiments are summarized in Tables 8–10. It will be seen that for all three sulphur amino acids there was some incorporation of labelled sulphur into protein, although the use of different uptake times precludes any quantitative comparisons.

Table 8. *Effects of* L-*ethionine and* DL-*methionine sulphone on the partition of* L-*methionine-sulphur in a brewer's yeast*

L-[$^{35}$S]Methionine 0·156 mM. L-Ethionine and DL-methionine sulphone 1·56 mM. Yeast 2% (wet weight pressed yeast/vol.). Temperature 30°. Uptake period 20 min. Figures in parentheses show the percentage decreases over the corresponding controls.

| | % Uptake of methionine into yeast | |
|---|---|---|
| Compound added | TCA-soluble fraction | TCA-insoluble fraction |
| None | 58·5 | 11·4 |
| L-Ethionine | 26·1 (55·4) | 10·8 (5·3) |
| None | 51·3 | 10·4 |
| DL-Methionine sulphone | 25·4 (50·5) | 9·0 (13·5) |

The effects of ethionine and methionine sulphone on methionine uptake were markedly to decrease the entry of sulphur-35 into the TCA-soluble fraction, while incorporation into protein was not significantly affected (Table 8). In the inhibition

of ethionine uptake by methionine (Table 9) quantitatively the major effect was again to decrease the entry of sulphur-35 into the TCA-soluble fraction, although the small incorporation of ethionine-sulphur into protein was also virtually abolished. The ability of reduced glutathione to enhance cysteine accumulation was likewise primarily an effect on the entry of sulphur-35 into the TCA-soluble fraction. In addition, there was an increase in appearance of sulphur-35 in the protein fraction (Table 10).

Table 9. *Effect of* L-*methionine on the partition of* DL-*ethionine-sulphur in a brewer's yeast*

DL-[$^{35}$S]Ethionine 0·156 mM. L-Methionine 1·56 mM. Uptake period 1 hr. Other details as in Table 8.

| | % Uptake of ethionine into yeast | |
|---|---|---|
| Compound added | TCA-soluble fraction | TCA-insoluble fraction |
| None | 27·8 | 11·4 |
| L-Methionine | 12·1 (56·5) | 0·8 (93·0) |

Table 10. *Effect of reduced glutathione on the partition of cysteine-sulphur in a brewer's yeast*

L-[$^{35}$S]Cysteine.HCl, 0·312 mM. Reduced glutathione, 3·12 mM. Uptake period 2 hr. Figures in parentheses show the percentage increases over the corresponding controls. Other details as in Table 8.

| | % Uptake of cysteine into yeast | |
|---|---|---|
| Compound added | TCA-soluble fraction | TCA-insoluble fraction |
| None | 14·9 | 9·7 |
| Reduced glutathione | 30·2 (104·1) | 13·9 (43·3) |

*Release of* S-*ethylcysteine-sulphur from yeast*

Attempts were made to examine the labelled sulphur released back into the medium following the accumulation of *S*-ethylcysteine by the yeast. Batches (10 ml.) of medium from flasks containing 0·312 mM *S*-ethyl-L-[$^{35}$S]cysteine which had been shaken with yeast (2% wet weight pressed yeast/vol.) for 2 hr. at 30° were pooled, centrifuged and loaded on to a column of Zeo Karb 225 resin ($H^+$ form). The column was washed twice with 25 ml. water and the eluates collected separately. The column was then eluted with N-HCl and the eluates collected in 25 ml. fractions in beakers, were evaporated to dryness and checked for radioactivity under a thin-window Geiger–Muller tube. Virtually all the radioactive material present passed straight through the column together with the glucose of the medium and appeared in the initial water washings. This contrasted with the behaviour of a 0·312 mM solution of labelled *S*-ethylcysteine in medium added directly to the column. In this case, the radioactivity was retained on the column, was not removed in the water washings, but appeared in the first three fractions together with the bulk of the salts following elution with acid. This suggested that after being shaken with yeast, the medium contained $^{35}$S-labelled material differing from the *S*-ethylcysteine initially present. Further experiments were carried out by paper chromatographic methods.

The centrifuged yeast-treated medium was streaked on to 3 in. wide strips of Whatman 3 MM paper and chromatographed with *n*-butanol + acetic acid + water (4 + 1 + 5 by vol.) as solvent. The chromatograms were then cut into 1 in. wide strips and scanned for radioactivity by using a windowless gas-flow counter. The strips showed two and sometimes three radioactive bands which were rather broad owing to the appreciable amounts of glucose and salts present in the medium. The bands were cut from the strips and eluted with water. The eluates, now largely separated from glucose and salts, were evaporated to small bulk under reduced pressure and chromatographed in the four solvents: *A*, *n*-butanol + acetic acid + water (4 + 1 + 5 by vol.); *B*, *n*-butanol + ethanol + acetic acid + water (10 + 10 + 2 + 5 by vol.); *C*, pyridine + methanol + water (1 + 20 + 5 by vol.); *D*, phenol + water (4 + 1 by vol.). When the strips were scanned for radioactivity they all revealed bands corresponding to unchanged *S*-ethyl[$^{35}$S]cysteine. In addition, in solvents *A* and *B*, there was a further major band and one minor band, both with higher $R_F$ values than *S*-ethylcysteine, indicating the presence of at least one new labelled compound in the medium. Chromatograms run in solvent *C* also showed a second major band running faster than *S*-ethylcysteine and a minor band of lower $R_F$ value. In solvent *D* no resolution occurred and only one band was obtained. Owing to shortage of material the second major labelled component of the medium has not so far been further examined.

## DISCUSSION

The ability of yeasts to accumulate amino acids has been described by a number of workers. The process requires an energy source such as the fermentation of glucose (Taylor, 1949; Davies, Folkes, Gale & Bigger, 1953; Halvorson & Cohen, 1958; Eddy & Indge, 1961), and aerobic conditions (Massin & Lindenberg, 1958). It is temperature dependent and is blocked by such metabolic inhibitors as azide and 2,4-dinitrophenol (Halvorson, Fry & Schwemmin, 1955; Halvorson & Cohen, 1958). In several studies amino acid accumulation has been shown to be inhibited by the presence of other amino acids (Taylor, 1949; Halvorson & Cohen, 1958; Massin & Lindenberg, 1958). The amino acids so far studied include arginine, glutamic acid, glycine, lysine, phenylalanine, tyrosine and valine, but no information appears to be available for the sulphur-containing amino acids. In the present work, experiments with a brewer's yeast and six $^{35}$S-labelled sulphur amino acids showed marked differences in the way these compounds were taken up. L-Methionine was accumulated more rapidly than any of the other compounds, and entry into the yeast continued until this amino acid was exhausted from the medium. In contrast, there was no detectable accumulation of L-cystine, and L-cysteine entered the yeast only very slowly unless present in the medium in high concentration. This would explain why cysteine and cystine are poor sources of sulphur for the growth of this yeast.

The uptake curves for *S*-methyl-L-cysteine and its analogue, *S*-ethyl-L-cysteine, showed that these compounds were rapidly accumulated by the yeast for a short period of time, after which a large fraction of the sulphur taken up was released back into the medium. Preliminary experiments with *S*-ethylcysteine indicated the presence of at least one compound distinct from *S*-ethylcysteine which contributed

to the released sulphur; the identity of this metabolite has not yet been established. The data on DL-ethionine illustrate a further mode of uptake. The yeast was able to remove only part of the amino acid from the medium, but the sulphur of this was retained by the organisms, in contrast to that of *S*-methylcysteine and *S*-ethylcysteine.

The accumulation of the sulphur amino acids was temperature dependent and sensitive to the presence of metabolic inhibitors. An appreciable amount of the sulphur of methionine, cysteine and ethionine which entered the yeast became associated with the protein fraction. In 2 hr. as much as 40% of the cysteine-sulphur appeared in this form; with methionine, 17% of its sulphur was incorporated within 20 min.

The effects of other sulphur compounds and amino acids on the accumulation of the sulphur amino acids under study suggest that the transport mechanisms involved are fairly specific, since in every case only compounds which were closely related in structure to a given sulphur amino acid produced any change in uptake, and then only when present in considerably greater concentration. Thus ethionine, methionine sulphoxide and methionine sulphone depressed methionine uptake appreciably, in contrast to such related compounds as *S*-methylcysteine and $\alpha$-aminobutyric acid, as well as a variety of other $\alpha$-amino acids. Again, methionine was the only effective inhibitor of ethionine uptake, and *S*-methylcysteine and *S*-ethylcysteine were likewise mutually inhibitory. These very restricted competitive effects may be compared with the conclusions of other workers (Taylor, 1949; Halvorson & Cohen, 1958; Massin & Lindenberg, 1958) who found that the amino acid-uptake mechanisms operative in the yeasts which they used were decidedly less specific.

The ability of reduced glutathione to enhance cysteine uptake seems unlikely to be simply a protection of the cysteine-thiol group against oxidation in the medium, since it was not produced by homocysteine. Furthermore, it appears to be a specific effect since reduced glutathione had no influence on the uptake of methionine, ethionine or *S*-methylcysteine. The inhibition of methionine accumulation by ethionine and methionine sulphone and of ethionine accumulation by methionine were primarily effects on the appearance of the labelled amino acids in the non-protein-sulphur fraction of the yeast, that is, they occurred at the stage of transport of the labelled amino acids into the cell. In addition, methionine was able to suppress almost completely the small incorporation of ethionine into protein. The increase in uptake of cysteine produced by glutathione was also primarily an effect on the transport process. This was associated with a significant increase in incorporation into protein, which might have been due to the increased availability of cysteine within the cell.

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# Stearic acid, an Essential Growth Factor for *Trypanosoma cruzi*

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SUMMARY

The function of serum in the composition of culture media for trypanosomes was investigated with *Trypanosoma cruzi*. Serum supplies stearic acid, essential for the growth of this trypanosome. A liquid heat-sterilizable medium, composed of peptone and known chemicals is described. The growth of *T. cruzi* in this medium reached $70 \times 10^6$ trypanosomes/ml.

## INTRODUCTION

Many aspects of the problem of the cultivation of trypanosomes remain unsolved. The trypanosomal stage, present in the blood, cannot be grown *in vitro*; the forms which multiply in culture are always these which develop in the invertebrate vectors. Each species has its own growth requirements and a successful defined medium has not yet been described; the presence of serum or whole blood is usually essential. The less exacting trypanosomes are parasites of cold-blooded vertebrates. Boné & Steinert (1956) described a heat-sterilizable medium in which *Trypanosoma mega*, parasite of *Bufo regularis*, multiplied freely. This medium, whose main constituents are a peptone and a liver extract, is limpid and stable and has been used in further investigations. This tryptose liver medium does not support the growth of trypanosome parasites of mammals, even of those which are the easiest to grow *in vitro*, namely the trypanosomes of the group *lewisi* and *cruzi*. In the present work, we have tried to improve the culture methods for *Trypanosoma cruzi* in order to obtain a limpid and stable medium and to identify some of the growth factors required. The media generally used for the cultivation of *T. cruzi* are biphasic: a liquid phase overlays a solid base; they all contain whole blood or egg (Novy & McNeal, 1904; Senekjie, 1943; Chang, 1947). Though excellent for stock cultures, these media are not convenient for experimentation because of their complexity and their heterogeneity due to protein precipitates and agar aggregates. By using a cellophan bag technique, Tobie & Rees (1948) showed that *T. cruzi* could grow in the liquid phase of a diphasic medium. A liquid medium was described by Warren (1960), but its complex composition (brain heart infusion and filtrate of coagulated whole blood) makes identification of the essential factors difficult. Partially defined media were used by Sampath & Little (1949) and Little & Oleson (1951), but agar and erythrocytes were still added. Citri & Grossowicz (1955) reported their results with a partially defined medium in which the only complex component was a casein hydrolysate. We tested this medium, but did not obtain

satisfactory growth. In the present work, starting from the medium described by Boné & Steinert (1956) for *T. mega*, we have found that the addition of calf serum or egg yolk is necessary for the growth of *T. cruzi*; the active principle has been identified as stearic acid.

## METHODS

*Organisms.* Our strain of *Trypanosoma cruzi* was obtained 5 years ago from the Institut de Médecine Tropicale, Antwerp, Belgium. It was maintained simultaneously in our tryptose liver serum medium with weekly subculture, and in the medium described by Chang (1947) with fortnightly subculture.

*Culture media.* Several media were used in this work. The tryptose liver medium of Boné & Steinert (1956) was used as the basal medium to be supplemented by various nutrients. Its composition was (g./l.): Bacto-Tryptose (Difco), 15; dehydrated liver infusion (Oxoid), 1; glucose, 2; haemin, 0·02; NaCl, 4; $Na_3PO_4.12H_2O$, 5; KCl, 0·4; dissolved in twice-distilled water. Tryptose liver serum medium was obtained by adding 1 vol. of partially deproteinized calf serum (prepared as described below) to 2 vol. of double-strength tryptose liver medium + 1 vol. twice-distilled water. The deproteinized calf serum was prepared by diluting calf serum with an equal volume of 0·1 M-$NaH_2PO_4$ adjusting to pH 5·6 and autoclaving for 30 min. at 115°. The protein precipitate was removed by centrifugation and the solution adjusted to pH 7·6. When stored in the frozen state, this solution keeps its activity.

Medium T 1 was the same as the tryptose liver medium with the exception that the liver infusion was replaced by: thiamine 1 mg./l. and folic acid 3 mg./l.

Medium T 2 was like medium T 1 but with addition of stearic acid. It therefore contained: Bacto-Tryptose, 15 g.; glucose, 2 g.; thiamine, 1 mg.; folic acid, 3 mg.; haemin, 20 mg.; sodium stearate, 25 mg.; NaCl, 4 g.; $Na_3PO_4.12H_2O$, 5 g.; KCl, 0·4 g.; twice-distilled water, 1·000 ml. Sodium stearate was added as a colloidal solution prepared for each batch of medium: 20 mg. stearic acid + 98·3 ml. distilled water + 1·7 ml. 0·1 N-NaOH were heated at 70° and stirred during 30 min.

The tryptose liver medium or T 1 medium were used in experiments for growth factor identification. They were supplemented with solutions of selected fatty acids dissolved in ethanol, 0·05 ml. volumes being added to each tube.

All the media were adjusted to pH 7·6, dispensed in cotton-wool-plugged vessels and sterilized by autoclaving at 115° for 10 min.

*Chemicals.* With the exception of arachidic and behenic acids which came from Fluka A.G., Buchs, Switzerland, all the fatty acids came from the Mann Research Laboratories, New York. The Tween 60 was supplied by the Atlas Powder Co, Wilmington, Delaware, U.S.A. and the butyl stearate by Schuchardt, Munich; the latter product was twice distilled under reduced pressure before use. Egg yolk, thiamine and folic acid were obtained from the General Biochemicals, Chagrin Falls, Ohio, U.S.A. Purified phospholipids were kindly given by Miss Faure, Institut Pasteur, Paris. All other reagents were of A.R. grade.

*Growth experiments.* Stock cultures were maintained in 200 ml. Erlenmeyer flasks containing 25 ml. medium. The experimental cultures were made in 15 × 125 mm. test tubes containing 4 ml. medium. Each tube was inoculated with 0·2 ml. of a 5 to 8 day culture containing 50–70 × $10^6$ flagellates/ml. When testing

the activity of sodium stearate, its concentration in the inoculum was decreased to a minimum by one transfer into a medium containing only 5 $\mu$g. sodium stearate/ml. After 5–8 days of incubation the trypanosomes were centrifuged down and resuspended in the required volume of T 1 medium. Cultures were maintained at 27° on a roller tube apparatus. When the medium was clear, the growth could be measured by optical density readings at 680 m$\mu$ with a Hilger Biochem spectrophotometer. Counts in a haemocytometer were made in the case of cloudy media and for the determination of growth curves on which each point recorded represented the mean value obtained by counting four cultures.

## RESULTS

The tryptose liver medium described by Boné & Steinert (1956) for *Trypanosoma mega* did not support the growth of *T. cruzi*, but it was used as a convenient basal medium for further experiments. With the addition to this medium of 2% (v/v) fresh calf serum, a rich growth was obtained. The active factor in the serum was heat stable: when the serum, diluted with an equal volume of buffer to pH 5·6, was heated for 30 min. at 115°, most of the protein precipitated and the activity remained in the slightly opalescent supernatant fluid. This deproteinized fraction of serum was used to supplement the tryptose liver medium.

The new medium, called tryptose liver serum, presented obvious advantages: it was clear, stable and heat sterilizable. It has been used for stock and experimental cultures. Our strain of *Trypanosoma cruzi* has been maintained in it during 150 transfers over a period of 3 years. The flagellates were subcultured every 8 days, at the end of exponential growth; they were still motile in 6-week-old cultures. The growth curve obtained with this medium is shown in Fig. 1: the maximum yield, reached after 11 days' incubation, varied from 35 and $45 \times 10^6$ organisms/ml. The tryptose liver serum medium contained three complex components namely, from muscle, liver and serum. We next tried to separate and identify the essential factors in each extract and to replace them by defined chemicals.

*Replacement of serum.* The isolation of the active principle present in serum was difficult because of its low concentration. We looked for a richer source of this factor and found it in egg yolk. By extracting lyophilized egg yolk with cold solvents, it was found that the active principle was very slightly soluble in water, soluble in methanol and insoluble in acetone. These solubility characteristics are those of lecithin, a component of egg yolk. Egg-yolk lecithin was tested and supported the growth of *Trypanosoma cruzi* when added to the tryptose liver medium at 500 $\mu$g./ml. Twenty-seven subcultures were made in this medium without any change in the growth rate of the flagellates. Other phospholipids were tested; a cardiolipid and phosphatidic acid were without action, while phosphatidyl inositol in the same concentration range showed the same activity as the egg-yolk lecithin.

As rather large amounts of lecithin or phosphatidyl inositol were required, we supposed that the activity was not due to the phospholipids as such, but to a common constituent of their molecules. Glycerol, choline and inositol had no action, but the fatty acids isolated by hydrolysing lecithin by the method of Hanahan, Turner & Jayko (1951) replaced the lecithin completely. From the composition of egg-yolk lecithin, we thought that stearic acid might be responsible

for the observed activity. We tested a series of saturated and unsaturated fatty acids (from $C_{12}$ to $C_{22}$): linoleic and arachidic acid were without action; lauric, myristic, palmitic, palmitoleic, linolenic, oleic and arachidonic acid had an adverse effect. Two acids only, stearic ($C_{18}$) and to a lesser extent behenic acid ($C_{22}$) promoted growth. When the tryptose liver medium was supplemented with 25 $\mu$g. stearic acid/ml., $70 \times 10^6$ organisms/ml. were counted, while only $40 \times 10^6$ organisms/ml. were obtained with 100 $\mu$g. behenic acid/ml.

The poor solubility of stearic acid made the preparation of media rather difficult; we looked thus for water soluble derivatives. The butyl ester was toxic but the results obtained with the sodium salt and with Tween 60 were very satisfactory (Fig. 1). The richest growth being obtained with sodium stearate, all further experiments were made with this salt.

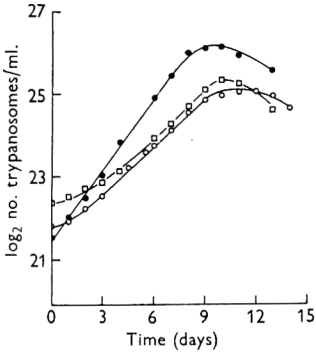


Fig. 1

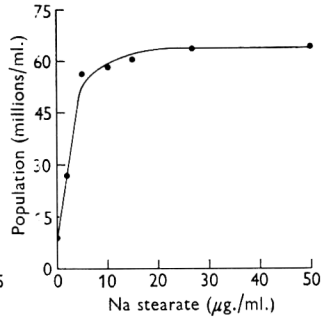


Fig. 2

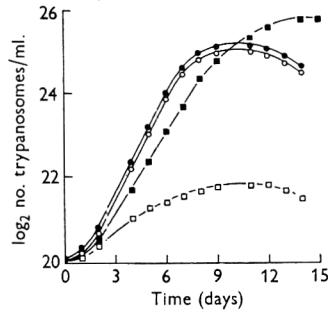


Fig. 3

Fig. 1. Growth of *Trypanosoma cruzi* in tryptose liver medium differently supplemented: ○, With serum; □, with 60 $\mu$g. Tween 60/ml.; ●, with 25 $\mu$g. sodium stearate/ml.

Fig. 2. Dose response of *Trypanosoma cruzi* to sodium stearate. Cultures were incubated until a stable population was reached.

Fig. 3. Influence of carbon dioxide on the growth of *Trypanosoma cruzi* without stearate (T 1 medium): in air, □; in air + $CO_2$ 5 % (v/v), ○. With stearate (T 2 medium): in air, ■; in air + $CO_2$ 5 % (v/v), ●.

To determine the relation between stearate concentration and growth, sodium stearate in aqueous solution was added to the T 1 medium (see below). In this medium, the liver extract, likely to contain fatty acids, had been replaced by thiamine + folic acid. Tryptose, a tryptic digest of muscle, was the only complex component still present. A maximum growth was obtained around 20 $\mu$g. stearate/ml. (Fig. 2) and a concentration of 25 $\mu$g. stearate/ml. was adopted in media for stock cultures.

*Replacement of liver extract.* Having found that serum could be replaced by sodium stearate, we examined the active factors present in the liver extract. We tested the activity of many substances, including those already identified as nutrients or growth factors for *Trypanosoma cruzi* (Senekjie, 1943; Sampath & Little, 1949; Little & Oleson, 1951; Citri & Grossowicz, 1955). When tryptose was the only undefined constituent of the medium, it appeared that two factors only needed to be supplied by the liver extract, namely, thiamine and folic acid. The optimum

concentration for thiamine was 1 $\mu$g./ml. and for folic acid 3 $\mu$g./ml. This new medium (T 2 medium) has supported the growth of *Trypanosoma cruzi* through 75 subcultures over a period of 18 months. An average population of $50–70 \times 10^6$ trypanosomes/ml. was reached at the end of the growth phase.

*Influence of carbon dioxide.* Carbon dioxide accelerated only slightly the growth of *Trypanosoma cruzi* in the T 2 medium, but it had a significant growth-promoting activity in media deprived of stearate. When the culture vessels were plugged with cottonwool with access to air, *T. cruzi* grew only poorly in the absence of stearate. When the cotton-wool-plugged tubes were kept in a rotating jar filled with air + 5% (v/v) $CO_2$ the growth became very abundant, comparable to the multiplication of *T. cruzi* in an optimum medium containing stearate (Fig. 3).

## DISCUSSION

Serum is a common constituent in culture media for trypanosomes. Its function has been ascribed either to some chelating or detoxifying effect or to the supply of essential nutritional factors. Our experiments with *Trypanosoma cruzi* support this last hypothesis: the serum supplies this trypanosome with an essential nutrient, stearic acid. A requirement for long-chain saturated fatty acids is unusual. It has, however, been already described for Paramecium, requiring stearic acid (Miller & Johnson, 1960) and for Trichomonas, which needs oleic + stearic acids (Shorb & Lund, 1959). The requirement for stearic acid, observed with *T. cruzi*, may prove to be common to many trypanosome parasites of mammals but conclusive evidence will be difficult to obtain since most species need additional growth factors present in erythrocytes. Some trypanosomes, however, are probably able to synthesize stearic acid: *T. mega*, parasite of an amphibian, does not need stearic acid as a nutrient (Boné & Steinert, 1956). Of the fatty acids tested, whether saturated or unsaturated from $C_{12}$ to $C_{22}$, only stearic acid and to a lesser extent behenic acid induced the growth of *T. cruzi*. The observed activity of the $C_{18}$ and $C_{22}$ saturated acids as compared with the absence of growth-promoting action by arachidic acid ($C_{20}$), seems hard to explain. We suspected an impure sample of behenic acid containing some stearic acid, but a paper chromatograph of our sample of behenic acid did not show stearic acid; amounts of stearic acid sufficient to give the amount of growth observed would have been detected. The lower final yield obtained with Tween 60 can be explained by the fact that it contains, in addition to stearic acid, appreciable amounts of palmitic, oleic and myristic acid (Shorb & Lund, 1959); these last three fatty acids, separately, are toxic for *T. cruzi*.

The role of stearic acid in the growth of *Trypanosoma cruzi* is unknown. The growth stimulating action of carbon dioxide has probably a bearing on this problem. With the two species of trypanosomes examined, this action seems to be linked to a requirement for stearic acid. Carbon dioxide was only stimulatory for *T. cruzi* in a medium lacking stearic acid but had no influence when this was supplied. With *T. mega*, which does not require stearic acid as a nutrient, carbon dioxide was also without effect. These observations accord with the present knowledge of the biosynthesis of fatty acids: the first step being the carboxylation of acetyl-CoA with carbon dioxide to give malonyl-CoA (Wakil, 1962).

Our T 2 medium is an improvement on the usual culture media for *Trypanosoma*

*cruzi*. All its constituents are commercial products and the tryptose is the only one of unknown composition. The medium can be autoclaved, it is limpid and allows growth determination by turbidimetry. The yield, $70 \times 10^6$ trypanosomes/ml., compares favourably with that obtained with the more complex culture media commonly used.

The initial stages of this work were undertaken in the Microbiology Unit, Department of Biochemistry, Oxford University. One of us (G.B.) is indebted to Professor D. D. Woods, F.R.S., for his hospitality, encouragement and helpful advice. The authors express their gratitude to the Institut belge pour l'encouragement de la Recherche Scientifique Outre-Mer (IBERSOM) for the substantial grant they both received.

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# On the Structural Transformations and Lysis of *Halobacterium salinarium* in Hypotonic and Isotonic Solution s

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SUMMARY

When the NaCl concentration of a suspension of *Halobacterium salinarium* is gradually lowered by adding water, the rod-shaped organisms are converted to spheres which lyse. The organisms do not change in size when transformed from rods to spheres. Chemicals which do not possess a strong net charge in aqueous solutions do not protect the structure of the organism when they replace NaCl in iso-osmolar concentions. The rod shape of the organisms is only maintained by very high concentrations of ions which interact weakly with common proteins; lithium and ammonium ions are exceptions to this rule. Ions which display strong interactions with common proteins, and chemicals which are believed to break secondary bonds between protein molecules, effect a transformation of rods to spheres, and frequently a lysis of the spheres, when brought in contact with organisms suspended in strong NaCl solution. The changes in structure in hypotonic and isotonic solutions are not affected by metabolic inhibitors. Isolated cell-wall fragments disintegrate into smaller units when exposed to conditions under which whole organisms lyse. It is concluded that the structural transformations and lysis of *H. salinarium* in hypotonic solutions are not caused by the action of enzymes and that osmotic phenomena play no, or only a minor, role. The observations support the contention that the globular lipoprotein particles, which constitute the bulk of the material of the cell wall of these bacteria, are bound together mainly by electrostatic forces and secondary bonds. When the cells are exposed to hypotonic solutions, or to ions which bind strongly to proteins, or to chemicals which are believed to break secondary bonds between protein molecules, the linkages binding the lipoprotein particles together are weakened so that the wall structure disintegrates. Only in the presence of high concentrations of sodium and chloride ions, or other ions which bind loosely to proteins, is it possible for the proteinaceous particles of the cell wall to associate in an orderly array.

## INTRODUCTION

Bacteria belonging to the genus *Halobacterium* Elazari-Volcani (*Bergey's Manual*, 1957) require at least 2·5 to 3 M-NaCl for growth; most species grow best in a medium containing 4 to 5 M-NaCl. The NaCl requirement for growth is specific; attempts to substitute other chemicals for NaCl have been unsuccessful. When cultivated in a medium giving good growth, the organisms normally are slender uniform rods of varying length. When the salt concentration of a suspension containing normal

rod-shaped organisms is gradually lowered by adding water, the organisms change their shape. At concentrations in the range 3 to 3·5M, most of the rods appear somewhat irregular; at concentrations in the range 2·5 to 3M, bulgy and apiculate structures are formed; at concentrations in the range 1·5 to 2·0M, most organisms appear as spheres. When the salt concentration is further lowered, the individual spheres suddenly lyse. Lysis normally occurs in the salt concentration range 1·0 to 1·5M (Klebahn, 1919; Petter, 1932; Christian, 1956; Abram & Gibbons, 1960). Evidence has been presented that the intracellular salt concentration in Halobacterium is very high, possibly as high as in the medium in which the organisms are grown (Christian, 1956; Christian & Ingram, 1959). Therefore, it seemed reasonable to ascribe the deformation and lysis of the organisms in hypotonic solutions to an osmotic effect. When the saline environment was diluted with water, an excess osmotic pressure would build up inside the organisms, resulting in the formation of spherical structures from the rod-shaped forms, and at higher dilutions in a disruption of the cell envelope (Christian, 1956; Ingram, 1957). Abram & Gibbons (1960, 1961) presented data which indicate that the deformation and lysis of Halobacterium in hypotonic solutions are not simply due to an osmotic effect. They suggest that Halobacterium requires a high sodium chloride content in its environment to maintain a rigid cell-wall structure. The present work was undertaken to examine these phenomena.

## METHODS

*Organism. Halobacterium salinarium* strain 1 was isolated in our laboratory from salt fish, and maintained on a yeast-autolysate tryptone salt medium (described below) to which 2% (w/v) agar (Difco) was added.

*Growth in liquid cultures.* The organisms were usually grown in 500 ml. flasks containing 100 ml. medium of the following composition (%, w/v): 25, NaCl; 0·5, $MgSO_4.7H_2O$; 0·02, $CaCl_2.6H_2O$; 0·25, tryptone (Oxoid); 5, yeast autolysate; in tap water; pH 7·0. The yeast autolysate was made by incubating a suspension of 500 g. baker's yeast in 500 ml. tap water at ≤5° for 24 hr. The suspension was then heated to boiling, adjusted to pH 7·0 with NaOH, and filtered. Tryptone and yeast autolysate were autoclaved separately from the salt components. The flasks were inoculated with 1·4 ml. of a 48 hr. culture and incubated at 37°on a reciprocating shaker (84 oscillations/min. of excursion 5 cm.).

