THE JOURNAL OF GENERAL MICROBIOLOGY

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THE JOURNAL OF GENERAL MICROBIOLOGY

The *Journal* will publish accounts of original research in general microbiology, i.e. the study of bacteria, microfungi, microscopic algae, protozoa, and viruses in their biological activities and, more particularly, the fundamental aspects of the study of these forms, including structure, development, physiology, genetics, cytology, systematics and ecology. Writers of papers on a specialized aspect of their subject should describe their work so that its relevance to their own science and to microbiology in general will be apparent to readers who may be unfamiliar with the particular aspect.

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'Easy reading's curst hard writing.'—*The Editors, J. gen. Microbiol.*

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(1) Papers must be written in English with the utmost conciseness consistent with clarity. The best English for the purpose of the *Journal* is that which gives the sense in the fewest short words.

(2) A paper should be written only when a piece of work is rounded off. Authors should not be seduced into writing a series of papers on the same subject *seriatim* as results come to hand. It is better, for many reasons, to wait until a concise and comprehensive paper can be written.

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Descriptions of new species of cultivable microbes should not be submitted unless an authentic specimen of a living culture has been deposited in a recognized culture collection

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Except for good reasons, micro-organisms should be designated by the names used in the works listed below. When other authorities are followed, they should be cited whenever obscurity might result from their use.

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

PLANT PATHOGENIC FUNGI AND PLANT DISEASES. *List of Common British Plant Diseases*, 1944. (Cambridge University Press.)

PLANT VIRUSES AND VIRUS DISEASES (1957). *Rev. appl. Mycol.* **35**, Suppl. 1–78.

BACTERIA. Author's preferences in naming are at present accepted provided that the designation is unambiguous and conforms with the International Bacteriological Code of Nomenclature (1949; *J. gen. Microbiol.* **3**, 444) and the Opinions issued by the International Committee on Bacteriological Nomenclature. If desired, a synonym may be added in brackets when a name is first mentioned.

J. gen. Microbiol. (1967), **48**, 1–8
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Alkali Metal Ions in Relation to the Growth of *Escherichia coli*: a Comparison with *Staphylococcus aureus*

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(*Accepted for publication* 30 *January* 1966)

SUMMARY

The effects of alkali metal ions on the growth of *Escherichia coli* have been compared with those for *Staphylococcus aureus* (*pyogenes*). Only rubidium could substantially support the growth of both organisms. The efficiency of rubidium as a growth promoter varied with both the potassium and rubidium content of the medium. The uptake of potassium and rubidium during the growth of *E. coli* was examined using radio-isotopes as tracers, and the velocities of uptake, the accumulation and internal concentrations of these cations were calculated. Potassium was taken up rapidly early in growth and this uptake was inhibited by rubidium, which itself was taken up, and in greater amounts than potassium. *Escherichia coli* differed from *S. aureus* in that the former readily leaked both cations when growth slowed down. Rubidium leaked out early in growth only to be taken up again. The two ions appeared to compete for influx and efflux.

INTRODUCTION

Bacteria require potassium for growth and rubidium can usually replace this requirement (Rothstein, 1959). The interactions of the alkali metal ions lithium, potassium, rubidium and caesium on *Staphylococcus aureus* (*pyogenes*) have been examined (Wyatt, 1963*a*) and found to be more complex than those reported for other organisms (e.g. Eddy & Hinshelwood, 1950). In this report we have examined *Escherichia coli* under similar experimental conditions, to see whether it resembles staphylococci.

Wyatt (1963*b*) showed that the uptake of potassium and rubidium during growth of the Gram-positive *Staphylococcus aureus* differed markedly from that of Gram-negative organisms such as *Alcaligenes faecalis* (Krebs, Whittam & Hems, 1957), *Escherichia coli* (Cowie, Roberts & Roberts, 1949) and fungi such as yeast (Conway & Duggan, 1958; Rothstein, 1959) and *Neurospora crassa* (Lester & Hechter, 1958). These differences might have been due to basic differences between Gram-positive and Gram-negative organisms, to different nutritional requirements, or to different experimental conditions and techniques. We have therefore examined the uptake of potassium and rubidium by *E. coli* under experimental conditions similar to those used for *S. aureus*. In the experiments reported here growth was limited by the concentration of potassium present and the potassium and rubidium concentrations were measured using the radio-isotopes ^{42}K and ^{86}Rb, respectively.

METHODS

The general methods and materials were similar to those of Wyatt (1963*a*, *b*). A medium containing 1% Lab-lemco, 1% peptone (Oxoid) and 0·15% glucose made deficient in cations was used. Cations were removed by passing a 5% Lab-lemco + 5% peptone solution down a column of IRC-50 (Na^+) and a 3% solution of glucose down IR-120(H^+). 'Specpure' salts, $MgSO_4.7H_2O$, $CaCl_2$, and $FeSO_4.7H_2O$ were added to the medium which had a Na^+ content of 50 mM.

Sterile ^{42}K and ^{86}Rb chlorides were obtained from the Radiochemical Centre, Amersham. Suitable dilutions of ^{42}KCl were made and the molarity determined by flame photometry (Wyatt, 1963*b*). The experimental tubes contained 23 μM-K^+ of which 3 μM was residual K^+ and the rest ^{42}K + carrier. Rubidium was added at two concentrations, 0·2 and 2·0 mM, of which only 0·2 μM was ^{86}Rb plus carrier and the rest 'Specpure' RbCl.

As it is difficult to measure ^{42}K and ^{86}Rb together duplicate series of tubes were used containing either ^{42}K or ^{86}Rb, but the same total amounts of K^+ and of Rb^+. The extent and rate of growth were almost identical in the duplicate series. After incubation, 5 ml. portions were removed, cooled to 0° and centrifuged at 1000*g* for 20 min. Samples (4 ml.) of the supernatant fluid were then diluted to 10 ml. for counting in a liquid GM counter, type M 6 H. Counting times gave a statistical accuracy of better than 1% for ^{86}Rb and 3% or better for ^{42}K, except for three samples with extremely low counts. The activities were corrected for decay (^{42}K) and for lost counts (^{86}Rb). A Spekker reading of 0·1 (with 1 cm. cells and H 508 filter) corresponded to a dry weight of 97 ± 7 mg./l. and gave 77×10^6 colonies per ml. suspension. Accumulations (internal concentration/external concentration) were calculated using the value of 3·2 ml. per g. dry wt., i.e. 4 ml./g. dry wt. less 26% interspace volume using the data of Roberts (1959) and Roberts *et al.* (1955).

Escherichia coli NCTC 8007 was used throughout.

RESULTS

Cation requirements for growth

The broth with 3 μM residual K^+ did not support the growth of either *Escherichia coli* or *Staphylococcus aureus*. We found no evidence of an arithmetic response by *E. coli* in low K^+ broth (see Roberts & Roberts, 1950). Growth of *E. coli* was proportional to the logarithm of the K^+ concentration up to about 0·1 mM-K^+ and addition of Rb^+ at different K^+ levels increased the cell yield (Fig. 1). The growth due to the K^+ in the broth was subtracted from the total growth, i.e. that due to K^+ plus Rb^+, and the remaining growth was assumed to be due to Rb^+. From the K^+ growth response curve (see for example Fig. 1 of Wyatt, 1963*a*) the additional K^+ concentration required to give the same growth as that due to Rb^+ was calculated. A series of Rb^+–K^+ equivalent curves similar to that shown by Wyatt (1963*a*, Fig. 3) was obtained. For Rb^+ concentrations up to 0·2 mM the relationship was $[Rb^+] = m[K^+]^n$, where $[K^+]$ is the equivalent concentration of K^+ and m and n are constants. From the graphs the calculated constants were $\log[Rb^+] = 1{\cdot}43\log[K^+] + 10{\cdot}3 - 0{\cdot}2[K^+]^*$ (*E. coli*). This is similar to the expression for *Staphylococcus aureus*:

$$\log[Rb^+] = 1{\cdot}25\log[K^+] + 1{\cdot}36 + 0{\cdot}5[K^+]^*$$

(Wyatt, 1963*a*), where $[K^-]^*$ is the concentration of K^+ in the medium.

The efficiency of Rb^+ as a growth promoter decreased with increasing Rb^+ concentration, but at low Rb^+ concentrations this efficiency was increased by raising the K^+ content of the broth (Fig. 2).

The addition of from 1 to 10 mM-Li^+ did not alter the cell mass obtained either with or without K^+ present, but Cs^+ gave increased growth with all levels of K^+ used—the efficiency of Cs^+ relative to K^+ varied from 0·2 to 5·0% (over a 1–10 mM-Cs^+ range). As with Rb^+, the efficiency of Cs^+ increased as the K^+ concentration was raised.

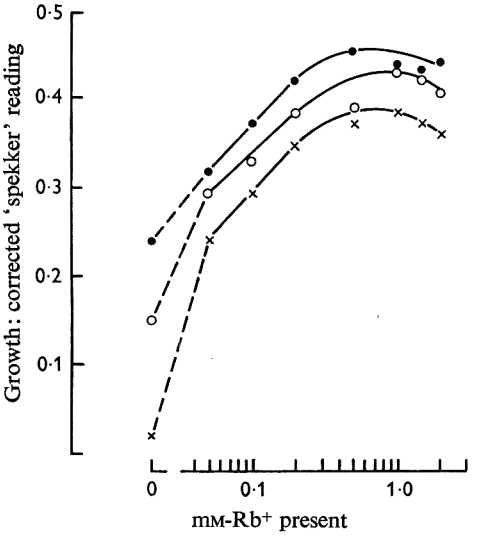

Fig. 1

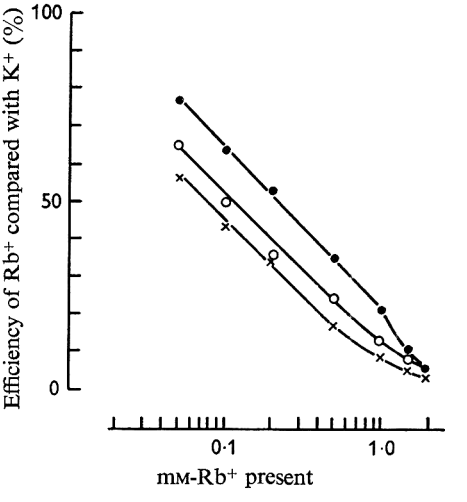

Fig. 2

Fig. 1. Effect of rubidium on the growth of *Escherichia coli*. ×, 0·003 mM-K^+; ○, 0·013 mM-K^+; ●, 0·023 mM-K^+. All tubes contained 0·2 mM-Mg^{2+} and 0·1 mM of both Ca^{2+} and Fe^{2+}. Growth was measured after incubation for 24 hr at 37°.

Fig. 2. Efficiency of rubidium as a growth promoter for *Escherichia coli*. For details see Fig. 1 and text.

The uptake and retention of potassium

There appeared to be logarithmic uptake of K^+ during the logarithmic period of growth (Fig. 3). From the plotted growth curves and the cumulative K^+ uptake the K^+ accumulation (Fig. 4) and rates of uptake were calculated. The rate of K^+ uptake decreased with decreasing extra-cellular K^+ (at 17·3 and 1·6 μM-K^+ the rates of K^+ uptake were 22·5 and 1·3 μmoles/100 mg. dry-wt/hr respectively). As the rates of uptake decreased during growth so the K^+ concentration per cell also decreased (Table 5). The cells leaked K^+ when growth ceased (Figs. 3, 4).

The effect of rubidium on growth

With added K^+ there was logarithmic growth for about 11 divisions with a mean generation time (M.G.T.) of 26 min. whether or not Rb^+ was added. When Rb^+ was added there then followed a further short logarithmic phase of increased M.G.T. With low K^+ (3 μM) no growth occurred unless Rb^+ was present and again the M.G.T. increased to 77 min. after 11 divisions.

The effect of rubidium on the uptake and leakage of potassium

The concentration of K^+ in the culture fluid was reduced to 1·6 μM from 23 μM in 7 hr in the absence of Rb^+, but when Rb^+ was present the rate of K^+ uptake was less and so also was the K^+ concentration per unit cell wt. When the initial $Rb^+:K^+$ ratio of the medium was increased by a factor of 10 to 88:1 there was no uptake of K^+ for the first 5 hr. Although the rate of K^+ uptake was less, the maximum K^+ uptake from the medium was greater, but as expected the time taken was longer (Table 1); although the K^+ concentration per cell was lower at this stage, the accumulation was greater (Fig. 4). Greater Rb:K ratios did not reduce the maximum K^+ uptake.

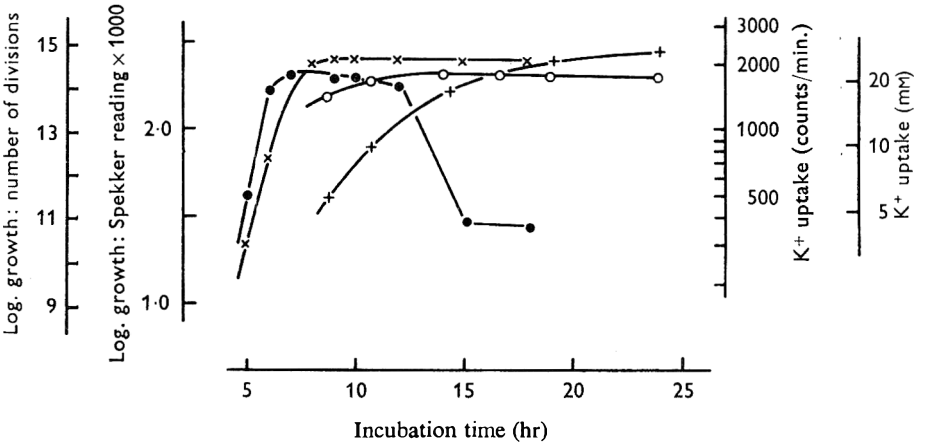

Fig. 3. Uptake of potassium during growth by *Escherichia coli* and *Staphylococcus aureus*. (*E. coli*: with 0·023 mM-K^+, 0·2 mM-Mg^{2+} and 0·1 mM of both Ca^{2+} and Fe^{2+}; ×, growth; ●, K^+ uptake. *S. aureus* (data taken from Wyatt, 1963*b*): with 0·021 mM-K^+, 1 mM-Mg^{2+}, 2·5 mM arginine and 0·1 mM of both Ca^{2+} and Fe^{2+}; +, growth; ○, K^+ uptake.

When the growth rate decreased there was a substantial net gain of K^+ by the medium but in the presence of Rb^+ this efflux was greatly reduced (Table 2) and consequently the final K^+ content per cell and the accumulation were greater (Table 3).

The uptake of rubidium

More Rb^+ than K^+ was taken up but this was due to the higher ratio of $Rb^+:K^+$ external concentrations. The maximum Rb^+ uptake was affected by the external concentrations of both K^+ and Rb^+ (Table 4). Initially Rb^+ was taken up more rapidly than K^+. With 0·2 mM-Rb^+ and 0·023 mM-K^+ the velocities of Rb^+ and K^+ uptake were 105 and 35 μmoles/100 mg. dry wt./hr respectively during the first 5 hr; with 2 mM-Rb^+ the corresponding values were 460 and 0 respectively. The pattern of Rb^+ uptake was more complicated than that shown by staphylococci (Wyatt, 1963*b*). In the four series studied, after the initial uptake between 0 and 5 hr, the cells lost some Rb^+. More Rb^+ was then taken up until, near the end of growth, it leaked out together with K^+. With low K^+, the Rb^+ concentration per cell wt dropped suddenly near the end of growth but with higher K^+ this was not so. This suggests that K^+ inside the cell was reducing the efflux of Rb^+ just as Rb^+ affected K^+ efflux.

The net rates of uptake of potassium and rubidium

When 0·023 mM-K^+ and 0·2 mM-Rb^+ were present together, the initial velocities of K^+ and Rb^+ uptake were lower than when either was present alone. The rate of K^+ uptake remained constant but that of Rb^+ fell. The ratio of the concentrations of Rb^+ to K^+ in the supernatant ($[S]_{Rb}$: $[S]_K$) increased until at 40:1 the rates of uptake of K^+ (decreasing) and Rb^+ (increasing) were equal. The ratio continued to increase until

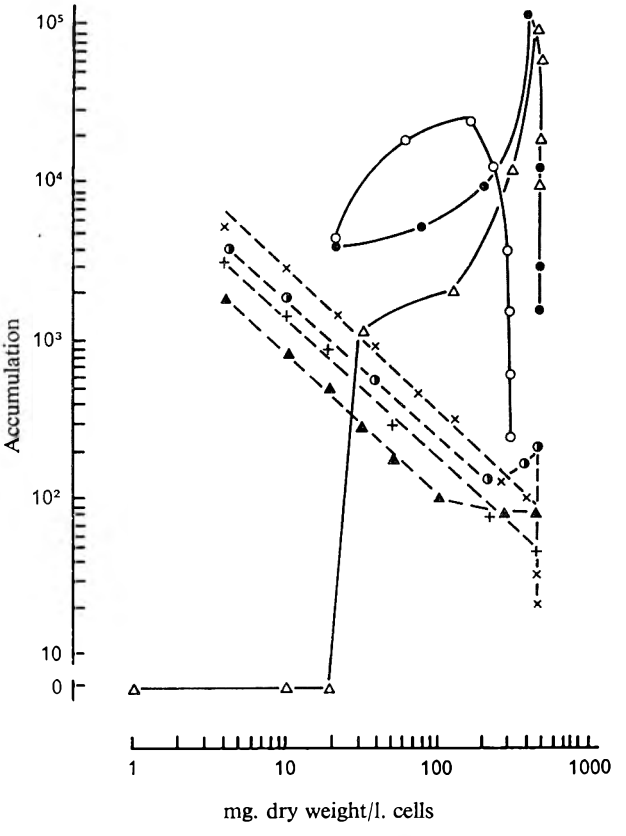

Fig. 4. Accumulation of K^+ and Rb^+ by *Escherichia coli*. For calculation of accumulations see Methods. All tubes contained 0·2 mM-Mg^{2+} and 0·1 mM of both Ca^{2+} and Fe^{2+}. Accumulation of K^+ (0·023 mM-K^+): ○, no Rb^+ added; ●, 0·2 mM-Rb^+; △, 2·0 mM-Rb^+. Accumulation of Rb^+ (0·2 mM-Rb^+): ×, 0·003 mM-K^+; ◑, 0·023 mM-K^+. (*c*) Accumulation of Rb^+ (2·0 mM-Rb^+): +, 0·003 mM-K^+; ▲, 0·023 mM-K^+.

at 1000:1 K^+ and Rb^+ leaked out. When Rb^+ was increased to 2·0 mM, K^+ uptake was completely inhibited for the first 5 hr during which Rb^+ uptake was rapid. When the external Rb:K ratio was 84:1, K^+ uptake began, and Rb^+ leaked out. At an $[S]_{Rb}$: $[S]_K$ of 85:1 the rates of K^+ and Rb^+ uptake were equal and almost zero. As with 0·2 mM-Rb^+ the supernatant Rb^+:K^+ ratio increased until at 100:1 the rates of uptake were once again equal. Finally K^+ was lost from the cell at a rate approximately one-fifth that of Rb^+.