For the study on cell walls, organisms were grown in a 25 l. carboy containing 10 l. medium of the composition described above. The inoculum (300 ml.) consisted of organisms grown for 48 hr. on the shaker. The culture was incubated with aeration (20 l./min.) at 37°; 4·0 ml. of autoclaved sperm whale oil were added to prevent foaming.

*Chemicals.* Guanidine nitrate was synthesized as described in *Organic Syntheses* (1947). The other chemicals were obtained commercially. Commercial monobromoacetic acid was distilled 3 times; commercial monochloroacetic acid and dichloroacetic acid were distilled twice. Commercial monoiodoacetic acid was recrystallized 5 times from light petroleum.

*Studies on whole organisms*

*Preparation of suspensions of organisms.* Organisms were harvested after 48 hr. (i.e. close to the end of the exponential growth phase) by centrifugation at 10,000 *g* for 5 min. in the cold. The pellets were washed once in the centrifuge with 4·3 M-NaCl, then resuspended in 4·3 M-NaCl to give a reading of 0·36 in an EEL Portable Colorimeter (Evans Electroselenium Ltd., Halstead, Essex) with 5 ml. standard cuvettes and filter no. 608. Such suspensions will be referred to as 'standard suspensions'. Some difficulty was encountered in suspending the fragile organisms without damage. The following procedure, by which only a small fraction of the organisms was structurally changed, was finally adopted: 5 ml. 4·3 M-NaCl was added to the pellet and the mixture homogenized by being carefully sucked into and blown out from a pipette 3–5 times.

*Exposure of organisms to hypotonic solutions.* Samples (0·1 ml.) of a standard suspension of organisms was pipetted at room temperature into a series of test tubes, each containing 0·9 ml. of a NaCl solution ranging in concentration from 0 to 4·3M.

*Substitution of salts and non-electrolytes for NaCl.* The main purpose of these experiments was to obtain information on the effect of various chemicals, substituted in iso-osmolar concentrations for NaCl, upon the shape of the organisms. Because of lack of data in the literature on the dissociation and osmotic pressure of strong salt solutions, osmolarity of the salts in solution was computed from the simple formula $(m+n)$M, in which $m$ and $n$ designate the valencies of the cations and the anions, respectively, and M the molar concentration. The values for osmolarity thus obtained are approximations only, but are considered sufficiently accurate for the present work.

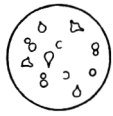
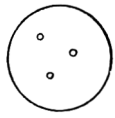
Samples (0·1 ml.) of standard suspension were pipetted at room temperature into a series of test tubes, each containing 0·9 ml. of a mixture of 8·6 osmolar NaCl and 8·6 osmolar solution of test substance in a proportion which varied from tube to tube. The solutions of test substances had previously been adjusted to pH 7·0 with NaOH or HCl; exceptions were the solutions containing $ZnCl_2$, $CdCl_2$ and $MnCl_2$ which were adjusted to pH 6·0–6·5, i.e. the limit at which insoluble hydroxides were formed.

*Experiments with metabolic inhibitors.* These experiments were designed to test the effect of metabolic inhibitors on the structural transformation patterns which were displayed by *Halobacterium salinarium* when the saline environment was diluted with water, or when other chemicals were substituted for NaCl in iso-osmolar concentrations. To a standard suspension was added metabolic inhibitor in concentration as listed on page 276. After incubation for 20 min. at room temperature 0·1 ml. of the suspension was pipetted into a series of test tubes, each containing 0·9 ml. of a hypotonic or an isotonic salt solution to which had been added inhibitor in the same concentration as in the standard suspension of organisms.

*Evaluation of structural changes.* A problem was to find a rational way of expressing the degree of structural deformation of the organisms when exposed to unfavourable conditions. In the past, measurements of turbidity or optical density have been used to register lysis (Christian, 1956). For the present work, this method was not satisfactory. We observed, as did Abram & Gibbons (1961), that when lysis of Halobacterium was brought about by changes in the concentration of certain salts,

there is no clear correlation between lysis and decrease in optical density. This might, in part, be due to the changes in refractive index of the suspending medium. Furthermore, measurements of optical density did not give satisfactory characterization of the structural changes taking place before lysis.

Table 1. *Numerical expression of different stages in the structural transformation of cells of* Halobacterium salinarium

| Structure number | Appearance of cells | Description |
|---|---|---|
| 1 |  | Mostly uniform rods, some slightly irregular rods |
| 2 |  | Mostly slightly irregular rods, some bulgy and apiculate forms |
| 3 |  | Mostly bulgy and apiculate forms, some spheres |
| 4 |  | Mostly spheres, some bulgy and apiculate forms |
| 5 |  | Spheres lysing rapidly |

In most of our experiments, the structural deformations were elicited by exposing non-proliferating organisms to chemicals in high concentrations. During the work, it became clear that when the organisms were deformed as a result of such exposures, the structural changes closely followed the pattern described by earlier workers for Halobacterium exposed to a gradual decrease of the salt concentration in the environment. The uniform rods were transformed, through transition forms, into spheres, and the spheres frequently lysed. The degree of structural alteration depended upon the severity, rather than the time, of exposure, e.g. the concentration of a given chemical. When exposed to such unfavourable conditions, the organisms would take on a new shape in less than 30 sec. Transition forms between rods and spheres were stable for several hours. Spheres appeared quite stable under some conditions; under other conditions they lysed more or less rapidly. The two different patterns of conversion from rods to spheres reported by Abram & Gibbons (1961) were not observed in our work. In view of the above considerations, it appeared most useful to express the degree of structural deformation by assigning a number to a certain structural state of the organisms (Table 1). In separate tests, it was shown that the degree of structural alteration resulting from a certain treatment

was reproduced with sufficient accuracy. In all experiments, the structural state of the organisms was observed in a phase contrast microscope 0·5–2 min. after exposure. The 'structure numbers' recorded were used in graphical representations of the results by plotting the numbers along an arbitrary scale.

### *Studies on cell-wall fragments*

*Isolation of cell-wall fragments.* When the culture in a 25 l. carboy was full-grown (70 hr.), the organisms were harvested with a Sharples centrifuge at room temperature. The following manipulations were then carried out at 0°. The organisms were washed carefully twice on the centrifuge with a standard salt solution containing (%, w/v): 25, NaCl; 0·5, $MgSO_4.7H_2O$; 0·02, $CaCl_2.6H_2O$; pH 7·0, and the sediment (17 g. wet weight) was suspended in 30 ml. of the standard salt solution. 50 ml. of glass beads (Ballotini, no. 12) were added and the suspension was treated for 5 min. in a MSE Homogenizer run at maximum speed. The glass beads were separated from the suspension by passage through a coarse sintered glass filter (no. 1). The filtrate was subjected to fractional centrifugation. As demonstrated by electron microscopy, cell-wall fragments were sedimented at 7,000–15,000 *g* (30 min.). The sediment was washed 15 times with 10 ml. of the standard salt solution by resuspension and centrifugation at 30,000 *g* for 40 min. Purity of the wall preparation was assessed by electron microscope examination of samples. Repeated washing was necessary to obtain a preparation of cell-wall fragments of satisfactory purity. The cell-wall fragments were finally suspended in 0·5 ml. of the standard salt solution and stored at 0°.

*Experiments with cell-wall fragments.* These experiments were designed to test the effect upon cell-wall fragments of substituting other chemicals for NaCl in isoosmolar concentrations, and of diluting the saline environment with water. In experiments of the first type, 0·01 ml. samples of the suspension of cell-wall fragments were pipetted into a series of test tubes, each containing 0·09 ml. of an 8·6 osmolar solution prepared by mixing the standard salt solution and an 8·6 osmolar solution of the chemical to be tested. The solutions of test chemicals had previously been adjusted to pH 7·0. Since the standard salt solution was 8·6 osmolar with respect to NaCl, the osmolar concentration of the sum of chemicals was near 8·6 in all the mixtures. In experiments on the effect of diluting the saline environment, 0·01 ml. of the suspension of cell-wall fragments was pipetted into a test tube containing 0·09 ml. water. The resulting suspensions were homogenized by being carefully sucked into and blown out from a Pasteur pipette 3 to 5 times. The experiments were made at room temperature. Samples were removed for examination of the cell-wall fragments in the electron microscope.

*Electron microscopy.* Carbon replicas of the cell-wall fragments were made by the methods of Dalitz (1953) and Roelofsen, Dalitz & Winjman (1953) for wall constituents of plants. The preparations were shadowed with platinum at 27° before being covered with a film of carbon. As shown in the present work, cell walls of Halobacterium dissolve when brought in contact with pure water. It was therefor unnecessary to use chromic acid for removal of the organic material which adhered to the carbon film; keeping the carbon film in distilled water for 2 hr. sufficed. The carbon replicas were examined in a Siemens Elmiskop I electron

microscope at accelerating voltage 80 kV. The salt contained in the suspensions of cell-wall fragments formed numerous crystals in the preparation of specimens for examination in the electron microscope. Fields which showed replicas of these crystals were avoided when photographing the specimens.

## RESULTS

### *Structural changes brought about by dilution of the saline environment with water*

The structural changes taking place when normal rod-shaped *Halobacterium salinarium* organisms were exposed to environments of progressively smaller salt content are shown in Fig. 1. The spheres which were formed at concentrations below 5 osmolar NaCl were surprisingly small as compared with the rods from which they originated. The average sizes of spheres and rods were computed on the basis of measurements of many individuals of each type. The results (Table 2) show that the average sphere had a volume about half of that of the average rod. In further experiments, the structural transformations of individual organisms were observed. Such observations could easily be made on a slide preparation of bacteria in 8·6 osmolar NaCl by adding a small drop of water to the edge of the cover slip. It appeared that a short rod normally gave rise to one sphere. Longer rods, however, normally gave rise to 2–4 spheres, depending upon the length of the rod. From observations on many organisms it was concluded that an average of about two spheres was formed from each rod. Within the limits of our simple technique, this figure seemed to be independent of the rate at which the rods were converted to spheres. Considering the average size of the spheres in relation to the average size of the rods (Table 2), it thus appears that the total volume of the spheres was close to the total volume of the rods from which the spheres were formed.

Table 2. *Comparison of size of normal, rod-shaped cells of* Halobacterium salinarium *and spheres formed from the rods by dilution of the saline environment*

The rod-shaped organisms were suspended in 8·6 osmolar NaCl. The spheres were produced by diluting the suspension with water to 4·0 osmolar NaCl. The figures are arithmetic means of measurements on 40 individuals of each type.

| | Rods | Spheres |
|---|---|---|
| Length | 4·3 $\mu$ | — |
| Width | 0·7 $\mu$ | 1·2 $\mu$ |
| Volume | 1·7 $\mu^3$ | 0·9 $\mu^3$ |

### *Structural changes by substitution of various chemicals for $Na^+$ and $Cl^-$*

*Replacement of $Cl^-$ by other anions.* The effects of several Na salts upon the shape of *Halobacterium salinarium* are graphically represented in Figs. 1–4. It appeared that the different anions had strikingly different effects. When chloride was replaced by nitrate or sulphate, the rods took on irregular shapes, the irregularity being greater the higher the degree of replacement (Fig. 1). However, these anions protected the organisms to some extent, since in their presence less deformation occurred than when the NaCl solution was simply diluted with water. On the other hand, when chloride was replaced by perchlorate or thiocyanate, a more extensive deformation of the organisms occurred than when the NaCl solution was simply

diluted with water (Fig. 1). The rod-shaped organisms changed to spheres which lysed rapidly at relatively low degrees of replacement, i.e. at NaCl concentrations still so high that no or only a slow lysis would take place if thiocyanate or perchlorate had not been present. Accordingly, it appeared that these anions enhanced the structural transformation.

The effect of fluoride could not be tested because of the low solubility of NaF. Of the other halides (Fig. 2), only chloride protected the rod shape of the organisms. When chloride was replaced by bromide, the organisms were transformed from rods to spheres as the chloride concentration decreased, at about the same chloride concentrations as if the suspension had been diluted with water. Bromide therefore gave no protection of the rod shape. On the other hand, the spheres that were formed in solutions containing bromide did not lyse even at very small chloride

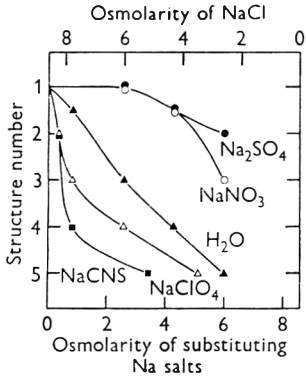



Fig. 1

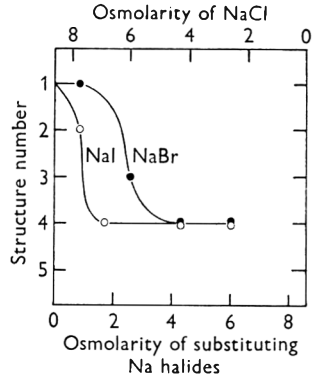



Fig. 2

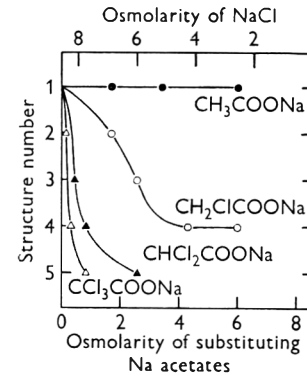



Fig. 3

**Fig. 1.** Structural transformations of *Halobacterium salinarium* by dilution of saline environment with water and by replacement of NaCl by various Na salts.

**Fig. 2.** Structural transformations of *Halobacterium salinarium* by replacement of NaCl by Na halides.

**Fig. 3.** Structural transformations of *Halobacterium salinarium* by substitution of Na chloroacetates for NaCl.

concentrations. Bromide therefore prevented lysis of the spheres. When chloride was replaced by iodide, the rods were transformed to spheres at chloride concentrations which would have protected the rod shape if iodide had not been present. Thus, iodide enhanced the transformation of rods to spheres. The spheres appeared to be relatively stable even at small chloride concentrations, but some lysis occurred upon prolonged incubation. In further experiments, the interesting observation was made that when a comparatively small amount (0·05 mg./ml.) of iodine was present in the iodide solution, the spheres formed would lyse rapidly.

In similar experiments where NaCl was replaced by Na salts of organic acids in iso-osmolar concentrations, the following anions gave good protection of the rod shape: formate, acetate, propionate, lactate, succinate; chlorinated derivatives of acetate, however, did not protect. The structural transformation took place at lower degrees of replacement the higher the number of chlorine atoms in the acetate ion (Fig. 3). The effect of trichloroacetate was especially striking: the organisms changed to spheres which lysed rapidly at comparatively very low degrees of

replacement of chloride by trichloroacetate ion. An analogous picture emerged when the effects of the various monohalogenated acetates were compared. The structural transformations took place at lower degrees of replacement the higher the atomic weight of the halogen (Fig. 4).

*Replacement of $Na^+$ by other cations.* The effect of replacing $Na^+$ by other cations upon the structure of *Halobacterium salinarium* was tested. As in the experiments with anions, strikingly different effects were observed, depending upon the chemical nature of the cation. Some of the results are shown in Fig. 5. The cations could be arranged in a series according to their effect upon the organisms: $Na^+$; $K^+$; $Mg^{2+}$, $Ca^{2+}$; $Mn^{2+}$; $Sr^{2+}$; $Zn^{2+}$; $Li^+$; $NH_4^+$; $Cd^{2+}$. When they replaced $Na^+$, the cations

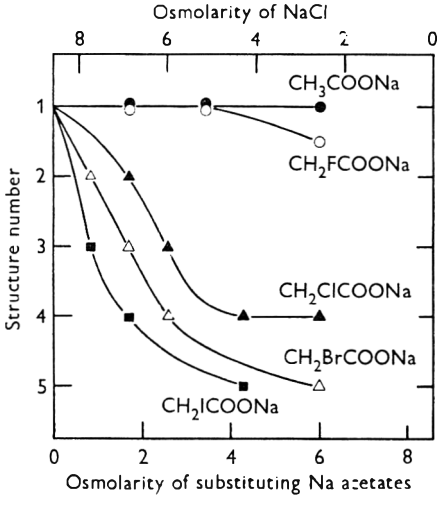



Fig. 4

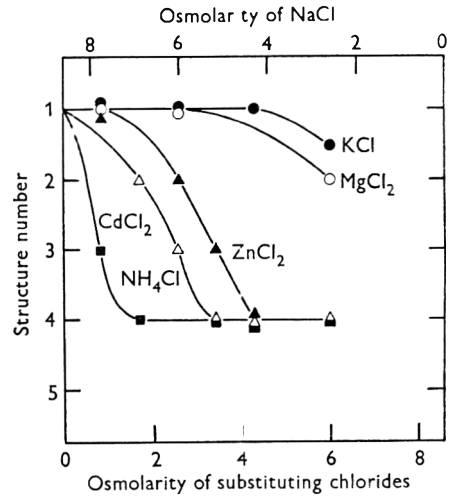



Fig. 5

Fig. 4. Structural transformations of *Halobacterium salinarium* by replacement of NaCl by Na halogenated acetates.

Fig. 5. Structural transformations of *Halobacterium salinarium* by replacement of NaCl by various chlorides.

listed to the left gave a good or fair protection of the rod shape. Decreasing degrees of protection were given by the cations as listed from the left to the right. The cations listed at the right end of the series not only did not protect the rod shape, but appeared to enhance the transformation of rods to spheres. For some of the cations, high degrees of replacement were precluded either by limited solubility of the chloride salts, or by aggregation of the organisms in their presence. In no case was lysis observed. Trimethylamine and guanidine which in solution at pH 7 have a strong positive charge, were tested in similar replacement experiments. Trimethylamine appeared to give a fair protection of the structure of the organisms; guanidine, however, enhanced the structural transformations.

*Replacement of NaCl by some non-electrolytes.* When NaCl was replaced by non-electrolytes, none of a number of compounds tested protected the rod shape of the organisms. Some of the compounds appeared to promote a transformation of rods to spheres; a few also caused lysis of the spheres. Some of the results are shown in Fig. 6. The compounds could be arranged in a series according to their effect:

ethyleneglycol, glycerol; acetamide; formamide; formaldehyde, ethanol, urea. When NaCl was replaced by ethylene glycol or glycerol, the structural transformations took place at about the same NaCl concentrations as when diluting with water. The other chemicals rendered the organisms susceptible to the structural changes, and with increasing intensity as listed from the left to the right in the above series.

*Effect of chemicals on organisms suspended in strong NaCl solutions*

The chemicals used in the replacement experiments reported above were dissolved in 8·6 osmolar NaCl and different amounts were added to organisms suspended in 8·6 osmolar NaCl. The picture which emerged from these experiments was as follows. Those chemicals which showed protection of the rod shape in the replacement experiments did not change the structure of organisms suspended in 8·6 osmolar

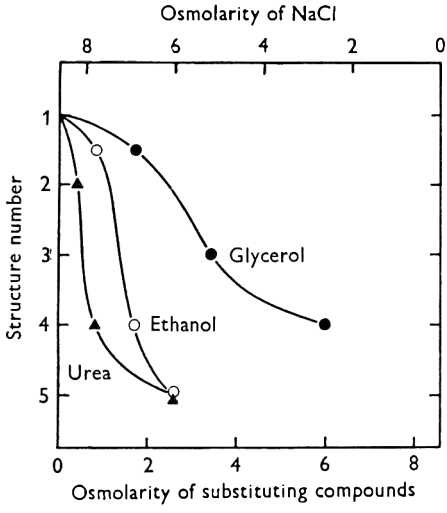



Fig. 6

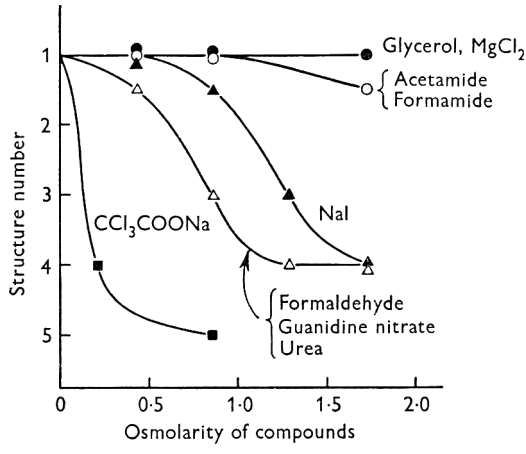



Fig. 7

Fig. 6. Structural transformations of *Halobacterium salinarium* by replacement of NaCl by various non-electrolytes.

Fig. 7. Structural transformations of *Halobacterium salinarium* when suspended in 8·6 osmolar NaCl and exposed to increasing concentrations of various compounds.

NaCl. The same applied to all chemicals which in the replacement experiments gave no protection of the organisms, in the sense that in their presence the organisms changed to spheres which lysed at the same NaCl concentrations as when the NaCl solution was diluted with water. However, all chemicals which enhanced the structural transformations in the replacement experiments did the same to organisms suspended in 8·6 osmolar NaCl, although less drastically, when applied in the same concentrations. Some of the results are given in Fig. 7. The curves should be compared with the corresponding curves in Figs. 2, 3, 5, and 6. It appears that NaCl in high concentrations tended to protect the organisms from chemicals which affected their normal shape.

The non-ionic detergents Tween 20 and Tween 80 only slightly affected the shape of organisms suspended in 8·6 osmolar NaCl when added in concentrations up to 3·5% (w/v). The anionic detergent Na dodecylsulphate is only slightly soluble

in 8·6 osmolar NaCl, and at 0·0035% (w/v) had no immediate effect upon the shape of the organisms. However, after incubation for 24 hr. at room temperature, the rods appeared irregular in shape and the optical density of the suspension had decreased to about one-third of the original value. The cationic detergent Cetavlon (a product of Imperial Chemical Industries Ltd, which consists of a mixture of dodecyl-, tetradecyl- and hexadecyl-trimethyl ammonium bromide) caused rapid lysis of the organisms when added to 0·01% (w/v). In contrast to other chemicals causing lysis, Cetavlon caused the rods to lyse without previous conversion to spheres. These observations with detergents essentially accord with those of Abram & Gibbons (1961) on *Halobacterium cutirubrum*.

*The effect on organisms of suspension in solutions of decreasing solute concentration*

*Halobacterium salinarium* organisms were centrifuged down and the sediments suspended in solutions of several chemicals made up in series of dilutions. The amount of NaCl introduced with the sediment gave a concentration of less than 0·02M, which was in itself far too little to protect the organisms from lysis. The results showed that chemicals which had displayed protection of the rod shape at 8·6 osmolar, displayed less protection the less their concentration. In most cases, the transformation of rods to spheres, and lysis, took place at molar concentrations higher than found for NaCl. In no case did a chemical protect the structure of the organisms at concentrations lower than found for NaCl. Na acetate appeared to be about as effective as NaCl, giving about the same degree of protection at any given molar concentration.

Mager (1959) reported that spermine in minute concentrations prevented lysis of *Achromobacter fischeri* in hypotonic solutions. We found that spermine did not affect the lysis pattern of *Halobacterium salinarium*. It is known, however, that Halobacterium organisms exposed to acidic conditions become 'fixed' so that they will not greatly change their structure upon dilution of the saline environment (Browne, 1922; Dussault, 1955). We found that acetic and hydrochloric acids were effective as fixing agents.

*Effect of metabolic inhibitors*

Most of the experiments reported above on structural transformations of *Halobacterium salinarium* by the replacement of NaCl by various chemicals were repeated in the presence of metabolic inhibitors. General and specific metabolic inhibitors were used, and in concentrations known to stop enzymic activities. The inhibitors used were (M): sodium iodoacetate, 0·1; sodium *p*-chloromercuribenzoate, 0·01; sodium arsenite, 0·01; sodium fluoride, 0·1; sodium fluoroacetate, 0·01; sodium arsenate, 0·01; sodium azide, 0·1; potassium cyanide, 0·01; hydroxylamine, 0·01; ethylurethane, 0·1; formaldehyde, 0·1; copper sulphate, 0·01; mercuric chloride, 0·02. None of the inhibitors effected a change in the structural transformation patterns.

*Effect of penicillin on proliferating organisms*

Na benzylpenicillin inhibited growth of *Halobacterium salinarium* markedly when added at 1000 units/ml. to exponentially growing shake cultures. In concentrations of 3000–4000 units/ml. Na benzylpenicillin effected a transformation of growing organism to spheres which then lysed.

*Effect of temperature*

Abram & Gibbons (1961) reported in detail on the structural changes which took place when suspensions of *Halobacterium cutirubrum* were exposed to elevated temperatures. Our observations on *H. salinarium* conform with their findings: when a suspension of normal organisms in 4·3 M-NaCl was heated to 65°, the rods were converted to spheres; on prolonged incubation at this temperature, the spheres lysed.

*Effect on cell-wall fragments of various chemicals substituted for $Na^+$ and $Cl^-$*

Plate 1, fig. 1, shows an electron micrograph of carbon replicas of cell-wall fragments. The preparation was made directly on material from a suspension of wall fragments in the standard salt solution described under Methods. An hexagonal arrangement of particles in the wall can be seen. A similar pattern was observed by Houwink (1956) in the wall of whole *Halobacterium halobium* organisms, and may possibly be a characteristic feature of all members of the genus. In the many preparations of this kind made, the hexagonal arrangement of particles was observed in every wall fragment examined, indicating that the pattern is expressed on both sides of the wall. The effect upon cell-wall fragments of various chemicals was tested, using concentrations known from the studies reported above to effect lysis of whole organisms. After exposure to 5·2 osmolar NaCl + 3·4 osmolar NaI + 0·05 mg./ml. $I_2$ (Pl. 1, fig. 2), or 6·9 osmolar NaCl + 1·7 osmolar Na trichloroacetate (Pl. 2, fig. 3), comparatively few cell-wall fragments were found in the suspension by electron microscopy and they had completely lost their hexagonal particle pattern. After exposure to 6·0 osmolar NaCl + 2·6 osmolar urea (Pl. 2, fig. 4), no cell-wall fragments could be seen. Under the conditions used, the chemical obviously effected a disintegration of the wall fragments into smaller units which formed a homogeneous layer on the specimens prepared for electron microscopy. For comparison, similar experiments were carried out on cell-wall fragments of a non-halophilic fluorescent pseudomonad. These fragments were suspended in water and exposed to NaI (+iodine), Na trichloroacetate, and urea of the same concentrations as used for the walls of *H. salinarium*. Examination in the electron microscope showed no alteration in the cell-wall structure.

*Effect on cell-wall fragments of dilute saline environments.* The suspension of cell-wall fragments in the standard salt solution was diluted ten times with water to a NaCl concentration of 0·86 osmolar. This concentration had been shown previously to cause lysis of whole organisms. Electron microscopy revealed the complete absence of cell-wall fragments in these diluted preparations. The dilution obviously effected a spontaneous disintegration of the wall fragments into smaller units.

## DISCUSSION

On the basis of studies of the effect upon *Halobacterium cutirubrum* of cations of the alkaline series, of urea, of detergents, and of heat, Abram & Gibbons (1961) proposed that the extreme fragility of this bacterium might be due to the cell wall being loosely held together by hydrogen bonds and electrostatic forces; only in the presence of high concentrations of NaCl are the electrostatic forces so screened that

the bonds hold the cell wall together in the shape of a rod. As far as the experiments can be compared, our results on *H. salinarium* agree very well with those of Abram & Gibbons. Further, we can offer evidence to strengthen their conclusion about the cause of the extreme fragility of Halobacterium cells and the lysis in hypotonic solutions.

If the structural transformations of Halobacterium in hypotonic solutions were due primarily to osmotic effects, one might expect an increase in the volume of the organisms upon dilution of the saline environment with water. Furthermore, one might expect that other chemicals, replacing NaCl in iso-osmolar concentrations, might prevent changes in structure. The experiments showed, however, that no measurable increase in the size of the organisms occurred on dilution of the environment, and that only few of the many chemicals used to replace NaCl protected the rod shape of the organisms. The results thus did not support the osmotic theory, which has prevailed in the literature in the past, to account for the structural transformations and lysis of Halobacterium in hypotonic solutions (Ingram, 1957). Recent experiments on a marine bacterium suggest that its lysis in hypotonic solutions might be the result of enzymic degradation of a cell-wall component (Brown, 1961). It seems highly unlikely that the structural transformations and lysis of *H. salinarium* in hypotonic and isotonic solutions are due to the action of enzymes, since our experiments showed that these transformations were unaffected by metabolic inhibitors.

From the experiments on the effect of ions upon *Halobacterium salinarium* an interesting relationship emerges. It appears that a high concentration of ions which are generally known to be poorly bound to, or which interact weakly with, common proteins protect the rod shape. Ions which are generally known to display strong interactions with proteins effect a transformation of the rods to spheres, and frequently also lysis of the spheres. These ions also effect a structural transformation of the organisms in strong NaCl solutions, but less drastically than when they replace NaCl. The latter observation shows that NaCl can counteract the detrimental effect of these ions to some extent. Ions known to interact moderately with proteins have moderate effects upon the cell structure. These rules hold strictly for all anions tested. In the case of the cations, however, lithium and ammonium ions are exceptions; they did not protect the rod shape of *H. salinarium* although they are known to interact only weakly with common proteins.

Polyalcohols did not maintain the rod shape when replacing NaCl. On the other hand, chemicals of this type did not promote the structural changes since when added to organisms suspended in strong NaCl solution they caused no change in structure, and when stepwise replacing NaCl, the structural transformations at any given NaCl concentration were about equal to those which occurred when the saline environment was simply diluted with water. Other organic chemicals, which are believed to break secondary bonds between protein molecules when used in high concentrations (e.g. urea, guanidine) did effect a structural transformation in strongly saline environments.

Proof that the structural transformations of these *Halobacterium salinarium* organisms were due to interference with the structure of the cell wall is given by the electron microscope studies of isolated cell-wall fragments. These studies showed that conditions which caused a structural transformation of whole organisms and lysis also caused a disintegration of cell-wall fragments. Smithies, Gibbons &

Bayley (1955) reported that cell walls of *H. salinarium* contain no, or only traces of carbohydrate. Analyses carried out in our laboratory (Lie, Tangen & Larsen, unpublished) did not show any simple sugars in hydrolysates; a small amount of amino sugar was detected. The bulk of the wall material is composed of lipid and protein; the amino acid composition, which includes diaminopimelic acid, closely conforms with that found for the walls of other Gram-negative bacteria. Weidel, Frank & Martin (1960) provided evidence that the cell wall of *Escherichia coli* is composed of three layers: an outer layer of lipoprotein particles; a middle layer of lipopolysaccharide particles of fibrous structure; an inner layer of protein particles tied together by chains of a molecular structure probably identical with the mucopeptide found in such abundant amounts in the walls of Gram-positive bacteria. The lack of simple sugars in hydrolysates of walls of *H. salinarium* can be taken as evidence that there is no polysaccharide fibre, or very little, in the walls of these bacteria. The penicillin-sensitivity of growing *H. salinarium* and the presence of amino sugar and diaminopimelic acid in wall hydrolysates indicate that a mucopeptide structure is present, but the low amino sugar content suggests that this mucopeptide is a minor component which does not contribute as much to the mechanical strength of the wall as is believed for most other bacteria. The dominating components of the wall of *H. salinarium*, proteins and/or lipoproteins, form globular particles of diameter about 130 Å. and are arranged in a hexagonal pattern (Pl. 1, fig. 1). It seems reasonable to attribute the disintegration of the wall by high concentrations of strongly binding ions to disturbances of the electrostatic forces between the proteinaceous particles. The disintegrating effect of guanidine, urea and compounds of similar properties can be interpreted in the light of the alleged tendency of such compounds to disrupt hydrogen and/or hydrophobic bonds between protein particles.

The proteinaceous sub-units of the walls of *Halobacterium salinarium* are held together in an orderly array only in the presence of high concentrations of ions which bind loosely to proteins. It is well known that loosely binding ions tend to decrease the solubility of proteins when applied in high concentrations, allegedly by decreasing the solubilizing interaction between water molecules and the polar groups of proteins (Taylor, 1953). Baxter (1959) suggested that the activating and protective effects of salts upon halophilic enzymes of Halobacterium are due to a reduction by the salts of electrostatic repulsions between charged groups in the enzyme proteins. It seems probable that sodium and chloride ions in high concentrations have similar effects upon the proteinaceous sub-units of Halobacterium walls, thereby making it possible for the units to associate to form a surface layer which possesses the properties necessary for normal cellular function.

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## EXPLANATION OF PLATES

Figs. 1–4 illustrate by electron micrographs of carbon replicas the disintegration of cell-wall fragments of *Halobacterium salinarium* when NaCl in the suspending solution was partially replaced by other chemicals in iso-osmolar concentrations. Magnification throughout × 60,000.

### PLATE 1

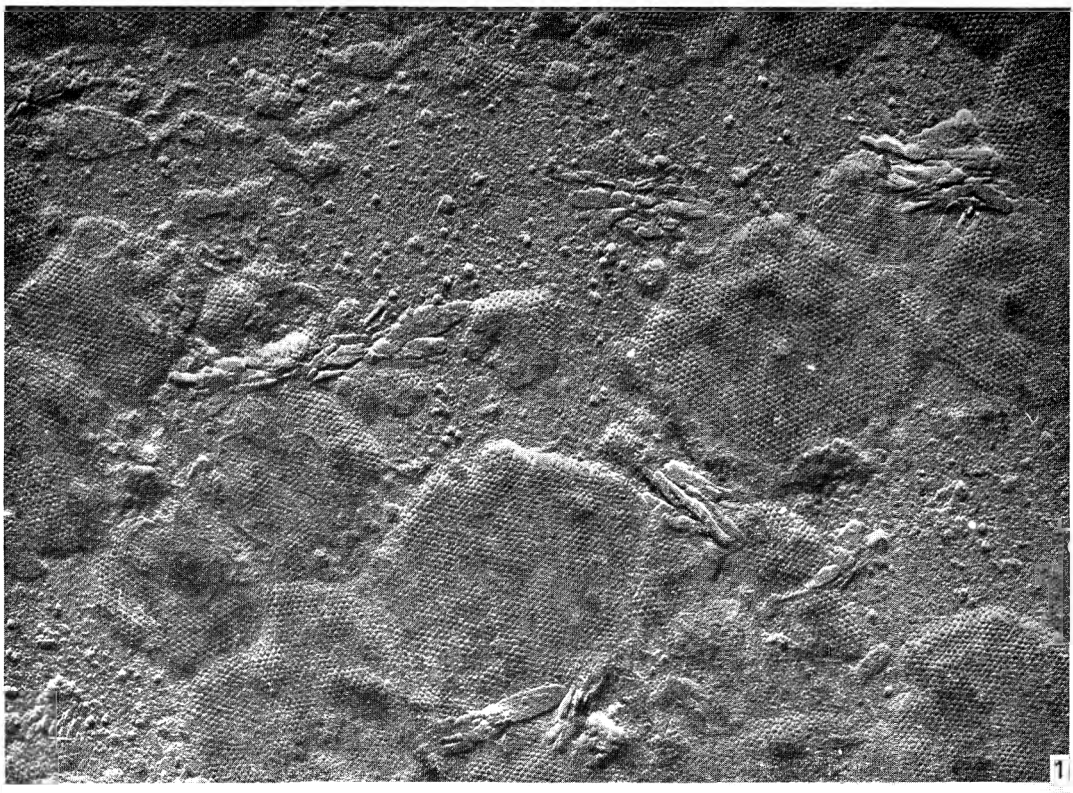
Fig. 1. Cell-wall fragments as prepared for electron microscopy from a suspension in 8·6 osmolar NaCl.

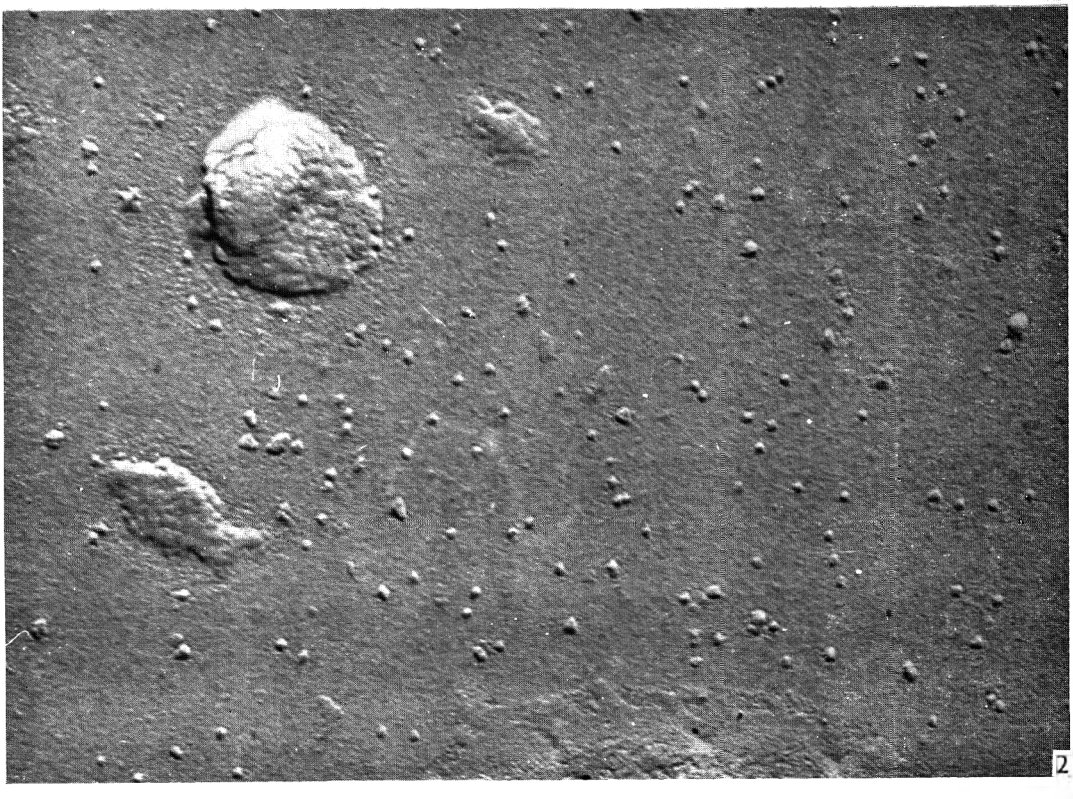
Fig. 2. After exposure of cell-wall fragments to a mixture containing 5·2 osmolar NaCl, 3·4 osmolar NaI and 0·05 mg. $I_2$/ml.

### PLATE 2

Fig. 3. After exposure of cell-wall fragments to a mixture containing 6·9 osmolar NaCl and 1·7 osmolar Na trichloroacetate.

Fig. 4. After exposure of cell-wall fragments to a mixture containing 6·0 osmolar NaCl and 2·6 osmolar urea.







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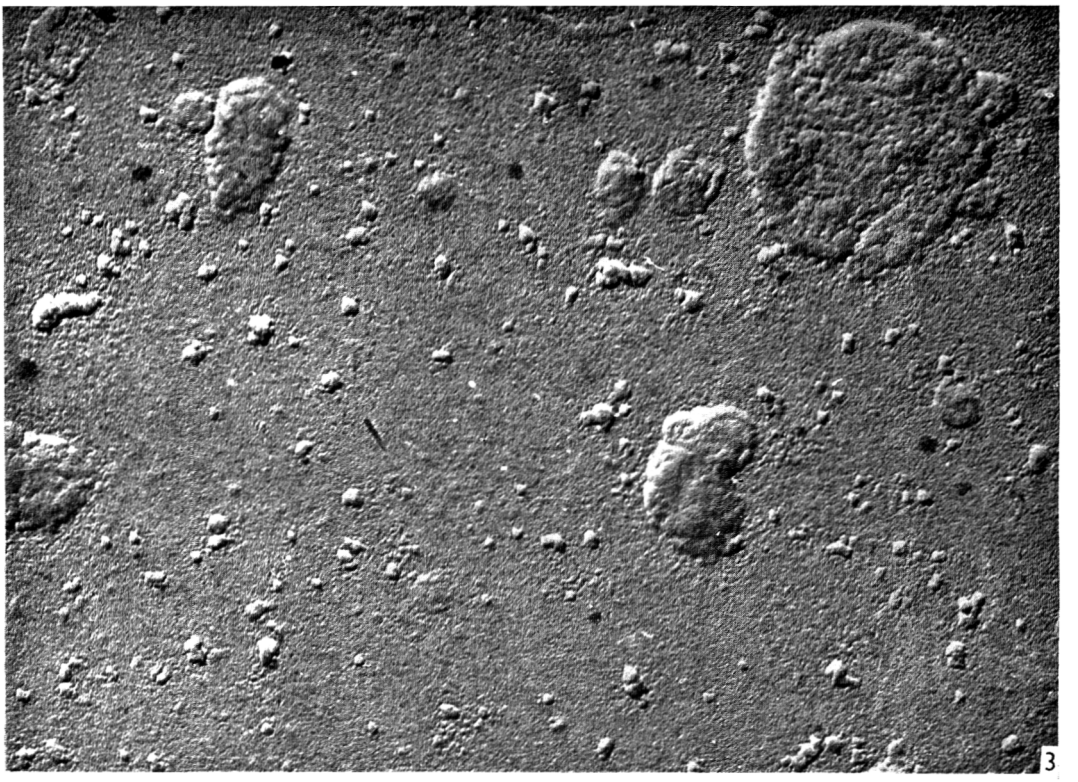

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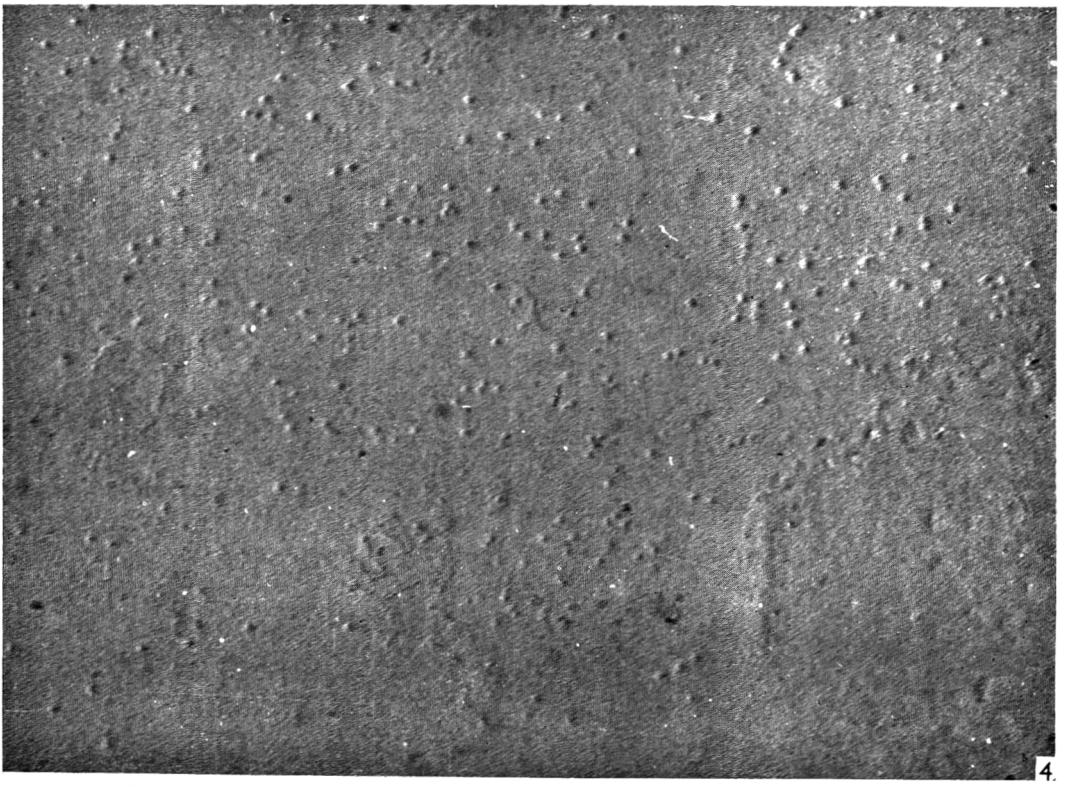

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# The Swarmers of *Bacillus cereus*

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SUMMARY

The production of swarmers by *Bacillus cereus* occurs naturally, especially in the case of 'mycoides' (i.e. morphologically rough) variants, but can also be induced by growth on media of low nitrogen content containing low concentrations of basic dyes. Very small coccobacilli, usually with one or two polar flagella, are released from the interior of the cells of the mother bacillus. The swarmers are Gram-negative or weakly Gram-positive. On further growth, or on subculture, they increase in size, and may simulate typical Gram-negative bacteria, or in some cases resemble granular, Gram-positive corynebacteria or mycobacteria. Growth of isolated swarmers is extremely slow. On continued subculture, a proportion of strains gradually revert to a Bacillus-like form, complete with heat-resistant endospore. Certain implications of this phenomenon are discussed.

## INTRODUCTION

The ability of bacteria to reproduce by a method common to many types of protista, the release of numerous small motile cells, variously described as swarmers and gonidia, has long been known but has been generally ignored. The accepted definition of bacteria (Schizomycetes) embodies the concept that they reproduce only by fission. Beijerinck's description of Rhizobium (1888) describes the phenomenon clearly, if not in detail, but later workers, while admitting the existence of a complex life-cycle, failed to understand the mode of origin of the swarmers, which was first illustrated by Bisset & Hale (1951), and the similarly, partly recognized phenomenon in Azobacter, by Bisset & Hale (1953), who pointed out the resemblance. In the case of Spirillum, the occurrence of swarmers has been frequently described; a good illustration was provided by Pease (1956). A general description and illustration of this type of reproduction in bacteria is given by Bisset (1955*a*).

The production of swarmers by Bacillus species has often been observed but seldom described in print; although Allen, Appleby & Wolf (1939) and Appleby (1939) record them in general terms. Our own experiences and numerous personal communications have convinced us that the phenomenon occurs with great frequency, but is usually regarded as evidence of contamination. The present investigation arises from our interest in the problem of the natural relationships of nitrogen-fixing bacteria. These exhibit so many Bacillus-like characters (Bisset, 1952, 1955*b*) that it was considered necessary to determine whether the distinctive character of their swarmer formation was shared by Bacillus; as, in fact, it is. The formation of swarmers and their morphology bear certain interesting inferences, and these are discussed.

## METHODS

Observations were made on thirty strains of *Bacillus cereus* isolated in this laboratory. Four of these, including one 'mycoides' variant, were mainly used and further variants arose in the course of the work. Swarmer production occurred during growth on nutrient agar at 20°, under normal conditions, but was induced by growing the bacteria upon mannitol-nutrient salts medium (Bisset & Hale, 1953), upon which were laid strips of filter-paper soaked in 0·5% (w/v) crystal violet or malachite green. Subcultures were made on nutrient agar, since the growth of this non-nitrogen-fixing species upon the nitrogen-deficient medium was very slight.

## RESULTS

The production of swarmers by *Bacillus cereus* strains was observed to occur under normal conditions of culture, but irregularly and unpredictably at any age or stage of growth. It was noted, however, that the 'mycoides' variants did so much more freely, and one strain observed produced swarmers consistently on almost every subculture, in sufficient numbers that these could usually be found by a careful search of any film. The appearance of swarmers and mother cells was exactly like that recorded many times for Rhizobium. The 'barred cells' with their thick, basophilic cross-walls, were clearly distinguishable, and so were the broken remnants of cells with emerging swarmers (Pl. 1, figs. 1, 2).

When grown in the presence of basic dyes on filter-paper strips, the results were very similar to those produced by penicillin used in a similar way. A variable area, 5–15 mm. wide, around the strip, produced no visible growth. In an intermediate zone of similar size, where L-forms might be expected in the analogous instance, was a thin irregular growth consisting mainly of organisms in the process of producing swarmers. Beyond this zone, growth was normal. By this means, both 'cereus' and 'mycoides' variants were induced to produce swarmers freely (Pl. 1, figs. 3, 4). The swarmers were of variable size, some being relatively long and slender and staining in a granular fashion that gave them a rather surprising resemblance to corynebacteria or mycobacteria (Pl. 1, figs. 5, 6). Characteristically, however, the swarmers were small Gram-negative coccobacilli, motile by means of one or two polar flagella (Pl. 1, fig. 7), and exactly resembling the swarmers of Rhizobium and Azotobacter (Bisset & Hale, 1951, 1953).

It is considered that the possibility of swarmers of this type being contaminants is obviated by the facts, first that their actual production could be observed, and secondly that nothing of the sort appeared upon control plates, either uninoculated or inoculated with 'subtilis'-type bacilli, which did not, in our experience, behave in this manner.

The growth of the cultures upon nitrogen-deficient medium was exceedingly slight, but on subculture on nutrient agar the swarmers could be isolated. Their growth and development was at first very slow, even on this medium, on which the parent strains grew freely. After several weeks of subculture, growth improved and the swarmers followed a fairly standard cycle of increase in size and gradual reversion to the staining reactions and morphology of the original bacilli. Endospores began to appear while size was still reduced, and Gram-positivity weak or

absent. Restoration of flagellar pattern was of interest. One strain, almost immediately on subculture from the swarmer stage, while still small, slender and asporogenous, produced a large number of peritrichous flagella, whereby it was able to swarm very actively on a moist agar plate (Pl. 2, fig. 8). In three other strains, as size increased, the flagella, while remaining few in number, became changed in position from a polar to a lateral position (Pl. 2, figs. 9, 10).

The last character to be regained in the strains examined was the original cultural appearance; and the revived strains, after several months of subculture, tended to be rather feeble by comparison with the originals. Such revivals of the original morphology were achieved by selection and subculture of progressively larger, more Gram-positive variants from the cultures of swarmers at various stages, except in the case of the first very tiny swarmers, which rarely retained this form for more than a single subculture, but rapidly increased in size. Beyond this point, it was possible to obtain fairly stable variants of the general appearance of Gram-negative, coliform bacteria.

## DISCUSSION

From these observations of swarmer production in *Bacillus cereus* strains under normal conditions of culture and when induced to do so by the presence of basic dyes, several interesting points arise. It is apparent that the relatively well-known reproduction of the nitrogen-fixing bacteria, especially Rhizobium, in this manner, is in complete accordance with the theory that these bacteria are closely related to the Bacillaceae. Even in such details as the barred appearance of the mother cells and the formation of relatively stable Bacterium-like variants from the tiny swarmers, they are exactly alike.

Further observations relative to the problem of phylogenetic relationships in bacteria arise from the appearance of the swarmers themselves, which are capable of simulating closely certain of the smaller Gram-positive and Gram-negative bacterial genera. The hypothesis has recently been proposed (Bisset, 1962) that the ancestral type of terrestrial bacterium was probably a form of Bacillus; and this concept is supported by the conclusion, drawn from electrically computed taxonomy, that Bacillus appears to be more closely related to certain Gram-negative genera than is any other Gram-positive genus (Sneath & Cowan, 1958). The observations reported in the present paper suggest one route whereby evolution might have occurred, through a process resembling neoteny. In this context, the claims of Csillag (1961) to have established a relationship between Bacillus and Mucobacterium are of interest, in view of the mycobacterial morphology of some of the swarmers of *Bacillus cereus* here illustrated. The hypothetically primitive condition of Bacillus is also confirmed by this retention of the power to produce swarmers, a character that is commonly found, among bacteria, only in the spirilla, which give evidence of linking the bacteria with other flagellate protista, where such swarmers are a commonplace (cf. Bisset, 1962). In more specialized bacteria this power has apparently been lost, together with other elaborate characters appertaining to their free-living ancestors.

Lastly, the phenomena of swarmer-production bear certain resemblances to the L-cycle (cf. Pease, 1956) which also occurs, at least upon occasion, by the intervention of a filamentous rough phase (Pease, 1962). True L-formation is unusual

in Bacillus, and penicillin has no such effect, in our experience, nor does it induce the formation of swarmers. There is, nevertheless, a distinct resemblance to penicillin in the action of the basic dyes described here, since both appear to act mainly upon the cell envelopes of Gram-positive bacteria. The large bodies of L-forms are now generally regarded as bacterial protoplasts that have discarded the cell wall, either by genetic accident or to avoid the action of penicillin (Pease, 1957). It is possible that the small bodies may represent a degenerate relict of swarmer production, and be simply a smaller grade of protoplast.

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## EXPLANATION OF PLATES

### PLATE 1

Figs. 1, 2. Barred mother cells and swarmers. Basic fuchsin. Photomicrographs. ×2500.

Figs. 3, 4. Mother cells and swarmers produced by exposure to crystal violet. Electron micrograph, gold-palladium shadowed. ×20,000 and 5000, respectively.

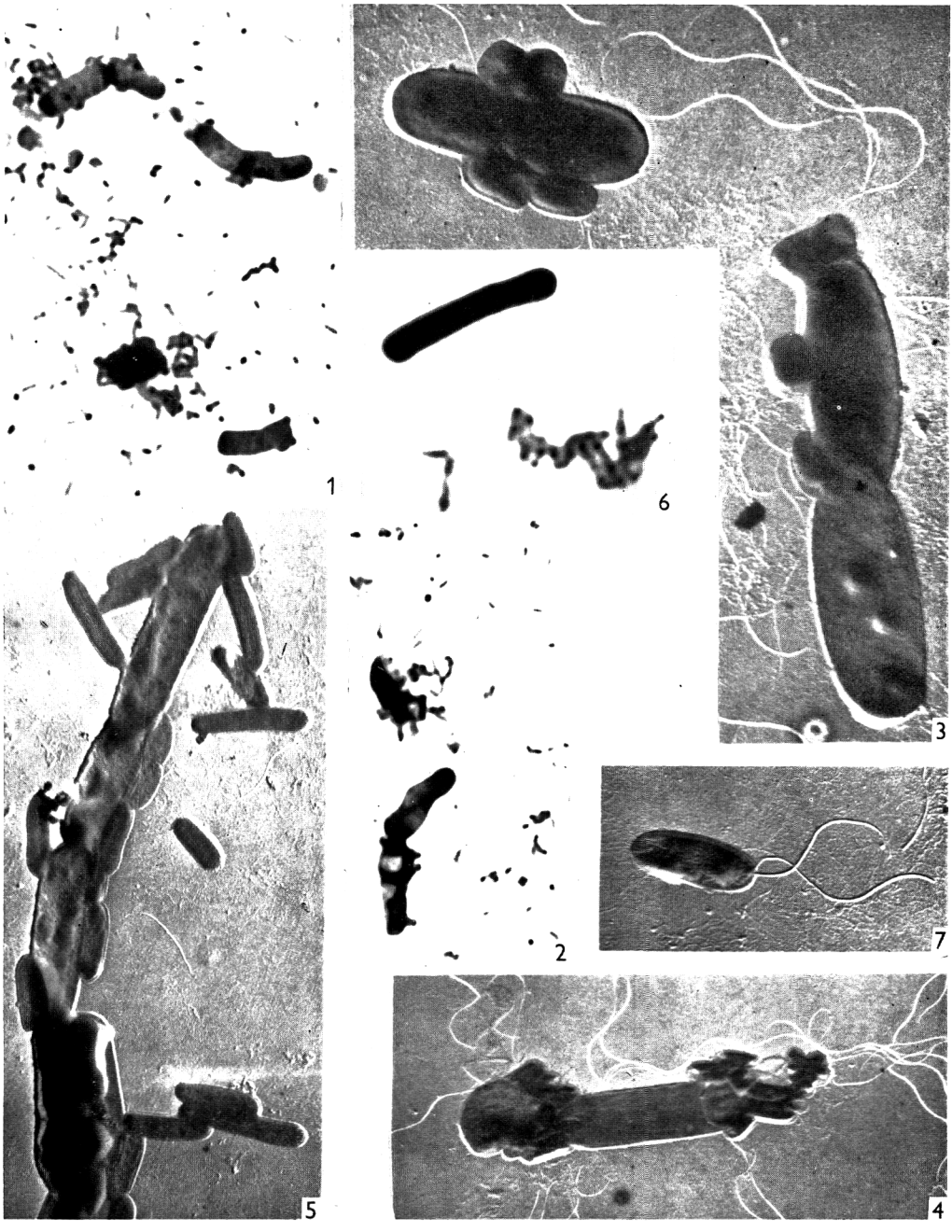
Figs. 5, 6. Mother cells and swarmers of 'mycoides' variant, the swarmers resembling corynebacteria. (5) Electron micrograph, gold-palladium shadowed. ×7000. (6) Photomicrograph, basic fuchsin. ×3000.

Fig. 7. Swarmer showing two polar flagella. Electron micrograph, gold-palladium shadowed. ×5000.

### PLATE 2

Fig. 8. Enlarged swarmer of unusual type, subcultured upon nutrient agar, showing numerous peritrichous flagella. Electron micrograph, gold-palladium shadowed. ×20,000.

Figs. 9, 10. Stages in the recovery of peritrichous flagellation and Bacillus-like morphology. Electron micrographs, gold-palladium shadowed. (9) ×20,000; (10) ×10,000.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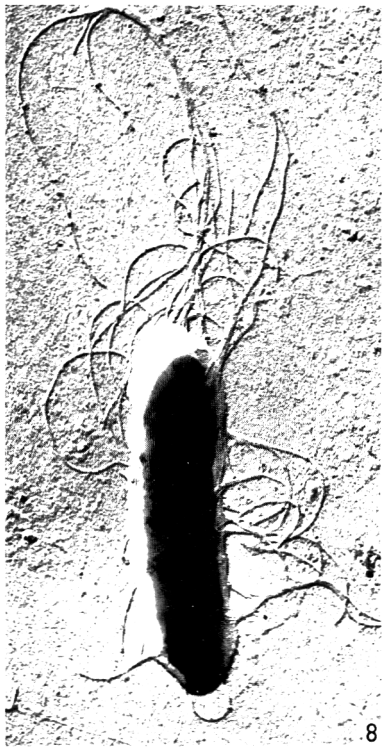

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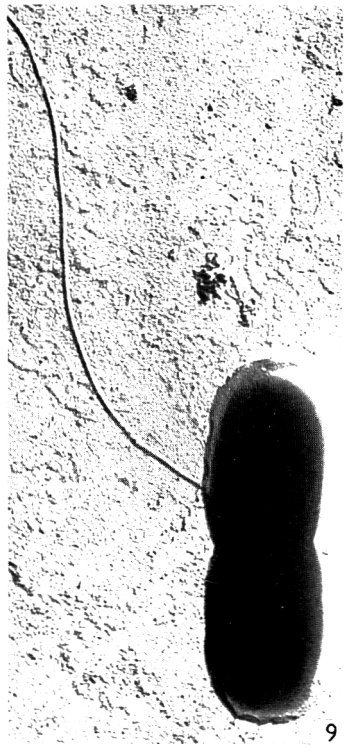

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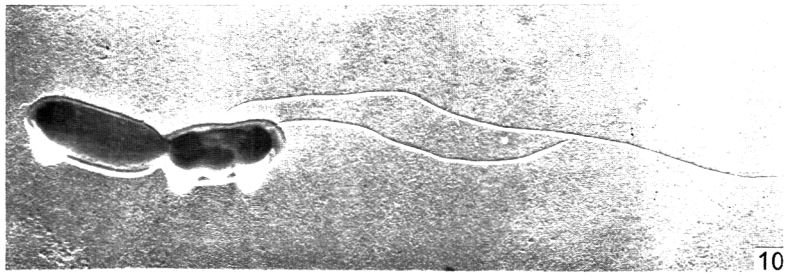

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# The Adaptive Metabolism of D-Galactose in *Aspergillus nidulans*

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SUMMARY

Methods are described for the growth of *Aspergillus nidulans* in submerged culture. In experiments with intact organisms D-galactose was oxidized by an inducible enzyme system; D-fucose was a poor inducer of this system. Mutants isolated by their failure to grow on galactose as sole carbon source were defective in the oxidation of galactose.