Table 1. *Effect of rubidium on the maximum uptake of potassium during growth of Escherichia coli*

	Rb^+ present (mM)*		
	0	0·2	2·0
Maximum K^+ uptake (μM) and percentage K^+ removed from medium	21·4 (93 %)	22·9 (99·5 %)	22·8 (99·1 %)
Time of maximum uptake (hr)	7	10	10
μmoles K^+/100 mg. dry wt at time of maximum uptake	13·3	4·8	4·8

* All tubes contained 23 μM-K^+, 0·2 mM-Mg^{2+} and 0·1 mM of both Ca^{2+} and Fe^{2+}.

Table 2. *Net efflux of potassium and rubidium in Escherichia coli*

	Rb^+ present (mM)		
	0	0·2	
K^+ concn. (mM)	V_K	V_K	V_{Rb}
0·003	—	—	0·7
0·023	1·7	0·12	0·2

Growth medium contained 0·2 mM-Mg^{2+} and 0·1 mM of both Ca^{2+} and Fe^{2+}. Velocities of efflux (V_K and V_{Rb}) were measured at 13 hr and are expressed as μmoles/100 mg. dry wt./hr.

Table 3. *Residual potassium after uptake has ceased*

	Staphylococcus aureus (*pyogenes*) (Wyatt, 1963*b*)		*Escherichia coli*	
	Rb^+ present (mM)		Rb^+ present (mM)	
	0	0·2	0	0·2
Time (hr)...	14 to 24	14 to 24	8½ to 10	9 to 11
Mean counts/min. ^{42}K, corrected	149 ± 8·9	96 ± 3·4	269 ± 30·5	9 ± 6·8
Standard error of means	4·8		16·4	
Difference of means	53, $P < 0·001$		260, $P < 0·001$	
Accumulation	72,000	101,000	8,000	119,000

Media contained: (*a*) *S. aureus*: 0·021 mM-K^+, 1·0 mM-Mg^{2+}, and 0·1 mM of both Ca^{2+} and Fe^{2+}; (*b*) *E. coli*: 0·023 mM-K^+, 0·2 mM-Mg^{2+} and 0·1 mM of both Ca^{2+} and Fe^{2+}.

Table 4. *Effect of initial potassium and rubidium concentrations on rubidium uptake during growth of Escherichia coli*

	Maximum Rb^+ uptake (μM) with K^+ (mM)	
Rb^+ present (μM)	0·003	0·023
200	25	56
2000	134	155

Growth media contained 0·2 mM-Mg^{2+} and 0·1 mM of both Ca^{2+} and Fe^{2+}. Maximum Rb^+ uptake expressed as the change in supernatant concentration.

Potassium and rubidium contents of Escherichia coli during growth

The potassium and rubidium contents of *Escherichia coli* were measured during growth in the presence of K^+ alone and in the presence of K^+ and Rb^+ (Table 5). Using the values for the efficiency of Rb^+ (in terms of growth, Fig. 2) the corresponding K^+ equivalents of the Rb^+ present in the cell were calculated and added to the existing K^+ content of the cell. The results expressed as K^+ uptake were fairly uniform for the different conditions of growth (Table 5).

Table 5. *Comparison of potassium uptake with potassium and rubidium uptake (expressed as potassium equivalents) during growth of Escherichia coli*

	Rb^+ present (mM)		
Time (hr)	0 K^+ uptake (μmoles/100 mg. dry-wt)	0·2 K^+ uptake + K^+ equivalents of Rb^+ uptake (Rb^+ efficiency 53·4%)	2·0 K^+ uptake + K^+ equivalents of uptake (Rb^+ efficiency 6·1%)
6	30·4	28·5*	14·1
7	13·3	14·0	11·3
8	8·8	10·0	8·3
9	7·7	10·5	6·7
10	7·3	10·0	6·5
11	6·8	10·5	6·4
12	6·4	5·5	6·3

Efficiencies calculated using Fig. 2; 0·023 mM-K^+ present.

* Example: K^+ uptake was 18·50 μmoles/100 mg. dry-wt

Rb^+ uptake was 18·80 μmoles/100 mg. dry-wt $\equiv \frac{18{\cdot}8 \times 53{\cdot}4}{100} \equiv 10{\cdot}02$ μmoles K^+/100 mg. dry-wt

K^+ uptake + K^+ equivalent of Rb^+ uptake $= 18{\cdot}50 + 10{\cdot}02 \equiv 28{\cdot}52$.

DISCUSSION

The efficiency of the alkali metal ions (excluding sodium) in promoting the growth of *Escherichia coli* and *Staphylococcus aureus* are potassium > rubidium $\gg$ caesium $\gg$ lithium. These results are consistent with those expected from enzyme studies; both rubidium and caesium are commonly alternative activators of potassium-requiring enzymes while lithium is not (Dixon & Webb, 1964). Rubidium competes with potassium for entry but caesium competition cannot be detected: it would be expected that caesium would, however, compete with rubidium for entry and so cause some inhibition of growth (Wright & Wyatt, in preparation). The interactions of potassium and rubidium are complicated as they represent the sum of the effects due to their mutually competitive inhibition of entry (Wyatt, 1963*b*; Wright & Wyatt, in preparation), their probable competition for exit (Wyatt, 1963*b*), their efficiency as enzyme activators and their relative stability constants for each chelate within the cell (Wyatt, 1964). Nevertheless the response of both staphylococcus and *E. coli* to alkali metal ions is very similar. Just as the growth responses and alkali ion requirements of *E. coli* and *S. aureus* are similar, so the uptakes of these ions (potassium and rubidium) have many

features in common. Potassium and rubidium were accumulated by both organisms during growth but while *S. aureus* appeared to have a tight membrane with respect to potassium, *E. coli* lost much of its accumulated potassium when growth slowed down: *S. aureus* maintained a 50,000-fold accumulation, but in *E. coli* the accumulation rapidly dropped to 200-fold.

In both organisms rubidium delayed the uptake of potassium and also reduced the rate of uptake, probably by a competitive inhibitory mechanism (Wright & Wyatt, 1965). Potassium similarly inhibited the entry of rubidium and probably also inhibited its efflux. The amount of potassium in the cell was lower when rubidium was present, and the presence of rubidium also brought about a decrease in the leakage of potassium from both organisms. In the presence of rubidium both organisms showed the same diphasic logarithmic growth, though this was more pronounced in *Staphylococcus aureus*. In conclusion, these results show that, contrary to previous data, *S. aureus* and *E. coli* behave similarly in their requirements for and their uptake of alkali metal ions, when tested under similar conditions.

We are grateful to Professor C. L. Oakley, F.R.S., for his advice and encouragement. Part of this work has been published as an abstract (Wright & Wyatt, 1963).

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On the Clumping of *Corynebacterium xerosis* as Affected by Temperature

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SUMMARY

Corynebacterium xerosis Strain NCIB 9956 (minimum temperature for growth about 20°) formed clumps in cultures or suspensions of bacteria from these cultures, when grown at 30° and rapidly cooled to 15° with fast stirring. Electron microscopy showed whole organisms to be connected by an adhesive material. Electron micrographs of thin sections showed that clumps appeared to be formed by single layers of bacteria collecting round gas bubbles. The extent of clumping was greatest around pH 3, and increased as the temperature was decreased from 30° to 15°. Substitution of nitrogen or oxygen for air as gas phase had little effect on the clumping. The clumping ability increased with the age of the culture from the mid-exponential to the late-exponential phase of growth, but thereafter declined. Salts were necessary for clump formation. The relative effects of different monovalent cations and anions depended on the positions of the ions in the Hofmeister series. Incubation of bacteria in buffered solutions of proteolytic enzymes decreased their clumping ability. The extent of clumping also decreased when bacteria were incubated in buffered solutions of guanidine HCl (5 M), urea (8 M) or in a solution of uranyl nitrate (10 mM) and NaCl (0·1 M). Isolated walls of the organism formed clumps when suspensions in phosphate buffer (pH 7·0) were incubated at temperatures between 35° and 5° with fast stirring. Pretreatment of walls with trypsin decreased clumping ability. The ability to form clumps when cultures were cooled to 5° with fast stirring was demonstrated with several but not all of the strains of *C. xerosis* tested.

INTRODUCTION

Strains of *Corynebacterium xerosis* have much higher minimum temperatures for growth (around 20°) than the majority of other mesophilic micro-organisms (5–10°; *Bergey's Manual*, 1957; Rose & Evison, 1965). During studies on the biochemical basis of the minimum temperature for growth of a strain of *C. xerosis*, mid-exponential phase cultures of the bacterium, grown at 30° in rapidly stirred cultures, were transferred to 15° with the object of analysing the bacteria after a period of incubation at the subminimum temperature. This shift in incubation temperature caused some of the bacteria in the culture to form clumps. The present paper describes experiments done to discover the basis of this clumping, and also reports on the ability of other corynebacteria and related organisms to form clumps at low temperatures.

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METHODS

Organisms. Most of the work described in this paper was done with a strain of *Corynebacterium xerosis* NCL from the culture collection of this Department (Rose & Evison, 1965); the strain has been deposited in the National Collection of Industrial Bacteria, and has been granted the accession number NCIB 9956. Several other bacteria were also examined for the ability to aggregate at low temperatures; the sources of these organisms are given in Table 1. All bacteria were maintained at 30° on nutrient agar slopes, and were subcultured every 24 hr. Stock cultures were preserved in the lyophilized state.

Experimental cultures. The bacteria were grown in a medium (pH 7·4) identical in composition with that described by Rose & Evison (1965) except that the concentration of $Na_2HPO_4.12H_2O$ was 1·5 g./l., and of KH_2PO_4 0·5 g./l. Portions (1150 ml.) of glucose-free medium were dispensed into 2 l. round, flat-bottom flasks, each of which was fitted with two ports, 2 cm. in diameter, to facilitate sampling of the culture. The ports and neck of each flask were plugged with cotton wool. The medium was sterilized by autoclaving at 115° for 15 min. and, after cooling, was supplemented with 100 ml. of sterile glucose solution (0·25 g./ml.). When larger crops of bacteria were required for preparing cell walls, 4 l. of medium were similarly dispensed into 5 l. round, flat-bottom flasks. In certain experiments, 6 ml. cultures were grown in Samco tubes covered with anodized aluminium caps (Oxo Ltd., Queen Street Place, London, E.C. 4; Northam & Norris, 1951). Inocula were prepared by suspending sufficient organisms from a 24-hr slope culture in 6 ml. phosphate buffer (0·1 M; pH 7·0; Gomori, 1955) to give the required concentration. Flasks containing 1250 ml. medium were inoculated with 6 ml. of a suspension containing the equivalent of 0·11 mg. dry-wt bacteria/ml. Smaller (6 ml.) portions of medium were inoculated with a proportionately smaller volume of this suspension. Larger volumes (4 l.) of medium were inoculated with 9 ml. of a suspension containing equiv. 0·60 mg. dry-wt bacteria/ml. Cultures were incubated in individual Perspex baths through which was circulated water at a constant temperature. Unless otherwise stated, the temperature of incubation was 30°. Particular attention was given to the maintenance of the incubation temperature since preliminary experiments indicated that growth of *Corynebacterium xerosis* NCIB 9956 was very sensitive to small changes in temperature. Larger (4 l. and 1250 ml.) cultures were agitated by using magnetic stirrers (Gallenkamp and Co. Ltd.; model SS420), and polytetrafluoroethylene (PTFE)-covered magnets (4 cm. long in 1250 ml. cultures; 6 cm. long in 4 l. cultures). After inoculation, the cultures were stirred slowly (about 100 rev. of magnet/min.) for 5 hr, after which time the rate of stirring was increased to about 1250 rev./min. The period of slow stirring immediately after inoculation shortened the length of the lag phase of growth. The content of bacteria in cultures was estimated gravimetrically. Triplicate portions of culture, containing about equiv. 3–5 mg. dry-wt bacteria, were filtered through membrane filters (Millipore Filter Corporation, Bedford, Massachusetts, U.S.A.; 25 mm. diam., 0·8 μ pore size) which had been dried at 100° to constant weight, and cooled in a desiccator over silica gel. The bacteria on a filter were washed with two portions (10 ml.) of distilled water, and the filter then removed from the holder and dried to constant weight at 100°. Growth of certain cultures was also measured turbidimetrically in glass cells (1 cm. light path) in the Hilger 'Spekker' absorptiometer (model H760)

with neutral green-grey H508 filters and a medium blank. Turbidity readings of *C. xerosis* NCIB 9956 cultures were related to dry weight of bacteria by using a calibration curve.

Harvesting of organisms. Except when otherwise stated, bacteria were harveste dfrom cultures by centrifugation at 1300 *g* for 20 min. at 0°. The supernatant fluid was rejected and the organisms washed twice with ice-cold phosphate buffer (pH 7·0). The organisms were then resuspended in buffer and stored at 4° until used. Storage of the bacteria at this temperature for up to 96 hr had no measurable effect on their clumping ability.

Measurement of clumping ability. The clumping ability of bacteria was measured in 350 ml. conical flasks by using portions (100 ml.) of bacterial culture or suspension which had been diluted, when necessary, to contain equiv. 0·35–0·40 mg. dry-wt bacteria/ml. However, preliminary experiments indicated that measurements of the clumping ability of bacteria from a given batch of organisms did not differ when they were made by using suspensions containing as little as equiv. 0·15 mg. dry-wt bacteria/ml. or as much as 0·70 mg./ml. The culture or suspension contained a PTFE-covered magnet (4 cm. long). The flasks were incubated in Perspex water baths through which was circulated water at the specified temperature. In most experiments, the temperature of the bath was maintained constant, although it was occasionally decreased during an experiment. The culture or suspension was allowed to equilibrate at the temperature of the bath for 5 min., during which time it was slowly stirred (about 100 rev. of magnet/min.); after this period of equilibration, the stirring rate was increased to 1250 rev./min. for 10 min.; this caused considerable aeration in the cultures or suspensions. The extent of clumping in the culture or suspension was then measured by transferring a portion (5 ml.) into a glass cell (1 cm. light path) and allowing it to stand in the cell holder of the Hilger 'Spekker' absorptiometer for 5 min. at room temperature (18–22°). During this period, any clumps of bacteria present in the culture or suspension rose to the surface. By measuring the turbidity of the underlying fluid, and comparing this with the turbidity of the culture or suspension before clumping occurred, calculations were made of the percentage dry weight of bacteria that had clumped. Except when otherwise stated, there was no further decrease in turbidity when the culture or suspension was allowed to stand in the cell holder for 10 min. This method for measuring the clumping ability of bacteria gave very consistent results with suspensions of organisms from the same culture and tested under the same conditions. There were, however, small variations in the clumping ability of *Corynebacterium xerosis* NCIB 9956, harvested from different cultures grown at 30° and at approximately the same stage of growth, and tested under the same conditions of temperature, pH value and concentration of organisms. The reason for this variation is not known; it may have been due partly to small differences in the age of the culture at the time of harvesting. For this reason, the effects of each environmental factor on clumping were examined by using bacteria from the same culture.

Preparation of cell walls. Cell walls of *Corynebacterium xerosis* NCIB 9956 were prepared by shaking a suspension of the organisms with glass beads in a cell homogenizer (B. Braun, Melsungen, West Germany; model MSK). Bacteria were harvested from cultures containing about equiv. 0·45 mg. dry-wt/ml., and were washed twice with ice-cold phosphate buffer (pH 7·0). Washed bacteria (equiv. 2·4 g. dry-wt) were suspended in 12 ml. phosphate buffer (pH 7·0) and the suspension placed in a 'Duran' Pyrex glass flask (40 ml. capacity with a false bottom) containing 25 ml. Ballotini

glass beads (grade 12). The flask was shaken in the homogenizer at 4000 rev./min. for 60 sec., during which time the bottle and its contents were cooled with a stream of CO_2. The bottle was then taken off the homogenizer, and the glass beads removed from the suspension by filtration through a sintered glass filter (Baird and Tatlock Ltd., London; porosity 1). Unbroken organisms were removed from the filtrate by centrifugation at 1300 *g* for 45 min. at 0°. Cell walls were separated from the supernatant fluid by centrifugation at 12,500 *g* for 30 min. at 2–5° in an M.S.E. High Speed '18' refrigerated centrifuge. The walls were washed twice with phosphate buffer at 2–5° and then with distilled water also at 2–5° until the extinction reading of the washings, at 260 mμ in a cuvette of 1 cm. light path, was not greater than 0·03. The cell walls were then suspended in distilled water and stored at 0° for short periods, or freeze-dried. The purity of the isolated cell walls was confirmed by examining shadowed preparations in the electron microscope.

Light microscopy. Drops of liquid cultures of *Corynebacterium xerosis* NCIB 9956 were put on glass slides, covered with cover slips, and examined with a Zeiss Photomicroscope; the no. 22 or 40 objective was used, and the illumination source was a high intensity lamp. Photographs were taken with Ilford HP 3 film. The total microscope magnification was × 64.

Electron microscopy. Bacteria which were to be examined as shadowed preparations in the electron microscope were harvested by filtering a portion of culture through a Millipore membrane filter (25 mm. diam.; 0·8 μ pore size). The organisms on the filter were washed twice with phosphate buffer (pH 7·0) and once with distilled water. They were then suspended in water, and one drop of the diluted suspension (containing less than equiv. 0·001 mg. dry-wt/ml.) was placed on a carbon-coated copper grid (3·05/3·10 mm.; 200-mesh; Smethurst High-Light Ltd., Bolton, Lancs.) laid on an aluminium block surrounded with a mixture of ethanol (95 %, v/v in water) and solid carbon dioxide. Drops of suspensions of washed cell walls (containing equiv. 0·001 mg. dry-wt/ml.) were similarly placed on the grids. The drops of suspension quickly froze on the grids, which were then freeze-dried. The grids were shadowed at an angle of $\cot^{-1} 3$ with gold + palladium before being examined with the electron microscope. Whole-organism preparations were examined in the Akashi Tronscope (model TRS 50) with a 50 μ objective and an accelerating voltage of 50 kV; photographs were taken at an instrument magnification of × 10,000. Cell wall preparations were examined in an EM 6 electron microscope (Associated Electrical Industries Ltd., Harlow, Essex) with a 50 μ objective and an accelerating voltage of 75 kV; photographs were taken at an instrument magnification of × 10,000.