## INTRODUCTION

Powerful techniques for genetic analysis have been developed for the mould *Aspergillus nidulans* (Pontecorvo *et al.* 1953; Pontecorvo & Käfer, 1958) but relatively little work has been done on the biochemistry of the organism (Hockenhull, 1950; Singh & Walker, 1956) and few attempts made to combine genetical and biochemical investigations (Shepherd, 1956). Following the elucidation of the Leloir pathway of galactose metabolism (reviewed by Kalckar, 1958) genetically determined lesions in the enzymes concerned were described in man (Kalckar, 1959), in *Saccharomyces cerevisiae* (Robichon-Szulmajster, 1958) and in certain enterobacteria (Kalckar, Kurahashi & Jordan, 1959; Fukasawa & Nikaido, 1961; Soffer, 1961). Regulation of the formation of the enzyme system has also been studied in some bacteria (Yarmolinsky, Jordan & Weismeyer, 1961; Buttin, 1961; Fukasawa, Jokura & Kurahashi, 1962). Mutants of *A. nidulans* which fail to grow on galactose have been isolated and analysed genetically (Roberts, 1963); the present paper describes investigations with intact organisms of the metabolism of galactose by the wild type organism and by the galactose mutants.

## METHODS

*Organisms. Aspergillus nidulans* strain *bi1;w3* (a biotin-requiring auxotroph with white conidia) was obtained from the Department of Genetics, Glasgow University (Pontecorvo *et al.* 1953). It has the wild type property of utilizing D-galactose as sole carbon source for growth and was the strain from which a number of mutants which did not grow on galactose were isolated following ultraviolet irradiation (Roberts, 1959, 1963). Cultures were maintained on slopes of malt extract agar.

*Media.* Malt extract agar: Malt extract, 20 g.; peptone 1 g.; glucose 20 g.; British Drug Houses agar, 25 g. were dissolved in tap water (1 l.) and autoclaved at 120° for 15 min.; the medium was then at pH 6·5–7·0.

The basal medium (BM) used was the standard minimal medium for *Aspergillus nidulans* (Pontecorvo *et al.* 1953) but prepared without a carbon source. A trace

salts solution was used in the present experiments, it contained (mg./100 ml.): $FeSO_4.7H_2O$, 100; $ZnSO_4.7H_2O$, 880; $CuSO_4.5H_2O$, 40; $MnSO_4.4H_2O$, 15; $Na_2B_4O_7.10H_2O$, 10; $(NH_4)_6Mo_7O_{24}.4H_2O$, 5. One litre of basal medium contained $NaNO_3$, 6·0 g.; KCl, 0·52 g.; $KH_2PO_4$, 1·52 g.; 1·0 ml. trace salts solution. This medium was adjusted to pH 6·5 before autoclaving (120°, 15 min.) and $MgSO_4$ (to 0·52 g./l.) added as a sterile solution when the medium had cooled, to avoid precipitation of magnesium phosphate. Biotin was supplied in excess at 0·5 mg./l. Carbohydrates were sterilized separately by autoclaving at 115° for 15 min. and added at a final concentration of 10 g./l.; conidia of *A. nidulans* are water repellent and the wetting agent Tween 80 was added to all liquid media ($1/10^4$) to bring conidia into suspension.

*Preparation of inocula.* A suspension of conidia was plated on malt extract agar to yield 25–100 colonies/plate and the plates incubated at 37° for 6 days by which time heavy sporulation had occurred. The conidia were harvested by drawing a sterile wire across the plate, collected in a screw cap bottle containing 0·85% NaCl solution + Tween 80 and shaken on a Microid shaker for 20 min. to break up spore clumps.

*Culture vessels.* Modified 1 l. and 2 l. conical flasks were used. Each flask had four internal baffles consisting of vertical invaginations of the wall of the vessel 1 cm. deep and 3 cm. high which just touched the surface of the medium when the flask was at rest. A water-repellent silicone film (Hopkins & Williams silicone fluid M.S. 1107) was applied to the internal surface of the flasks. The baffles dispersed the mycelial pellets and aided aeration, while the silicone film prevented accumulation of pellets above the wash line of the medium.

*Culture conditions.* Flasks containing one-fifth their volume of medium were inoculated with suspensions of conidia to a final concentration of about $3 \times 10^6$ conidia/ml. medium. They were incubated at 30° with vigorous swirling (200 rotations/min.) on a gyrorotary shaker (New Brunswick Instrument Co.). These conditions of culture yielded dense suspensions of small pellets of mycelium about 0·05 mm. in diameter which were formed by aggregation of germinating conidia. The suspensions can be handled quantitatively by pipetting, but variations observed in measuring rates of gas exchange (Table 1) might have been due to differences in the size and texture of the pellets.

*Preparation of suspensions of organisms.* Mycelial pellets were harvested on a sintered glass filter (No. 2 grade), resuspended in three changes of deionized water, washed twice with distilled water and finally suspended in 0·04 M-potassium phosphate buffer (pH 6·5). Dry weights were determined after washing samples on the filter and drying overnight at 105° in aluminium foil cups. The suspensions used contained 1·0–2·5 mg. dry weight organisms/ml.

*Estimations.* Manometric assays of $O_2$ uptake or $CO_2$ production were done by conventional methods (Umbreit, Burris & Stauffer, 1949). The manometer flasks contained 2·0 ml. suspension (equiv. 2·0–5·0 mg. dry weight organism) in the main compartment, 0·5 ml. 0·01M substrate solution (or 0·5 ml. water for controls) in the side arms, and 0·2 ml. of 20% KOH in the centre well (when $O_2$ uptake was measured). The temperature was 30°. There was a linear relationship between the rate of oxygen uptake and dry weight of organisms up to at least 7·5 mg. dry weight organism/flask.

The disappearance of sugar from the medium was followed under conditions simulating as far as possible those in the manometer flasks. An open 100 ml. conical flask containing 20 ml. of organism suspension and 5·0 ml. of 0·01 M substrate was shaken in the manometer bath and samples taken periodically. Organisms were removed by filtration and the amount of reducing sugar in the filtrate estimated by the arsenomolybdate method (Nelson, 1944).

*Chemicals.* D-Galactose was obtained from T. Kerfoot and Co., (Vale of Bardsley, Lancs.). Glucose contamination of the galactose was estimated by the glucose oxidase method (Huggett & Nixon, 1957) and did not exceed 2% in any sample. D-Fucose was obtained from L. Light and Co. (Colnbrook, Bucks., England).

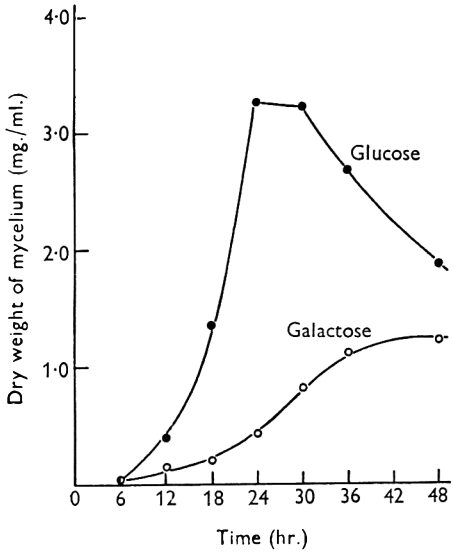


Fig 1

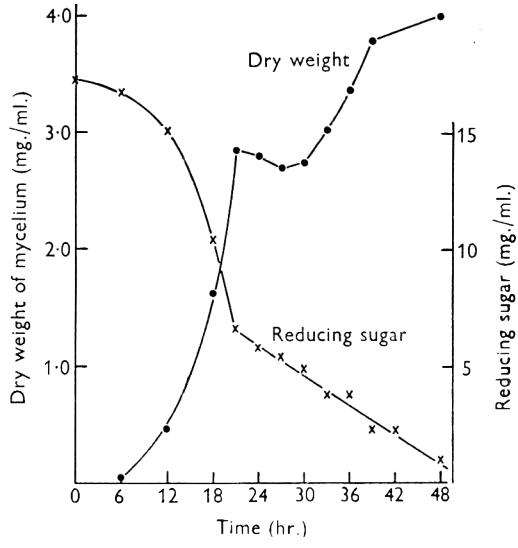


Fig. 2

Fig. 1. Growth of *Aspergillus nidulans* strain *bi1;w3* in submerged culture grown in basal medium (BM)+biotin with 1% glucose (●—●) or 1% galactose (○—○) as carbon source. Samples were taken for dry weight determinations at the times shown.

Fig. 2. Diauxie of *Aspergillus nidulans* strain *bi1;w3* grown in submerged culture with a mixture of 0·75% glucose and 0·75% galactose. Dry weights (●—●) and total reducing sugar (×—×) in the medium (calculated as galactose) were determined at the times shown.

## RESULTS

### *Growth of* Aspergillus nidulans *strain* bi1;w3 *with glucose or galactose as carbon source*

Galactose is a comparatively poor carbon source for *Aspergillus nidulans*, the rate of growth and the yield of organism being substantially less than with glucose (Fig. 1). The long lag period in growth on galactose and the typical diauxie (Monod, 1942) observed when a culture was supplied with a mixture of glucose and galactose suggested that galactose is utilized adaptively (Fig. 2).

*Uptake and oxidation of glucose and galactose by* Aspergillus nidulans *strain* bi1;w3

Organisms were grown with glucose or galactose as carbon source and then tested for ability to take up and to oxidize these sugars. Glucose is metabolized by a constitutive enzyme system and galactose by an inducible system, for organisms grown on galactose immediately take up and oxidize both sugars, whereas organisms grown on glucose metabolize glucose but cannot oxidize galactose and only remove it from the medium at a low rate (Table 1). Differences in specific rates of oxidation and sugar uptake are probably due to variation in the size and texture of the mycelial pellets. The rate of oxidation of galactose by galactose-grown organisms was about 40% that for glucose oxidation; the rate of galactose uptake was 60% that of glucose uptake.

Table 1. *Adaptive uptake and oxidation of* D-*galactose by* Aspergillus nidulans, *strain* bi1;w3

Organisms were grown in basal medium (BM) + biotin with 1 % glucose or 1 % galactose as carbon source. Washed organisms were suspended in 0·04 M-phosphate buffer (pH 6·5) and the rates of oxygen consumption and sugar uptake determined as described in Methods.

| | | Rate of oxygen uptake ($\mu$l./hr./mg. dry weight organism) | | | Rate of sugar uptake ($\mu$moles/hr./mg. dry weight organism) | |
|---|---|---|---|---|---|---|
| | | | Increase over endogenous | | | |
| Carbon source for growth | Growth period (hr.) | Endogenous | Glucose added | Galactose added | Glucose | Galactose |
| Glucose | 16 | 16 | 45 | 1 | — | — |
| | 15 | 14 | 69 | 3 | — | — |
| | 15 | 11 | 48 | 5 | — | — |
| | 15 | 16 | 61 | 2 | 1·82 | 0·04 |
| | 15 | 14 | 47 | 0 | 1·78 | 0·11 |
| | 15 | 19 | 64 | 4 | 1·96 | 0·15 |
| | 15 | 20 | 37 | 0 | 1·54 | 0·07 |
| | Mean | 16 | 53 | 2 | 1·78 | 0·08 |
| Galactose | 16 | 11 | 44 | 20 | — | — |
| | 18 | 15 | 67 | 18 | 2·20 | 1·10 |
| | 21 | 17 | 55 | 25 | 1·50 | 1·00 |
| | Mean | 14 | 55 | 21 | 1·85 | 1·05 |

Endogenous rates of oxygen uptake were high and the respiratory quotient (0·7 for glucose-grown organisms, 0·8 for galactose-grown organisms) are consistent with a fat being the substrate (cf. Singh & Walker, 1956). Starvation of organisms by incubation in buffer or in a medium without a carbon source resulted in parallel decrease of endogenous and exogenous rates. During the oxidation of glucose and of galactose the R.Q. was 1·0.

A substantial proportion of the substrate was assimilated. This was studied by two methods, both based upon the assumption that complete oxidation of the substrate requires 6 equivalents of oxygen. In the first method the oxygen consumed in the oxidation of a known quantity of substrate was measured, corrected for endogenous respiration, and the proportion of substrate assimilated estimated from the difference between observed and expected quantities of oxygen consumed

Table 2. *Assimilation of substrates by* Aspergillus nidulans, *strain* bi1;w3

Organisms grown on glucose or galactose were washed and suspended in 0·04 M-phosphate buffer (pH 6·5). The $O_2$ consumed (column 4) when 5 $\mu$moles substrate was added was determined manometrically, as was the endogenous $O_2$ uptake (column 5) in the same time. The figures in columns 8 and 10 are taken from Table 1. Calculation of the percentage of substrate assimilated is described in the text.

| | | $O_2$ uptake ($\mu$l.) | | | | | Rate of sugar uptake ($\mu$mole/hr./mg. dry weight organism | Rate of $O_2$ uptake ($\mu$l./hr./mg. dry weight organism | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | Observed | | | | | | | |
| Carbon source for growth | Substrate (5 $\mu$moles) | Expected | Total | Endogenous | Net | Substrate assimilated | | Expected | Observed | Substrate assimilated |
| Column ... 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 |
| Glucose | Glucose | 672 | 286 | 184 | 102 | 85 % | 1·78 | 239 | 53 | 78 % |
| Galactose | Glucose | 672 | 214 | 88 | 126 | 82 % | 1·85 | 248 | 55 | 78 % |
| | Galactose | 672 | 204 | 108 | 96 | 86 % | 1·05 | 141 | 21 | 86 % |

(Table 2, columns 3–7). In the second method the rate of oxygen uptake expected from the rate of sugar uptake was calculated and the proportion of substrate assimilated estimated from the difference between the expected and observed rates of oxygen uptake (Table 2, columns 8–11). The two methods yielded results in close agreement; about 80% of added glucose was assimilated and about 85% of the galactose.

*Induction of the galactose oxidation system in* Aspergillus nidulans *strain* bil;w3

The galactose-oxidizing system was induced by incubation of organisms in a growth medium in the presence of galactose. Organisms were grown in basal medium (BM)+glucose for 15 hr., washed aseptically and resuspended in fresh

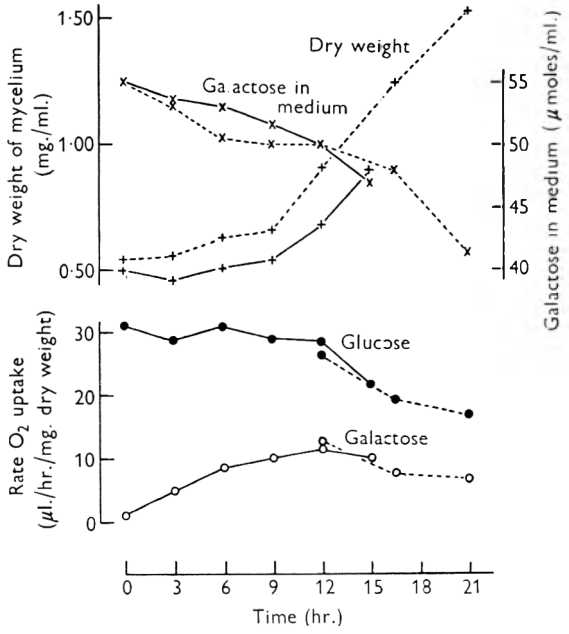


Fig. 3. Induction of the galactose oxidation system in *Aspergillus nidulans* strain *bil;w3*. Organisms were grown for 15 hr. in basal medium (BM)+biotin with 1% glucose as carbon source, harvested, washed aseptically by centrifugation and resuspended in fresh basal medium (BM)+biotin, with 1% galactose and incubated at 30° on the shaker. Samples were taken periodically and the dry weight yields of organisms and the galactose remaining in the medium determined. Rates of oxidation of glucose (●—●) and galactose (○—○) were determined manometrically and are shown after subtraction of the endogenous values. The curves were derived from two separate experiments, one for the induction period 0–15 hr. (solid lines) and one for the period 12–21 hr. (broken lines).

BM+galactose. The results of two such induction experiments are shown in Fig. 3. There was a lag in growth for about 9 hr. which corresponds to the lag previously observed in the growth of conidia on galactose (Fig. 1) and in diauxie on a mixture of glucose and galactose (Fig. 2). During this lag period there was a slow uptake of galactose and a steady increase in the specific rate of galactose oxidation to a maximum at about 12 hr., when it was 40% of that of glucose oxidation. The de-

crease in rates of oxidation after incubation for 15 hr. (30 hr. total incubation) probably resulted from limitation of $O_2$ uptake by the size of the pellets and the accumulation of inert material such as walls of empty mycelium.

D-Fucose (6-deoxy-galactose) is an inducer of the enzymes for galactose utilization in *Escherichia coli* (Buttin, 1961). *Aspergillus nidulans* does not utilize D-fucose for growth and organisms grown in the presence of fucose (with glycerol as carbon source) do not oxidize the sugar. When *A. nidulans* strain *bil;w3* was grown with glycerol + fucose or galactose there was significant induction of the galactose oxidation system in both cases; but fucose was only one-third as effective as galactose as an inducer (Table 3). This was also the case when the organism was grown on glucose and then incubated in a fresh growth medium with glycerol + fucose or galactose.

Table 3. *Induction of the galactose oxidation system in* Aspergillus nidulans *strain* bil;w3 *by* D-*fucose*

In Experiments 1 and 2 organisms were grown for 18 hr. in basal medium (BM) + biotin with 1% glycerol and inducer as shown. In Experiment 3 organisms were grown for 15 hr. in basal medium (BM) + biotin with 1% glucose; they were then harvested, washed aseptically by centrifugation and resuspended in basal medium (BM) + biotin + the carbohydrates shown, and incubated for 15 hr. Rates of galactose oxidation were determined manometrically.

| | Rates of $O_2$ uptake ($\mu$l./hr./mg. dry weight organism) | |
|---|---|---|
| Organisms grown on | Endogenous | Increase when galactose added |
| Experiment 1 | | |
| Glycerol | 13·8 | 1·8 |
| Glycerol + 0·01 M-fucose | 14·1 | 3·1 |
| Glycerol + 0·01 M-galactose | 13·9 | 7·6 |
| Experiment 2 | | |
| Glycerol | 17·2 | 1·4 |
| Glycerol + 0·05 M-fucose | 17·1 | 3·9 |
| Glycerol + 0·05 M-galactose | 16·6 | 12·4 |
| Experiment 3 | | |
| Glucose 15 hr. then: | | |
| Glycerol | 22·7 | 1·2 |
| Glycerol + 0·05 M-fucose | 22·8 | 2·6 |
| Glycerol + 0·05 M-galactose | 19·7 | 6·9 |
| 0·05 M-galactose | 21·5 | 9·8 |

*Metabolism of galactose by galactose mutants of* Aspergillus nidulans *strain* bil;w3

The genetic analysis of a number of ultraviolet-induced galactose mutants of *Aspergillus nidulans* has resulted in recognition of five loci which control the metabolism of galactose in this organism (Roberts, 1963). These galactose mutants all grow normally with glucose as carbon source and are of two distinct phenotypes when tested for growth on galactose: one does not grow at all, while the other grows slowly, yielding non-sporing colonies which are morphologically abnormal. These slow-growing mutants are different from partial ('leaky') mutants which have been isolated by their poor growth on other sugars (e.g. maltose, lactose) in

Table 4. *Uptake and oxidation of galactose by galactose mutants of* Aspergillus nidulans *strain* bi1;w3

Organisms were grown for 15 hr. at 30° in basal medium (BM) + biotin with 1 % glucose as carbon source. They were harvested and washed twice aseptically by centrifugation, and resuspended in fresh basal medium (BM) + biotin with 1 % galactose. Organisms were assayed after 15 hr. incubation at 30°.

| | | Induced organisms | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | Oxygen uptake ($\mu$l./hr./mg. dry weight organism) | | | Sugar uptake ($\mu$mole/hr./mg. dry weight organism) | | Relative rates | | | |
| | | | Increase over endogenous | | | | Ratio of rates of oxidation Galactose† / Glucose | | Ratio of rates of uptake Galactose / Glucose | |
| Strain | Growth on galactose (solid media) | Endogenous | Glucose added | Galactose added | Glucose | Galactose | | | | |
| *bi1;w3** | Wild type | 13 | 43 | 19 | 1·64 | 0·70 | 0·44 | 100 % | 0·43 | 100 % |
| *bi1;w3; gal1* | No growth | 19 | 32 | 0 | 1·10 | 0·12 | 0·03 | 7 % | 0·16 | 37 % |
| | | 23 | 38 | 2 | 1·46 | 0·27 | — | — | — | — |
| *bi1;w3; gal2* | Slow growing | 16 | 26 | 6 | 0·91 | 0·36 | 0·23 | 53 % | 0·40 | 93 % |
| *bi1;w3; gal3* | Slow growing | 19 | 34 | 7 | 1·22 | 0·43 | 0·20 | 46 % | 0·35 | 82 % |
| *bi1;w3; gal4* | Slow growing | 14 | 29 | 6 | 0·88 | 0·37 | 0·21 | 48 % | 0·42 | 98 % |
| *bi1;w3; gal5* | No growth | 10 | 35 | 4 | 1·36 | 0·22 | 0·05 | 12 % | 0·15 | 35 % |
| | | 12 | 38 | 0 | 1·20 | 0·16 | — | — | — | — |

* Mean value of three experiments.
† Less endogenous rates.

which hyphae grow at the same rate as the wild type but are far fewer in number and form characteristic sparse colonies. All the galactose mutants at a locus are of the same phenotype and five mutants representing each of the loci (called *gal 1* to *gal 5*) were tested for their ability to metabolize galactose after induction of glucose-grown organisms (Table 4).

As expected the mutants all metabolized glucose at rates much the same as the original strain but showed defects in their uptake and oxidation of galactose. These defects may be correlated with the phenotype of the mutants. Differences between the mutants and wild type in the metabolism of galactose are most clearly shown when activities are expressed as the ratios of the rates of galactose to glucose uptake or oxidation (Table 4). Total mutants (*gal 1, gal 5*) took up galactose at about one-third the rate of the wild type and oxidized the sugar at markedly slower rates. The slow-growing mutants (*gal 2, gal 3, gal 4*) took up galactose at the same rate as the wild type but oxidized the sugar at about one-half the rate of the wild type.

## DISCUSSION

High endogenous metabolic rates and the assimilation of substrate are general features of mould metabolism (Cochrane, 1958); *Aspergillus nidulans* conforms to this pattern. The mycelial habit has made moulds unpopular material for biochemical work but in the case of *A. nidulans* small mycelial pellets are satisfactory for whole cell experiments and are good starting material for the preparation of cell-free extracts.

Comparison of these results for *A. nidulans* with the classical work of Stephenson on *Escherichia coli* (Stephenson, 1949) and *Saccharomyces cerevisiae* (Stephenson & Yudkin, 1936) reveals similarity of the overall metabolism of galactose in all three organisms. Relative to glucose, galactose is a poor carbon source for growth; it is oxidized by galactose-grown organisms at about one half the rate of glucose and the enzyme systems are inducible. However, D-fucose, which is a good inducer in *E. coli* (Buttin, 1961) is only one-third as effective as equimolar concentrations of D-galactose in *A. nidulans*. A constitutive galactose permease has been described in *E. coli* (Horecker, Thomas & Monod, 1960). The rapid initial uptake of galactose by adapted organisms and the slow uptake by non-adapted organisms suggests an inducible permease in *A. nidulans* but requires direct investigation by using $^{14}C$ galactose.

All of the galactose mutants examined metabolize glucose normally as would be expected from their normal growth on it. Isolation of mutants which fail to grow upon a specific carbohydrate may therefore be expected to exclude organisms with a defect in their general metabolism of carbohydrates, as found among bacterial mutants isolated by a negative fermentation reaction on eosin methylene-blue agar (Lederberg, Lederberg, Zinder & Lively, 1951; Lederberg, 1960).

The two distinct phenotypes found among the *Aspergillus nidulans* galactose mutants may probably be correlated with the position of the metabolic lesion. The slow-growing mutants take up galactose at the same rate as the wild type but oxidize it at about half the wild-type rate. About 80% of exogenous galactose is assimilated and if this assimilation takes place through uridine diphosphogalactose (Kalckar, 1958) it is possible that the slow growers are defective in a metabolic step

between this compound and glucose-1-phosphate. On the other hand, the non-growing mutants, which take up galactose at about one-half the wild type rate but only oxidize it at 10% of the rate of the latter, may be defective in a metabolic step before uridine diphosphogalactose.

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# The Infection of *Escherichia coli* with Lambda ($\lambda_{22}$) Phage and the Establishment of Lysogeny

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SUMMARY

Infection of *Escherichia coli* by phage $\lambda_{22}$ in 0·02 M-$Sr(NO_3)_2$, $CaCl_2$ or $MnSO_4$ occurred as readily as in 0·02 M-$MgSO_4$. The response of bacteria infected in the presence of $Sr^{2+}$, $Ca^{2+}$ and $Mg^{2+}$ was predominantly lysogenic, but infection in 0·02 M-$Mg^{2+}$ gave a higher lysogenic response (90%) than with the other cations tested. With $Mn^{2+}$ in optimal conditions, at least 25% of the infected bacteria always gave the lytic response. When the newly infected bacteria were transferred to a growth medium, a period of about 40 min. had to elapse before the prophage could be detected by zygotic induction by mating with a non-lysogenic $F^-$ organism. A period of about 100 min. was required before all the bacteria giving the lysogenic response could be induced by ultraviolet radiation.

## INTRODUCTION

Lambda ($\lambda$) phage will absorb well to washed organisms of *Escherichia coli* in the presence of magnesium sulphate (Kaiser, 1957), and for *E. coli* strain K112 and phage $\lambda_{22}$ conditions have been found (0·02 M-$MgSO_4$ at about pH 6·5) in which about 99% of the bacteria were infected (Fry, 1959); 10% of the infected bacteria eventually lysed and 90% became lysogenic for phage $\lambda_{22}$. The factors which control the decision of whether a bacterium gives a lytic or lysogenic response and the biochemical events which lead to the establishment of the prophage in those bacteria which give a lysogenic response are largely unknown. It appeared from earlier work that the composition of the adsorption medium may affect the response of the cell to infection with phage $\lambda_{22}$ (Fry, 1959). Since more than one type of cation will aid the irreversible adsorption of various T phages to *E. coli* strain B (Puck, Garen & Cline, 1951), the adsorption of phage $\lambda_{22}$ in the presence of ions other than $Mg^{2+}$ was therefore studied.

After infection with a temperate phage, the metabolism of bacteria which are to become lysogenic seems to be profoundly disturbed and when the bacteria are placed in a growth medium one reflexion of this disturbance is a long delay before division occurs (Lieb, 1953; Fry & Gros, 1959). Moreover some bacteria may divide more than once before the prophage is fully established when no more non-lysogenic progeny segregate (Luria, Fraser, Adams & Burrows, 1958). It would be of interest to know how soon after infection the genetic material of the phage is established as a prophage on the bacterial chromosome. By using $\lambda$ phage labelled with $^{32}P$ and then studying the effect of $^{32}P$ decay on the establishment of lysogeny, Stent & Fuerst (1956) found that 30 min. after infection the genome of the phage

was in a state such that it was no longer susceptible to $^{32}P$ decay. However, this does not prove directly whether the prophage has in fact become associated with bacterial chromosome at this time. In order to establish when the latter event has taken place, other properties of the prophage might be exploited. For example, samples of the bacteria can be taken at intervals after infection and irradiated with ultraviolet (u.v.) light in order to determine whether those bacteria which ultimately give the lysogenic response are in an inducible state at the time of sampling. Use may also be made of the phenomenon of zygotic induction (Jacob & Wollman, 1956), which is observed when an Hfr strain lysogenic for phage $\lambda$ is mated with a non-lysogenic $F^-$ strain, since on entry into the $F^-$ organism, the prophage undergoes spontaneous induction. Jacob & Wollman (1956) established that only phage genetic material attached to the bacterial chromosome, i.e. in the form of prophage, can be transferred to the $F^-$ strain. By using such techniques, studies have now been made to see how soon after infection the establishment of the prophage can be recognized.

## METHODS

*Organisms.* *Escherichia coli* strains K112, HfrC-Wollman, W678 and the indicator organisms for phage $\lambda$ (strains C600 $Sr^r$ and K112 $Sr^r$, streptomycin resistant) were from the culture collection of Drs F. Jacob and E. Wollman of the Pasteur Institute, Paris. Strain HfrC-Wollman requires thiamine and is sensitive to streptomycin and phage $T_6$ (HfrC $B_1^-$ $Sr^s$ $T_6^s$); strain W678 is $F^-$, requires threonine + leucine + thiamine, and is resistant to streptomycin and to phage $\lambda$ (W678 $T^-$ $L^-$ $B_1^-$ $Sr^r$/$\lambda F^-$); a mutant resistant to phage $T_6$ ($T_6^r$) was isolated by the author; strain K112 is $F^+$.

*Phage.* The $\lambda$ phage used was a wild type designated as $\lambda_{22}$ (see Fry, 1959).