For the examination of carbon replicas of bacteria in the electron microscope, a drop of suspension of organisms (containing equiv. 0·001 mg. dry-wt/ml.) was freeze-dried on a Formvar-coated copper grid. The organisms were then coated with carbon and the Formvar removed by solution in chloroform. The coated bacteria were then treated with a solution of $KMnO_4$ in sulphuric acid according to the method of Bradley & Williams (1957). The carbon replicas were shadowed with gold + palladium at an angle of $\cot^{-1} 3$, and examined in the EM 6 electron microscope (50 μ objective aperture and an accelerating voltage of 75 kV). Photographs were taken at an instrument magnification of × 10,000.

Bacteria were fixed for the preparation of thin sections by adding 6 ml. glutaraldehyde solution (25 %, w/v) to 94 ml. culture containing the equivalent of about 25 mg.

dry-wt bacteria. The suspension was maintained at 30° for 15 min. and then cooled to 5° for 15 min. It was then centrifuged and the bacteria washed twice with cold (5°) phosphate buffer (pH 7·0) after which they were fixed for 2 hr at 0° in buffered osmium tetroxide (0·5 %, w/v, in phosphate buffer, pH 7·0). The suspension was again centrifuged, and the bacteria washed twice in phosphate buffer (pH 7·0) and then dehydrated by suspending in graded concentrations of methanol in water (50, 75, 95 %, v/v; 5 min. in each) followed by two periods each of 5 min. in absolute methanol. The dehydrated organisms were then suspended for 30 min. in 3 ml. of a solution of uranyl nitrate (30 %, w/v, in absolute methanol). The stained organisms were centrifuged and washed three times with absolute methanol. They were finally dehydrated by suspending in 3 ml. 1,2-epoxypropane for 15 min., followed by suspension in a further 3 ml. for 15 min. The organisms were then suspended in a mixture (1 + 1, by vol.) of Araldite mixture + 1,2-epoxypropane for 1 hr. Araldite mixture was prepared by mixing Araldite CY 212 (20 ml.), resin HY 964 (20 ml.), benzene dimethylamine (accelerator BDMA; 0·6 ml.), and dibutyl phthalate (2 ml.). The organisms were centrifuged from the Araldite + epoxypropane and resuspended in Araldite mixture alone in gelatin capsules (No. 0; Eli Lilly and Co. Ltd., Basingstoke, England); the Araldite was polymerized by incubation at 60° for 48 hr. Sections were cut with a Huxley ultramicrotome (manufactured in the Engineering Laboratories, Cambridge University) with glass knives. Only sections showing silver or gold interference patterns (60–100 mμ thick) were examined. Sections were examined in the Akashi Tronscope (model TRS-50) with an objective aperture of 50 μ and an accelerating voltage of 50 kV; photographs were taken at an instrument magnification of × 15,000.

Thin sections of clumps of bacteria were prepared by embedding stained preparations in methacrylate. This technique was used since it gave greater contrast in the material than the technique used for examining sections of individual bacteria. Fixing and staining of the clumps were done as described for individual bacteria. After washing three times in absolute methanol, the dehydrated material was suspended for 15 min. in a mixture (1 + 1) of absolute methanol + monomer mixture (*n*-butyl and methyl methacryates, 6 + 1, by vol.), followed by suspension for 3 hr in pure monomer mixture. Finally, the clumps were suspended for 30 min. in a mixture of the monomers (6 + 1, by vol.) which had been partially polymerized with benzoyl peroxide (2 %, w/v) as described by Mercer & Birbeck (1961). The clumps were then centrifuged, and small pieces of the pellet transferred to gelatin capsules (no. 0) containing the methacrylate mixture. Polymerization was done by maintaining the capsule at 60° for 48 hr. Sections were cut with a Huxley ultramicrotome using glass knives. Sections (60–100 mμ thick) were examined in the Akashi Tronscope (model TRS-50) with an objective aperture of 50 μ and an accelerating voltage of 50 kV. Photographs were taken at an instrument magnification of × 2500.

Chemicals. All chemicals and reagents used were of the highest purity available commercially. Where possible, AnalaR chemicals were used. Casamino acids (technical) was obtained from Difco Laboratories Inc., Detroit, Michigan, U.S.A. Hyaluronidase (ex. bovine testes), lipase (ex. wheat germ), lysozyme chloride (ex. egg white), pancreatin (ex. hog pancreas), papain (ex. papaya latex), pepsin (ex. hog stomach mucosa; crystalline), trypsin (ex. bovine pancreas; recrystallized twice), vitamins and glutaraldehyde solution (25 %, w/v, in water) were obtained from Koch-Light Laboratories Ltd., Colnbrook, Buckinghamshire. Amino acids and reagents used for reacting with

proteins were supplied by British Drug Houses, Ltd., Poole, Dorset. Araldite CY212 and resin HY964 were obtained from Ciba (A.R.L.) Ltd., Duxford, Cambridge. Benzene dimethylamine (accelerator BDMA) was obtained from Shell Chemicals Ltd., 15–17, Great Marlborough Street, London, W. 1. Nystatin ('Mycostatin') was purchased from E. R. Squibb and Sons, New Brunswick, New Jersey, U.S.A.

RESULTS

Ability of coryneform bacteria to form clumps at 5°

Clumping at temperatures below the minimum for growth was first observed in cultures of *Corynebacterium xerosis* NCIB 9956 described by Rose & Evison (1965). It was decided to examine the ability of related bacteria, particularly other coryneform organisms, to form clumps at low temperatures, and to discover whether this ability was found only in bacteria with high minimum temperatures for growth. The results of this survey are given in Table 1. Two of the bacteria examined, namely *C. xerosis* ATCC 7091 and *Corynebacterium* sp. NCMB 8, were unable to grow in the medium used. *C. xerosis* ATCC 7711 grew in the form of aggregates in cultures incubated at 30°, which made it difficult to detect the formation of clumps at low temperatures. All of the other bacteria examined grew as discrete organisms at 30°. Several of the corynebacteria formed clumps when cultures were rapidly stirred and cooled to 5°. However, not all of these bacteria had high minimum temperatures for growth.

Table 1. *Ability of various coryneform bacteria to form clumps at 5°, in relation to the minimum temperatures for growth*

Bacteria were grown in 1250 ml. cultures as described under Methods. Mid-exponential-phase cultures were cooled with slow stirring to 5°, and clump formation measured at this temperature. The approximate minimum temperatures for growth were determined with 6 ml. cultures in Samco tubes (see Methods). Cultures were incubated at low temperatures for 2 weeks.

Species	Strain	Ability to form clumps at 5°	Approx. min. temp. for growth (°)
Corynebacterium aquaticum	NCIB 9460	+	5–10
C. manihot	NCIB 9097	−	5–10
C. mediolanum	NCIB 7206	+	10–15
C. ovis	Laboratory strain	+	20–25
C. rubrum	NCIB 9433	−	5–10
C. xerosis	NCIB 9956 (Rose & Evison, 1965)	+	15–20
C. xerosis	ATCC 373	+	10–15
C. xerosis	ATCC 9061	+	15–20
C. xerosis	ATCC 9755	−	20–25
C. xerosis	NCTC 7243	+	
Corynebacterium sp.	NCMB 12	−	5–10
Corynebacterium sp.	NCMB 16	−	5–10
Corynebacterium sp.	NCMB 31	+	5–10
Coryneform	NCIB 8179	−	5–10
Coryneform	NCIB 8180	−	0–5
Coryneform	NCIB 8181	−	5–10
Microbacterium flavum	NCIB 8707	−	10–15
M. lacticum	NCIB 8540	−	15–20
M. lacticum	NCIB 8541	−	5–10

Microscopic examination of clumps formed by Corynebacterium xerosis NCIB *9956*

Attempts to obtain photomicrographs of intermediate stages in clump formation, during the cooling of cultures of *Corynebacterium xerosis* NCIB 9956 from 30° to 15° with fast stirring, were unsuccessful; this indicated that the formation of clumps from discrete bacteria occurred rapidly. A photomicrograph of a clump formed in a culture cooled to 15° with fast stirring is shown in Pl. 1, fig. 1. Plate 1, fig. 2, is an electron micrograph of bacteria that had been freeze-dried on the electron microscope grid and then shadowed. It shows bacteria connected by presumably adhesive material which does not appear to be localized but to be distributed over the bacterial surface. A carbon replica of *C. xerosis* NCIB 9956 that had been freeze-dried on the electron microscope grid is shown in Pl. 1, fig. 3. The surfaces of the bacteria are seen to be covered by wart-like lumps. An electron micrograph of a thin section of a clump of *C. xerosis* NCIB 9956 is shown in Pl. 1, fig. 4. Each clump was hollow and appeared to have been formed by a single layer of bacteria collecting round a gas bubble. The ability of the bacteria in the clumps to remain at least partly in contact after preparation of the material for electron microscopy testified to the mechanical strength of the adhesive material. Plate 1, fig. 5, is an electron micrograph of a thin section through bacteria stained with uranyl nitrate, showing patches of electron-dense material on the surfaces of the bacteria.

Effect of environmental factors on clumping of Corynebacterium xerosis NCIB *9956*

Hydrogen-ion concentration. Since the clumping of *Corynebacterium xerosis* NCIB 9956 involved contact and adhesion at the surface of the organisms, it seemed likely that the pH value of the suspending fluid might affect the degree of clumping. Figure 1 shows that, at 15°, the clumping ability was greatest when the bacteria were suspended in buffer at pH 3·0. Between pH 3 and 5, there was a decline in the percentage of bacteria that clumped, but clumping ability was little affected by pH values between 5 and 10. At pH 2·0, the percentage of bacteria that clumped was lower than at pH 3·0. However, when the suspensions of bacteria in buffer at pH 2·0 were stood in the absorptiometer cell holder for 10 min. instead of 5 min., the turbidity of the suspension decreased further with the result that the extent of clumping then appeared greater than at pH 3·0. This longer period of standing had no effect on the percentage of bacteria clumping in buffers at or above pH 3·0. The clumping ability at several pH values was examined using two different buffering systems, and was shown not to be affected by the chemical composition of the buffers at the concentrations used.

Temperature. The clumping ability of bacteria harvested from exponential-phase cultures and suspended in buffer increased as the temperature of the suspension was decreased below 30°. The extent of clumping at any one temperature depended on the pH value of the buffer (Fig. 2). At pH 7·0, clump formation was not measurable at 30°, but at pH 3·0 it was appreciable at 40°. The shapes of the Arrhenius curves at pH 7·0 and 3·0 were similar over the range 25°–10° but, at temperatures above 30°, the pH 3·0 curve deviated markedly from that for suspensions at pH 7·0. Bacteria harvested from late exponential-phase cultures formed a few clumps at 30° in pH 7·0 buffer (see below). No differences could be detected in the clumping ability of bacteria grown at 35° or 40° as compared with bacteria grown at 30°, when measured under the same conditions.

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Nature of gas phase. Substitution of nitrogen or oxygen for air as the gas phase had little effect on the clumping ability of bacteria at 15° (Table 2). However, when hydrogen was used as the gas phase, there was a decrease in the clumping ability.

Age of culture. The clumping ability of the bacteria increased with the age of the culture from the mid-exponential to the late-exponential phases of growth, but declined as the culture entered the stationary phase of growth (Fig. 3). The difficulties

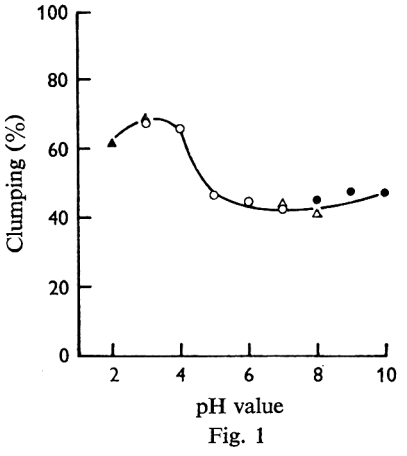

Fig. 1

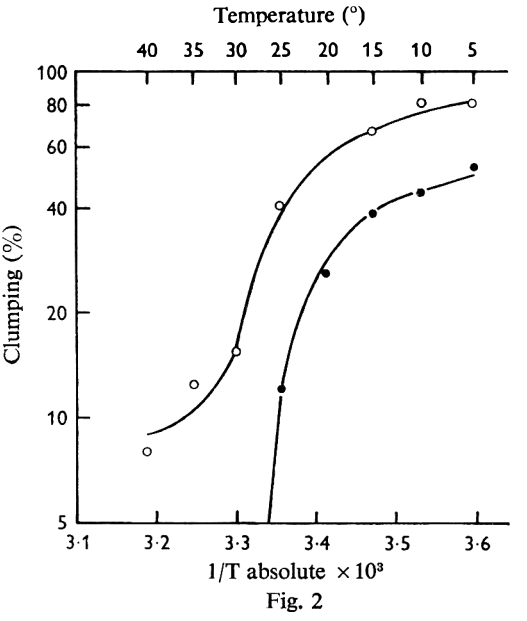

Fig. 2

Fig. 1. Effect of pH value on clumping of *Corynebacterium xerosis* NCIB 9956. The buffering systems used were HCl+KCl (▲; Bower & Bates, 1955), citric acid+Na_2HPO_4 (○; McIlvaine, 1921), $Na_2HPO_4+NaH_2PO_4$ (△; Gomori, 1955), and borate+KCl+NaOH (●; Bower & Bates, 1955). Bacteria were harvested from cultures containing equiv. 0·2–0·3 mg. dry-wt bacteria/ml. The extent of clumping was measured as described in Methods.

Fig. 2. Arrhenius plots for the clumping of *Corynebacterium xerosis* NCIB 9956 at pH 3·0 (○; HCl+KCl; Bower & Bates, 1955) and pH 7·0 (●; $Na_2HPO_4+NaH_2PO_4$; Gomori, 1955). Bacteria were harvested from cultures containing equiv. 0·2–0·3 mg. dry-wt bacteria/ml. The extent of clumping at the different temperatures was then measured.

of harvesting bacteria from very early exponential-phase cultures prevented the examination of the clumping ability of these bacteria. Bacteria from mid exponential-phase cultures did not form clumps at 30° in pH 7·0 buffer, but organisms from late exponential-phase cultures did so to a small extent (less than 10%).

Salts. Bacteria did not clump in distilled water at 15° when the suspension was rapidly stirred, but when salts were present clumps formed. In the presence of NaCl, clump formation was greatest in solutions with an ionic strength of about 0·10 (Fig. 4). An examination of the effects of different monovalent cations and anions showed that these effects could be correlated with the positions of the ions in the Hofmeister or lyotropic series (Young, 1963; Table 3). Divalent (Mg^{2+}) and trivalent (Fe^{3+}) ions were also effective.

Table 2. *Effect of different gas phases on the clumping ability of Corynebacterium xerosis* NCIB *9956 at 15°*

Bacteria were harvested from cultures grown at 30° and containing equiv. 0·2–0·3 mg. dry-wt bacteria/ml. The bacteria were washed twice in phosphate buffer (pH 7) and re-suspended in this buffer to a concentration of equiv. 35 mg. dry-wt/ml. Phosphate buffer (99 ml.; pH 7·0) was placed in a 350 ml. conical flask, containing a PTFE-covered magnet and fitted with a rubber bung through which was passed a gas sparger and an outlet tube. The flask was placed in the water bath at 15°, and the gas from a cylinder was then blown through the buffer for 15 min. during which time the buffer was stirred slowly. The sparging jet was then raised to about 1 cm. above the level of liquid in the flask, and 1 ml. of the suspension of bacteria added to the buffer. The clumping ability of the bacteria was then determined as described under Methods. Gas was flushed into the flask while the suspension was rapidly stirred.

Nature of gas	Extent of clumping at 15° (%)
Air	49·5
Nitrogen (oxygen-free)	47·0
Oxygen (nitrogen-free)	49·3
Hydrogen	38·5

Table 3. *Effect of salts on the clumping ability of Corynebacterium xerosis* NCIB *9956 at 15°*

Bacteria were harvested from cultures grown at 30° and containing equiv. 0·2–0·3 mg. dry-wt bacteria/ml. The bacteria were washed twice in pH 7·0 phosphate buffer and twice in distilled water, and resuspended in the salt solution to a concentration equiv. 0·35 mg. dry-wt/ml. The suspensions were cooled at 15° with slow stirring, and the extent of clumping determined. Each ion was tested at an ionic strength of 0·10. Cations were present as chlorides; anions as potassium salts.

Ion	Extent of clumping at 15° (%)
Cations: K^+	47·8
Na^+	40·7
Li^+	36·3
Anions: Cl^-	41·7
NO_3^-	42·5
I^-	46·2
CNS^-	47·8

Effect of different treatments on the clumping of Corynebacterium xerosis NCIB *9956*

Information about the chemical nature of the cell-surface components responsible for the increased clumping of the corynebacterium at low temperatures was sought by examining ability to form clumps after the organisms had been subjected to various treatments. Boiling a suspension of the bacteria in phosphate buffer (pH 7·0), or repeated freezing and thawing of a suspension in the same buffer, had no detectable effect on ability to form clumps at 15°. Other treatments which did not affect ability to clump at 15° included extraction with a hot mixture of chloroform + methanol (3 + 1 by vol.; Kates, Adams & Martin, 1964), incubation for 15 min. in buffer (pH 7·0) containing 0·1 M-sodium periodate (Hotchkiss, 1948) or 0·25 mg. nystatin/ml. (Lampen, Arnow, Borowska & Laskin, 1962). However, the clumping ability of bacteria suspended at 15° in NaCl (0·1 M) containing uranyl nitrate (10 mM) quickly

decreased, although some of these bacteria were able to clump even after incubation in the solution for 60 min. (Table 4). Bacteria suspended in a solution containing only uranyl nitrate (10 mM) did not form clumps under these conditions.