*Growth medium.* Nutrient broth (medium A), pH 7, contained (%, w/v): 1, Oxoid peptone; 0·1, Liebig's meat extract; 1, NaCl. For plating procedure, 1% agar (Parke Davis) was added (medium B). For the detection of lysogeny (medium C), medium B was supplemented with 1% lactose and an indicator solution (2 ml./100 ml. medium). The indicator solution contained 5 mg. thymol blue, 12·5 mg. methyl red, 60 mg. bromthymol blue/100 ml. Defined medium, for the assay of $T^+L^+$ recombinants in the mating experiments, was that described by Hayes (1957) supplemented with 100 $\mu$g. streptomycin/ml.

*Experimental techniques.* The detection of response of $\lambda$-infected bacteria, preparation of phage stocks and of washed bacterial suspensions and the measurement of phage adsorption have been described previously (Fry, 1959). The response to infection was calculated from the mean of two plates, and refers in each experiment to the same dilution of the original suspension. The percentage response is expressed relative to the total number of bacteria giving the lytic + lysogenic + refractory response. The mating experiments for obtaining zygotic induction followed the procedures of Jacob & Wollman (1956). In the u.v. irradiation of bacteria, a Hanovia Chromatolite low pressure Hg. lamp (Hanovia Ltd., Slough, Bucks.) without the filter, was used at 75 cm. from the sample (about 7 ml.) contained in an open Petri dish (9 cm. diam.). Immediately after irradiation, some of the bacteria appear to be in an unstable state, and unless they are allowed to metabolize in broth for a period, the outcome of the irradiation procedure can be reversed, e.g. by plating. Thus

bacteria which would otherwise appear to be induced, will survive, remain viable and form lysogenic colonies. Consequently, after irradiation, samples were usually not plated immediately but incubated in broth for 45 min. in order to stabilize the response to u.v. irradiation (cf. Weigle & Delbrück, 1951). The procedure for single step curves was that of Ellis & Delbrück (1939). Concentrations of solutes refer to final concentrations. All incubations were done at 37°.

## RESULTS

### *Assay of response to infection with λ phage*

Bertani (1960) suggested that plate assays for the detection of response to phage infection would be upset if an appreciable number of the bacteria occurred in chains of two or more organisms. If, for example, only one organism of a chain were infected and gave the lytic response and lysed on the plate, one or more of the remaining bacteria of the chain might thus undergo multiple infection and these are conditions which favour the establishment of lysogeny. In these circumstances a true lytic response would in fact be recorded as a lysogenic response. Examination of cultures of *Escherichia coli* K112 at the time of harvesting for the preparation of washed

Table 1. *Response of* Escherichia coli K112 *to infection with λ phage in different concentrations of* $MnSO_4$, $SrNO_3$ *and* $CaCl_2$.

In each experiment samples of the same washed suspension (about $2 \times 10^8$ bacteria/ml.) of *E. coli* K112 were infected with λ phage in $MnSO_4$, $SrNO_3$ or $CaCl_2$ at final concentration and input multiplicities shown in the table. Adsorption period: 10 min. Numbers are the mean of three plates and refer to same dilution of original suspension of infected bacteria. Percentage response is expressed relative to the total number of cells giving the lytic, lysogenic and refractory response. Expt. 4 shows effect of multiplicity of infection (in 0·02 M-$MnSO_4$) on type of response. Multiplicities of infection shown in parentheses and calculated from phage remaining unadsorbed at end of 10 min. in sample from mixture with lowest input multiplicity (found: 14% phage unadsorbed) and assuming that bacteria giving refractory response did not adsorb λ phage.

| Expt. no. | Infection medium | Input multiplicity (λ particles/ bacterium) | Type of response: Lytic No. | Lytic % | Lysogenic No. | Lysogenic % | Refractory No. | Refractory % |
|---|---|---|---|---|---|---|---|---|
| 1 | 0·02 M-$MgSO_4$ | 10 | 7 | 6 | 104 | 91 | 3 | 3 |
| | 0·02 M-$MnSO_4$ | 10 | 54 | 46 | 52 | 45 | 11 | 9 |
| | 0·01 M-$MnSO_4$ | 10 | 41 | 38 | 58 | 54 | 9 | 8 |
| | 0·004 M-$MnSO_4$ | 10 | 9 | 7 | 11 | 9 | 101 | 84 |
| 2 | 0·02 M-$MgSO_4$ | 17 | 6 | 6 | 85 | 90 | 4 | 4 |
| | 0·02 M-$Sr(NO_3)_2$ | 17 | 15 | 15 | 82 | 81 | 4 | 4 |
| | 0·01 M-$Sr(NO_3)_2$ | 17 | 13 | 12 | 63 | 58 | 32 | 30 |
| | 0·004 M-$Sr(NO_3)_2$ | 17 | 3 | 3 | 8 | 7 | 97 | 90 |
| 3 | 0·02 M-$MgSO_4$ | 12 | 21 | 17 | 100 | 79 | 6 | 4 |
| | 0·02 M-$CaCl_2$ | 11 | 32 | 22 | 109 | 76 | 3 | 2 |
| | 0·01 M-$CaCl_2$ | 11 | 56 | 37 | 88 | 58 | 7 | 5 |
| | 0·004 M-$CaCl_2$ | 11 | 9 | 5 | 3 | 2 | 166 | 93 |
| 4 | 0·02 M-$MnSO_4$ | 16·8 (16·4) | 31 | 34 | 50 | 54 | 11 | 12 |
| | | 8·4 (8·0) | 20 | 21 | 63 | 69 | 9 | 10 |
| | | 4·2 (4·4) | 22 | 23 | 56 | 58 | 19 | 19 |
| | | 0·8 (1·8) | 9 | 9 | 32 | 31 | 62 | 60 |

suspensions showed that less than 5% of the total count of organisms was in chain form. Further evidence that the plate assay technique used gave a true record of response was obtained by determining the response by plate assay, and also by incubating a sample in liquid medium at 37°, and taking samples at intervals for a standard one-step curve of the number of plaque-forming units present. Phage released by lysis of bacteria in the second growth tube (nomenclature of one-step curve technique) should remain free and could be assayed accurately. If an appreciable number of the bacteria present were in chains and were giving a true lytic response, but appeared as lysogenic on the lactose + indicator plates (medium C) or if samples of the first growth tube were plated on medium B, then one would

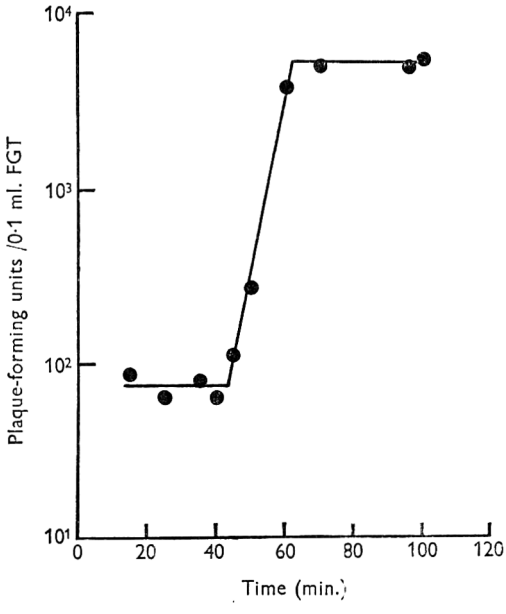


Fig. 1

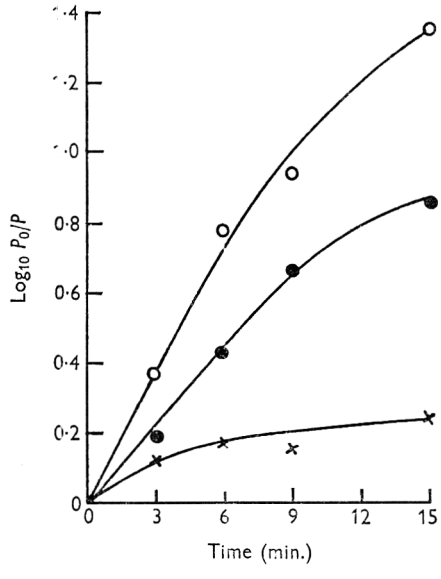


Fig. 2

Fig. 1. Determination of yield of phage particles/bacterium giving the lytic response. Washed suspension of *Escherichia coli* K112 ($1 \times 10^3$ bacteria/ml.) infected in 0·02 M-$MgSO_4$ with $\lambda$ phage ($1{\cdot}6 \times 10^8$ particles/ml.). After 10 min., sample of infected bacteria transferred to $\lambda$ phage antiserum in broth (10 min.) and then first and second growth tubes prepared (dilutions of $10^{-4}$ and $2 \times 10^{-6}$ respectively). Samples taken at intervals and plated with indicator bacteria on medium B. Results are expressed as plaque-forming units corresponding to 0·1 ml. of first growth tube (FGT). Overall response to infection in an equivalent sample determined after 20 min. showed (bacteria/sample) 90 lytic, 820 lysogenic and 20 refractory.

Fig. 2. Rate of adsorption of phage $\lambda$ to *Escherichia coli* K112 in 0·01 M-$MnSO_4$ (×), 0·02 M-$MnSO_4$ (○) or 0·02 M-$MgSO_4$ (●). Washed suspension ($2{\cdot}5 \times 10^8$ bacteria/ml.) mixed at 37° with equal volume of phage suspension in $MnSO_4$ or $MgSO_4$ solution. Samples taken at intervals to determine unadsorbed phage, $P$, at time $t$; $P_0$ = concentration of phage at time 0. Bacteria + phage suspension at pH 6·6; input multiplicity 2. Bacteria lytic:lysogenic:refractory response (%) at 15 min. was: in 0·02 M-$Mn^{2+}$, 46:19:35; in 0·02 M-$Mg^{2+}$, 18:64:28; in 0·01 M-$Mn^{2+}$, 17:14:69.

expect that the calculated yield of phage particles/bacterium giving the lytic response would be greater than normal, since the number of lytic bacteria would be underestimated. In fact the yield was found to be 84 $\lambda$ particles/bacterium (Fig. 1),

which is similar to that found by other workers (Jacob & Wollman, 1954). After infection, the decision of a bacterium to give the lysogenic response or the lytic response may be altered by cultural conditions (cf. Lieb, 1953) and it is possible that the small percentage of bacteria which show the lytic response after infection in 0·02 M-$MgSO_4$ (cf. Table 1) is in fact due to bacteria which would become lysogenic if they were maintained in liquid medium and not subjected to the temperature shocks, etc., involved in plating procedures. However, the production of free phage by such a population of newly infected bacteria during their first 80 min. in broth, and the order of the phage yield/bacterium giving the lytic response, together provide evidence for the conclusion that the plate assay gives a true measure of the response to infection with phage λ.

*Response to adsorption of phage λ in the presence of* $Mn^{2+}$, $Sr^{2+}$ *and* $Ca^{2+}$

Samples of the same washed bacterial suspension were infected with λ phage in the presence of different concentrations of $Mn^{2+}$ or in 0·02 M-$MgSO_4$ (control). About 90 % of the bacteria were infected when the absorption medium was 0·02 M- or 0·01 M-$MnSO_4$, and 16 % when the concentration was 0·004 M (Table 1, expt. 1). After adsorption in the presence of $MgSO_4$, 91 % of the bacteria were lysogenic but only about half of the bacteria infected in MnSO gave a lysogenic response; the remainder gave the lytic response (Table 1). Irrespective of the multiplicity, infection in the presence of $Mn^{2+}$ always yielded an appreciable percentage of bacteria which gave the lytic response (Table 1, expt. 4). Studies of the rate of uptake of phage λ in the presence of $MnSO_4$ (0·01 M and 0·02 M) compared with uptake in the presence of 0·02 M-$MgSO_4$, showed that uptake in 0·02 M-$MnSO_4$ took place more quickly (60 % faster) than in 0·02 M-$MgSO_4$ (Fig. 2). Once again the bacteria infected in $Mn^{2+}$ tended to give the lytic response whereas those infected in $Mg^{2+}$ were mainly lysogenic. Uptake of λ phage in the presence of 0·01 M-$MnSO_4$ was comparatively poor (Fig. 2). The effect of different concentrations of $Sr^{2+}$ (as $SrNO_3$) and of $Ca^{2+}$ (as $CaCl_2$) was also studied. Bacteria infected in presence of either of these cations tended to respond like bacteria infected in $Mg^{2+}$, i.e. when the cation was 0·02 M about 95 % of the bacteria were infected and most of these (75–80 %) gave the lysogenic response (Table 1, expts. 2 and 3), when exposed to a high multiplicity of λ phage. There has always been the possibility that infection in the presence of a cation other than $Mg^{2++}$ might yield a lysogenization rate of nearly 100 %, but conditions for this ideal situation have not yet been found. No evidence has been found that the type of anion affects the adsorption of λ phage.

*Use of zygotic induction to detect when prophage becomes established in bacteria giving a lysogenic response*

Organisms of *Escherichia coli* strain HfrC $B_1^-$ $Sr^s$ $T_6^s$ were infected with phage $\lambda_{22}$ in conditions such that a large proportion of the infected bacteria gave the lysogenic response (assayed by the usual method; the response was found to be 9 % lytic, 80 % lysogenic, 11 % refractory). The infected bacteria were then incubated in broth + antiserum to λ phage and samples taken at intervals and incubated with *E. coli* w678-$T^-L^-B_1^-Sr^rT_6^sF^-$ in broth + λ phage antiserum and mating allowed to continue for 20 min. A sample of the mating mixture was then transferred to

broth +0·02 M-KCN + λ phage antiserum. After 30 min., samples were diluted and plated (*a*) for $T^+L^+$ recombinants and (*b*) with the indicator organism *E. coli* K112 $Sr^sF^+$ for plaques. Meanwhile other samples of the infected Hfr bacteria were taken at intervals, transferred directly to broth + KCN + λ phage antiserum for 30 min., and then diluted and plated with indicator organisms to give the number of plaques produced by unmated Hfr bacteria. The number of $T^+L^+$ recombinants produced by uninfected HfrC bacteria taken through the same experimental procedure

Table 2. *Zygotic induction with* Escherichia coli *strain* HfrC *in which lysogeny for λ phage is being established*

Washed suspension ($1 \times 10^8$ bacteria/ml.) of *E. coli* HfrC infected in 0·02 M-$MgSO_4$ with λ phage (multiplicity 10 λ particles/bacterium), and after 10 min. a sample transferred to broth + λ phage antiserum (culture A). Samples (0·1 ml.) of latter taken at intervals and mated with $F^-$ strain in broth + antiserum (0·9 ml. containing $2 \times 10^7$ *E. coli* W678/ml.). After mating for 20 min., a sample was transferred to broth + KCN + antiserum for 30 min., and then diluted in saline and plated for (*a*) plaque formation, (*b*) recombinants ($T^+L^+$) able to synthesize threonine and leucine. Uninfected = $T^+L^+$ recombinants produced by uninfected Hfr bacteria taken through same procedure. Samples of infected Hfr bacteria taken at intervals from culture A, incubated in broth + KCN + antiserum for 30 min., and then plated for plaque formation. Time after infection is the time from when infected bacteria were placed in culture A. Plaques and $T^+L^+$ recombinants = no./plate. Other details in text.

| Bacteria mated | | | Bacteria not mated | | Plaques due to zygotic induction (A − B) |
|---|---|---|---|---|---|
| Time after infection (min.) | Plaques (A) | $T^+L^+$ recombinants | Time after infection (min.) | Plaques (B) | |
| 0 | 123 | 65 | 20 | 150 | — |
| 10 | 121 | 140 | 30 | 145 | — |
| 20 | 172 | 164 | 40 | 197 | — |
| 30 | 146 | 174 | 50 | 135 | 9 |
| 40 | 182 | 223 | 60 | 71 | 111 |
| 50 | 203 | 251 | 70 | 64 | 139 |
| 60 | 177 | 295 | 80 | 65 | 112 |
| | Uninfected | 198 | | | |

(i.e. incubation in 0·02 M-$MgSO_4$, mated for 20 min. and then cyanide broth treatment), was used to control the effect of infection with λ phage on the mating properties of strain HfrC. The broth containing KCN was used to eliminate Hfr bacteria containing mature phage particles (method of Doerman, 1952; used for λ phage by Weigle & Delbrück, 1951), since in preliminary experiments without this step, lysis of some of the Hfr bacteria giving the lytic response occurred during the plating procedure and caused unexpectedly large numbers of plaques on the plates. Plating $Sr^s$-Hfr bacteria infected with λ phage without mating (column B, Table 2), leads to the formation of some plaques, even in presence of streptomycin. This was not due to mating on the plate, and it is concluded that phage development in some Hfr bacteria was not halted by plating on streptomycin media. The mating period was 20 min. in nutrient broth, and during this time it is reasonable to expect that development of λ phage still continues in Hfr bacteria giving the lytic response. Consequently, plaque formation due to zygotic induction is taken as the difference between the number of plaques produced by mating a sample of the Hfr bacteria taken *t* min. after infection and the number produced by unmated Hfr bacteria

taken $t+20$ min. after infection. If the prophage is established in the Hfr bacteria very soon after infection (i.e. within a few minutes), then the number of plaques produced by zygotic induction after a mating period of 20 min. should be maximal, and should not vary with the time after infection at which the Hfr sample was taken for mating with the $F^-$ organism. If, however, an appreciable period must elapse after infection before the prophage is established in the Hfr bacteria, then the number of plaques should increase to a maximum, as the samples of Hfr bacteria are taken for mating at progressively longer intervals after infection. The number of plaques reached a maximum when the Hfr bacteria had been allowed to metabolize for some 40 min. in broth before being mated with the $F^-$ organism (Table 2). After 10 min. in broth, the infected bacteria yielded 70 % of the number of $T^+L^+$ recombinants produced by uninfected bacteria (Table 2). It is therefore concluded that the mating properties of Hfr bacteria newly infected with phage λ were not disturbed to any appreciable extent.

*Effect of ultraviolet radiation on bacteria in which lysogeny for λ phage is being established*

When the genome of λ phage in an infected bacterium has reached the prophage state then, provided the aptitude of the bacterium has not been upset (Lwoff, 1953), it is to be expected that exposure of such a bacterium to u.v. radiation would lead to the induction of phage development. Irradiation of the bacteria after infection with phage $\lambda_{22}$, but before they were placed in a growth medium, showed that the effect of u.v. radiation was to cause a decrease in the number of bacteria which gave the lysogenic response and, especially for the lower doses of u.v. radiation, most of this decrease was correlated with an increase in the number of bacteria which gave the lytic response (Table 3). Though at first sight this result might appear to be due to the induction of truly lysogenic bacteria, this is regarded as being unlikely, since irradiation of newly infected bacteria after they had been for 20 min. in a growth medium showed that although the irradiation caused a decrease in the number of bacteria which gave the lysogenic response, this was only correlated with a relatively small increase in the number of bacteria which gave the lytic response (Table 3). Moreover it has already been shown (Fry, 1959) that when the bacteria are u.v. irradiated before infection, then depending on the dose received, some of the bacteria which are no longer capable of giving the lysogenic response when infected with phage $\lambda_{22}$ give a lytic response. It is therefore likely that the effect of u.v. irradiation on the phage + bacterium complex in newly infected bacteria before transfer to a growth medium is the same as that found when the bacteria are first u.v. irradiated and then immediately infected with phage $\lambda_{22}$, i.e. in both cases, the u.v. radiation acts on a target in the bacterium which is distinct from phage material, and thus disposes the bacterium to the vegetative reproduction of phage.

In a further experiment, bacteria infected in 0·02 M-$MgSO_4$ with phage $\lambda_{22}$ were transferred after 10 min. to complete medium containing λ phage antiserum. Samples were taken at intervals and u.v.-irradiated with a dose known to give 90–100 % induction of the parent *Escherichia coli* K112 lysogenic for phage $\lambda_{22}$. Inspection of Table 4 reveals that not until 100 min. had elapsed did the number of bacteria giving a lytic response after irradiation become equivalent to the number

Table 3. *Effect of exposure of λ-infected* Escherichia coli *to ultraviolet radiation immediately after infection with λ phage and after a short period in complete medium*

Washed suspension of *Escherichia coli* K112 ($2{\cdot}6 \times 10^8$ bacteria/ml.) mixed with an equal volume of λ phage ($2{\cdot}7 \times 10^9$ particles/ml.) in 0·02 M-$MgSO_4$ (average multiplicity 10 λ phages/bacterium). After 10 min. sample A of infected bacteria was taken and irradiated with doses of u.v. radiation and then incubated in λ phage antiserum in broth for 45 min., diluted in broth and plated. Meanwhile sample B of the infected bacteria was transferred to broth + antiserum, incubated for 20 min. and then diluted 1/10 with mineral salts medium and irradiated as required. Samples were taken and incubated in broth for 45 min. and then plated. Unirradiated controls were not incubated for the additional 45 min. Platings were made with indicator bacteria on medium C. 'Induction' is percentage of lytic responses relative to lysogenic bacteria in corresponding unirradiated control. The effects of the same dose of u.v. radiation on the survival of uninfected *E. coli* K112 and on the induction of *E. coli* K112 ($\lambda_{22}$) in equivalent conditions are given for comparison.

| Time of u.v. irradiation (sec.) | Type of response | | | Survival of lysogenic bacteria (%) | 'Induction' (%) | Survival of strain K112 (%) | Induction of strain K112 ($\lambda_{22}$) (%) |
|---|---|---|---|---|---|---|---|
| | Lytic | Lysogenic | Refractory | | | | |
| Sample A | | | | | | | |
| 0 | 9 | 115 | 2 | — | — | — | — |
| 10 | 34 | 77 | 10 | 67 | 30 | 84 | 17 |
| 20 | 60 | 51 | 4 | 44 | 52 | 69 | 34 |
| 30 | 52 | 35 | 13 | 30 | 45 | 61 | 58 |
| 60 | 87 | 5 | 6 | 4 | 76 | 34 | 97 |
| Sample B | | | | | | | |
| 0 | 6 | 103 | 2 | — | — | — | — |
| 10 | 13 | 84 | 2 | 82 | 13 | — | — |
| 20 | 20 | 63 | 11 | 61 | 19 | — | — |
| 30 | 22 | 52 | 2 | 51 | 21 | — | — |
| 60 | 44 | 22 | 2 | 21 | 43 | — | — |

Table 4. *Effect of optimal induction dose of u.v. radiation on* Escherichia coli K112 *in which lysogeny for λ phage is being established*

Washed suspension of *Escherichia coli* K112 ($2{\cdot}6 \times 10^8$ bacteria/ml.) mixed with an equal volume of phage $\lambda_{22}$ suspension ($2{\cdot}3 \times 10^9$ particles/ml.) in 0·02 M-$MgSO_4$ (average multiplicity 9 λ phages/bacterium), and after 10 min. sample of infected bacteria transferred to λ phage antiserum in broth and incubated. Samples of the latter were diluted at appropriate times in broth and one portion diluted further and plated immediately ('unirradiated' in table) whilst another portion was irradiated with a dose of u.v. radiation which would normally induce at least 90 % of strain K112 ($\lambda_{22}$). The irradiated bacteria were transferred to broth + antiserum, incubated for 45 min. to stabilize response to irradiation and then plated. Other details as in Table 3.

| Time (min.) | Unirradiated | | u.v. irradiated | | 'Induction' (%) |
|---|---|---|---|---|---|
| | Lytic | Lysogenic | Lytic | Lysogenic | |
| 10 | 10 | 77 | 38 | 11 | 49 |
| 35 | 7 | 85 | 44 | 2 | 52 |
| 60 | 19 | 97 | 61 | 1 | 63 |
| 80 | 16 | 121 | 95 | 1 | 79 |
| 100 | 5 | 140 | 139 | 0 | 99 |
| 120 | 4 | 202 | 206 | 4 | 102 |
| 140 | 0 | 348 | 386 | 8 | 111 |
| 160 | 0 | 606 | 562 | 4 | 93 |

of bacteria giving the lysogenic response in the unirradiated samples. Thus an appreciable time appears to be required before the prophage is in an inducible state in all the bacteria which become lysogenic. With samples taken earlier than 100 min. the decrease in the number of lysogenic bacteria in the irradiated culture was less than the increase in the number of bacteria which gave the lytic response. This indicates that for about 80 min. after infection, some bacteria which gave the lysogenic response were in such a state that u.v. irradiation produced a lethal effect and did not induce bacteria in the normal manner.

*Effect of chloramphenicol on the establishment of lysogeny for λ phage*

In certain systems, treatment with chloramphenicol is reported to alter the response of bacteria infected with temperate phages. Thus with *Salmonella typhimurium* and phage P22, chloramphenicol added early after infection caused 'curing' of the bacteria, but when added several minutes later, it brought about an increase in the number of bacteria which became lysogenic (Ting, 1960). With

Table 5. *Effect of chloramphenicol on the establishment of lysogeny in* Escherichia coli K112

Washed suspension of *Escherichia coli* K112 infected with λ phage in 0·02 M-$MgSO_4$ and one sample transferred to broth and one to broth containing chloramphenicol (30 μg./ml.). Samples taken at appropriate times, diluted ten times with 0·9% NaCl and one portion plated immediately whilst another was irradiated and then incubated for 45 min. in broth to stabilize response and then plated. All broths contained λ phage antiserum. Other details in table. Dose of u.v. radiation for 60 sec. will give 90–100% induction of *E. coli* strain K112 ($\lambda_{22}$) in similar conditions.

| Treatment after infection | Time of u.v. irradiation (sec.) | Type of response | | |
|---|---|---|---|---|
| | | Lytic | Lysogenic | Refractory |
| Broth (10 min.) | 0 | 2 | 76 | 3 |
| | 60 | 9 | 22 | 1 |
| Broth + chloramphenicol (45 min.) | 0 | 64 | 14 | 2 |
| | 60 | 45 | 17 | 2 |
| Broth + chloramphenicol (45 min.) then broth (10 min.) | 0 | 18 | 49 | 3 |
| Broth (30 min.) | 0 | 1 | 73 | 3 |
| | 60 | 9 | 26 | 2 |
| Broth (30 min.) then broth + chloramphenicol (45 min.) | 0 | 4 | 56 | 5 |
| | 60 | 1 | 28 | 3 |
| | 120 | 3 | 10 | 2 |

*Shigella dysenteriae* infected with phage P1, the addition of chloramphenicol at the time of infection or 10–15 min. later caused the prompt establishment of lysogeny (detected by immunity to superinfection by phage $T_1$) in those bacteria which gave the lysogenic response to infection (Christensen, 1960). Studies of the effect of chloramphenicol on *Escherichia coli* infected with phage $\lambda_{22}$ did not show any comparable effects. When *E. coli* infected in 0·02 M-$MgSO_4$ were transferred to broth + chloramphenicol (+ λ phage antiserum) for 45 min. and then plated to determine their response to infection, although there was no curing of the bacteria from phage infection, there was a marked increase in the number of bacteria which gave the lytic response and a decrease in those which were lysogenic (Table 5). This result indicates that immediately after infection, the bacteria were

in a labile state in the sense that environmental conditions could still affect their type of response. When newly infected bacteria were transferred to broth (i.e. with no chloramphenicol) and plated after only 2 min., the lytic response was greater than with samples taken after 5 min. or later. (A period less than 2 min. in broth is difficult to study, since sufficient time must be allowed for inactivation of unadsorbed phage by antiserum before plating.) Evidently when bacteria can metabolize normally, a change occurs in the first few minutes which stabilizes the decision to become lysogenic and nullifies the adverse effects of the plating procedure. The predominantly lytic response of bacteria plated immediately after exposure to broth + chloramphenicol for 45 min. might mean that either this change had not taken place in these bacteria or it had not been irreversibly established. Since chloramphenicol inhibits protein synthesis but not the synthesis of ribo- and deoxyribonucleic acids (Gros & Gros, 1958), it may tentatively be concluded that the first step in the decision to become lysogenic involves the synthesis of protein. Treatment with chloramphenicol for 45 min. immediately after infection had no long lasting detrimental effect, since such bacteria after incubation for only 10 min. in broth in the absence of chloramphenicol showed the expected predominantly lysogenic response (Table 5). Treatment of infected bacteria with chloramphenicol for 45 min. after they had first been incubated in broth alone for 30 min. was without significant effect; most of the bacteria still give the lysogenic response on plating. When u.v. irradiation is used to detect an established prophage, then it is clear that treatment of bacteria with chloramphenicol even 30 min. after infection did not lead to the immediate establishment of lysogeny, since although the irradiation caused a decrease in the number of bacteria which gave the lysogenic response, this was not correlated with a corresponding increase in those which gave the lytic response (Table 5). Indeed the results were identical with those discussed previously (p. 303). Furthermore, it may be concluded that even after 30 min. in a complete growth medium, the processes which enable the bacteria giving the lysogenic response to become fully inducible could not take place (or continue) in the presence of chloramphenicol.

## DISCUSSION

The experiments concerned with the adsorption of phage $\lambda_{22}$ to *Escherichia coli* in the presence of inorganic cations showed that $Sr^{2+}$, $Ca^{2+}$ and $Mn^{2+}$ can replace $Mg^{2+}$ (Table 1). It is well known that although these ions are related and have several properties in common, the similarities between $Mg^{2+}$, $Sr^{2+}$ and $Ca^{2+}$ are greater than those between $Mg^{2+}$ and $Mn^{2+}$. It is therefore not surprising that whilst all of them will aid the infection of *E. coli* by phage $\lambda_{22}$, the results with $Sr^{2+}$ and $Ca^{2+}$ are more akin to those obtained with $Mg^{2+}$ than with $Mn^{2+}$. However, whereas 75–80% of the bacteria infected in the presence of $Ca^{2+}$ and $Sr^{2+}$ become lysogenic, infection in optimal conditions in the presence of $Mg^{2+}$ usually gives a 90% lysogenic response, and $Mg^{2+}$ is still the cation of choice for infection in defined conditions. Adsorption of phage $\lambda$ to sensitive bacteria can take place in broth (cf. Jacob & Wollman, 1954), which usually contains $Mg^{2+}$, though not at a concentration of 0·02 M. Unpublished experiments by the author with single amino acids at different concentrations have not shown any amino acid which by itself promoted the adsorption of phage $\lambda_{22}$ to *E. coli* strain K112.

Bacteria which are becoming lysogenic for λ phage show a delay in cell division and disturbance of their metabolism (Fry & Gros, 1959). These findings could be explained on the basis that they reflect the time required for the prophage to become established or, that the prophage may be established soon after infection but the events connected with this process cause a profound upset in the metabolism of the host bacterium which takes an appreciable time to recover. From the results of the chloramphenicol experiments (Table 5) it is tentatively suggested that in the decision to become lysogenic there is a primary reaction which involves protein synthesis rather than the synthesis of nucleic acid.

The two tests used here to detect establishment of the prophage, namely zygotic induction and induction of prophage by u.v. irradiation, are admittedly indirect. Meaningful results are only obtained if, immediately after establishment of the prophage, the physiological state and metabolism of the host bacteria are not abnormal. The mating potential of newly infected Hfr bacteria is somewhat less than that of non-infected bacteria as shown by the yield of $T^+L^+$ recombinants. Nevertheless, 10 min. after infection it is 70% of that produced by the uninfected control bacteria, and since the prophage is carried at a definite locus on the bacterial chromosome (Jacob & Wollman, 1958), it is difficult to understand how, if the prophage were established very soon after infection (i.e. within 5–10 min.), transfer of prophage genetic material could not take place whilst recombination for $T^+L^+$ genes proceeded more or less normally. From the zygotic induction experiment it is therefore concluded that establishment of the prophage takes place about 40 min. after infection (Table 2). The time is not precise, because prophage material may be added to the Hfr chromosome during the mating period up to the time of entry of the prophage locus into the $F^-$ organism. The results of the zygotic induction experiment would be more convincing if all the Hfr bacteria could have been eliminated from the sample plated with the indicator organism, so that any plaque formation observed in such conditions would unequivocally be due to phage development in the $F^-$ bacteria alone. This was attempted by using a phage $T_6^s$ Hfr strain and a w678 $T_6^r$ $F^-$ mutant, and treating samples of the mating cultures with a high multiplicity of phage $T_6$ (sufficient to cause lysis from without) in the presence of λ phage antiserum (method of Hayes, 1957) before plating for recombinants and plaque formation. However when this method was used in trial experiments with strain $T_6^s$ HfrC lysogenic for phage $\lambda_{22}$, the number of plaques due to zygotic induction was markedly less than in the control where the procedures of Jacob & Wollman (1956) were followed and platings were made directly on media containing streptomycin. There are two possible explanations of these results. With this Hfr strain the gene for $T_6$ sensitivity precedes entry of genetic material of the prophage into the $F^-$ bacterium (Jacob & Wollman, 1958), and a large number of the $F^-$ bacteria may thus become sensitive to phage $T_6$ and would consequently be eliminated by the Hayes-$T_6$ method before being plated. However, if this were so, one would also expect the number of $T^+L^+$ recombinants to show a significant decrease in the $T_6$-treated samples, and this was not observed. It is therefore possible that the $T_6$ phages inject their DNA into the $T_6^s$ $F^-$ organism, and although incapable of $T_6$ production, this DNA interferes with the vegetative development of λ phage in those bacteria in which zygotic induction is taking place (cf. Weigle & Delbrück, 1951).

In the induction experiment, bacteria which showed the lysogenic response were not all inducible until about 100 min. after infection. Though this time differs from that obtained in the mating experiment, it is in keeping with the observation (Fry & Gros, 1959) that bacteria in which lysogeny is being established do not grow and metabolize normally until 60–80 min. after infection. So that, even though the prophage may be established in about 40 min., detection by induction might not be possible because the aptitude of at least some of the bacteria for induction and phage development was not normal (cf. Lwoff, 1953). The mating experiment and the induction experiment were done with different strains of bacteria, and it is possible that if they had been done at the same time with the same bacterial suspension closer agreement in times for the establishment of the prophage might have been found.

Chloramphenicol did not seem to promote the establishment of lysogeny (determined by inducibility of prophage), even when added after 30 min. growth in complete medium (Table 5). On the other hand, with *Shigella dysenteriae* infected with phage $P_1$, the addition of chloramphenicol immediately after infection or within 10–15 min., caused the prompt establishment of lysogeny (Christensen, 1960). However, here the detection of lysogeny depended on the expression of immunity to infection by phage $T_1$, and it is possible that immunity is expressed before the prophage is in fact established. The relationship of immunity to establishment of the prophage for phage $\lambda$ is being studied in *Escherichia coli* and will be reported separately.

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# The Influence of Some Metal Ions and pH on the Inactivation of Vaccinia Virus by Heat

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SUMMARY

The addition of metal ions to the suspending medium of vaccinia virus preparations made them more resistant to heat. Monovalent metals were more effective than divalent, but a mixture of the two kinds was the most effective. Heavy metals rapidly destroyed virus infectivity. The protective effect is probably due to the formation of metal-protein complexes with increased resistance to heat denaturation.

## INTRODUCTION

Earlier work (Kaplan, 1958) showed that the inactivation by heat of vaccinia virus does not follow first order kinetics to completion. The inactivation curve has a sharp inflexion where the inactivation rate suddenly decreases. In those experiments the virus preparations used were all suspended in dilute phosphate + citric acid buffer (McIlvaine, 1921) pH = 7·2, $Na_2HPO_4 = 4 \times 10^{-3}$M. Kaplan & Micklem (1961) reported that the stability of smallpox vaccine was enhanced by the addition to the suspending fluid of $10^{-1}$M-$Na_2HPO_4$. In the work reported here suspending fluids of different compositions and various molarities were used, and the influence of hydrogen ion concentration on the inactivation of vaccinia virus infectivity was determined.

## METHODS

*Virus.* The experiments were done with the Lister Institute vaccine strain adapted to the chick embryo chorioallantoic membrane. A suspension (L. 13) was prepared by inoculating the chorioallantoic membranes of ten 12-day chick embryos with about $10^6$ pock forming infectious units (i.u.) of virus. After incubation for 2 days at 35·5° the infected chorioallantoic membranes were harvested with sterile precautions, washed in cold sterile dilute McIlvaine buffer and extracted in 50 ml. dilute buffer +10 ml. Arcton 113 (trifluorotrichloroethane, I.C.I.; Gessler, Bender & Parkinson, 1956) by mechanical disruption in the chilled, stainless steel chamber of a Servall 'Omnimix'. The extract was centrifuged at 2,000 rev./min. for 5 min. in the 240 head of an International Centrifuge, size 1, type SBV. The supernatant fluids of this centrifugation were re-extracted with another 10 ml. of Arcton and centrifuged again. The second supernatant fluid of suspension L. 13 was kept at 4° for a week, during the titration of potency, and then stored at −70° in 1 ml. volumes in sealed ampoules.

*Infectivity titrations* were made by pock count in the chorioallantoic membranes

of 12-day chick embryos (Westwood, Phipps & Boulter, 1957). All dilutions were made in dilute McIlvaine buffer.

*Heat inactivation experiments.* Virus was thawed, and diluted in the appropriate suspending fluid to give an initial titre of about $10^7$ i.u./ml. at the pH value or metal ion concentration required. One ml. volumes were then dispensed into small test tubes (50 × 12 mm.) which were placed in a water bath whose temperature was maintained to within ±0·05°. Tubes were removed at intervals and chilled in melting ice. Residual virus was then titrated and from the results the velocity constant ($K$) of the inactivation was calculated from the formula $\log V_0/V = Kt/2{\cdot}3$, where $V_0$ = initial virus titre, and $V$ = residual virus titre at any time $t$. Only the velocity constants ($K_f$) from the fast inactivation portion of the curve were compared (Kaplan, 1958) since differences in heat resistance were more obvious here than in the slow inactivation part ($K_s$). Some of the experiments were done at only one temperature; where possible, however, three temperatures were used. Gard (1959) emphasized the need for many repetitions of the same experiment to ensure a reliable estimate of the mean inactivation rate of a virus under any given conditions. Most of the tests were repeated four to ten times.

*Suspending fluids.* In most of the experiments virus was suspended in McIlvaine's phosphate + citric acid buffer, pH 7·2; $Na_2HPO_4$ = $10^{-1}$M, $10^{-2}$M or $10^{-3}$M. In some, solutions of other salts of sodium or solutions of salts of other metals were used. Analar grade reagents were dissolved in deionized water.

## RESULTS

### *Influence of metal ion concentration*

At pH 7·2 the reaction velocity of the inactivation decreased as the concentration of $Na_2HPO_4$ in the buffer increased (Table 1(*a*)). To determine whether this effect was attributable to the cation or the anion, the experiment was repeated at 50° with virus suspended in sodium acetate and sodium chloride (Table 1(*b*)). Clearly, the cation was responsible for the stabilization of infectivity. Potassium (Table 1(*c*)) replaced sodium. Two experiments only were done with lithium; the results suggest that this element, too, could replace sodium.

Divalent metal ions were also investigated (Table 1(*d*)). At 50° $10^{-1}$M-$Mg^{2+}$ and $10^{-1}$M-$Ca^{2+}$ were less effective than $10^{-1}$M-$Na^+$. There was no indication of an increased rate of inactivation. At 55° and 60° $10^{-1}$M-$Mg^{2+}$ was less effective than $10^{-1}$M-$Na^+$ but more effective than $10^{-3}$M-$Na^+$. A mixture of $10^{-1}$M-$Na^+$ and $10^{-1}$M-$Mg^{2+}$ stabilized vaccinia virus infectivity more effectively than either ion separately (Table 1(*e*)).

A few experiments were done with salts of the heavy metals, copper, cobalt and iron. These inactivated virus very rapidly. The experiments were complicated, however, by the toxicity of these heavy metals for the chick embryo chorioallantoic membrane, so that the data were insufficient for calculation of reaction velocities.

### *Influence of* pH *value*

Virus was heated in McIlvaine buffer, $Na_2HPO_4$ = $10^{-1}$M and $10^{-3}$M, pH = 6 and 7·2 at each molarity (Table 2). The inactivation rate was clearly increased in the acid medium.

Table 1. *The influence of various ions and temperatures on the reaction velocities ($K_f$) of heat inactivation of vaccinia virus*

| Substance | Concentration (M) | $K_f$ (min.$^{-1}$)* 50° | 55° | 60° |
|---|---|---|---|---|
| (*a*) $Na_2HPO_4$ | $10^{-1}$ | 0·07 | 0·37 | 2·3 |
| | $10^{-2}$ | 0·08 | n.t. | n.t. |
| | $10^{-3}$ | 0·10 | 0·79 | 5·8 |
| Deionized water | | 0·18 | n.t. | n.t. |
| (*b*) $CH_3COONa$ | $10^{-1}$ | 0·07 | n.t. | n.t. |
| NaCl | $10^{-1}$ | 0·07 | n.t. | n.t. |
| (*c*) KCl | $10^{-1}$ | 0·06 | 0·42 | n.t. |
| NaCl | $10^{-1}$ | 0·07 | 0·48 | n.t. |
| (*d*) $CaCl_2$ | $10^{-1}$ | 0·12 | n.t. | n.t. |
| $MgSO_4$ | $10^{-1}$ | 0·11 | 0·46 | 3·2 |
| $MgSO_4$ | $10^{-2}$ | 0·15 | n.t. | n.t. |
| (*e*) NaCl + $MgSO_4$ | $10^{-1}$ + $10^{-2}$ | 0·05 | n.t. | n.t. |
| NaCl + $MgSO_4$ | $10^{-1}$ + $10^{-1}$ | 0·006 | n.t. | n.t. |

* Each value is the mean of four to ten tests.
n.t. Not tested.

Table 2. *The influence of* pH *value and sodium concentration on the reaction velocity ($K_f$) of vaccinia virus inactivation*

| Sodium conc. (M) | Temp. | $K_f$ (min.$^{-1}$) at pH 6 | pH 7·2 |
|---|---|---|---|
| $10^{-1}$ | 50° | 0·2 | 0·07 |
| | 55° | 0·61 | 0·37 |
| $10^{-3}$ | 50° | 0·24 | 0·1 |
| | 55° | 1·1 | 0·79 |

## DISCUSSION

Several authors (Burnet & McKie, 1930; Adams, 1949; Lark & Adams, 1953; Northrop, 1955) have shown that the heat stability of bacteriophage suspensions depends on the concentrations of cations in the suspending fluids. Stability was generally increased when small concentrations of divalent cations were added to a basic concentration of monovalent cation, although monovalent cations by themselves conferred less protection than distilled water (Burnet & McKie, 1930). Wallis, Yang & Melnick (1962) reported that 2 M-$Na^+$ completely protected vaccinia virus for 4 hr. at 50° and at least 24 hr. at 37°; M-$Na^+$ was less effective, while M-$Mg^{2+}$ and M-$Ca^{2+}$ increased the rate of destruction of infectivity. The results of Wallis *et al.* (1962) are not comparable with ours, since the concentrations of cations tested were so different. It is noteworthy, however, that the highest concentrations of $Mg^{2+}$ ($10^{-1}$ M) in our experiments had an appreciable protective action at 55° and 60°, although at 50° there was no significant difference between the inactivation rates in $10^{-3}$ M-$Na^+$ and in $10^{-1}$ M-$Mg^{2+}$ or $Ca^{2+}$. Since Wallis *et al.* (1962)

did not record the provenance or mode of preparation of the vaccinia virus they used, comparison with their results would in any case be unrewarding.

The findings in our experiments agree, in general, with those of workers with bacteriophage. However, the mode of action of the cations is not entirely clear. Woese (1960) suggested that, at least at high temperatures, heat inactivation of viruses might as well be due to denaturation of nucleic acid as to denaturation of protein. Rice & Doty (1957) found that a highly polymerized preparation of deoxyribonucleic acid (DNA) began to change viscosity at 85° but not at lower temperatures in the absence of substances (e.g. urea) effective in denaturing proteins. If it be assumed that native DNA behaves similarly, then at the temperatures at which we were working, denaturation of DNA was probably not a factor in the destruction of infectivity. Eichhorn (1962) found $Co^{2+}$ to be a stabilizer of DNA. In our experiments $Co^{2+}$ and two other heavy metals $Cu^{2+}$ and $Fe^{2+}$ greatly accelerated the rate of destruction of virus infectivity, suggesting that the most important factor in heat inactivation is protein denaturation. The increased inactivation rates at pH 6 also indicate that the virus protein is the primary target in heat inactivation. The protective action of some metal ions on the infectivity of vaccinia virus may, therefore, be exerted by a bonding of the ions to polar groups of the protein with consequent strengthening of its molecular structure against heat denaturation—a familiar enough concept in protein studies.

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# Generation Times of Individual Bacteria: Some Corroborative Measurements

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SUMMARY

Measurements of individual generation times were carried out on three species of bacteria, each in two different growth media. They confirmed in every respect conclusions already reached about the broad features of the generation time pattern. In addition, they supported two important inferences the existing evidence for which was no more than suggestive: (i) complex media give rise to a greater dispersion of generation time than do simple media; (ii) there is positive association between the generation times of second cousins, i.e. organisms whose nearest common ancestor is three generations removed.

## INTRODUCTION

The observations of bacterial generation times recorded in this paper were intended to supplement those of Powell (1958). We wished particularly to resolve doubts concerning two inferences, of some interest and importance, which were suggested but only weakly supported by Powell's data. These were (i) that the coefficient of variation of generation times is greater, the greater the chemical complexity of the growth medium; (ii) that the properties of a given organism affect the individual generation times of its progeny up to at least the third generation.

The experiments were conducted in the same way as Powell's (1958); the organisms were grown on a cellophane membrane in a culture chamber (Powell, 1956*b*) which permitted observation under phase-contrast illumination while the aerated growth medium was continuously circulated under the cellophane. A large number (800–900) of measurements of individual generation times were made for each combination of organism and medium. Perhaps as a result of increasing skill and experience it was found possible to maintain sensibly constant growth rate until overcrowding became intolerable, i.e. often up to six mean generation times. It was thus possible to obtain a much higher proportion of observations on complete sets of eight second cousins—the third generation progeny of single ancestors ('$C_2$-octads' Fig. 1). In addition, records were kept in such a way as to preserve the original topology of the family trees. It results from the repeated transverse binary fission of rod-like organisms that those belonging to any one generation from a common ancestor fall into a natural order, shown schematically in Fig. 1 A, even though the spatial order actually realized may be altered through mechanical or temporal displacement (the same applies to Streptococcus; but Staphylococcus

and Gaffkya, for example, give rise to more complex, though still definite, arrays). It may be suspected then, that the properties of an organism may be to some extent dependent on its position in the chain to which it belongs; for example, the cell wall of an ancestor (Fig. 1A (*a*) and Fig. 3) may be for the most part transmitted to the terminal organisms of the chains into which it develops. Accordingly all observations were recorded in the form of trees (Fig. 1B) corresponding in their spatial order with the development of the organisms they represented.

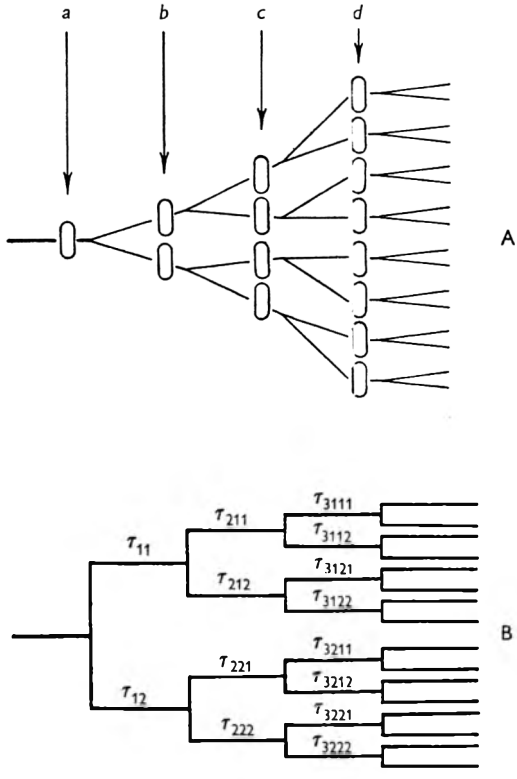


Fig. 1A. A typical family tree. The groups of organisms in each generation are referred to as: *a*, ancestor; *b*, *s*-dyad; *c*, $C_1$-tetrad; *d*, $C_2$-octad. Schematic: because of the dispersion of generation time not all the organisms in *c* and *d* are necessarily contemporaries. B. Method of recording results. The horizontal segments are labelled with the $\tau$ of the corresponding organisms in A.

## ORGANISMS AND MEDIA

Three species of organisms were used: one strain each of *Pseudomonas aeruginosa*, *Proteus vulgaris* and *Escherichia coli*. Previous experience had suggested that Proteus does not readily produce elongated swarming organisms on cellophane over a flowing medium (Powell, 1955), and in fact a rather crude test (Powell, 1958) showed that the growth rate was satisfactorily constant over the experimental periods.

Two series of experiments were carried out for each species, one with tryptic meat broth ('TMB') and one with a chemically defined medium ('H 12P'). The

chemically defined medium was made up from 'Analar' chemicals and demineralized water in the following way:

Solution (i): $NaH_2PO_4.2H_2O$, 31 g. (0·2 M); $(NH_4)_2HPO_4$, 238 g. (1·8 M); $K_2SO_4$, 70 g. (0·4 M); water to 1 l.

Solution (ii): Concentrated HCl, 50 ml. (0·58 M); MgO, 10 g. (0·25 M); $CaCO_3$, 2 g. (0·02 M); $FeCl_3.6H_2O$, 5·4 g. (0·02 M); $ZnSO_4.7H_2O$, 1·44 g. (0·005 M); $MnSO_4.4H_2O$, 1·11 g. (0·005 M); $CuSO_4.5H_2O$, 0·25 g. (0·001 M); $CoSO_4.7H_2O$, 0·28 g. (0·001 M); $H_3BO_3$, 0·062 g. (0·001 M); $Na_2MoO_4/2H_2O$, 0·49 g. (0·002 M); water to 1 l.

Solutions (i) (50 ml.) and (ii) (5 ml.) were mixed, made up to 1 l. with water and autoclaved.

Solution (iii): Citric acid, 4·2 g. (0·02 M); glucose, 3·6 g. (0·02 M); L-glutamic acid, 2·94 g. (0·02 M); succinic acid, 1·18 g. (0·01 M); these components were mixed in a little water (*ca.* 50 ml.) and neutralized with NaOH, then $Na_2S_2O_3.5H_2O$, 1·24 g. (0·005 M) was added.

This mixture was filtered, with sterility precautions, into 1 l. of the previously autoclaved salt solution.

For the growth of *Proteus vulgaris* solution (iii) was fortified with 1 mg. of nicotinic acid (Medium 'H 12P*').

Of the addenda in solution (iii), the citric acid mainly served to prevent the precipitation of metal phosphates. The other substances had each been found to increase the growth rate of one or more of the organisms with which the medium had been tested.

Table 1. *Synopsis of experiments*

The reference numbers are used in later tables to distinguish the several series of experiments.

| Series reference no. | Organism | Medium | No. of experiments | Total no. of generation times |
|---|---|---|---|---|
| Pa1 | *Pseudomonas aeruginosa* | TMB | 29 | 963 |
| Pa2 | *P. aeruginosa* | H12P | 12 | 887 |
| Pv1 | *Proteus vulgaris* | TMB | 19 | 874 |
| Pv2 | *P. vulgaris* | H12P* | 10 | 850 |
| Ec1 | *Escherichia coli* | TMB | 25 | 854 |
| Ec2 | *E. coli* | H12P | 13 | 815 |

Before a series of experiments was begun, the organism was subcultured at least three times on the chosen medium, and after the culture chamber had been inoculated, a period of incubation was allowed before any observations were made (Powell, 1958). All experiments were carried out at 35°. Generation times were recorded to the nearest 1 min., but it should be understood that the personal error of estimation is probably greater than this—perhaps 2–5 min., depending on the growth rate. For the most part, observations were continued on the progeny of selected organisms up to an equal number of generations in every line of descent. When because of overcrowding it became necessary to break off observations on certain branches of a family, the decision to do so was taken in such a way as not to bias the observations in favour of short-lived organisms (cf. Powell 1955).

Table 1 gives a synopsis of the experiments undertaken.

## ANALYSIS OF OBSERVATIONS

In view of the similarity of the experimental conditions, it will not be necessary to repeat Powell's (1958) lengthy discussion of the effects of experimental error. But it is desirable to review one feature of our results which is in fact common to all extended investigations in this subject: the lack of statistical homogeneity in series of replicate experiments. It has invariably been found that analysis of variance applied to such series has disclosed a highly significant inter-experiment variance suggestive of imperfect replication. It is often obvious to inspection that the mean generation times in individual experiments differ much more than would be expected from the variances and sample sizes. Neither improvements in technique nor increase in sample size have mitigated the discrepancy. It seems therefore that the heterogeneity should be accepted as a genuine feature of the pattern of generation times. Powell (1958) explained it as a result of the association between generation times of closely related organisms, coupled (in his experiments, and the same applies to ours) with the method of selecting organisms for observation.

We subjected our results, series by series, to conventional two-way analyses of variance in order to obtain estimates of the 'true' (within-experiment) variance $\sigma_\tau^2$ of the generation times ($\tau$) and of the between-experiment variance $\sigma_E^2$ supposedly introduced by imperfect replication. On the assumption that the coefficient of variation of $\tau$ is a stable property, not sensibly affected by minor changes in experimental conditions, the results of the analysis were used to compute corrected coefficients of variation $c_0$; if $\mathbf{a}$, $\sigma^2$, and $c$ are respectively the grand mean, variance and coefficient of variation in a given series of experiments, $c = \sigma/\mathbf{a}$; the variation of the mean from experiment to experiment introduces a coefficient of variation $c_j = \sigma_E/\mathbf{a}$; and (Powell, 1955)

$$c_0^2 = (c^2+1)/(c_j^2+1) - 1 \qquad (1)$$

(see Table 2). If we admit that the apparent inter-experiment variance is in part due to lack of reproducibility and in part contributed by a real property of the generation time distribution, we can say that the true coefficient of variation lies between $c$ and $c_0$, and similarly that any other parameter is likely to be over-corrected by an adjustment derived from the analysis of variance.

Table 2. *Estimates of variances*

Estimates of the apparent within-experiment ($\sigma_\tau^2$) and between-experiment ($\sigma_E^2$) variances, derived from 2-way analyses of variance, together with the crude ($c$) and corrected ($c_0$) coefficients of variation of $\tau$. The grand mean ($\mathbf{a}$) and estimated variance ($\sigma^2$) of the observations are also given. The coefficient of variation ($c_j$) for the fluctuation of experiment means is used here and elsewhere in correcting other parameters. (Unit of time, 1 min.)

| Series | a | Est. $\sigma^2$ | Est. $\sigma_\tau^2$ | Est. $\sigma_E^2$ | $c^2$ | $c^2$ | $c_0^2$ |
|---|---|---|---|---|---|---|---|
| Pa 1 | 32·22 | 48·11 | 43·30 | 5·618 | 0·04634 | 0·005412 | 0·04071 |
| Pa 2 | 38·86 | 54·29 | 41·98 | 13·66 | 0·03594 | 0·009047 | 0·02665 |
| Pv 1 | 19·29 | 20·54 | 19·13 | 1·593 | 0·05521 | 0·004282 | 0·05071 |
| Pv 2 | 43·64 | 55·11 | 52·87 | 2·625 | 0·02894 | 0·001378 | 0·02752 |
| Ec 1 | 19·79 | 21·50 | 20·60 | 1·064 | 0·05509 | 0·002715 | 0·05223 |
| Ec 2 | 29·02 | 27·37 | 24·80 | 1·698 | 0·03250 | 0·002017 | 0·03042 |

*The distribution of generation times*

The foregoing remarks, added to a steadily accumulating general experience, suggest that the fitting of frequency functions to the pooled results of a series of experiments is less dubious than it seemed in the earlier days of the subject. It is still best, however, to regard any fitted function as a convenient description, merely useful for algebraic and statistical manipulation.

Kubitschek (1962), moved apparently by a not unusual prejudice in favour of the normal distribution, found that the distribution function of the reciprocal of the generation time gave a nearly linear plot on arithmetic-probability paper, but he gave no statistical test of the fit. Davies (1947) says of this procedure '. . . a fair straight line can be obtained with data which are far from normal. . . a histogram gives a better idea of the shape of the distribution, and is less likely to lead to false conclusions'. Since Kubitschek himself found that his generation time measurements could be adequately fitted to a Pearson Type III distribution

$$f(\tau) = \frac{\tau^{g-1}e^{-\tau/m}}{m^g\Gamma(g)} \tag{2}$$

it follows that $1/\tau$ must have been distributed approximately in a Pearson Type V, which is more skew than the corresponding Type III and in that respect less nearly normal. Since also the normal distribution is inappropriate because of its unlimited range, we see no reason to accord it any special place at this stage.

We therefore graduated our measurements by fitting three convenient singly terminated distributions:

(i) the Pearson Type III (Equation (2));

(ii) the Pearson Type V,

$$f(\tau) = \frac{m^g\tau^{-g-1}e^{-m/\tau}}{\Gamma(g)}; \tag{3}$$

(iii) a distribution of intermediate form suggested by Ash and Powell (1962):

$$f(\tau) = \frac{\tau^{-5/2}\exp\left\{-\frac{r}{2}\left(\frac{\tau}{m}+\frac{m}{\tau}\right)\right\}}{2m^{-3/2}K_{3/2}(r)}. \tag{4}$$

(Here $K_{3/2}(r)$ is the modified Bessel function of the second kind. We refer to this frequency function, for convenience, as a '$K$-distribution'). The parameters $g$ (in (2) and (3)) and $r$ (in (4)) are measures of intrinsic dispersion—functions of the coefficient of variation; $m$ is a scale parameter. The results of fitting by the method of maximum likelihood are shown in Table 3. Only for one series (Ec2) does none of the functions provide an adequate fit, and inspection of the figures suggests that this is due to a ragged histogram, presumably accidental (Fig. 2). The sample skewness and kurtosis (Table 4) for series Ec2 were not extreme.