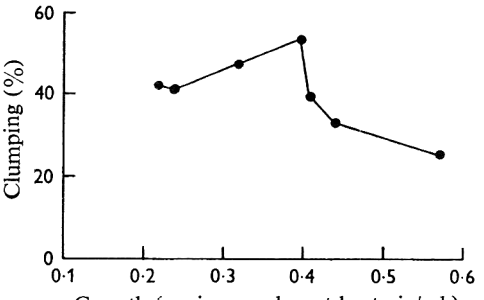

Fig. 3

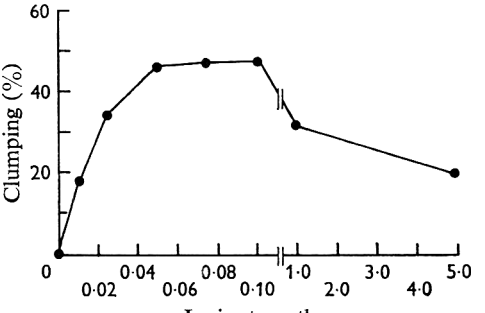

Fig. 4

Fig. 3. Effect of culture age on clumping of *Corynebacterium xerosis* NCIB 9956 at 15° and pH 7·0. Observations were made during the late-exponential phase (equiv. 0·2–0·4 mg. dry-wt/ml.) and early stationary phase (equiv. 0·4–0·55 mg. dry-wt/ml.) of cultures. Bacteria were harvested from cultures grown at 30° by filtration through a membrane filter, and were washed on the filter with phosphate buffer (pH 7·0). The extent of clumping was then measured.

Fig. 4. Effect of NaCl concentration on clumping of *Corynebacterium xerosis* NCIB 9956 at 15°. Bacteria were harvested from cultures grown at 30° and containing equiv. 0·2–0·3 mg. dry-wt bacteria/ml. The extent of clumping was then measured.

Table 4. *Effect of uranyl nitrate on the clumping ability of Corynebacterium xerosis* NCIB *9956 at 15°*

Bacteria were harvested from cultures grown at 30° and containing equiv. 0·2–0·3 mg. dry-wt. bacteria/ml. The bacteria were washed twice in pH 7·0 phosphate buffer and twice in distilled water, and resuspended at 15° in NaCl (0·1 M) containing uranyl nitrate (10 mM) to a concentration equiv. of 0·35 mg. dry-wt/ml. The suspension was stirred slowly for the times indicated and the clumping ability of the bacteria then determined.

Duration of incubation in NaCl-uranyl nitrate solution at 15° (min.)	Extent of clumping at 15° (%)
0	63·5
5	40·5
15	28·0
30	28·0
45	28·0
60	30·0

Incubation of a suspension of bacteria at 37° in phosphate buffer (pH 7·0) containing individual enzymes (500 μg./ml.) was also studied. Incubation in solutions of hyaluronidase, lipase, lysozyme chloride or phospholipase D had no measurable effect on ability to clump at 15°. Incubation in solutions of proteolytic enzymes had some effect (Table 5), and the ability to form clumps was completely removed by suspending bacteria for 1 hr in pH 7·0 buffer containing papain (500 μg./ml.). Incubation of suspensions of bacteria in pH 7·0 buffer containing trypsin (500 μg./ml.) led to the disappearance of much of the adhesive material that surrounded shadowed preparations of bacteria on electron micrographs (Pl. 1, fig. 2).

Experiments were also done with reagents that react with different groups in proteins to discover which groups in the cell-surface protein were involved in clump formation. Because of the need to use relatively mild conditions only a few reagents could be tested. As shown in Table 6, only guanidine-HCl and urea of the reagents tested had an effect on the clumping ability of the bacteria.

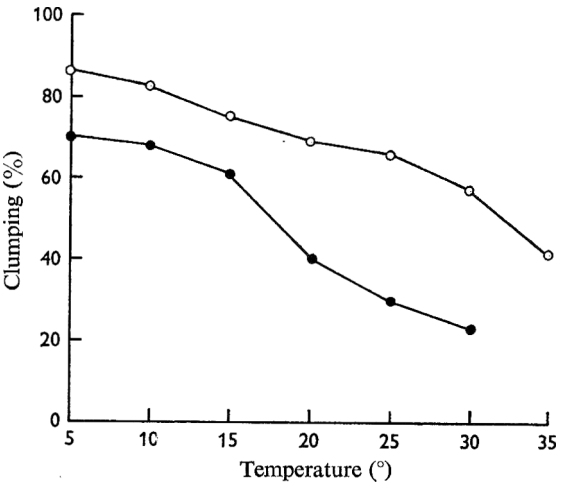

Fig. 5. Effect of temperature on clumping of isolated cell walls of *Corynebacterium xerosis* NCIB 9956. Cell walls were prepared as described in Methods. ● indicates the response of walls after treatment with trypsin (500 μg./ml.) in pH 7·0 phosphate buffer for 4 hr, as described in Table 5; ○ indicates the response of untreated walls. The extent of clumping was measured as described in Methods, except that the wall suspensions were stood for 20 min. in the absorptiometer cell holder.

Table 5. *Effect of proteolytic enzymes on the ability of Corynebacterium xerosis* NCIB *9956 to form clumps at 15°*

Bacteria were harvested from cultures grown at 30° and containing equiv. 0·2–0·3 mg. dry-wt. bacteria/ml. The bacteria were washed and resuspended to a concentration equiv. 5 mg. dry-wt/ml. in buffer (20 ml.) at pH 2·3 (HCl+KCl; Bower & Bates, 1955), pH 4·5 (acetic acid sodium acetate; Walpole, 1914), or pH 7·0 (phosphate 0·1 M; Gomori, 1955) in the presence and absence of the proteolytic enzyme (500 μg./ml.). The suspensions were shaken at 176 rotations/min. in a New Brunswick Gyrotary shaker-incubator (model G-25) for 1 hr. The suspensions were then passed through a membrane filter (Oxoid), the bacteria washed with 20 ml. phosphate buffer (pH 7·0) at 37°, and resuspended in phosphate buffer (pH 7·0; 100 ml.) to a concentration equiv. 0·38 mg. dry-wt bacteria/ml. The extent of clumping at 15° was then measured.

Enzyme	pH value of buffer	Extent of clumping at 15° (% of control)
Pancreatin	7·0	27·9
Papain	4·5	0·0
Pepsin	2·3	34·6
Trypsin	7·0	20·9
None (control)	2·3	100·0
	4·5	100·0
	7·0	100·0

Clumping of isolated walls of Corynebacterium xerosis NCIB *9956*

Isolated cell walls of *Corynebacterium xerosis* NCIB 9956 formed clumps when suspensions of the walls in phosphate buffer (pH 7·0) were incubated at temperatures between of the walls in phosphate buffer (pH 7·0) were incubated at temperatures between 35° and 5° with rapid stirring (Fig. 5). Clumps of walls took 20 min. to rise to the surface of the suspensions in the absorptiometer cells. Pretreatment of the walls with trypsin decreased clumping ability (Fig. 5). Trypsin was preferred to papain as the proteolytic enzyme for digestion since it contained less contaminating material and gave cleaner electron micrographs. The adhesive material that surrounded intact organisms which had been freeze-dried on the electron microscope grid (Pl. 1, fig. 2) could not be seen in electron micrographs of shadowed preparations of freeze-dried walls (Pl. 1, fig. 5).

Table 6. *Effect of different reagents on the clumping ability of Corynebacterium xerosis* NCIB *9956 at 15°*

Bacteria were harvested from cultures grown at 30° and containing equiv. 0·2–0·3 mg. dry-wt bacteria/ml. The bacteria were washed and resuspended at equiv. 0·36 mg. dry-wt/ml. in buffer (pH 7·0; 100 ml.) containing the reagent at the concentration stated. The suspensions were then placed in the water bath, stirred slowly at 15° for 15 min., and the extent of clumping was then measrued.

Reagent	Concentration	Extent of clumping at 15° (% of control)
p-Chloromercuribenzoate	0·01 M	100
L-Cysteine	0·20% (w/v)	100
Formaldehyde	0·20% (w/v)	100
Guanidine-HCl	5·0 M	72·5
2-Mercaptoethanol	0·20% (w/v)	100
Urea	8·0 M	42·7
None (control)	—	100

DISCUSSION

Clumping of *Corynebacterium xerosis* NCIB 9956 in rapidly stirred cultures or suspensions appeared to be caused by a cell-surface or microcapsular component, which became increasingly sticky as the temperature was decreased from 40° to 5°, causing the organisms which collected around a gas bubble to form a clump. The inability of the bacteria to clump in slowly stirred cultures or suspensions is presumably explained by the absence of large numbers of air bubbles. Electron micrographs of shadowed preparations of bacteria and of thin sections of clumps provided the best evidence for the cell-surface nature of the causative agent. Additional evidence came from the demonstration that suspensions of isolated cell walls formed clumps under the same conditions, although we were unable to show the adhesive material in electron micrographs of shadowed preparations of walls, possibly because the walls were flattened. The electron micrographs of shadowed preparations of bacteria also showed that the adhesive material was distributed over most of the surface of the bacteria. Conceivably, the electron-dense patches seen on the edges of thin sections of bacteria stained with uranyl nitrate represented adhesive material, particularly since uranyl ions, which react specifically with phosphate groups (Rothstein & Meier, 1951), caused a decrease in the clumping ability.

Clumping of several different micro-organisms has been shown to be associated with different types of cell-surface component, including carbohydrate (e g. Sneath & Lederberg, 1961), protein (Taylor, 1964) and hyaluronic acid (Warren & Gray, 1955), but the clumping of those organisms has not been reported to be influenced by temperature in the manner found with *Corynebacterium xerosis* NCIB 9956. The ability of proteolytic enzymes to decrease and even to remove the clumping ability of *C. xerosis* NCIB 9956, together with the inability of lipid solvents or of enzymes and reagents which react specifically with carbohydrates, hyaluronic acid, mucopeptides or sterols, to affect this ability, indicates that a cell-surface protein is involved in the clumping. The presence of protein in corynebacterium walls has been reported by Hewitt (1947) and Cummins (1954), and Stanley (1966) showed that the walls of *C. xerosis* NCIB 9956 contain about 30 % by weight of protein. Nevertheless, our data do not preclude the possibility that the cell-surface protein on *C. xerosis* NCIB 9956 is linked to other cell wall or microcapsular components not involved in clump formation. Presumably, those corynebacteria that were unable to clump under the conditions used, and which were present in greatest proportions in early exponential-phase and late stationary-phase cultures, did not have the cell-surface protein or at least not sufficient of it to allow the corynebacteria to clump.

The inhibitory effect of uranyl nitrate on the clumping ability of the corynebacteria suggested that cell-surface phosphate groups were involved in clump formation. The absence of any effect with cysteine, 2-mercaptoethanol (Cecil & McPhee, 1957), or *p*-chloromercuribenzoate (Fraenkel-Conrat, 1957), suggested that disulphide bridges and sulphydryl groups did not contribute to the stickiness of the bacteria. Formaldehyde, which reacts with amino and sulphydryl groups in proteins (Fraenkel-Conrat, 1957), also had no effect. Our data indicated, however, that α-carboxyl groups may contribute to the stickiness of the cell-surface component, since clumping ability was greatest around pH 3, which is the pK value of the α-carboxyl group (Cohn & Edsell, 1943).

Any hypothesis concerning the mechanism of clump formation in *Corynebacterium xerosis* NCIB 9956 must include an explanation of the way in which the bacteria aggregate at the gas-bubble/water interface. Since what appears to be the adhesive material can be seen surrounding bacteria that have been freeze-dried on the electron microscope grid, it is evident that the bacteria can become sticky at low temperatures in the absence of gas bubbles. It seems likely that, in liquid cultures or suspensions, gas bubbles provide a suitable interface at which the bacteria can collect. Since only a single layer of bacteria collected round each gas bubble, and substituting nitrogen or oxygen for air as the gas phase had no marked effect on clumping ability, it is conceivable that the collection of bacteria at the gas-bubble/water interface is a 'hydrophobic effect' which might depend upon the presence of exposed hydrophobic amino acid residues in a cell-surface protein (Brandts, 1967). *C. xerosis* NCIB 9956 and several of the other strains of *C. xerosis* collected in the foam layer when nutrient broth cultures were vigorously aerated (S. O. Stanley and A. H. Rose, unpublished observations). We suggest, therefore, that the ability to collect at the gas-bubble/water interface may not be a result of low-temperature conformational changes in the cell-surface protein, but that clumps were formed only at low temperatures when bacteria which have collected at these interfaces became sticky.

Our data give little information about the nature of the adhesive forces that bind the

bacteria in the clumps. Since treatment with urea and guanidine-HCl, reagents which are thought to prevent the formation of hydrogen bonds (Kauzmann, 1959), did not completely prevent clump formation, it is possible that other types of secondary bonds in addition to hydrogen bonds are involved. This conclusion may be premature, for Brandts (1967) has stressed how little is know about the effects of urea and guanidine-HCl on hydrogen-bond formation, particularly at low temperatures. The finding that clumps were formed only in the presence of salts suggests that the stickiness may have been caused in part at least by the formation of salt bridges between protein groups on the surfaces of adjacent bacteria.

The limited survey of the clumping ability of coryneform bacteria showed that clump formation, under the conditions used, was confined to species of the genus *Corynebacterium*. However, not all of the corynebacteria with high minimum temperatures for growth formed clumps at 5°, which suggests that the physiological basis of these high minimum growth temperatures is not directly associated with clumping behaviour. Bacteria are known to aggregate and to adhere to particulate matter in oceans and lake waters at low temperatures. Barber (1966) suggested that the formation of some of these aggregates in sea water involves the deposition of bacteria round air bubbles, possibly, therefore, the aggregation of marine bacteria round air bubbles has a similar physiological basis to that observed in the present study with *Corynebacterium xerosis* NCIB 9956.

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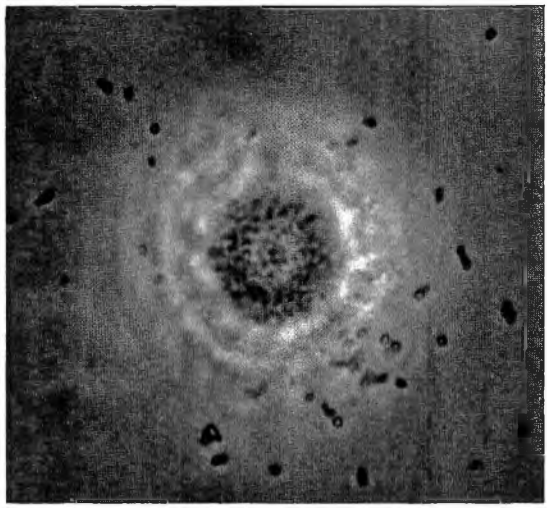


Fig. 1

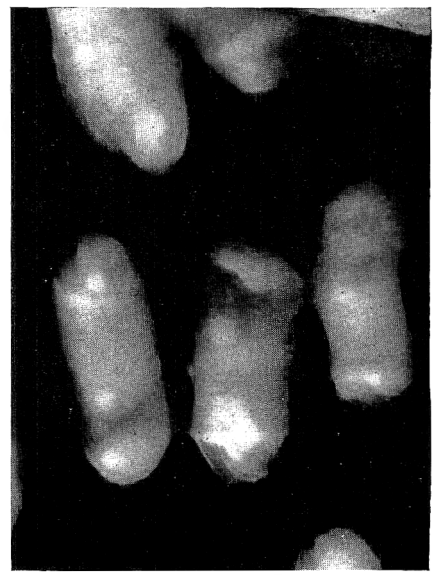


Fig. 2

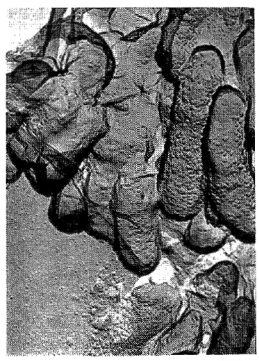
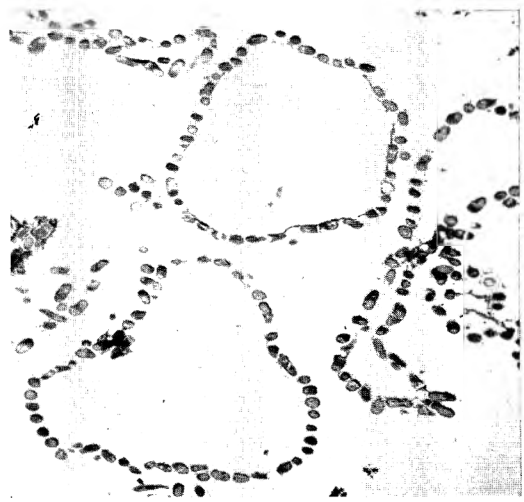


Fig. 3



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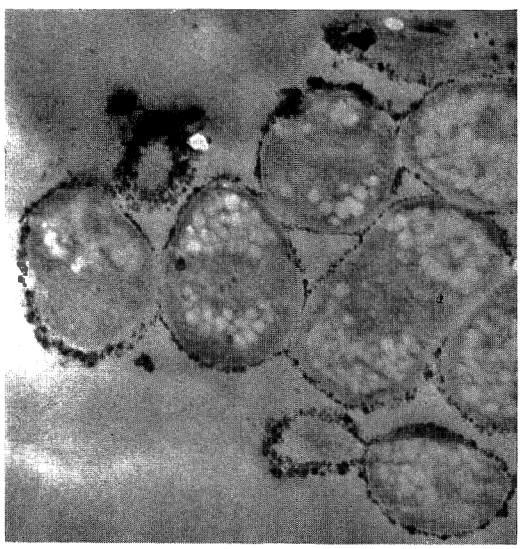

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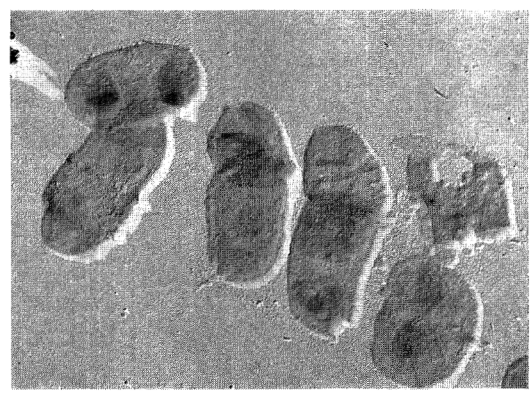

Fig. 6

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EXPLANATION OF PLATE

Fig. 1. Photomicrograph of a clump of *Corynebacterium xerosis* NCIB 9956 from a culture grown at 30° and cooled to 15° with fast stirring. × 2100.

Fig. 2. Electron micrograph of *Corynebacterium xerosis* NCIB 9956, freeze-dried on the electron microscope grid, and shadowed with gold + palladium. × 20,000.

Fig. 3. Carbon replica of *Corynebacterium xerosis* NCIB 9956. × 15,500.

Fig. 4. Electron micrograph of a thin section through a clump of *Corynebacterium xerosis* NCIB 9956 formed on cooling a culture from 30 to 15° with fast stirring. × 5000.

Fig. 5. Electron micrograph of a thin section through *Corynebacterium xerosis* NCIB 9956 stained with uranyl nitrate. The bacteria were clumped by cooling a culture from 30° to 15° with fast stirring. × 30,300.