In spite of the usually good fit so obtained, the sample skewness and kurtosis of each series corresponded to points in the Type IV area of the Pearsonian $\beta_1$, $\beta_2$ diagram (see, for example, Pearson, 1930). We ascribe the excessive kurtosis to the few observations of large $\tau$ in the upper tails of the distributions, which on account of the necessity for grouping do not greatly affect the values of $\chi^2$. It is at all events

Table 3. *Frequency function parameters and goodness of fit by the $\chi^2$ test*

| | | Series | | | | | |
|---|---|---|---|---|---|---|---|
| | | Pa1 | Pa2 | Pv1 | Pv2 | Ec1 | Ec2 |
| Type III (eq. (2)) | $g$ | 24·3 | 29·9 | 18·0 | 35·6 | 18·0 | 31·4 |
| | $m$ | 1·32 | 1·30 | 1·07 | 1·23 | 1·10 | 0·92 |
| | $\chi^2$ | 46·3 | 49·6 | 16·9 | 28·8 | 28·2 | 53·6 |
| | $n$ | 28 | 29 | 19 | 30 | 20 | 22 |
| | $P(\chi^2)$ | 0·017 | 0·010 | 0·60 | 0·53 | 0·11 | 0·000 |
| Type V (eq. (3)) | $g$ | 25·7 | 31·1 | 17·1 | 35·0 | 14·3 | 29·5 |
| | $m$ | 797 | 1171 | 312 | 1483 | 266 | 827 |
| | $\chi^2$ | 33·5 | 26·4 | 40·0 | 27·9 | 67·9 | 116·5 |
| | $n$ | 26 | 28 | 19 | 30 | 22 | 23 |
| | $P(\chi^2)$ | 0·15 | 0·55 | 0·003 | 0·57 | 0·000 | 0·000 |
| $K$-distribution (eq. (4)) | $r$ | 24·3 | 29·8 | 16·8 | 34·6 | 15·6 | 29·7 |
| | $m$ | 33·5 | 40·2 | 20·4 | 44·9 | 21·1 | 30·0 |
| | $\chi^2$ | 34·1 | 34·0 | 28·5 | 25·5 | 43·6 | 77·9 |
| | $n$ | 27 | 28 | 20 | 29 | 21 | 22 |
| | $P(\chi^2)$ | 0·16 | 0·20 | 0·07 | 0·65 | 0·003 | 0·000 |

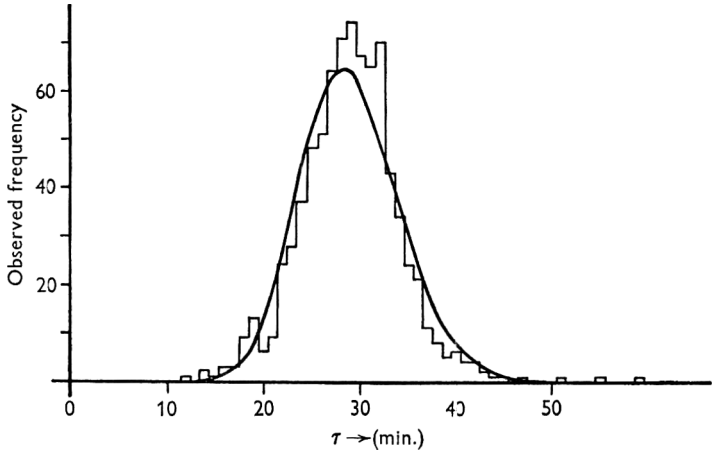


Fig. 2. A histogram of generation times in *Escherichia coli* (Series Ec2). The continuous line is a Pearson Type III distribution fitted by the method of maximum likelihood.

possible that the scattered values of $\gamma_2$ (Table 4) are associated with the variability of the mean in replicate experiments.

Schaechter, Williamson, Hood & Koch (1962) found that the skewness in small samples of generation time measurements was statistically non-significant. This is not surprising; but they did not look for a general tendency. Their belief that the skewness is negligible is in accordance with the Koch & Schaechter (1962) model of the statistics of cell division, which predicts that the generation time distribution $f(\tau)$ will be symmetrical, or nearly so. We therefore think it worth while to describe a test of skewness which is independent of errors of replication.

It can be shown by the same method as was used by Powell (1955) in deriving

equation (1), that if the true distribution of $\tau$ is symmetrical with coefficient of variation $c_0$, the observed third moment of $f(\tau)$ will be

$$\mu_3 = \mu_3(j)\,(3c_0^2+1)+6c_0^2c_j^2\mathbf{a}^3$$

where $\mu_3(j)$ and $c_j$ are respectively the third moment and coefficient of variation of the distribution of the experiment means. Thus, the true third moment may be zero even if the observed skewness is positive, as it is always found to be in the pooled results.

In a positively skew distribution the median lies below the mean: in a group of small samples from such a distribution we should expect more than half to show an excess of observations in the range below the mean. Accordingly, in each of the experiments comprised in a series the number of observations falling above and below the experiment mean was counted; the corresponding signs of the difference (mean − median) are collected in Table 4. The numbers of experiments are too small to allow of attaining a high level of statistical significance in any one series, but the evidence is uniformly in favour of the hypothesis that the true median lies below the mean i.e. that the skewness is really positive. However, the test is much too crude to show that the Koch & Schaechter model is untenable.

The general type of $K$-distribution proposed by Ash & Powell (1962) contains an extra parameter in a factor $\tau^{g-1}$ instead of the fixed power $\tau^{-5/2}$ of (4), and it includes the Pearson Types III and V as limiting cases. It is thus adequate to describe the generation time distribution for most purposes.

Table 4. *Sample values of the skewness, $\gamma_1$, and kurtosis, $\gamma_2$, of the crude distribution $f(\tau)$, together with the numbers of experiments classified according to the sign of (mean − median)*

| Series | Est. $\gamma_1$ | Est. $\gamma_2$ | No. of experiments in which the sign of (mean − median) was Positive | Negative |
|---|---|---|---|---|
| Pa1 | 1·84 | 10·00 | 18 | 7 |
| Pa2 | 1·10 | 2·63 | 10 | 2 |
| Pv1 | 0·47 | 0·72 | 10 | 5 |
| Pv2 | 0·72 | 1·40 | 7 | 3 |
| Ec1 | 0·78 | 2·65 | 15 | 4 |
| Ec2 | 0·44 | 2·26 | 8 | 4 |

### *Non-viable organisms*

Only six organisms could be fairly certainly classified as non-viable, among the 5200 observed. This confirms Powell's (1958) estimate, that the index of viability $\alpha$ is usually at least 0·99 in unhampered growth, but with so small a number no further generalization is possible.

### *The dissymmetry of fission*

Málek has for long held that of the two so-called sisters arising from the fission of a single organism, one should properly be regarded as the progeny of the other (see e.g. Málek, 1955).

In general the two organisms have different generation times; there may be detectable differences in a few biochemical properties; one but not the other may

occasionally turn out to be non-viable: such inequalities would not be remarkable in siblings of any species, and may well be random in character. But whether or not there is intrinsic inequality in the division of nuclear and cytoplasmic material, there is one respect in which fission is certainly unsymmetrical, namely in the manner of growth and inheritance of the cell wall. The main features of this process are most admirably described by Cole & Hahn (1962); they dealt with only one species, but other evidence (e.g. Bisset, 1951), including the mode of inheritance of flagella (Stocker, 1956) and vital staining of cell walls with an optical bleach (Dr G. C. Ware, private communication), suggests the same general picture.

This picture is the same for all species that have been examined, apart from minor variations due to differences in relative timing of the component processes (Fig. 3). Cell-wall material is not uniformly secreted over the cell membrane, but

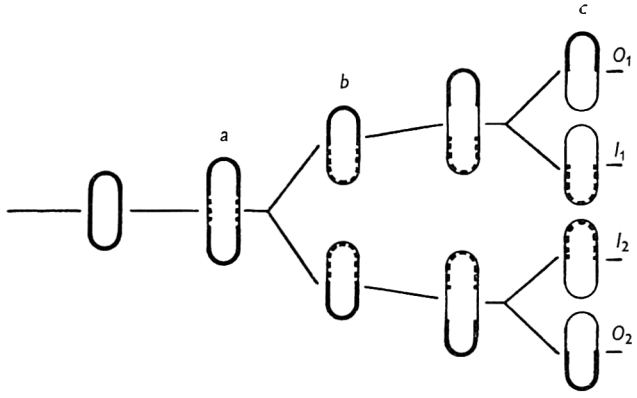


Fig. 3. Mode of inheritance of cell wall. During the growth of the ancestor, new cell wall is laid down in a central zone (*a*). The two daughters (*b*) carry partly new cell wall, partly cell wall which was already formed at the inception of the ancestor. In a $C_1$-tetrad (*c*) the outer organisms ($O_1$, $O_2$) inherit the older cell wall, the inner ($I_1$, $I_2$) that which was laid down during the development of the ancestor.

is laid down in a zone overlying the site of an incipient fission (Fig. 3*a*). Each of a pair of sisters carries, on one half, cell wall already formed at the inception of the mother, on the other, cell wall laid down during the growth of the mother (Fig. 3*b*). Because of dissymmetry in the mother the two organisms at (*b*) are not alike. It results that if the descent for two generations of a set of first cousins ('$C_1$-tetrad'; Fig. 3*c*) is known, they can be labelled unambiguously as 'inner' ($I_1$, $I_2$) and 'outer' ($O_1$, $O_2$) organisms; the 'outer' organisms carry cell wall of a greater age than do the 'inner'. As the cell wall ages, it hardens; this can be shown directly in favourable cases (Powell, 1956*b*). There is thus a possibility that the generation times of a pair of sisters of known descent might differ systematically according to their relative position in the family tree. The method of recording results which we have described enabled us to classify the organisms of most *S*-dyads as 'inner' (*I*) or 'outer' (*O*) in this sense.

For each series, the number of instances in which the generation time of the inner sister ($\tau_I$) exceeded that of its outer sister ($\tau_O$), and *vice versa*, were counted, with the result shown in Table 5. In five series the frequency of $\tau_O > \tau_I$ exceeded that of

$\tau_I > \tau_o$, but reached significance (probability $< 0{\cdot}05$) in only two (Pv 1, Ec 2). For the grand total, the difference $(\tau_o > \tau_I) - (\tau_I > \tau_o)$ was 129, about six times the standard deviation

$$\sqrt{(2079)}/2 = 22{\cdot}8,$$

on the hypothesis of symmetry. The probability of such a discrepancy is about 0·005. Other methods of comparison were tried, e.g. the outermost organisms of $C_2$-octads were compared with the six inner organisms. Results of similar uniformity and significance were obtained.

There is thus good evidence that the generation time of an organism is in part determined by its position in the family tree, and so perhaps by the history of the cell wall which it bears. The effect is detectable only in large samples, however, and is probably due to nothing more than mechanical constraint.

Table 5. *Generation times of inner* ($\tau_I$) *and outer* ($\tau_0$) *sisters compared*

| Series | Instances of $\tau_o > \tau_I$ | Instances of $\tau_I > \tau_o$ | Total instances |
|---|---|---|---|
| Pa 1 | 213 | 204 | 417 |
| Pa 2 | 165 | 169 | 334 |
| Pv 1 | 194 | 145 | 339 |
| Pv 2 | 176 | 158 | 334 |
| Ec 1 | 184 | 163 | 347 |
| Ec 2 | 172 | 136 | 308 |
| Sums ... | 1104 | 975 | 2079 |

*The coefficient of variation of generation time*

The crude ($c$) and corrected ($c_0$) coefficients of variation of $\tau$ are set out in Table 2.

It can be seen that for each organism both the $c$ and the $c_0$ associated with the complex medium are greater than those associated with the chemically simpler defined medium. This finding adds considerable weight to Powell's (1958) data in support of his suggestion—an extension of Kendall's (1948) hypothesis—that 'the dispersion of generation time is dependent on the number of synthetic processes the organism is called upon to perform in order to convert pabulum into living matter'.

The range of media and species which have so far been investigated is very small, however. Clearly a much more expeditious method of determining $c$ is necessary if the range is to be greatly extended. An indication of such a method is to be found in Kendall's (1948) discovery, that under certain assumptions $c$ is approximately equal to the coefficient of variation of the number of organisms in colonies of the same age developed from single organisms. But it is not yet known to what extent Kendall's theorem may be falsified by the associations between generation times of related organisms.

*Correlations between generation times*

Five product-moment correlation coefficients were computed from the data (see Fig. 1B):

(i) Between the $\tau$ of mothers and daughters, $\rho(H_1)$. Typical pairs are $\tau_{11}$, $\tau_{211}$; $\tau_{11}$, $\tau_{212}$.

(ii) Between the $\tau$ of grandmothers and granddaughters, $\rho(H_2)$. Typical pairs are $\tau_{11}$, $\tau_{31ab}$ ($a$, $b$ = 1, 2).

(iii) Between the $\tau$ of sisters, $\rho(S)$. A typical pair is $\tau_{11}$, $\tau_{12}$.

(iv) Between first cousins, $\bar{\rho}(C_1)$; in each $C_1$-tetrad the mean of the $S$-dyad on one side of the family was compared with the mean of that on the other. A typical pair is

$$\tfrac{1}{2}(\tau_{211}+\tau_{212}),\ \tfrac{1}{2}(\tau_{221}+\tau_{222}).$$

(v) Between second cousins, $\bar{\rho}(C_2)$; in each $C_2$-octad the mean of the $C_1$-tetrad on one side of the family was compared with the mean of that on the other. A typical pair is

$$\tfrac{1}{4}\Sigma\tau_{31ab},\ \tfrac{1}{4}\Sigma\tau_{32ab} \quad (a, b = 1, 2).$$

The advantage of the special coefficients $\bar{\rho}(C_1)$, $\bar{\rho}(C_2)$ is that they can show up a general similarity between the two branches of a family unobscured by the variations among the $\tau$ in each branch. Our larger sample sizes enabled us to prefer them to the less sensitive intra-class coefficients adopted by Powell (1958), in spite of their greater susceptibility to sampling errors when corrected for inter-experiment variation. (Powell used a slightly different notation for these coefficients, with dashed $C$ instead of dashed $\rho$; the present form is easier to print).

Table 6. *Crude* ($\rho$) *and corrected* ($\rho_0$) *product-moment correlation coefficients between mothers and daughers* ($H_1$), *grandmothers and granddaughters* ($H_2$), *between sisters* ($S$), *between first cousins* ($C_1$) *and between second cousins* ($C_2$)

Number of pairs of observations: $n$.

| | Series | | | | | |
|---|---|---|---|---|---|---|
| | Pa1 | Pa2 | Pv1 | Pv2 | Ec1 | Ec2 |
| $\rho(H_1)$ | +0·125 | +0·108 | −0·017 | −0·090 | −0·198 | +0·119 |
| $n$ | 742 | 753 | 689 | 724 | 680 | 674 |
| $\rho(H_2)$ | +0·126 | +0·203 | +0·015 | −0·126 | +0·050 | −0·110 |
| $n$ | 370 | 500 | 288 | 436 | 393 | 427 |
| $\rho(S)$ | +0·513 | +0·632 | +0·596 | +0·624 | +0·402 | +0·478 |
| $n$ | 470 | 433 | 427 | 417 | 397 | 401 |
| $\bar{\rho}(C_1)$ | +0·499 | +0·522 | +0·175 | +0·446 | +0·317 | +0·374 |
| $n$ | 204 | 178 | 177 | 180 | 161 | 177 |
| $\bar{\rho}(C_2)$ | +0·438 | +0·554 | +0·349 | +0·496 | +0·143 | +0·299 |
| $n$ | 58 | 53 | 55 | 61 | 43 | 57 |
| $\rho_0(H_1)$ | +0·009 | −0·193 | −0·099 | −0·145 | −0·261 | +0·060 |
| $\rho_0(H_2)$ | +0·010 | −0·066 | −0·068 | −0·182 | +0·001 | −0·183 |
| $\rho_0(S)$ | +0·449 | +0·508 | +0·562 | +0·606 | +0·371 | +0·444 |
| $\bar{\rho}_0(C_1)$ | +0·400 | +0·309 | +0·086 | +0·412 | +0·266 | +0·316 |
| $\bar{\rho}_0(C_2)$ | +0·266 | +0·251 | +0·220 | +0·450 | +0·040 | +0·201 |

The values of $\rho(H_1)$ and $\rho(S)$ (Table 6) were comparable with previous experience: in spite of the high values of $\rho(S)$—near 0·5—$\rho(H_1)$ was nowhere strikingly different from zero. The individual values of $\rho(H_1)$ obviously differed among themselves, but no general pattern was discernible. The same was true of $\rho(H_2)$, and in particular it was not everywhere positive nor systematically nearer to 0 than $\rho(H_1)$ (cf. Fieller's suggestion in Powell, 1958). Corrected values of these three coefficients, $\rho_0(S)$, $\rho_0(H_1)$, $\rho_0(H_2)$ were obtained by application of Powell's (1958), formula, but

no greater regularity appeared (Table 6). It must be admitted, then, that we can offer at present no quantitative description of the manner in which generation times are inherited.

That generation times are inherited in some sense is made evident by the correlations $\bar{\rho}(C_1)$, $\bar{\rho}(C_2)$. The effect of inter-experiment variance on the presumptive true values is given by Powell; a transposition of his formulae yields:

$$\bar{\rho}_0(C_1) = \bar{\rho}(C_1) - \{1 - \bar{\rho}(C_1)\}\frac{2c_j^2}{c^2\{1+\rho(S)\} - 2c_j^2},$$

$$\bar{\rho}_0(C_2) = \bar{\rho}(C_2) - \{1 - \bar{\rho}(C_2)\}\frac{4c_j^2}{c^2\{1+\rho(S)\}\{1+\bar{\rho}(C_1)\} - 4c_j^2}.$$

Both the crude and corrected values (Table 6) were not only positive throughout, but surprisingly large in several cases. We can conclude that the influence of an ancestor is transmitted through at least three generations. The series of coefficients $\rho(S), \bar{\rho}(C_1), \bar{\rho}(C_2)$ diminish on the whole so slowly as to suggest that the two branches of the family tree stemming from a common ancestor retain their similarity for a long time, though this similarity is obscured by variability in the individual $\tau$.

By isolating single cells from large and small colonies of *Escherichia coli*, and allowing them to develop, Hughes (1955) inferred that growth rate could be inherited over many generations. Powell (1958) criticized Hughes on the ground that he had not taken sufficient account of the effect of the initial conditions on the development of colonies. While we still consider Powell's objection to be valid, our figures for the correlation coefficients tend to support Hughes's conclusion.

## DISCUSSION

The general sense of the above results is such as uniformly to support earlier work on the pattern of generation times. In spite of the large sample sizes, they do not suggest any appreciable refinement of previous descriptions. In this section, therefore, we wish only to advance some remarks on the generation time distribution.

We shall assume that the proportion of non-viable organisms thrown off by a steadily growing culture is negligible, i.e. that the index of viability ($\alpha$) is unity. The adjustments necessary when this assumption does not hold are indicated in Powell (1956*a*). We shall also assume that the correlation coefficients $\rho(H_1)$, $\rho(H_2)$ and similar coefficients of higher order are small enough not to affect the growth rate appreciably.

The integral defining the number growth rate, $\nu$, namely

$$2\int_0^\infty e^{-\nu\tau} f(\tau)\, d\tau = 1, \tag{5}$$

implies very little restriction on the nature of $f(\tau)$ beyond its definition as a frequency function:

$$\int_0^\infty f(\tau)\, d\tau = 1, \quad f(\tau) \geqslant 0.$$

The last equation in fact shows that the factor $e^{-\nu\tau}$ in (5) cannot be everywhere as much as unity, and therefore that $\nu$ must have a positive non-zero value. Thus even when a possibly finite proportion of non-viable organisms is excluded the mean and all higher moments of $f(\tau)$ can be infinite. An example will make this clear.

Suppose that $\tau$ is distributed in a Pearson Type V (Eq. (3)). For this distribution the mean and variance are

$$\mu_1' = \frac{m}{g-1}, \quad \mu_2 = \frac{m^2}{(g-1)^2(g-2)},$$

and the growth rate is given by

$$\nu^{g/2}K_g\{2\sqrt{(m\nu)}\} = 2^{-g}m^{-g/2}\Gamma(g),$$

where $K_g$ is the modified Bessel function of the second kind (Powell 1958). If $g$ is as small as 2 and if for convenience we take $m = 1$, the appearance of the frequency function (Fig. 4) is not at all unusual, but while the mean is finite ($\mu_1' = 1$), the variance and higher moments are infinite. The growth rate, so far from being near to $(\log 2)/\mu_1'$ is about 50 % greater ($\nu = 1{\cdot}03$). This is a somewhat extreme example, but

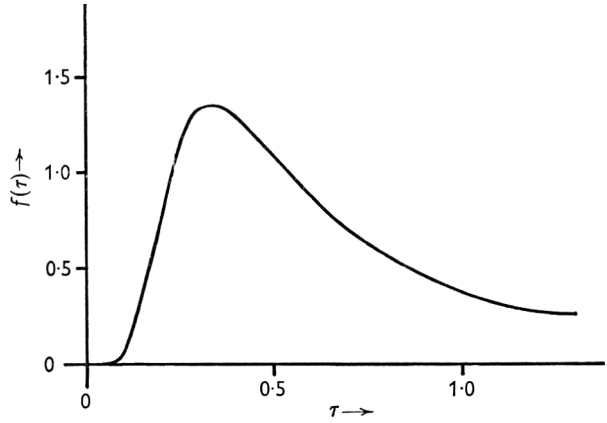


Fig. 4. Pearson Type V distribution with parameters $m = 1$, $g = 2$.

a re-examination of Powell's (1955) figures for *Bacillus mycoides* shows that they could be fairly well represented by a Type V distribution with $g$ near 5. When $g = 5$, the kurtosis $\gamma_2$ is 42: the sampling variance of the variance is about 20 times that of a normal distribution with the same second moment. In most unicellular organisms, the frequency function is much more nearly symmetrical near the mode, but the upper tail of the distribution does not fall to zero as rapidly as is required by conventional functions which fit well over the range where the ordinates are large; and it is the tail which contributes so much to the higher moments.

Whatever explanation can be found to account for the variability of small samples (as evinced by analysis of variance), it may well be true that the whole population of $\tau$ ought to be represented by a frequency function whose higher moments are infinite. It is impossible to prove this by sampling alone, since all the moments of a finite sample are necessarily finite. Nevertheless, if in the future a particular function of this kind should be proposed, its definition will lead to inferences about sampling variance which will serve as a test of its truth.

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# The Three Factors of Anthrax Toxin: their Immunogenicity and Lack of Demonstrable Enzymic Activity

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SUMMARY

A lethal mixture of the three factors (I, II, III) of anthrax toxin had none of the following enzyme activities: adenosine triphosphatase, alkaline or acid phosphatase, catalase, collagenase, ribonuclease, desoxyribonuclease, gelatinase, hyaluronidase, lecithinase, lipase, proteinase. The mode of action of anthrax toxin is unknown. Although factor II is the only factor which is immunogenic when injected alone, the addition of factor I results in an increased immunizing activity; however, the activity of this mixture can be decreased by adding factor III. The possible effect of these results on preparations used to immunize against anthrax is discussed.

## INTRODUCTION

The specific, lethal and oedema-forming toxin of *Bacillus anthracis* (Smith, Keppie & Stanley, 1955; Harris-Smith, Smith & Keppie, 1958; Thorne, Molnar & Strange, 1960) consists of three components (Smith *et al.* 1956; Thorne *et al.* 1960; Stanley, Sargeant & Smith, 1960; Sargeant, Stanley & Smith, 1960; Stanley & Smith, 1961; Smith & Stanley, 1962). The three factors (I, II, III) have been purified. The final preparation of factor I appeared to be a single serological component but factors II and III were serologically heterogeneous. The three factors were neither lethal nor oedema-producing when injected alone. When mixed together, all three acted synergistically in lethality tests in mice, but factor III inhibited to some extent the otherwise large oedema-producing activity of mixtures of factors I and II; a summary of the relationship between these factors in toxicity tests was given in Table 8 of Stanley & Smith (1961). The present paper describes the failure to show in mixtures of the three purified factors any significant enzymic activity which might be the basis for their toxicity. It also describes the ability of these factors and mixtures of them to immunize guinea pigs against infection with *B. anthracis*, and summarizes attempts to separate the major serological component of purified factor II (the main immunogen).

## METHODS

*Analytical methods (including ultracentrifugation and paper electrophoresis).* These were described by Stanley & Smith (1961).

*Purified factors I and III of the anthrax toxin.* These were prepared as described by Stanley & Smith (1961) and Smith & Stanley (1962).

*Purified factor II of the anthrax toxin.* This was prepared from filtrates of cultures

containing the toxin of *Bacillus anthracis* (Thorne *et al.* 1960) by two methods. (*a*) By the method of Strange & Thorne (1958) which involves ammonium sulphate precipitation, precipitation at pH 4·2 and chromatography on an alumina $C_\gamma$-Celite column. The properties of this immunogenic material were described by Strange & Thorne (1958) and Sargeant *et al.* (1960); it appeared to contain two serological components B and C. (*b*) By fractionation on diethylaminoethyl (DEAE) cellulose. Culture filtrate (4 l.) was dialysed (18 hr.) against 0·01 *I* phosphate buffer (pH 7·4) and applied to a column (4·5 cm. diam.; 4 cm. long) of DEAE cellulose (5 g.) which had been equilibrated with 0·1 *I* phosphate buffer (pH 7·4). No pressure was applied and the flow rate was about 30 ml./min. The column was eluted with: (1) 0·1 *I* phosphate buffer (60 ml.; pH 7·4); (2) 0·15 *I* phosphate buffer (90 ml.; pH 7·4) and (3) 0·2 *I* phosphate buffer (60 ml.; pH 7·4). Factor II (60 ml., 0·04 % protein) was eluted by buffer (2). The final preparation was concentrated by dialysis against Carbowax (20 M) and showed no evidence for heterogeneity in the ultracentrifuge (0·5 % (w/v) dialysed against 0·05 *I* phosphate buffer (pH 7·4)) and on paper electrophoresis (1 mg. examined in 0·2 *I* barbitone buffer, pH 8·6)). After exhaustive dialysis against water the freeze-dried product had N 14·2 % (cf. Strange & Thorne, 1958). This preparation of factor II was indistinguishable from the preparation described above in: (1) the assay for factor II activity (Stanley *et al.* 1960), (2) gel-diffusion against 'spore' and 'antigen' antisera which indicated the presence of two serological components (Sargeant *et al.* 1960) and (3) tests for immunogenicity (Smith & Gallop, 1956).

*Serological precipitation in gels.* The methods and antisera ('spore' H533 and 'antigen' H25) used were described by Sargeant *et al.* (1960).

*Test for immunogenicity.* Guinea pigs were immunized as described by Smith & Gallop (1956); the total amount of preparation given in the three equal doses of the immunization course has been quoted in the tables below together with the number of animals surviving subsequent challenge with 1000 lethal doses (LD) of *Bacillus anthracis*, strain N.P., spores. In many experiments, no unvaccinated control animals survived this challenge.

### *Tests for enzymic activity*

A mixture of purified factor I (4 μg.), purified factor II (40 μg.) and purified factor III (9 μg.) was examined in the following tests for enzyme activity, in each of which a known active enzyme preparation was included for comparison.

*Adenosine triphosphatase.* By the method of Kielley & Meyerhof (1950) with adenosine triphosphate (Boehringer & Soehne, Mannheim) as substrate and the enzyme of red cells for comparison.

*Acid and alkaline phosphatase.* By the method of King (1951) with disodium phenylphosphate (British Drug Houses Ltd.) as substrate and the phosphatases of red cells and serum for comparison.

*Catalase.* This was detected by its ability to decompose hydrogen peroxide; crystalline catalase (Boehringer & Soehne, Mannheim) was used for comparison.

*Collagenase.* By the method of Oakley *et al.* (1946) with hide powder coupled with azocoll as substrate and the filtrate of a 72 hr. culture of *Bacillus cereus* for comparison.

*Desoxyribonuclease.* By the method of Kunitz (1950) with desoxyribonucleic acid

(prepared from calf thymus by our colleague S. Lovett) as substrate and a sample of desoxyribonuclease (Light and Co., Ltd.) for comparison.

*Ribonuclease.* By the method of Kunitz (1946) with a sample of ribonucleic acid (Boehringer & Soehne, Mannheim) as substrate and a preparation of ribonuclease (Light and Co., Ltd.) for comparison.

*Gelatinase.* By the method of Strange & Thorne (1958) on X-ray film, with a filtrate of a 72-hr. culture of *Bacillus cereus* for comparison.

*Hyaluronidase.* By the method of Alburn & Whitley (1951) with potassium hyaluronate (Allen & Hanbury, Ltd.) as substrate and hyaluronidase (Evans Medical, Ltd.) for comparison.

*Lecithinase.* By the method of Macfarlane & Knight (1941) with egg yolk media as the substrate and filtrate from a 72-hr. culture of *Bacillus cereus* for comparison.

*Lipase.* By the method of Bier (1955) with Tween 20 as the substrate and a sample of lipase (Light and Co., Ltd.) for comparison.

*Proteinase.* By the method of Kunitz (1947) with Bacto isoelectric casein (Difco Laboratories) as substrate and crystalline trypsin (Armour Laboratories) for comparison.

## RESULTS

### *Lack of demonstrable enzymic activity in mixtures of factors I, II and III of the anthrax toxin*

In tests, in which known enzyme preparations were active, a mixture of factors I, II and III of anthrax toxin had no adenosine triphosphatase, phosphatase (acid or alkaline), catalase, collagenase, desoxyribonuclease, gelatinase, hyaluronidase, lecithinase, lipase, proteinase or ribonuclease activity. Hence we have not identified any enzymic basis for the toxic activity of these factors. A mixture of factors I and II obviously increased capillary permeability since it produced oedema when injected intradermally. Addition of factor III decreased the local oedema production by a mixture of factors I and II (Stanley & Smith, 1961). It seemed possible that factor III was a spreading factor leading to a quick dispersal of factors I and II, hence to a diminution in their local effect. However, no evidence for the presence of a spreading factor (by the method of Hechter, 1947) or of a permeability increasing substance (by the method of Miles & Wilhelm, 1955) was obtained when factor III was examined in rabbits.

### *Immunogenicity for guinea pigs of mixtures of factors I, II and III of anthrax toxin*

When the three factors were injected singly, only factor II protected guinea pigs against subsequent infection with *Bacillus anthracis* (Tables 1, 2). However, the addition of factors I and III to factor II produced some interesting results. These mixtures were not given in sufficient strength to kill any guinea pigs and since all vaccinations were done subcutaneously there were no overt signs of oedema production. The results in Tables 1 and 2, which were analysed by our colleague Mr S. Peto, show that addition of factor I to factor II increased the immunizing power of the latter, but that addition of factor III to a mixture of factor I and II decreased their combined activity. On the other hand, addition of factor III to factor II did not seem to affect the immunizing activity of the latter (Table 2).

It was also possible that a mixture of factors I and III had some immunizing activity (Table 2).