Fig. 6. Electron micrograph of isolated cell walls of *Corynebacterium xerosis* NCIB 9956, freeze-dried on the electron microscope grid and shadowed with gold + palladium. × 20,000.

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Ultrastructure of Cell Envelopes of Large Cells, Small Cells and Cysts of *Azotobacter chroococcum*

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SUMMARY

Vegetative organisms of *Azotobacter chroococcum*, as seen by electron microscopy of ultra-thin sections, had a single, very thin wall, surrounded by a capsule which appears to correspond structurally to an outer wall. The smaller gonidial forms had double walls and no capsule. Cysts had a spore-like multiple wall, and give evidence of being produced within the cell.

INTRODUCTION

The cytology of *Azotobacter chroococcum* was described by Bisset & Hale (1953) and Bisset (1955), who mainly used light microscopy of stained preparations, and it was concluded that these organisms had affinities with the Bacillaceae. The present study is intended to advance this information by means of electron microscopy of ultra-thin sections. Sections of Azotobacter were illustrated by Socolofsky & Wyss (1961, 1962), but most of the envelope structures in the vegetative organism described here, and the mode of formation of gonidia, were not demonstrated.

METHODS

Three strains of *Azotobacter chroococcum*, newly isolated in this laboratory from soil, were grown in the fluid medium described by Bisset & Hale (1953), concentrated by centrifugation, fixed in 1% osmium tetroxide solution buffered to pH 7·4 with veronal+acetate for 3 days, embedded in methacrylate and then sectioned. The period of fixation in the buffered osmium tetroxide gave adequate electron opacity to the cell envelopes; the visualization of cytoplasmic and nuclear structures was not sought. There is some disagreement between authors with respect to the validity of results obtainable by different methods in the preparation of sections for electron microscopy, but the vacuolated structure of the cytoplasm shown in these preparations is similar to that seen in light-microscope preparations by Bisset & Hale (1953) and Bisset (1955), so it may be assumed that gross distortion has been avoided.

RESULTS

Samples taken at 2–30 days from cultures were examined. The large forms had the characteristic appearance of the genus, being oval, almost spherical, contained numerous large lipid droplets (Bisset & Hale, 1953; Socolofsky & Wyss, 1961) and were embedded in a relatively thick capsule (Pl. 1, fig. 1). The cell wall was single, very

electron-dense and extremely fine (*a* in Pl. 1, fig. 2, Pl. 2, fig. 3). The capsule was demarcated by a firm outline, and often appeared as a septum between two cells (*b* in Pl. 1, fig. 2; Pl. 2, figs. 3, 4). No apparent membrane surrounded the lipid droplets (*c* in Pl. 1, fig. 2). Small forms, apparently developing gonidia (Bisset & Hale, 1953; Bisset & Hale-McCaughey, 1967), were observed in most cultures (*d* in Pl. 1, fig. 2) and occasionally composed the majority (Pl. 2, figs. 5, 6), although usually reverting to the large phase in 2–3 days. They also contained lipid droplets; these were approximately the same size in the large and small forms, although much less numerous in the latter. The envelopes of the small forms were entirely distinct in appearance, giving the impression of a wall surrounding a slightly plasmolysed membrane (*d* in Pl. 1, fig. 2; Pl. 2 figs. 5, 6).

Cysts (Pl. 3, figs. 7, 8) appeared in cultures of all ages. They were enclosed in a thick, outer wall with four or more inner layers of varying opacity. Occasional organisms showed appearances suggestive of the formation of cysts internally (Pl. 2, fig. 9).

DISCUSSION

The envelopes of the large vegetative Azotobacter organisms seem to be of a form not hitherto described. They do not resemble any of the types of Gram-negative wall listed by Murray, Steed & Elson (1965), but if the so-called capsule may be regarded as a greatly expanded cell wall, which it strongly resembles, then the pattern is much more like that of the wall surrounding a membrane that has been recorded for several Gram-positive bacteria (Glauert & Hopwood, 1961; Mazanec, Kocur & Martinec, 1965; Bisset, 1966). The appearance of the non-capsulated small-phase elements supports this view, as also does the resemblance between the cysts illustrated here, and sections of endospores published by Mayall & Robinow (1957) and many others. Although Socolofsky & Wyss (1962) deny the resemblance between cysts and spores pointed out by Bisset (1955), the existence of a heat-resistant 'corpuscle' in Azotobacter has been confirmed by Garbosky & Giambiagi (1963); and the spore-like appearance of the cysts figured in the present paper is most marked. Also, the appearance of an endospore-like method of production, seen in some cells, supports the concept of an affinity with the Bacillaceae (Bisset, 1955).

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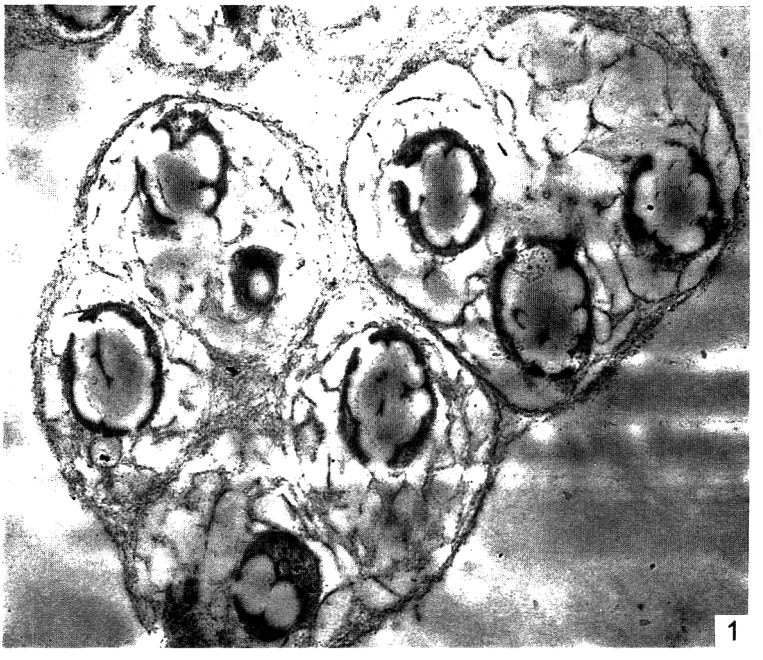

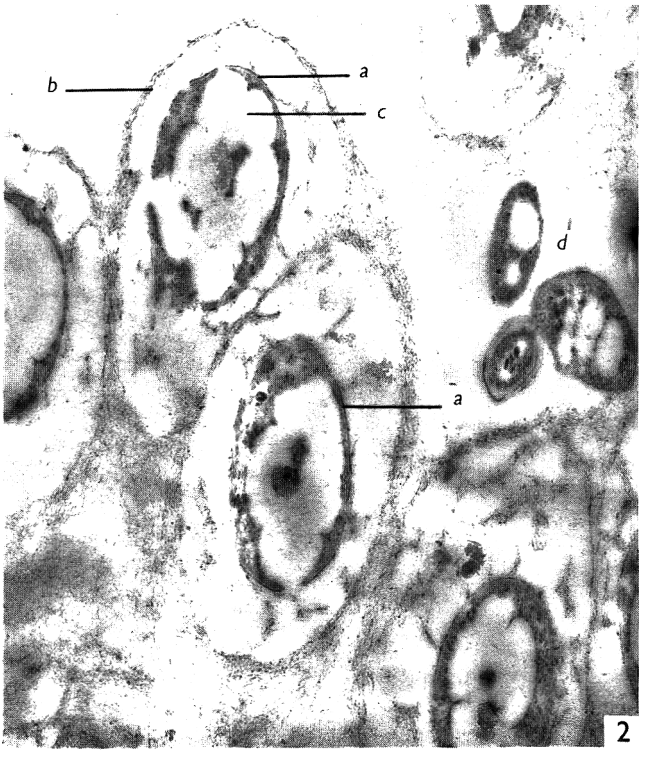

K. A. BISSET

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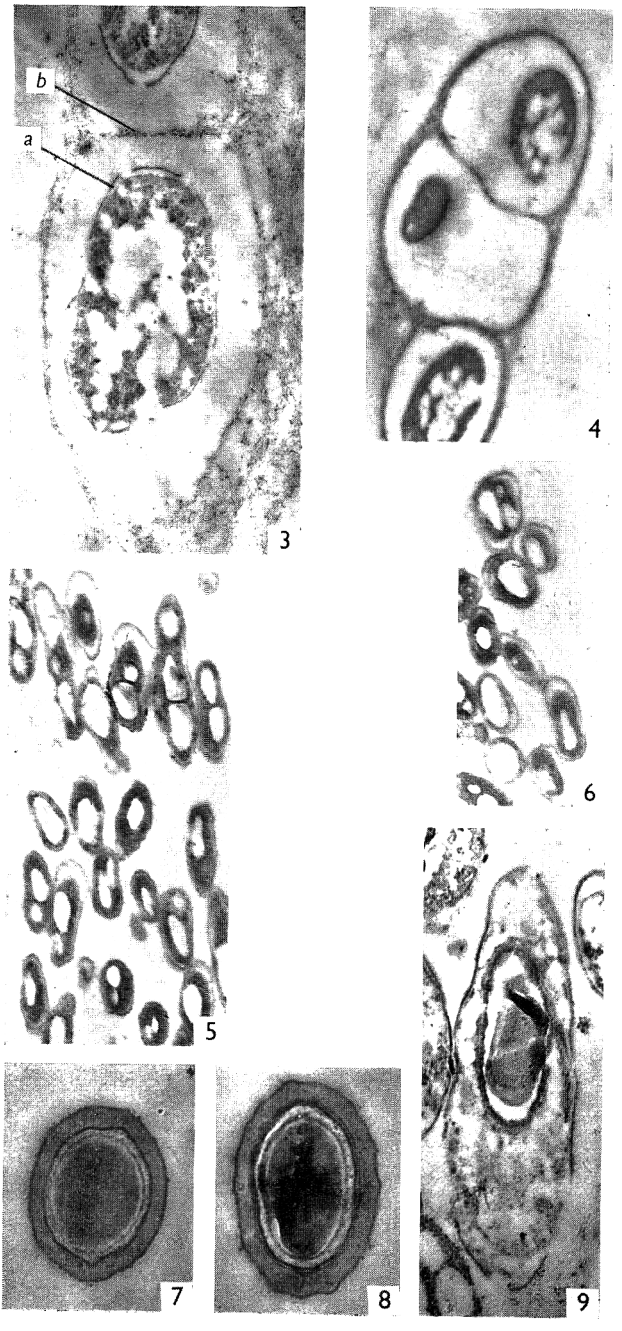
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EXPLANATION OF PLATES

Electron micrographs of sections of *Azotobacter chroococcum*; all at × 14,000 except where stated otherwise.

PLATE 1

Fig. 1. Group of large organisms in capsule. × 7000.

Fig. 2. Large and small organisms. *a*, envelope, probably semi-permeable membrane; *b*, capsule boundary, probably representing true wall; *c*, lipid droplets; *d*, small cells, lower left showing double envelope.

PLATE 2

Fig. 3. Large organism showing slightly disrupted membrane (*a*), and marked septum in capsule-wall between cells (*b*).

Fig. 4. Large organisms showing obvious cell wall appearance of the capsule. × 7000.

Figs. 5, 6. Small cells, many showing double envelope.

Figs. 7, 8. Cysts, showing thick wall and multiple inner envelopes.

Fig. 9. Longitudinal section of large cell, showing appearance suggestive of a cyst forming internally.

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Gonidium Production in *Azotobacter chroococcum*

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

SUMMARY

Electron microscopy of sections of *Azotobacter chroococcum* show that gonidia *c.* 0·05 μ are produced by or near the cell envelopes, increasing in size to *c.* 1 μ and filling the lumen of the cell before release, which occurs by rupture of the envelopes.

INTRODUCTION

Reproduction of various bacterial genera by the release of motile gonidia has been recognized since the paper by Beijerinck in 1888; the process was described, by the use of stained preparations, phase-contrast and electron microscopy of entire organisms in Rhizobium by Bisset & Hale (1951), in Azotobacter by Bisset & Hale (1953), in Spirillum by Pease (1956) and in Bacillus by Bisset & Hale (1963). General descriptions have been given by numerous workers, mainly of these same genera. Because of the exceedingly small size of the gonidia, and the electron-opacity of bacterial cell walls, convincing detail has been hard to obtain. The object of the present work was to attempt to overcome these problems, by using the technique of electron microscopy of ultra-thin sections. Sections of the gonidial swarmers of *Bacillus cereus* were obtained by Bisset (1966), but the mode of formation was not demonstrated, and sections through what were probably developing gonidia of spirochaetes were illustrated by Bladen & Hampp (1964).

This type of reproduction is, of course, a commonplace among other types of flagellate protista; its theoretical importance in bacteria was pointed out by Bisset & Hale (1963) and by Bisset (1966), who suggested that it might represent the primitive state of the process whereby L-forms and possibly mycoplasma are produced in structurally degenerate genera.

METHODS

Two strains of *Azotobacter chroococcum*, isolated in this laboratory, were cultured in the fluid medium described by Bisset & Hale (1953). Old samples were examined microscopically between 4 and 6 weeks for signs of gonidium production. Suitable cultures were prepared and examined, as described by Bisset (1967).

RESULTS

Mother-cells in an early stage of gonidium production were only slightly larger than the vegetative forms. They appeared partially empty, with a thick wall on or near which the gonidia, about 0·05 μ in diameter, were situated (Pl. 1, figs. 1–3). There was much unidentifiable debris both within and outside such forms. Larger mother-cells, presumably near maturation, were less commonly seen, and were 10 μ or more in

diameter, containing fully developed small-phase vegetative forms (Bisset & Hale, 1953; Bisset, 1967). These were about 1 μ in diameter, were detached from the envelopes and appeared to fill the lumen of the mother-cell (Pl. 1, figs. 5, 6). Some mother-cells contained large (lipid?) vacuoles also (Pl. 1, fig. 4). Frequently, the latter stages were represented by disrupted cell walls, associated with debris and a few small forms (Pl. 1, fig. 4).

DISCUSSION

The evidence provided by electron microscopy of ultra-thin sections of Azotobacter shows that gonidia develop within the lumen of specialized mother-cells, as described from stained preparations by Bisset & Hale (1951, 1953), and confirms the role of the cell envelopes in the formation of the new material, as suggested by Bisset & Hale (1951). The size of the early stages is so small that, even by the ultra-thin section technique, they show little or no structure, but the mature gonidia or small vegetative forms (Bisset & Hale, 1953; Bisset, 1967) are manifestly small bacteria. Earlier studies (Bisset & Hale, 1953) show them to have polar flagella in common with all other known bacterial gonidia. Apart from the nitrogen-fixing genera, *Azotobacter* and *Rhizobium*, gonidia have been properly demonstrated in *Bacillus* and in spiral bacteria, and according to the theory of bacterial evolution suggested by Bisset (1962) these are all relatively primitive bacteria. Indeed, gonidium-formation may reasonably be regarded as a primitive character in bacteria, linking them with an ancestral form, little removed from other flagellate protista.

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EXPLANATION OF PLATE

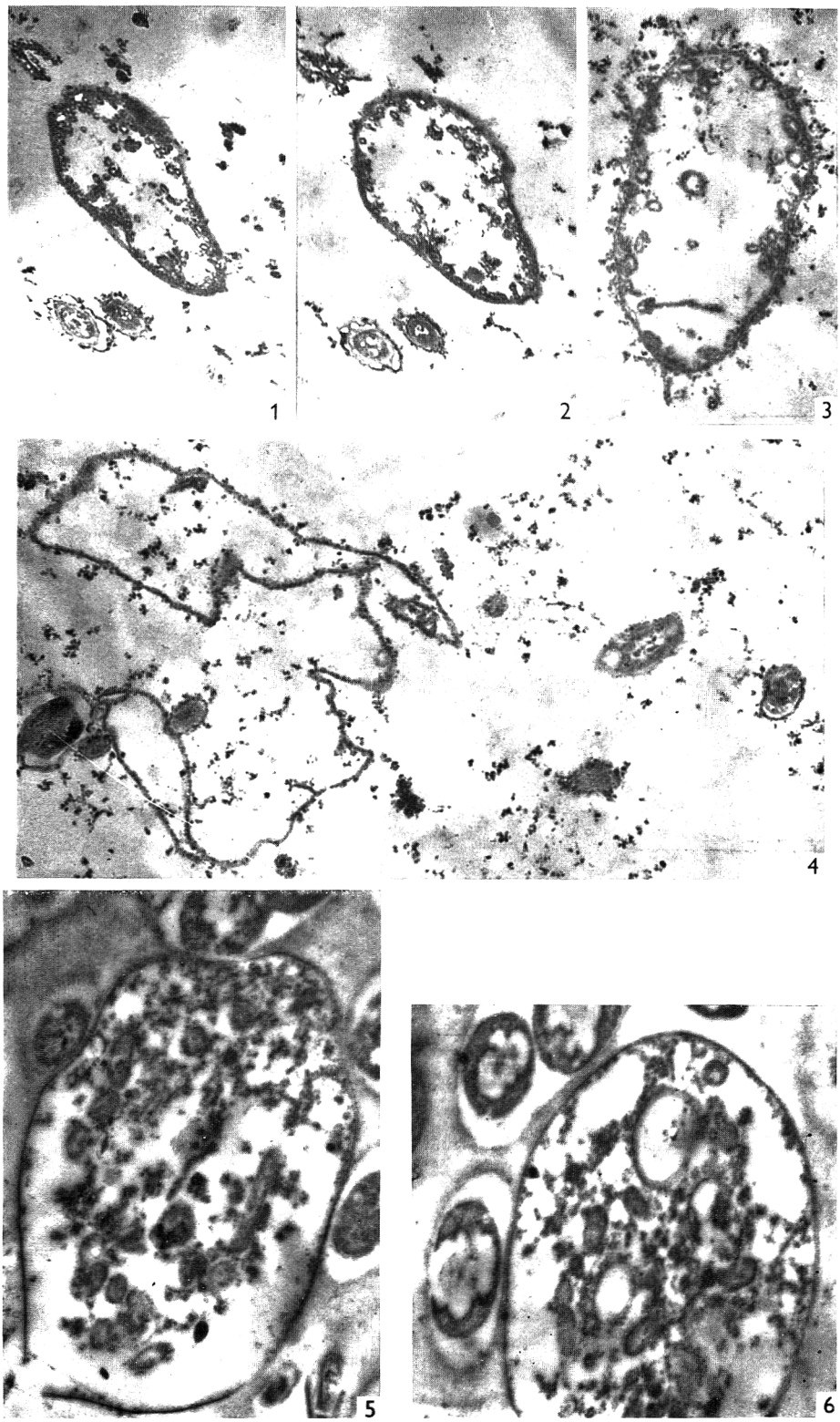
All electron micrographs of unstained sections

Figs. 1, 2. Adjacent sections through cell in early stages of gonidium formation, showing very small bodies, mainly attached to, or near, the cell envelopes. Comparison of figs. 1 and 2 shows that they are short and randomly arranged. Sections through two small cells appear below. × 14,000.

Fig. 3. A slightly more advanced mother-cell showing some larger bodies. × 21,000.

Fig. 4. Burst wall of mother-cell, with debris and small cells (i.e. mature gonidia). × 14,000.

Figs. 5, 6. Mature mother-cells containing well-formed gonidia, resembling small bacteria; also a few (lipid?) globules and debris. Gonidia are no longer attached to the cell envelopes, but lie in the lumen of the enlarged cell. A few large vegetative cells are seen for comparison. × 7,000.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Nucleic Acid Changes in Bacteroids of *Rhizobium lupini* During Nodule Development

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(*Accepted for publication* 17 *October* 1966)

SUMMARY

The nodules of yellow lupin plants (*Lupinus luteus* L.) increased logarithmically in wet weight, leghaemoglobin and rhizobial numbers. Bacteroids were isolated from such nodules and the changes in concentration of protein, RNA and DNA per organism were followed as a function of nodule age. Six weeks after emergence, DNA and RNA per organism had fallen to 40 % and 13 %, respectively, of the values found in the bacteria from 1-week-old nodules. No further changes in RNA and DNA were found after this time. The decreases are discussed with reference to previous reports of loss of nuclear material during bacteroid formation.

INTRODUCTION

Several investigations of changes in the nuclear material of organisms isolated from the nodules of legumes have been made in an attempt to explain the low viability of such cells on laboratory media (Almon, 1933).

Rautanen & Saubert (1955), in a chemical study, emphasized the fact that both soybean and cowpea bacteroids contained considerably less nucleic acid than the respective laboratory grown rhizobia, the difference being most marked with soybean organisms. Such differences, however, may be due to changes in composition of the laboratory grown cells with stage of growth.

Various studies with stained material under the light microscope also suggest losses of nuclear material during bacteroid formation. Schaede (1941) noted that bacteroids from *Pisum sativum* and *Vicia faba* did not give a Feulgen reaction, while those from lupin gave a diffuse reaction decreasing when the cells underwent digestion. Heumann (1952) showed that pea bacteroids gave a Feulgen reaction from 'nucleoids' in only about 40 % of the cells. Indications that nuclear material was diffuse in bacteroids from young nodules and only appeared as nucleoids later in their development were obtained with fluorescence microscopy (Chizhik, 1959). Bergersen (1955) observed that the amount of nuclear material apparently decreased with nodule age with bacteroids from subterranean clover stained by various nuclear stains.

In bacteroids from soybean, Bergersen (1958) concluded that it was unlikely that cessation of division of the bacteroids was due to lowered nucleic acid content. No significant changes occurred in the total nucleic acid of bacteroids from nodule initiation to maturity, and no differences were found between bacteroids and laboratory-grown cells in their nucleic acid content. Since the methods used do not distinguish

between RNA and DNA, changes in DNA affecting capacity for division could have been obscured.

Electron microscopy of clover and medic nodules (Dart & Mercer, 1963) showed that the fibrillar material of the nucleoid region of rod-shaped cells in the infection thread decreased after release into host cells during nodule formation.

The literature cited indicates that definite losses of nuclear material occur during bacteroid formation in legume nodules, though the report of Bergersen (1958) is at variance with other chemical and microscopic evidence. However, whether the nuclear degeneration is accompanied by loss of DNA is still unknown.

METHODS

Plants. Seeds of *Lupinus luteus* L. were inoculated with strain of D25 of *Rhizobium lupini* maintained according to Parker & Oakley (1963), and sown in the field in sand given the following fertilizer treatment: 1 g. per square foot of a mixture of commercial superphosphate, rock phosphate, KCl and $MgSO_4.7H_2O$ mixed in a ratio of 2:4 2:1 (w/w), and 0·035 g. per square foot of a mixture of commercial $CuSO_4.5H_2O$, $ZnSO_4.7H_2O$, Na_2MoO_4 and $MnSO_4.4H_2O$ mixed in the ratio 2:2:1:2 by weight.

All times are recorded from emergence.

Isolation of bacteroids. The term 'bacteroids' used here refers only to rhizobia obtained from nodules. Bacteroids from lupin nodules rarely exhibit the extreme deformation of shape commonly seen with bacteroids from clover and medic nodules, though they are enlarged compared to laboratory-grown cells.

One week after emergence, nodules were visible only as 1 mm. foci in the root cortex, with no detectable swelling of the root. Lengths of these roots (2 cm.) were washed and then homogenized in 0·1 M-potassium phosphate buffer (pH 7·1) in a 20 ml. Perspex homogenizer and filtered through a sintered glass funnel (porosity 0) to remove plant debris. This procedure was only necessary for weeks 1 and 2.

In later weeks, the nodules were large enough to be conveniently removed from the roots. Between weeks 3 and 8, these were sufficiently soft to be homogenized directly but from week 9, this was unsatisfactory and the hardened cortex was pared away with a scalpel before homogenizing and filtering.

The filtrate was then centrifuged, first at 300 *g* for 5 min. to remove starch grains and plant material, then at 2700 *g* for 15 min. to sediment bacteroids. These were washed once with 0·1 M-phosphate (pH 7·1) and resuspended in the same buffer. All operations after the removal of nodules (or root segments) were performed at 4°.

Counting. Bacteroids were counted under dark-field illumination at a magnification of 400, in a Hawksley-Thoma counting chamber. A minimum of 1,000 cells was counted at several dilutions.

Estimation of protein and nucleic acids. Soluble cell protein was measured by the biuret method. Bacteroid samples were treated with 2 vols of 5% (w/v) trichloracetic acid (TCA) and centrifuged. The cells were resuspended in biuret reagent (Dawson, Elliott, Elliott & Jones, 1959) and colour measured after 30–60 min. at room temperature and a centrifugation at 20,000 *g* for 10 min.

Nucleic acids were measured as ribose and deoxyribose, after degradation according to Volkin & Cohn (1954), but omitting the extractions with organic solvents. Ribose

was estimated by a modification of the orcinol method (Oliver & Blumer, 1964) using yeast RNA (type XI, Sigma Chemical Co., St Louis, U.S.A.) as a standard. Deoxyribose was estimated by the diphenylamine reaction (Burton, 1956) using a standard of salmon sperm DNA (California Foundation for Biochemical Research, Los Angeles, U.S.A.) hydrolysed by heating for 15 min. at 70° in 0·5 N-perchloric acid.

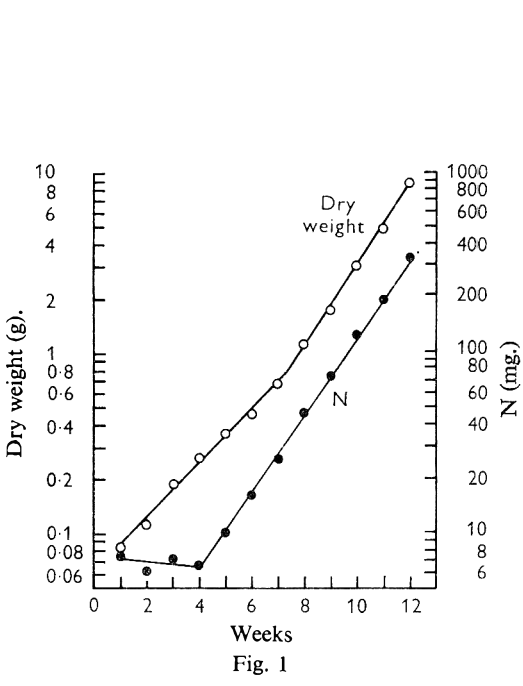

Fig. 1

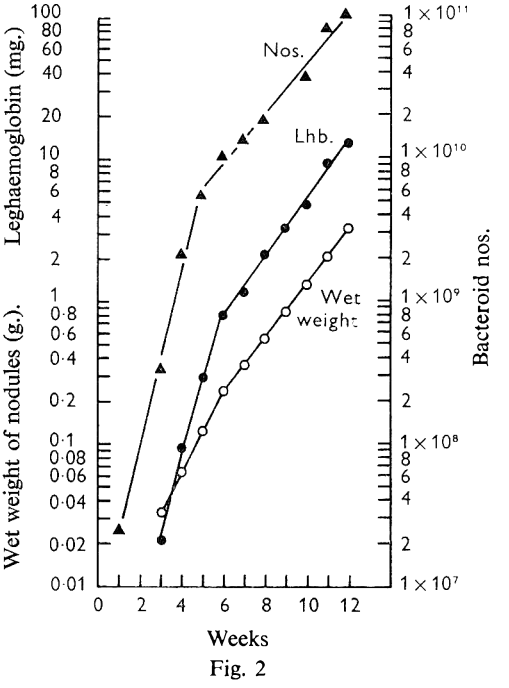

Fig. 2

Fig. 1. Semi-log plot of increase in plant dry weight (○) and total nitrogen (●) as a function of time. All lines of positive slopes are regressions with $P < 0{\cdot}001$.

Fig. 2. Semi-log plot of changes in bacteroid numbers, (▲), leghaemoglobin (●) and wet weight of nodules (○) per plant. All lines are regressions with $P < 0{\cdot}01$ for the initial parts and $P < 0{\cdot}001$ thereafter.

Leghaemoglobin. Leghaemoglobin was measured in the supernatant from the bacteroids following centrifugation at 25,000 *g* for 20 min. at 5°. The solution was diluted in 0·1 M-potassium phosphate (pH 7·1), reduced with dithionite, and the leghaemoglobin estimated from its absorption at 555 mμ using an extinction coefficient of 7·9 for 1 % (w/v) solution.

RESULTS

Curves for the increase in dry weight and nitrogen of the plants are shown in Fig. 1. Since the plants were growing in sand, fixation of N_2 is apparently contributing to plant growth from about 4 weeks after emergence.

Data for increases in bacteroid numbers, wet weight of nodules, and leghaemoglobin per plant are presented in Fig. 2. The main feature appears to be a discontinuity in the rate of increase of all three parameters at about 5–6 weeks. Apparently this did not

affect N_2 fixation since the nitrogen increase of the whole plants shows no such effect.

The results of the nucleic acid analyses are presented in Fig. 3. There is a marked decline in both RNA and DNA per bacteroid, with a simultaneous fall in protein. It is apparent that the falls in RNA and DNA cease after about 6 weeks, after which relatively constant levels are obtained. A second experiment showed similar falls in RNA, DNA and protein over a period of 5 weeks. The experiment of Fig. 3 was discontinued at 12 weeks with the onset of flowering, in order to avoid possible complications introduced by nodule senescence.

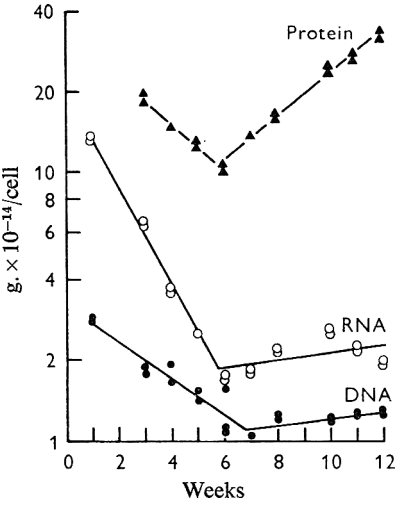

Fig. 3. Semi-log plot of bacteroid protein (▲), RNA (○) and DNA (●) as a function of time. All lines of negative slope are regressions with $P < 0{\cdot}001$. Of the lines of positive slope, those for protein and DNA are regressions with $P < 0{\cdot}001$ and $0{\cdot}05$, respectively.

DISCUSSION

The morphology of the lupin nodule has been discussed by Kidby & Goodchild (1966). Because only a collar of nodule tissue is formed from the initial inoculation, it is possible to obtain material of uniform age and development. No senescence is observed in such nodules up to flowering in contrast to the nodules formed by the same rhizobial strain nodulating *Ornithopus sativus*. This suggests that the changes observed in the bacteroids cannot be due to nodule breakdown but are typical of normal nodule development, a conclusion supported by the consistent increase in nodule weight over the period.

The marked fall in RNA suggests that protein synthesis by bacteroids could be impaired. However, protein per bacteroid increased after 6 weeks, probably as a result of cell enlargement, indicating that protein synthesis was continuing.

The decline in bacteroid DNA has obvious implications for bacteroid viability and appears to confirm observations on the disappearance of nuclear material made by light microscopy (Bergersen, 1955; Heumann, 1952; Schaede, 1941), by chemical analysis (Rautanen & Saubert, 1955) and by electron microscopy (Dart & Mercer, 1963). These results are in contrast to those of Bergersen (1958). In view of the relatively short time (about 4 weeks) required for the soybean nodules to reach maximum

size, compared with the steady increase still proceeding at 12 weeks in the lupin nodule, it seems possible that the changes reported here for nucleic acids may well have taken place very rapidly in the soybean nodules examined. In that case, the relatively constant nucleic acid levels found in soybean bacteroids might correspond to the constant levels found in lupin bacteroids from nodules 6–12 weeks old.

The simultaneous decline in RNA and DNA may, however, be a reflexion of a marked change in growth rate. With *Salmonella typhimurium*, Schaechter, Maaløe & Kjeldgaard (1958) found that the function log (DNA/cell) was linear with the growth rate. Similarly, Lark (1966) showed that the growth rate of *Escherichia coli* governed its DNA content, with a maximum value some four times the value reported for the non-replicating chromosome (Cairns, 1963). In the lupin nodule, the change in rate of increase of bacteroid numbers seen in Fig. 2 may govern the levels of RNA and DNA found in these cells.

The flat portions of both the RNA and DNA curves are presumably reached when the proportion of actively-dividing rhizobia is small compared to the mass of non-proliferating cells in which decline in RNA and DNA has ceased.

The breaks in slope in the curves at about 6 weeks are at the moment unexplained. However, the fact that the changes in slope are to lower values of nodule parameters suggests a control mechanism on the rate of growth of the nodule relative to that of the plant.

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Increased Toxin Titres Obtained by the Addition of Selected Carbon Sources to Shaken Cultures of *Corynebacterium diphtheriae*

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(*Accepted for publication* 6 *February* 1967)

SUMMARY

The rate of utilization of maltose in shaken cultures of a strain of *Corynebacterium diphtheriae* Park Williams 8 was much higher than that of the N source. Considerable gain in toxin yields was achieved when maltose or some intermediates of its metabolism (glucose, glycerophosphate, lactate, acetate) were added to the cultures between 20 and 32 hr of incubation. When, however, the initial concentration of these carbon sources in the medium was increased above the usual value (25 mg./ml.) a decrease in toxin formation resulted. The carbon intermediates were active at concentrations up to 15 times lower than that of maltose. Their action on the toxin titre was immediate, as contrasted to the lag of 3–4 hr which followed the addition of maltose.

INTRODUCTION

The introduction of submerged cultures (Linggood & Fenton, 1947; Linggood *et al.* 1955) instead of surface cultures for large-scale production of diphtheria toxin yielded not only more toxin but primarily a much greater bacterial mass. This fact led us to the assumption that some of the nutrients which were present in excess in the surface cultures might in submerged cultures reach suboptimal concentrations that limited the further increase in toxin titres. Preliminary studies have shown that the rate of utilization of the carbon source (maltose) was much higher than that of the nitrogen source. The present paper deals therefore with the results of a study aimed to achieve higher toxin yields by adding to the medium maltose or some other carbon sources which are intermediates of maltose metabolism.

METHODS

Organism. A culture of the Park Williams no. 8 strain of *Corynebacterium diphtheriae* (CN2000 from the National Institute of Public Health, Utrecht, Netherlands, by the courtesy of Dr A. Tasman) was used throughout.

Medium. The organisms were cultivated on a medium based on papain digest of beef muscle as described by Linggood *et al.* (1947) with the exception that our routine medium contained 2·5 % (w/v) maltose. The medium was sterilized by filtration through Seitz filters and steamed for 20 min.

Toxin. Toxin was estimated by the flocculation method (Ramon, 1922) with a

purified antitoxin calibrated against the standard antitoxin for flocculation of the National Institutes of Health, Bethesda, U.S.A.

Growth. The extinction of washed and diluted samples of bacterial culture was determined with a Beckman B spectrophotometer at 540 mμ.

Maltose. Maltose utilization was estimated in the supernatant fluid of centrifuged cultures by the method of Somogyi (1945).

Amino-nitrogen. The amino-nitrogen content of the supernatant fluids of centrifuged cultures was estimated by the method of Pope & Stevens (1939).

Total nitrogen. The method of Ma & Zuazaga (1942) was used to estimate total N in the supernatant fluids of centrifuged cultures.

Toxin production. Toxin was produced in 1 l. Erlenmeyer flasks, each containing 100 ml. medium. Each flask was inoculated with 5 ml. of an 18–24 hr. shaken culture and mounted on a reciprocating shaker (120 strokes/min.) which could accommodate 56 bottles, and shaken for 48 hr in an incubator at 36–37°. In kinetic studies, samples of two to seven flasks were removed at intervals, examined microscopically to test the purity of the cultures, pooled and submitted to the tests described.

RESULTS

Influence of added carbon sources on the toxin titres

Preliminary experiments have shown that the rate of maltose utilization in shaken cultures of *Corynebacterium diphtheriae* was so high that in many cases it disappeared almost completely from the medium within 48 hr (duration of the process), whereas a large excess of the N source always remained. In the 18 experiments given in Table 1 the percentage of total nitrogen utilized varied from 1·1 to 31·2%, average 20·95%. For amino-N the values were 3·7–54·4%, average 30·8%, while for the maltose values were 29·6–99%, average 82·1%.

It was assumed that because of the high rate and extent of maltose utilization, the concentration of this nutrient in the medium might become (after decreasing below a certain value) suboptimal for toxin formation. When, however, the initial concentration of maltose in the medium was increased above the usual value (25 mg./ml.), an unexpected decrease (of about 25%) in toxin formation resulted. The timing of maltose addition was therefore changed, and it was added to the cultures at a phase when a considerable amount of maltose was already used up and the rate of toxin formation was high. Indeed such a treatment resulted in considerable improvement in toxin yields. Concentrations of added maltose ranging from 1 to 25 mg./ml. were investigated. The lowest concentration which still caused a limited but reproducible increase in toxin titre was 5 mg./ml. On an average the best results were obtained when maltose 15 mg./ml. were added to the cultures any time between 20 and 32 hr of incubation.