Table 1. *Effect of factor I of anthrax toxin on the immunogenicity of factor II*

This table includes the results of five experiments; sections *A*, *B* and *C* include the results of two similar experiments and sections *D* and *E* the results of five similar experiments.

| | Antigens injected* (μg.) | | Immunizing activity in guinea pigs. No. of survivors / No. injected = % | |
|---|---|---|---|---|
| | Factor I | Factor II | | |
| *A* | 120 | — | 0/15 | Nil |
| | 30 | — | 0/25 | Nil |
| | 10 | — | 0/25 | Nil |
| | 3 | — | 0/25 | Nil |
| *B* | — | 600 | 14/25 | 56 |
| | 30 | 600 | 16/25 | 64 |
| *C* | — | 200 | 10/25 | 40 |
| | 10 | 200 | 17/25 | 68 |
| *D* | — | 60 | 22/60 | 37 |
| | 3 | 60 | 27/40 | 67 |
| *E* | — | 6 | 18/60 | 30 |
| | 3 | 6 | 24/40 | 60 |

* For details of tests of immunizing activity in guinea pigs see Methods; the total dose of antigen (given subcutaneously in 3 equal doses) is quoted; animals were challenged with 1000 lethal doses of virulent *Bacillus anthracis* strain N.P. No control animals survived.

*Attempts to separate the major serological component in factor II of anthrax toxin*

The final preparation of factor II was a mixture of two serological components, B and C (Sargeant *et al.* 1960); B appeared to be associated with factor II activity but the relative proportions of components B and C in the final preparation was unknown (see Sargeant *et al.* 1960). Since this preparation of factor II was the only factor of the anthrax toxin which was immunogenic when injected alone, attempts were made to separate the two components so that each could be examined for factor II activity and immunogenicity. These attempts, described below, were only partially successful. Component *C* was obtained free from component B by the purification of fractions discarded during the fractionation of factor I (Stanley & Smith, 1961); it proved to have no factor II or immunogenic activity at the con

centrations examined. This supported the view of Sargeant *et al.* (1960), that component B was responsible for the factor II activity and indicated that component B was also the immunogen.

The preparation of component C was sufficiently pure to be used for estimations of the relative proportions of components B and C in factor II by comparative titrations on serological diffusion plates. Component B proved to be the major

Table 2. *Effect of factor III of anthrax toxin on the immunogenicity of factors I and II and their mixture*

This table includes the results of four similar experiments for each comparison of factors I and II (or their mixture) with and without the addition of factor III. For details of tests of immunizing activity in guinea pigs see Methods.

| Antigens injected* ($\mu$g.) | | | Immunizing activity in guinea pigs.* | |
|---|---|---|---|---|
| I | II | III | $\frac{\text{No of survivors}}{\text{No. injected}}$ | = % |
| — | — | 300 | $\frac{0}{10}$ | Nil |
| — | — | 100 | $\frac{0}{10}$ | Nil |
| — | — | 30 | $\frac{0}{10}$ | Nil |
| — | — | 3 | $\frac{0}{29}$ | Nil |
| 3 | — | — | $\frac{0}{35}$ | Nil |
| 3 | — | 3 | $\frac{7}{50}$ | 14 |
| — | 60 | — | $\frac{15}{40}$ | 38 |
| — | 60 | 3 | $\frac{10}{29}$ | 34 |
| — | 60 | 30 | $\frac{7}{20}$ | 35 |
| — | 6 | — | $\frac{19}{78}$ | 24 |
| — | 6 | 3 | $\frac{21}{79}$ | 27 |
| — | 6 | 9 | $\frac{5}{20}$ | 25 |
| 3 | 60 | — | $\frac{28}{40}$ | 70 |
| 3 | 60 | 3 | $\frac{23}{40}$ | 58 |
| 3 | 60 | 30 | $\frac{2}{20}$ | 10 |
| 3 | 6 | — | $\frac{35}{60}$ | 58 |
| 3 | 6 | 3 | $\frac{20}{49}$ | 41 |
| 3 | 6 | 9 | $\frac{4}{30}$ | 13 |

* See Table 1.

component in the preparation of factor II but all subsequent attempts to decrease significantly its contamination with component C failed.

*Preparation of component C.* The serological detection and characterization of component C has been described elsewhere (Sargeant *et al.* 1960); in gel-diffusion plates it precipitated with 'antigen' (H25) antiserum but not with 'spore' (H 533) antiserum. Although, during the original filtration of toxic cultures, component C passed the filters to be present in the final preparation of factor II, some component C was retained on the filters to be eluted with factor I. It was from fractions discarded during the fractionation of the latter (see Stanley & Smith, 1961; the 0·1 *I* and 0·12 *I* phosphate buffer eluates from the DEAE-cellulose column), from two batches of filter eluate ($2 \times 200$ ml. $\equiv 2 \times 50$ l. original culture) that component C was obtained. The fractions, containing component C and some component B and factor I. were bulked and left at 0° for 16 days. A precipitate formed and after removal of this, the soluble material contained component C but had a much decreased content of component B. The solution was diluted with water (3 vol) and applied to a column (2 cm. diam., 1 cm. length) of DEAE-cellulose (0·2 g.) which had been equilibrated with 0·1 *I* phosphate buffer (pH 7·4). Component C was eluted with 0·2 *I* phosphate buffer (pH 7·2) containing 0·2 M-NaCl (4 ml.). A sample of this material was dialysed against water and freeze-dried to obtain the content of non-dialysable matter.

For paper electrophoresis (pH 8·6, 0·2 *I* barbitone buffer) the final solution was concentrated by dialysis against carbowax; 1 mg. of material showed a single band migrating to the anode. In serological diffusion plates, the final preparation produced the C line against 'antigen' antiserum (see below) but 4 $\mu$g. produced no B line and this indicated (by comparative titration with factor II which contained serological component B) that contamination with component B was less than 5%. The preparation of component C contained 104% protein (calculated with reference to an ovalbumin standard) and on serological diffusion plates 0·24 $\mu$g. was the minimum quantity that formed the C line against 'antigen' (H25) antiserum. It (40 $\mu$g.) had no activity in the assay for factor II, and in the test for immunogenicity total doses of 60 and 6 $\mu$g. did not protect guinea pigs (cf. factor II in Table 1).

*Estimation of content of component C in the final preparation of factor II.* The difficulty was that the B and C lines formed by factor II with 'antigen' (H25) antiserum overlaid one another so that individual titrations could not be observed directly (Sargeant *et al.* 1960). Estimations were done by two methods using antigen (H 25) antisera: (1) wells containing serial descending dilutions of factor II were alternated with those containing the preparation of component C (1 $\mu$g.) which formed a line which ran into the line formed by the higher concentrations of factor II. The amount of factor II in the first dilution which showed the absence of component C (i.e. the C line from adjacent wells crossed the B line of the factor II preparation and continued to the factor II wells) was noted and compared with the amount of component C preparation (0·12 $\mu$g.) which just did not form a C line. (2) An antiserum (R-1; Sargeant *et al.* 1960) which inhibited the formation of the B line by preparations of factor II (see Sargeant *et al.* 1960) but had no effect on the formation of the C line (by comparative titrations with the preparation of component C), was added (equal volumes) to serial dilutions of the preparation of factor II. The minimal amounts of factor II which formed a C line under these conditions

were compared with the corresponding amounts of the preparation of component C examined under the same conditions. Both methods of estimation agreed in indicating that the amount of component C in several batches of the final preparation of factor II was not more than 15–30 %. The second method of estimation was used in subsequent work.

*Attempts to decrease the amount of component C in preparations of factor II.* Exhaustive attempts to reduce significantly the content of component C in the final preparation of factor II by fractionating the latter on diethylaminoethyl DEAE-cellulose, hydroxyapatite or on alumina Cγ celite columns failed. Since components B and C were so alike in chemical behaviour attempts were made to avoid the necessity of separating them by obtaining initial culture filtrates with a lower relative content of component C. Filtrates of cultures, taken after different times of incubation were examined in the hope that components B and C might be formed at significantly different rates or at different stages in growth. Several flasks of medium (250 ml.; Thorne *et al.* 1960) were inoculated with *Bacillus anthracis*, Sterne strain, $2{\cdot}5 \times 10^7$ spores, and incubated at 37°. After 8, 12, 18, 24 and 36 hr., samples of the culture were filtered. Immediately each sample (at 8 hr., 1 l.; at 12 hr., 500 ml.; at 18, 24 and 36 hr., 250 ml.) was dialysed for 4 hr. against phosphate buffer (pH 7·4, 0–2°) and fractionated for factor II on DEAE-cellulose (2 g.) as described in Methods. Subsequent serological analysis indicated that the factor II preparations obtained from the 8, 12, 18, 24 and 36 hr. filtrates contained not more than 30, 30, 12, 17 and 17 %, respectively, of component C. Although the content of component C could be varied by varying the time of incubation, it was not significantly decreased from the normal figure (15–30 %) for various preparations of factor II obtained from 24 hr. filtrates.

## DISCUSSION

Although some knowledge is now available about the chemistry of the components of the anthrax toxin, their mode of action is unknown. We have been unable to identify any enzymic activity in a mixture of the three factors of anthrax toxin which might explain its lethal effect. There is no explanation for the synergic activity of the three factors in lethality tests (see Stanley & Smith, 1961; table 8), for the dramatic effect of mixtures of factors I and II on the capillaries *in vivo* which results in the production of so much local oedema, or for the effect of factor III on the mixture of I + II in decreasing its local oedema production and increasing its lethality. It would be satisfying to think that the complexing action of factor I on metals had some connexion with its role in the toxic mixture but its non-replacement by EDTA (Stanley & Smith, 1961) was discouraging in this respect. The three factors of the toxin do not appear to be complexed in any way, at least when they appear extracellularly. Thus, the individual factors could be detected (in gel diffusion plates against 'spore' antiserum) in the plasma of guinea pigs dying of anthrax and in cultures *in vitro* which had not been filtered (which separates the factors) but only centrifuged to remove most bacteria and concentrated for examination by dialysis against Carbowax.

The immunizing activities of the various factors of the anthrax toxin are important in relation to practical vaccination against anthrax. Although the factor II preparation was the only factor which was immunogenic when injected

alone, it was clear that the addition of the other factors influenced the overall immunogenicity, e.g. addition of factor I increased the immunizing activity of factor II but the combined activity of I+II was decreased by adding factor III. However, the addition of factor III alone to factor II seemed to have no significant effect on the immunizing activity at the concentrations examined. It is almost impossible to say what is the optimum mixture of the three factors which will produce the maximum immunogenicity, for the following reasons. First, the large amount of material and the number of animals which would be needed for comprehensive titrations of various mixtures of the three factors (cf. toxicity tests, Stanley & Smith, 1961); the unforeseen results which might emerge from such titrations are indicated by the appearance of some immunizing activity in mixtures of factors I and III (see Table 2). Secondly, the low slope of the dose-response curve in these tests for immunogenicity (cf. a similar response with products from *Pasteurella pestis*, Keppie *et al.* (1960) and from *Brucella abortus*, Smith *et al.* (1962)). The preparation used at present for vaccinating man against anthrax is prepared from a culture filtrate (Belton & Strange, 1954) which contains factors II and III. It would appear that addition of factor I (which is removed during the filtration) and removal of factor III would improve the vaccine, if the resulting mixture could be detoxified. The latter should not present much difficulty in view of the extreme lability of the toxic manifestations of factors I and II (Stanley *et al.* 1960; Stanley & Smith, 1961). In this respect, it is interesting that an impure preparation containing factor I of anthrax toxin produced *in vivo* (Stanley *et al.* 1960) was a good immunogen (60 $\mu$g. protected over 50% of the guinea pigs in the standard test for immunogenicity), and later Sargeant *et al.* (1960) showed it to contain, in addition to factor I, component B (i.e. the immunogen in the factor II preparation) in a detoxified but antigenic form.

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# THE SOCIETY FOR GENERAL MICROBIOLOGY

*The Society for General Microbiology held its thirty-sixth General Meeting in the University of Sheffield, on Monday and Tuesday, 7 and 8 January* 1963. *The following communications were made:*

## SYMPOSIUM: SOME COMPARATIVE ASPECTS OF INTERMEDIARY METABOLISM IN MICRO-ORGANISMS

MONDAY, 7 January

**Comparative Aspects of Alcohol Formation.** By E. A. DAWES (*Department of Biochemistry, University of Glasgow*)

**The Microbiological Degradation of Aromatic Compounds.** By W. C. EVANS (*Department of Biochemistry and Soil Science, University College of North Wales, Bangor*)

**The Breakdown and Biosynthesis of Glutamic Acid.** By D. S. HOARE (*Department of Microbiology, University of Sheffield*)

**The Assimilation of 1-C Compounds.** By J. R. QUAYLE (*Department of Biochemistry, University of Oxford*)

**The Assimilation of 2-C Compounds Excluding Acetate.** By J. G. MORRIS (*Department of Biochemistry, University of Leicester*)

**Storage Products.** By J. F. WILKINSON (*Department of Bacteriology, University of Edinburgh*)

Abbreviated versions of these contributions will shortly be published in the *Journal of General Microbiology.*

## ORIGINAL PAPERS

TUESDAY, 8 January

**Methanol Oxidation in *Pseudomonas* sp. M27.** By C. ANTHONY and L. J. ZATMAN (*Department of Microbiology, University of Reading*)

*Pseudomonas* sp. M27, isolated from soil, grows aerobically on methanol as sole source of carbon and energy; it is very similar to *Pseudomonas* sp. AM1 of Peel & Quayle ((1961), *Biochem. J.* **81**, 465). Oxygen uptake in the presence of methanol by washed suspensions of methanol-grown M27 is completely inhibited by $10^{-5}$ M-*p*-nitrophenylhydrazine, $10^{-4}$ M-phenylhydrazine and $5 \times 10^{-3}$ M-EDTA, without affecting the catalase activity (oxygen production from $H_2O_2$). Conversely, methanol oxidation is inhibited only 50% by azide or hydroxylamine ($4 \times 10^{-3}$ M), whilst catalase activity is completely inhibited. These results appear to preclude the catalatic mechanism of methanol oxidation proposed by Harrington & Kallio ((1960), *Canad. J. Microbiol.* **6**, 1). With methanol as substrate, cell-free sonicates catalyse reduction of phenazine methosulphate (PMS) anaerobically in Thunberg tubes. In the presence of PMS the reaction can be followed either manometrically with oxygen as final acceptor or spectrophotometrically with 2,6-dichlorophenolindophenol (PIP) or cytochrome *c* as the final acceptor. Partially purified enzyme preparations lose some activity on dilution and all activity is lost on dialysis against 0·05 M-phosphate buffer

(pH 7·0). Activity of dialysed preparations is restored completely by $5 \times 10^{-3}$ M-ammonium salts and partially by methylamine hydrochloride; salts of Li, Na, K, Rb, Mg or Ca, and of dimethylamine, triethylamine, tetramethylamine, aniline or choline have no effect. The reaction rate in the PMS/PIP system is directly proportional to enzyme and PIP concentrations and is maximal at pH 9·0. There is no inhibition by cyanide, EDTA or phenylhydrazine, all at $10^{-3}$ M. Contrary to the findings of Kaneda & Roxburgh ((1959), *Canad. J. Microbiol.* **5**, 187) no reduction of NAD or NADP in the presence of methanol has been observed. Our present data thus suggest that the oxidation of methanol in *Pseudomonas* sp. M27 involves a new alcohol dehydrogenase which does not require a nicotinamide nucleotide coenzyme.

**The Relation between Growth and Oxygen Consumption in Micro-organisms.** By A. M. Whitaker and S. R. Elsden (*A.R.C. Unit for Microbiology, Department of Microbiology, University of Sheffield*)

We have measured the dry weight of cells produced and the amount of oxygen consumed by cultures of micro-organisms growing in media in which the energy source is the factor limiting growth. Sterile Warburg flasks containing sterile medium and 0·2 ml. 10% (w/v) NaOH in the centre well were inoculated and the oxygen uptake during growth measured. The end of growth was indicated by a fall in the rate of oxygen consumption to a 'resting' level. At this point the cell density was estimated either turbidimetrically or by determining the total perchloric acid-precipitable organic matter by the method of Johnson ((1949), *J. biol. Chem.* **181**, 707). Growth was proportional to the amount of oxygen consumed and the coefficient: g. dry wt./mole $O_2$ ($Y_{O_2}$) was constant. In general the value for $Y_{O_2}$ increased with the complexity of the energy source. Thus for *Pseudomonas fluorescens* KB1, growing upon acetate, succinate and citrate, the mean values for $Y_{O_2}$ were 14·9, 20·7 and 26·4, respectively. When this organism was grown on benzoate or nicotinate, the values for $Y_{O_2}$ were less than those obtained when the organism was grown on equimolecular mixtures of the products of ring fission. The values for benzoate and for succinate + acetate were 17 and 21·9, respectively; for nicotinate and fumarate + formate 11 and 17·6, respectively. These results are in keeping with the view that the oxygen used for ring fission does not participate in oxidative phosphorylation.

*Escherichia coli*, *Torula utilis* and *Acetobacter suboxydans* 621 growing on glucose gave values for $Y_{O_2}$ of 40·4, 42·3 and 9·8, respectively. These results suggest that *A. suboxydans* uses oxygen less efficiently than either *E. coli* or *T. utilis*.

**Photoassimilation of Acetate by the Green Sulphur Bacterium *Chlorobium thiosulphatophilum*.** By J. Gibson and D. S. Hoare (*Agricultural Research Council Unit for Microbiology, University of Sheffield*)

Growing cultures of *Chlorobium limicola* assimilate acetate into cell material provided sulphide, as a source of reducing power, and bicarbonate are present (Sadler & Stanier (1960), *Proc. Nat. Acad. Sci., Wash.* **46**, 1329). The effect of acetate on cell yield has been investigated with the closely related bacterium *Chlorobium thiosulphatophilum*. Low concentrations of acetate increase the rate of growth and final cell yield with maximum effects at 0·005 M acetate. With resting cell suspensions, the presence of acetate decreased the rate at which radioactive carbon dioxide was assimilated during photosynthesis in 95% $H_2$ + 5% $CO_2$. Resting cell suspensions assimilate [1-] and [2-$^{14}$C] acetate provided the system is illuminated, a source of reducing power, such as hydrogen, is available, and carbon dioxide is present. The finding that bicarbonate is essential for the assimilation of acetate by resting cell suspensions of *C. thiosulphatophilum* contrasts with similar experiments with *Rhodospirillum rubrum* (Stanier, R. Y. *et al.* (1959), *Proc. Nat. Acad. Sci., Wash.* **45**, 1246) where it was found that acetate is photoassimilated in the absence of bicarbonate to poly-$\beta$-hydroxybutyrate. Poly-$\beta$-hydroxybutyrate has not been found either in cultures grown in the presence of acetate or in resting cell suspensions exposed to acetate in the

light. Carbon dioxide present during the photo-assimilation of [1-] and [2-$^{14}$C] acetate by resting cell suspensions did not contain radioactivity after 2 hr. exposure in the light, suggesting that acetate is not oxidized under these conditions. The crude protein fraction obtained by the procedure of Roberts *et al.* ((1955), *Studies of Biosynthesis in* Escherichia coli. Carnegie Instn. of Washington Publication 607, Washington D.C.), has been hydrolysed and amino acids have been separated and identified by paper electrophoresis and paper chromatography coupled with radioautography. Both [1-] and [2-$^{14}$C] acetate appear to be incorporated into all the common amino acids. The distribution of radioactivity in the amino acids is being investigated by unequivocal degradation procedures.

**The Metabolism of Erythritol by a Virulent Strain of *Brucella abortus*.** By J. D. Anderson and H. Smith (*Microbiological Research Establishment, Porton, Wiltshire*)

Foetal erythritol appears to explain the preferential growth of *Brucella abortus* in foetal tissue during bovine brucellosis. The polyol (0·001 $\mu$mole/ml.) stimulated the growth of *Br. abortus* in bovine phagocytes and laboratory media and enhanced an infection *in vivo* (Smith, H. *et al.* (1962), *Nature, Lond.* **193**, 47). Erythritol was known to be the best of nine carbohydrates when used as sole carbon source for *Br. abortus* in a simple medium (McCullough, N. B. & Beal, G. A. (1951), *J. Infect. Dis.* **89**, 266). However, our interest in the function of erythritol *in vivo* prompted an investigation into its metabolism by *Br. abortus* (strain 544) growing (37°, shake flasks, 5 % $CO_2$/air) in a complex defined medium (based on that of Rode, L. J., Oglesby, G. & Schuhardt, V. T. (1950), *J. Bacteriol.* **60**, 661) and in the presence of adequate carbohydrate (38 $\mu$mole/ml. D-glucose). Kinetic studies and erythritol determinations during growth in the latter medium showed that erythritol (0·005–1·0 $\mu$mole/ml.) stimulated growth of the organism approximately twofold and that low concentrations of erythritol were only effective for small populations. Despite the high D-glucose concentration in these experiments, *Br. abortus* used more than its own weight of the polyol during growth. Radiotracer investigations confirmed these conclusions and showed that as soon as 0·5 $\mu$mole/ml. of U-[$^{14}$C]erythritol had disappeared from the medium after growth of *Br. abortus* from about $2 \times 10^7$ to about $1 \times 10^9$ cells/ml., the distribution of [$^{14}$C] was approximately: carbon dioxide 45 %, bacteria 25 % and medium 30 %. The nature of material remaining in the medium is under investigation.

**Erythritol, its Effect on the Growth of Various Strains of *Brucella abortus* and its Possible Significance in Infections with *Brucella melitensis* and *Brucella suis*.** By A. E. Williams, J. Keppie and H. Smith (*Microbiological Research Establishment, Porton, Wiltshire*)

The predilection of *Brucella abortus* for bovine foetal fluids and placenta is probably due to the presence in these tissues of erythritol—a growth stimulant for this organism (Smith, H. *et al.* (1962), *Nature, Lond.* **193**, 47). This work in the bovine was done with a single virulent strain of *Br. abortus*. It remained to be seen whether different strains of *Br. abortus* would be similarly stimulated by erythritol and whether the presence of erythritol could explain the localization of *Br. melitensis* in goats and sheep and *Br. suis* in pigs. When a number of strains of *Br. abortus* were examined for the effect of erythritol on their growth, not only were other virulent strains stimulated but there was a good correlation between degree of virulence (for guinea-pigs) and the extent of growth-stimulation by erythritol. Thus, in a mixture of tryptic meat broth (1 part) and Locke solution (1 part), erythritol stimulated the growth of seven virulent smooth strains to a greater extent than it did four attenuated smooth strains and one avirulent smooth strain. The relevance of these findings to the pathogenesis of bovine brucellosis is being studied. With regard to brucellosis in other hosts, significant amounts of erythritol were found in the placenta of animals, such as goats, sheep and pigs, that in brucellosis suffer a heavy infection of the placenta, but not in human placenta or in that of the rat, rabbit or guinea-pig which do not show the characteristic placentitis. Furthermore, the growth of virulent strains of *Br. melitensis* and

*Br. suis* was significantly stimulated by adding erythritol to cultures in a serum medium and a tryptic meat broth for *Br. melitensis*, and a defined medium (McCullough, N. B. & Dick, L. A. (1943), *Proc. Soc. exp. Biol., N.Y.* **52**, 310) for *Br. suis*.

**The Isolation and Composition of Flagella from *Crithidia (Strigomonas) oncopelti*.** By J. K. CHESTERS (*Sub-Department of Chemical Microbiology, Department of Biochemistry, University of Cambridge*)

The trypanosomid flagellate, *Crithidia oncopelti*, used in this investigation, was a strain of the organism first isolated in bacteria-free culture by Noguchi & Tilden ((1926), *J. exp. Med.* **44**, 307) and named *Herpetomonas oncopelti*.

Suspensions of the organism ($10^9$ cells per ml.) in 0·25 M sucrose solution were homogenized for short periods of time in a loosely fitting Potter homogenizer. In this way flagella were detached from the cells with little cell breakage and then isolated by differential centrifugation through sucrose gradients.

The degree of contamination of preparations of flagella with other cell fragments was assessed by phase contrast and electron microscopy; the possibility of contamination with adsorbed soluble materials, liberated by cell breakage, was investigated using $^{14}C$-labelled cells.

Analysis showed the general composition of the flagella to be: protein, 72 %; lipid, 27 %; nucleic acid, 0·5 % and carbohydrate, 0·5 %. Thirteen per cent of the lipid was shown to be phospholipid, the sole nitrogenous base being ethanolamine. Gas-liquid chromatography of the fatty acids of the lipid identified four major components: palmitic, 15-methyl-hexadecanoic, oleic and a highly unsaturated $C_{18}$ acid. In this respect, the lipid from flagella is very similar to that extracted from intact cells.

The amount of nucleic acid in preparations of flagella varied considerably but never exceeded 1·0 %. Experiments with $^{14}C$-adenine-labelled cells suggest that this nucleic acid may arise from contamination with cytoplasm rather than form an integral part of the flagellum.

**The Incorporation of $^{14}C$ Labelled Methionine into Bacterial Flagellin.** By D. KERRIDGE (*Sub-Department of Chemical Microbiology, Department of Biochemistry, University of Cambridge*)

Flagella from *Salmonella typhimurium* have been shown to contain the unusual amino acid εN-methyl lysine (NML) (Ambler & Rees (1959), *Nature, Lond.* **186**, 56). In cultures of *S. typhimurium* this amino acid can be labelled with either $^{14}C$ lysine or $^{14}C$ methyl labelled methionine. The incorporation of the labelled methyl group from methionine into NML gives a satisfactory measure of the rate of formation of flagella by cultures of *S. typhimurium*.

The methionine analogues, D-methionine and DL-methionine sulphoxide have no effect on the growth rate of *Salmonella typhimurium* in a minimal medium, but competitively inhibit the isotopic labelling of the methyl group of NML. DL-ethionine and DL-methionine sulphone also inhibit the methyl labelling of NML.

Flagellin can be detected serologically in the soluble protein fraction of *Salmonella typhimurium* (cf. Weinstein, Koffler & Moskowitz (1960), *Bact. Proc.* p. 63) but using the methyl labelling of NML as a marker it has not been possible to show intracellular precursors of flagella.

**Glycine Metabolism in Relation to the Synthesis of Methionine by *Escherichia coli*.** By F. G. BULL and D. D. WOODS (*Microbiology Unit, Department of Biochemistry, University of Oxford*)

Suspensions of *Escherichia coli* PA 15 (a serine/glycine auxotroph) convert homocysteine to methionine with serine as source of the methyl group. Organisms grown with glycine formed twice as much methionine as those grown with serine and in addition could use glycine as $C_1$ donor (Gibson, F. & Woods, D. D. (1960), *Biochem. J.* **79**, 160). Serine-grown

organisms produced equimolar quantities of glycine and methionine; glycine-grown organisms, however, formed only about one-third as much glycine as methionine from serine.

Using [$^{14}$C-2] serine it has now been shown that C-2 of serine is incorporated into the methyl group of methionine by organisms grown with glycine, though not with serine. This result shows that C-2 of glycine (formed from serine by serine hydroxymethylase) can also act as source of the $C_1$ unit for methionine synthesis. Dialysed ultrasonic extracts of glycine-grown organisms in the presence of tetrahydrofolate formed $C_1$ units (isolated as [$^{14}$C]formaldmethone) from [$^{14}$C-2]glycine; only $^{14}CO_2$ was formed from [$^{14}$C-1]glycine. Chromatography on TEAE-cellulose of products with [$^{14}$C-1] or [$^{14}$C-2]glycine as substrate showed that radioactive formaldehyde and tetrahydrofolate derivatives were formed only from [$^{14}$C-2]glycine. These results are consistent with those of Pitts, J. D. & Crosbie, G. W. ((1962), *Biochem. J.* 83, 35P).

Growth in the presence of serine (even if glycine is also present) represses the formation of the enzyme system for the production of $C_1$ units (at the formaldehyde level of oxidation) from glycine. Relief from repression occurs on transfer of serine-grown organisms to media containing glycine only and is inhibited by chloramphenicol. When glycine-grown organisms are transferred to a medium containing serine, glycine metabolism is repressed and ability to synthesize methionine (serine as $C_1$ donor) also decreases at a rate consistent with the dilution out of a mechanism for the utilization of C-2 (though not of C-3) of serine.

**Amino Acid Accumulation as a Consequence of Respiration in *Bacillus megaterium*.** By R. E. MARQUIS (*Chemical Biology Unit, Zoology Department, Edinburgh University*)

There is a great deal of evidence suggesting that the transport of certain molecules into cells can be an energy-requiring process and so must be coupled to some exergonic reaction. Such a couple can be demonstrated in *Bacillus megaterium* between respiration and the uptake of alpha-aminoisobutyrate, a metabolically inert amino acid that enters these cells by the normal pathways for glycine and alanine. In the absence of respiration, at 4° or under a nitrogen atmosphere, this amino acid is taken up passively only, the uptake being essentially the same at 4° as at 30°. During respiration, uptake occurs also by mechanisms linked to oxygen utilization and, therefore, metabolic degradation of compounds such as glucose, succinate or lactate leads to increases in amino acid uptake in proportion to the extent that oxygen uptake is accelerated. This respiration-dependent uptake can be completely reversed by placing the cells in a nitrogen atmosphere or by allowing them to deplete their oxidizable substrates.

Since amino acid uptake by this organism is directly related to oxygen uptake, it can be stimulated by agents such as arsenate or dinitrophenol, when these speed up respiration, and inhibited by agents such as cyanide that block oxidation. The coupling between amino acid uptake and respiration is of a nature that changes in the rate of respiration are reflected by amino acid movement only after a brief time lag. That is to say, the primary asymmetry between the cell and its environment is caused by respiration, amino acid accumulation representing only a response to this unstable condition. The rate of oxygen utilization required to maintain a particular concentration differential between interior and exterior of the cell is a function of the work involved in the concentration process. Some details of this function will be presented.

**The Effect of Antibiotics on Fixation of Penicillin by Sensitive Bacteria.** By P. E. REYNOLDS (*Sub-Department of Chemical Microbiology, Department of Biochemistry, University of Cambridge*)