To see whether the effect of maltose on the toxin titres was due specifically to maltose or to the elimination of some partial deficiency in a readily available carbon source at a critical period, the same treatment was repeated with some intermediates of maltose metabolism; glucose, lactate, glycerophosphate, acetate. (Lactate and acetate are ingredients of the routine culture medium.) The intermediates acted in a similar way to maltose; their addition at 0 hr caused a decrease in toxin titres, whereas when added to the growing cultures between 20 and 32 hr, they caused a comparable

rise in toxin yields. A significant difference was, however, observed: the concentrations of the intermediates which were as effective as maltose were up to 15 times less than that of maltose.

Table 1 summarizes the results of some of the experiments in which the carbon sources were added to the incubating cultures. The values given in Table 1 were determined on pooled cultures. The number of cultures pooled in each experiment varied between 2 and 10 according to the number of variations tested and according to whether or not cultures were also taken at intermediate times of incubation. The fact that in some cases only few cultures were pooled does not detract from the significance of the results obtained because this was done only in kinetic studies in which the effects were measured and verified on many samples at consecutive periods of time (see Figs. 1–3). Since within the given period, the exact time of addition was not found to be critical, only those experiments are given in Table 1 in which the additions were made at 24 hr of incubation. For convenience almost all the experiments in which the effect of only one kind of carbon source was compared with untreated controls are omitted from Table 1. In spite of this selection, only in the case of maltose and glucose 1 mg./ml. were there slight deviations from the overall average gains in titre. The overall gain as a consequence of adding maltose was 25% instead of 23% as in the experiments listed in Table 1, whereas in the case of glucose 1 mg./ml. the overall gain was 23% instead of 25% in the experiments listed in the table.

The variation in toxin yields of control cultures was considerable. This can be attributed to differences in the quality of meat used for preparing the digest and/or to variations in the degree of deferration of the different batches of media. It is also difficult to exclude slight variations in the physiological state of the inocula. Experience has shown that in course of routine production variations in titres are not infrequent and vary from laboratory to laboratory. Therefore it seems to be relevant that, following the addition of carbon sources, increased toxin titres could be obtained in each experiment regardless of the toxin yield in control cultures.

It can be seen from Table 1 that the best results were obtained with acetic acid 0·97 mg./ml. About 41% gain in toxin yield was achieved in this case which represented about 80 Lf/ml. as compared to 36% (about 70 Lf/ml.) in the case of glucose 2·5 mg./ml. and about 50 Lf/ml. in the case of maltose 15 mg./ml. or glucose 1 mg./ml.

The addition of maltose always caused a decrease in final pH values, which was not general in the case of the intermediates. The addition of acetic acid 0·97 mg./ml. decreased the pH value by about 1 unit at the time of addition, but in later phases of incubation even higher pH values than those of the controls were obtained. This phenomenon, as pointed out by Linggood *et al.* (1947) was probably due to the conversion of acetate to carbonate and water. Too high concentrations of added glucose or acetic acid delayed toxin formation or (in cases when values below pH 6 were obtained) even caused the destruction of the toxin formed before these additions.

Kinetics of the influence of some of the carbon sources on the rate of toxin formation

The finding that the maltose metabolism intermediates influenced the toxin titre at much lower concentrations than did maltose itself pointed to possible differences in the respective kinetics of their action on toxin formation. The rate of toxin formation immediately after the additions at 24 hr was therefore studied. Treated and control

Table 1. *Effect of maltose or of some of the intermediates added to growing shaken cultures of C. diphtheriae (CN2000) on final toxin titres and pH values*

(Lf./ml. (A) and pH (B) values at the end of the experiment (48 hr).)*

Additions at the 24th of growth hr (mg./ml.)	Exp. 77		Exp. 79		Exp. 82		Exp. 84		Exp. 85		Exp. 86		Exp. 104		Exp. 105		Exp. 107	
	A	B	A	B	A	B	A	B	A	B	A	B	A	B	A	B	A	B
None	280	8·85	340	8·6	240	7·5	280	8·75	280	8·75	260	8·8	180	7·4	240	7·6	120	7·2
Maltose																		
15	—	—	360	8·1	260	7·0	360	7·8	320	7·4	320	7·5	250	7·1	320	6·55	180	6·5
Sodium lactate																		
1	300	9·0	380	7·75	—	—	320	8·7	300	8·4	300	8·8	—		—		—	
3	310	9·1	380	8·65	—	—	—	—	—	—	—	—	—	—	—	—	—	—
5	—	—	—	—	—	—	—	—	—	—	320	8·95	—	—	—	—	—	—
Lactic acid																		
0·88	—	—	—	—	280	7·9	300	8·55	320	8·5	300	8·8	—	—	—	—	—	—
1·76	—	—	320	8·5	—	—	—	—	—	—	—	—	—	—	—	—	—	—
2·64	—	—	—	—	270	7·9	—	—	—	—	—	—	—	—	—	—	—	—
3·52	—	—	360	8·65	—	—	—	—	—	—	—	—	—	—	—	—	—	—
4·40	—	—	—	—	—	—	—	—	—	—	320	8·95	—	—	—	—	—	—
Glycerophosphate																		
1	—	—	—	—	260	7·0	320	8·7	300	8·5	280	9·0	—	—	—	—	—	—
3	—	—	—	—	250	7·0	—	—	—	—	—	—	—	—	—	—	—	—
5	—	—	—	—	—	—	—	—	—	—	300	9·0	—	—	—	—	—	—
Glucose																		
1	—	—	—	—	—	—	—	—	—	—	—	—	200	7·3	280	7·7	220	7·35
2·5	—	—	—	—	—	—	—	—	—	—	—	—	240	7·5	260	7·9	270	7·2
5·0	—	—	—	—	—	—	—	—	—	—	—	—	nf†	5·5	160	7·0	—	—
7·5	—	—	—	—	—	—	—	—	—	—	340	8·6	nf†	5·3	80	6·4	—	—
10·0	—	—	—	—	—	—	—	—	—	—	—	—	—	—	nf†	5·25	—	—
Acetic acid																		
0·97	—	—	—	—	—	—	—	—	—	—	—	—	280	7·7	320	8·1	260	7·25
1·94	—	—	—	—	—	—	—	—	—	—	—	—	nf†	5·7	220	8·1	nf†	5·6

	Exp. 108		Exp. 109		Exp. 123		Exp. 124		Exp. 127		Exp. 130		Exp. 131		Exp. 132		Exp. 133	
	A	B	A	B	A	B	A	B	A	B	A	B	A	B	A	B	A	B
None	120	6·7	90	7·2	320	8·85	180	8·65	300	8·25	180	8·4	300	8·3	260	8·2	200	8·2
Maltose																		
15	180	6·5	180	6·82	360	8·5	220	8·5	—	—	260	7·3	320	7·0	300	7·2	225	7·2
Sodium lactate																		
1	—	—	140	6·9	—	—	—	—	—	—	—	—	—	—	—	—	—	—
3	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
5	—	—	140	8·0	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Glucose																		
1	150	7·5	—	—	—	—	220	8·6	340	8·45	290	6·7	320	8·35	300	8·1	280	8·1
2·5	—	—	220	7·5	—	—	—	—	—	—	260	7·7	320	8·2	290	8·15	280	7·85
5·0	—	—	—	—	—	—	—	—	—	—	240	7·8	250	8·0	—	—	—	—
Acetic acid																		
0·97	220	7·2	220	7·7	380	8·9	—	—	360	8·3	220	8·0	330	8·4	—	—	260	8·1
1·94	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Sodium acetate																		
1	—	—	—	—	—	—	—	—	—	—	240	8·5	320	8·5	—	—	240	8·3
2	—	—	—	—	—	—	—	—	—	—	270	8·65	350	8·5	—	—	250	8·2
3	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	260	8·25

* Values obtained from pooling several cultures (see text). † No flocculation.

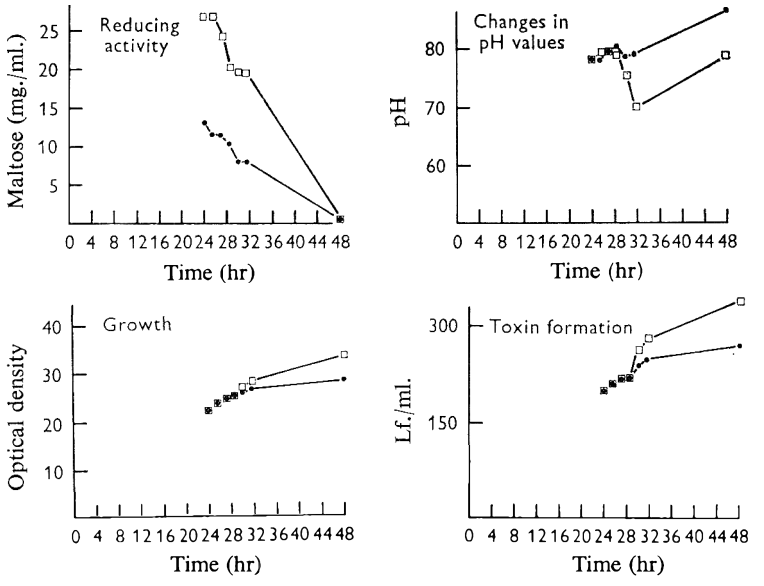

Fig. 1. Expt. 122. Effect of adding maltose 15 mg./ml. at the 24th hr of incubation to shaken cultures of *C. diphtheriae* (CN 2000). ●—●, No addition; □—□, addition of maltose 15 mg./ml.

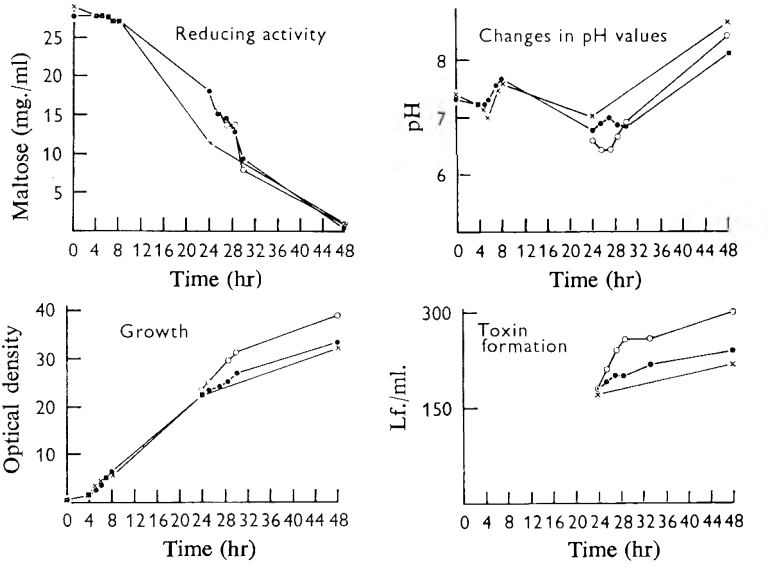

Fig. 2. Expt. 119. Effect of adding glucose 1 mg./ml. at 0 hr or at the 24th hr of incubation to shaken cultures of *C. diphtheriae* (CN 2000). ●—●, no addition; × — ×, addition at 0 hr 1 mg./ml. glucose; ○—○, addition at 24 hr 1 mg./ml. glucose.

flasks were removed from the shaker at intervals of 1–1·5 hr and their contents examined.

The results are presented in Figures 1–3.

After adding 15 mg. maltose/ml. (Fig. 1), more than 4 hr elapsed before the titre in the treated cultures started to increase above the value in the controls. It can be

seen from the curves of reducing activity that about 13 mg. maltose/ml. were present in the medium at the time of addition and that the addition caused an increase in the rate of maltose utilization. Final maltose concentrations were identical in both treated and control cultures. The pH value decreased because of conversion of maltose to acids and remained lower than that of the controls until the end of the experiment. In general growth was promoted and more nitrogen source was used up as a consequence of the addition. There were, however, exceptions in which the curves of the growth or nitrogen sources did not follow this pattern.

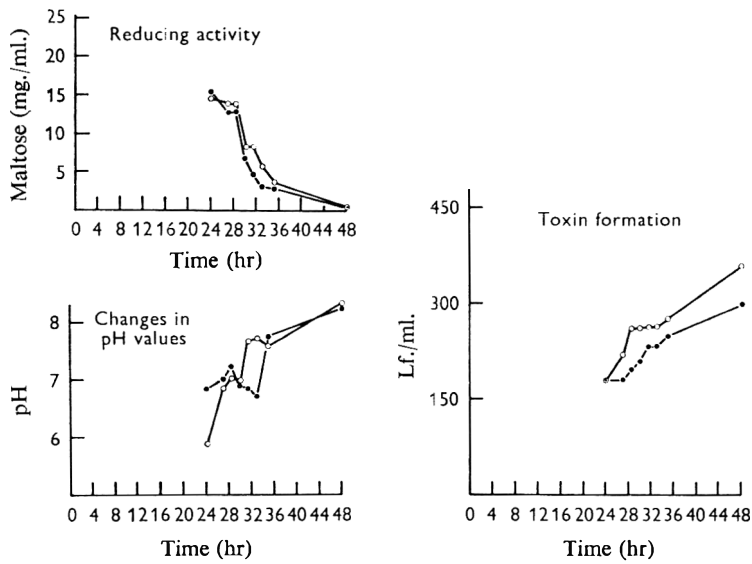

Fig. 3. Expt. 127. Effect of adding acetic acid 0·97 mg./ml. at the 24th hr of incubation to shaken cultures of *C. diphtheriae* (CN 2000). ●—●, no addition; ○—○, addition of 0·97 mg./ml. acetic acid.

In Fig. 2 the effect of adding glucose 1 mg./ml. at 0 hr or at 24 hr is shown. As a consequence of the addition at 0 hr the rate of toxin formation was retarded and the pH value in the first hours of growth decreased to lower values than those of the controls.

In contrast to what was observed on adding maltose at the 24 hr, the toxin titre started to increase without a considerable lag period when glucose was added. The pH value decreased immediately, but then it started to increase indicating a high rate of oxidative processes. As previously stated, the differences between the final pH values of control and treated cultures did not follow a uniform pattern. At the time of addition of glucose more than 15 mg. maltose/ml. were found in the culture. The rate of utilization of the maltose was not affected by the treatment. In general, growth and the utilization of N sources were somewhat promoted but more deviations from this pattern were observed when glucose was added than with maltose. A short time after its addition, glucose could not be detected in chromatograms of the culture fluid.

Figure 3 depicts the effect of adding acetic acid 0·97 mg./ml. to the cultures at 24 hr of incubation when the culture still contained about 15 mg. maltose/ml. The general pattern is similar to that shown in the case of the addition of glucose.

DISCUSSION

It has been shown that the rate of toxin formation can be increased when certain carbon sources are added to cultures which still contain considerable amounts of maltose and which produce toxin at a high rate. The explanation offered for this finding is that, with increasing bacterial mass, the concentration of the maltose becomes suboptimal for toxin production. The fact that the cultures are able to metabolize an additional 15 mg. maltose/ml. seems to support this view. The question of how 15 times less glucose or acetic acid can cause the same or even higher increase in titres requires further investigation. It is known (Tasman & Brandwijk, 1936) that maltose is split by *C. diphtheriae* to glucose at a much slower rate than the further metabolization of glucose. Edwards (1960) could not find any sugar other than maltose in his culture filtrates possibly because the intermediate glucose was used immediately after its formation. In our experiments too, the added glucose disappeared from the cultures almost immediately after its addition. The lag in response to the addition of maltose as compared to the immediate rise in toxin titre following the addition of glucose or acetic acid, might be connected with these findings.

The fact that maltose and certain metabolism intermediates, when added at the beginning of the incubation, caused a delay in toxin formation, indicates that the ratio of concentration of nutrients to bacterial mass is of importance for high toxin formation and that nutritional requirements may change according to the physiological age of the culture. Perhaps our system was more sensitive to slight changes in pH value at the beginning of incubation rather than later. Our findings, according to which glucose, when added to the medium at 0 hr, retards toxin formation is in accordance with those of Linggood *et al.* (1947). In their system, however, much more than 1 mg. glucose/ml. was needed to obtain a pronounced adverse effect on toxin formation. This can be explained by the fact that they used much smaller and older inocula than we did, and therefore even at a high rate of glucose consumption per micro-organism the acid formation at low glucose concentrations could not reach values which would significantly influence the pH value of the cultures.

From a practical point of view, the gain in toxin yield that can be achieved by the method described, seems to be remarkable if one takes into account the low cost involved. About a hundred experiments have so far been made with the different carbon sources without the method having once failed. Fermentors offer easy technical solution for the problem of adding nutrients to growing cultures; preliminary experiments carried out on 4-litre fermentors indicate that the application of the method to such systems is feasible.

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Immunological Relationships Among the Lower Trypanosomatidae

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SUMMARY

By the use of Freund's complete adjuvant, high-titre agglutinating antisera were prepared to nine trypanosomatids: *Crithidia fasciculata* (Anopheles strain), *C. fasciculata* (Culex strain, Wallace isolate), *C. fasciculata* (Culex strain, Nöller isolate), *C. luciliae*, *Crithidia* sp. from *Euryophthalmus davisi*, *Leptomonas* sp. from *Dysdercus suturellus*, *L. mirabilis*, and *Blastocrithidia leptocoidis*. The three *C. fasciculata* strains (Culex, Wallace isolate: Culex, Nöller isolate: Anopheles strain) and *C. oncopelti* are immunologically similar according to agglutination tests. *Crithidia* sp. from Euryophthalmus and *C. luciliae* are distinct from each other and from *C. oncopelti*. The two Leptomonas species are distinct from each other and are not closely related immunologically to Crithidia species. The trypanosomatids tested here are not separable into groups according to original host (hemiptera or diptera).

INTRODUCTION

The first studies of serological relationships among the Trypanosomatidae (Noguchi, 1926) have served as a basis for consideration of species and genus affinities by later workers. Noguchi (1926), who used sera diluted to only 1/20 concluded that the two strains of *Herpetomonas culicidarum*, isolated from different hosts were identical and that *H. oncopelti* did not cross-react with either *H. culicidarum* strain. Clark (1958), using antisera of higher titre, confirmed Noguchi's results and in addition, noted the Nöller isolate of *Crithidia fasciculata* (Culex strain, ATCC 12857) is immunologically different from the two *C. fasciculata* strains. These organisms are all species of the genus *Crithidia*. The *H. culicidarum* strains are *C. fasciculata* (*Anopheles quadrimaculatus* strain) ATCC 11745 and *C. fasciculata* (*Culex pipiens* strain) ATCC 12857. *Herpetomonas oncopelti* is *C. oncopelti*, endosymbiote-infected (ATCC 12982). A *C. oncopelti*, cured of endosymbiote, is now available (ATCC 16422).

More recently, McGhee & Hanson (1963) used white leghorn chickens for producing anti-trypanosomatid antibody. Their chickens always contained small quantities of natural antibody against all trypanosomatids tested and the resultant chicken antisera were of considerably lower titre than the rabbit antisera used by Clark (1958): eight out of nine antisera produced against *Crithidia fasciculata* (Culex strain) had homologous agglutinating titres of 1/160–1/640, three antisera against *Crithidia* sp. from Euryophthalmus had homologous titres of 1/640–1/1280, and only one antiserum against *C. fasciculata* (Culex strain) had a homologous titre of 1/5120 (McGhee & Hanson, 1963). Sera with titres of 1/2560 and lower are considered low-titred antisera.

In contrast to Noguchi's findings (1926), McGhee & Hanson (1963) found 100% agglutinating cross-reaction between *Crithidia oncopelti* and *C. fasciculata* (Culex strain) and also between *C. luciliae* and *C. fasciculata* (Culex strain) but only 12–25% cross-reaction between *C. fasciculata* (Culex strain) and *Crithidia* sp. from Euryophthalmus. On the basis of their agglutinating cross-reactions, McGhee & Hanson (1963) separated the trypanosomatids into two groups according to whether they had been isolated from hemiptera or diptera; they found high cross-reactions between organisms from the same host type and low cross-reactions between organisms isolated from different host types. Such a sharp division was surprising since there is no such division of these organisms into categories on the basis of general nutritional requirements (Guttman & Wallace, 1964), ability to use various carbohydrates as the main growth substrate (Guttman, 1963), ability to synthesize methionine (Guttman & Wallace, 1964) or pattern of catalase activity (Wertlieb & Guttman, 1963). Indeed more recent experiments (McGhee, Schmittner & Hanson, 1965) on the ability of various Crithidia isolates to infect either hemiptera or diptera supported the conclusion that taxonomic groupings should not be based on the original host type, since experimental infection of the other host type was frequently possible. Simultaneously with the appearance of these studies on experimental infection, we (Vitetta & Guttman, 1965) pointed out the potential danger of identification of species on immunological data obtained with low-titred sera from chickens. We also presented preliminary immunological results which disagreed with the earlier work of McGhee & Hanson (1963) and supported the later conclusions of McGhee *et al.* (1965).

This study presents methods for the preparation of high-titred sera against whole or disrupted cells of several lower trypanosomatids and data on the relationships of the various organisms as determined by agglutination tests.

METHODS

Preparation of whole-cell antigens. All trypanosomatids used in these studies (Table 1) were grown in 2800 ml. Fernbach flasks containing 1000 ml. of undefined medium (Guttman & Eisenman, 1965). Each flask was inoculated with 5·0 ml. of log phase culture of the particular trypanosomatid and incubated at room temperature until the logarithmic growth phase. After harvesting by centrifugation at 5000 *g* at 10° for 10 min., the organisms were washed twice with distilled water and the packed organisms distributed into several Wasserman tubes and stored in a freezer at −20° until needed. (Lower trypanosomatids may be suspended in distilled water for considerable periods of time without cell lysis.)

Preparation of disrupted-cell antigens. Trypanosomatids to be disrupted were grown and harvested as described above. After washing, 10–15 ml. of packed organisms were placed in a 150 ml. beaker, diluted 1/3 (v/v) with 0·85% (w/v) saline and disrupted by one of the following methods:

(*a*) *Pressure.* 10 ml. of the diluted suspension were cooled in an ice bath for 30 min. and then rapidly passed through a French cell three times at 10,000 lb. pressure.

(*b*) *Sonically.* 50–70 ml. of the diluted suspension in a 150 ml. beaker were packed in an ice bath and the organisms were disrupted by treatment for 5 min. with a Branson S-110 Sonifier equipped with a microtip and adjusted to a setting of 4.

Suspensions were examined microscopically after disruption to insure the absence

of whole organisms and the presence of small particles. There was no further gradation of particle size. These suspensions ('disrupted antigen') were then distributed into Wasserman tubes and frozen until used.

Preparation and assay of antibodies. Methods are described in detail since they embody modifications and combinations of those outlined by Boyd (1956), Kabat & Mayer (1961), Campbell *et al.* (1963), Crowle (1963) and Nussenzweig (personal communications).

Rabbits were used because they produce antibodies physicochemically more homogeneous than those produced by horses or chickens (Crowle, 1963; Kabat & Mayer, 1961).

Since there was considerable difficulty involved in obtaining high-titre antisera to trypanosomatids, and also because of the low titres of trypanosomatid antisera obtained by others (Clark, 1958; McGhee & Hanson, 1963) in the absence of adjuvant, we used antigen mixed with Freund's complete adjuvant, which produces a granulomatous reaction at the injection site (Fishel *et al.* 1952) and hence produces a better antibody response.

Table 1. *Organisms used as antigens*

Organism	ATCC	Host type	Isolator	Year
Crithidia fasciculata (Anopheles strain)	11745	Diptera	Noguchi & Tilden	1926
C. fasciculata (Culex, Wallace)	12857	Diptera	Wallace	1942
C. fasciculata (Culex, Nöller)	12858	Diptera	Nöller	1917
C. oncopelti (with endosymbiote)	12982	Hemiptera (?)	Noguchi & Tilden	1926
C. luciliae	14765	Diptera	Wallace	1958
Crithidia sp. *from Euryophthalmus davisi*	14766	Hemiptera	McGhee	1958
Leptomonas sp. from *Dysdercus suturellus*	—	Hemiptera	Wallace	1959
L. mirabilis	—	Diptera	Wallace	1963
Blastocrithidia leptocoridis	—	Diptera	Wallace	1964

Before immunization, all rabbits were tested and found to contain no natural agglutinating antibodies against the trypanosomatids to be used in these studies. Animals were then immunized with a series of three subcutaneous injections at 1-week intervals. Each injection of 1·0 ml. contained either 0·2 ml. of packed whole organisms + 0·3 ml. of saline or 0·5 ml. of disrupted antigen suspension, emulsified with 0·5 ml. of Freund's complete adjuvant (Baltimore Biological Laboratories, Baltimore, Md.). No animals were injected with complete adjuvant only (see Discussion).

From 3 to 7 days after the last injection and then at intervals of 4 to 7 days for 30 to 70 days, blood was collected from the marginal ear vein and the serum tested for agglutinating antibody titre. When the homologous titre reached a peak, 20 ml. of blood were collected by cardiac puncture and the serum was tested for cross-reactions. After the titre had reached its peak, animals were bled every 3–7 days from the marginal ear vein and the serum separated by centrifugation at 400 *g* and stored at 0°.

Agglutination tests. Dilutions of antisera (against both whole and disrupted cells) from 1/10 to 1/10240 were prepared in 0·85% (w/v) saline. Dilutions were routinely run to the 1/10240 mark to ensure that the equilibrium region had been reached to obviate prezone effects (Kabat & Mayer, 1961). To each Wasserman tube containing 0·5 ml. of diluted antiserum, 0·1 ml. of whole antigen, adjusted to an optical density

of 2·0 with saline, was added. The tubes were then shaken vigorously and incubated at 55° for 30 min. along with a control tube containing 0·5 ml. saline + 0·1 ml. antigen. The serum-saline control was omitted after initial tests showed no effect after incubation at 55°. Since endpoints were difficult to read when anti-disrupted-cell serum was tested against disrupted antigen (probably because of the small-sized particles), whole cells were used in all agglutination tests.

Incubation was at 55° rather than lower temperatures in order to inactivate complement and assure maximum contact between serum and antigen by thermal agitation. When complement is not inactivated by either heating or ageing, it may mask true agglutination reactions (Gray, 1964) and may cause non-specific cross-reactions in our particular system (see Discussion).

Results of homologous agglutination tests were read to the nearest dilution; cross-reactions were expressed as '% cross-reaction' where 100% = homologous agglutinating titre. Since the doubling dilution method was used in tests, sera were tested only at levels which would determine, 100, 50, 25, 12, and 6% cross-reaction. Organisms which cross-reacted 50–100% were considered immunologically similar.

RESULTS

The number of our antisera (prepared against whole and disrupted organisms) reaching high agglutinating titres (1/2560 or greater) and the methods used by various other investigators to generate anti-trypanosomatid antibodies are listed in Tables 2 and 3. Both the highest titre sera and the greatest number of high-titre sera were produced using the adjuvant method described here.

Table 2. *Comparison of anti-trypanosomatid antisera preparation and agglutinating potency*

Animal used for antibody	Route and frequency of injections	No. of days between first injection and antisera collection	Max. titre for agglutination tests; incubation temp.	Ref.
Rabbit	i.v.*; 4 at 4-day intervals	25	1:20 at 55°	(Noguchi, 1926)
Rabbit	i.v.; 5 at 5 to 7-day intervals	33–44	1:5120 at room temp.	(Clark, 1958)
Chicken	i.v.; 3 at 2-day intervals	16	1:5120 at 37°	(McGhee & Hanson, 1963)
Rabbit	s.c. mixed with Freund's complete adjuvant; 3 at 7-day intervals	22–140	1:10240 at 55°	This report

* i.v. = intravenous: s.c. = subcutaneous.

With all but one antigen preparation (Table 3), peak titres of 1/2560–1/10240 were reached 20–60 days after the final injection. After the peak was reached, the titre usually dropped somewhat and then remained level for at least 140 days. A slow rise in titre followed by a long-lasting plateau is typical of the response in adjuvant-injected animals (Uchitel & Khasman, 1965). One exception to this pattern was found with a serum against a disrupted *Crithidia* sp. from Euryophthalmus; the titre reached a peak of 1/2560 and then fell rapidly to zero.

Agglutinating cross-reactions with sera which had peak homologous titre or plateau past the peak titre, reveal certain relationships within the genus *Crithidia*. The

three *C. fasciculata* strains (Culex, Wallace isolate, Culex, Nöller isolate, and Anopheles) and *C. oncopelti* are immunologically very similar since agglutinating cross-reactions for all are in the 50–100 % range (Table 4). *C. luciliae* and *Crithidia* sp. from Euryophthalmus are immunologically distinct from the three *C. fasciculata* strains and

Table 3. *Number of days to reach peak homologous titre*

Antiserum to	No. days	Titre at peak
C. fasciculata (Anopheles)		
Whole	65	1:5120
Disrupted	40	1:2560–5120
C. fasciculata (Culex, Wallace)		
Whole (2 sera)	40	1:5120; 1:10240
Disrupted (2 sera)	30; 50	1:5120, 1:10240
C. fasciculata (Culex, Nöller)		
Whole	60	1:10240
Disrupted	50	1:5120
C. luciliae		
Whole	40	1:5120
Crithidia sp. from *Euryophthalmus davisi*		
Whole	30	1:1280
Disrupted (2 sera)	35; 45	1:2560; 1:2560–5120
Leptomonas sp. from *Dysdercus suturellus*		
Whole	60	1:10240
Disrupted (2 sera)	35; 50	1:1280-2560; 1:10240
L. mirabilis		
Disrupted	35	1:10240
Blastocrithidia leptocoridis		
Whole	60	1:10240

Table 4. *Percentage agglutinating reaction among lower Trypanosomatidae tested at or after peak homologous agglutinating titre*

	Antiserum to									
	(Anopheles)		*Crithidia fasciculata* Culex–Wallace		Culex–Nöller	*Crithidia* from Euryoph-thalmus	*Leptomonas* from Dysdercus		*L. mirabilis*	*Blastocrithidia leptocoridis*
Antigens	W*	D	W	D	D	D	W	D	D	W
C. fasciculata										
Anopheles	100	100	50	50; 100	100	—†	—	50	—	—
Culex–Wallace	100	—	100	100	50	25	25	25	50	25
Culex–Nöller	50	—	50	50	100	—	—	25	—	—
Crithidia sp. from Euryophthalmus	25	—	6; 25	12; 25	25	100	25	12	25	12
C. oncopelti	50	—	50	—	100	—	—	25	—	—
C. luciliae	—	12	6	25	24	—	—	25	—	—
Leptomonas sp. from Dysdercus	6	—	6; 50	25	25	12	100	100	25	—
L. mirabilis	—	—	50	12; 25	—	25	25	—	100	—
Blastocrithidia leptocoridis	—	—	25	12	—	12	50	—	—	100

*W = whole; D = disrupted. †— = Not tested.

C. oncopelti as well as from each other as evidenced by their low agglutinating cross-reactions (Table 4). Agglutinating cross-reactions between *Crithidia* sp. and *Leptomonas* sp. were low with four exceptions (one between *C. fasciculata* Culex–Wallace and *Leptomonas* sp. from Dysdercus and three between *C. fasciculata* Culex–Wallace and *L. mirabilis*) (Table 4). The two Leptomonas species show no strong agglutinating cross-reactions comparable to that seen among certain Crithidia species (Table 4).

Table 5. *Biochemical comparisons of six Crithidia spp.*

	Fasciculata					
	Anopheles	Culex, Wallace	Culex, Nöller	From Eury-ophthalmus	*Luciliae*	*Oncopelti*
Differences in % AT between nuclear and kinetoplastic DNA (moles %) (Mandel, 1965; Schildkraut *et al.* 1962)	15	24	—*	—	—	—
% AK after 24 hr acriflavin treatment (Guttman & Eisenman, 1965)	22±5·0	59±2·1	49±2·9	—	—	41±1·0
Ability to synthesize methionine (+at 28° C.) at 23–26° C (Guttman & Wallace, 1964)	0	+	0	+	+	—
Effect of temp. increase (26° to 33°) on catalase activity (Wertleb & Guttman, 1963)	Increase	Decrease	Decrease	Slight decrease	Increase	Increase
Carbohydrates used for growth† (Guttman, 1963)						
1 D-xylose	+	0	0	+	Slight	—
2 D-xylitol	+	0	Slight	0	0	—
3 β-CH_3-D-xyloside	+	0	0	0	—	—
4 D-fructose	+	0	0	0	0	—
5 α-D-glucoheptose	+	0	+	0	—	—
6 mannitol	+	0	Slight	0	+	—
7 D-arabitol	+	0	Slight	0	—	—
8 L-arabitol	+	0	Slight	0	—	—
9 sorbitol	+	+	+	0	+	—
10 D-galacturonic acid	—	—	+	0	+	—
11 L-sorbose	0	0	0	0	+	—

* —Not tested.

† Differences in ability to ferment carbohydrates have also been reported but these data are not tabulated here.

DISCUSSION

The difficulty in generating high-titre antisera to trypanosomatids is apparent from the reports in which low-titred antisera were used (Noguchi, 1926; Clark, 1958; McGhee & Hanson, 1963). The efficacy of mixing Freund's complete adjuvant with the antigen is clearly demonstrated (Tables 2 and 3) by the incidence of high-titre (1/2560–1/10240) sera produced. Although adjuvants containing mycobacteria (complete adjuvants) are more effective in stimulating an immune response than are the same preparations without mycobacteria (incomplete adjuvants) (Fishel *et al.* 1952), investigators are reluctant to use complete adjuvants in field immunization because of the granulomatous response produced at the inoculation site and presence of the mycobacteria *per se*. At the present time, therefore, the goal is to develop an adjuvant

which would contain no mycobacteria but still produce a sufficiently elevated immune response.

A possible complication to the interpretation of our high anti-trypanosomatid titre was posed by Konopka, Goble & Lewis (1961) in an abstract which noted that mice infected with *Leishmania donovani* exhibited prolonged survival to subsequent infection by *Mycobacterium tuberculosis*. It was subsequently found (Goble, personal communication) that cross-reaction between mycobacteria and *L. donovani* resided in the complement fraction and in our tests complement was inactivated. In addition, formation of non-specific antibodies to mycobacteria would be reflected in our finding non-specific agglutinating cross-reactions even at peak homologous titres but this was not the case (Table 4).

Our immunological data (Table 4) plus various biochemical differences (Table 5) among the Crithidia strains provide enough criteria to justify calling them separate species. The closest relationships exist among the four organisms currently called *Crithidia fasciculata* (Anopheles, Culex–Wallace, and Culex–Nöller) and *C. oncopelti*, but differences in such a primary response as induction of akinetoplasty (which depends upon reaction with the organisms' mitochondrial DNA itself) (Guttman & Eisenman, 1965) set these organisms apart as separate species.

All other organisms used in these studies were immunologically distinct as evidenced by the low agglutinating cross-reactions. Thus, our results clearly do not support the separation of organisms into immunologically related groups according to whether they were isolated from hemiptera or diptera. If such a relationship were to hold, we would expect *C. luciliae* to show a high agglutinating cross-reaction with *C. fasciculata* strains and *Leptomonas* sp. from Dysdercus to show a high cross-reaction with *Crithidia* sp. from Euryophthalmus. Our data show that this is not the case (Table 4). In addition, the biochemical characteristics of the organisms (Table 5) form no pattern which separates organisms isolated from diptera and hemiptera.

We hope that nomenclatural reform will follow shortly or else we may look forward to increased confusion about the precise nature of organisms used in biochemical studies (Carter, Gaver & Yu, 1966; Guttman & Eisenman, 1965).

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Uptake and Utilization of Acetate by Mycoplasma

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SUMMARY

Mycoplasma laidlawii and *M. gallisepticum* were found to incorporate [^{14}C]acetate from the growth medium mainly into their polar lipids, whereas *M. hominis* and *M. orale* incorporated the acetate mainly into their neutral lipids. *M. mycoides* var. *mycoides*, *Mycoplasma* sp. strain 14 and *M. fermentans* did not incorporate acetate, although they were found to possess acetokinase activity. Most of the radioactivity of incorporated acetate was found in the fatty acid fraction of *M. laidlawii* lipids. Palmitic and stearic acids almost completely inhibited acetate incorporation by this organism, whereas oleic acid did not, indicating that the major part of the acetate incorporated by *M. laidlawii* was used for the synthesis of saturated fatty acids. Washed *M. laidlawii* required glucose, coenzyme A (CoA) and Mg^{2+} for acetate uptake. The uptake process was temperature-dependent and pH-dependent and was inhibited by several metabolic inhibitors, in particular iodoacetate. Pyruvate considerably enhanced acetate incorporation into *M. laidlawii* lipids without raising the low degree of radioactivity in the cell fraction soluble in cold trichloroacetic acid. Pyruvate did not replace glucose as an energy source for acetate uptake. Propionate and butyrate markedly decreased the acetate uptake, probably by inhibition of the acetokinase activity of the organisms.

INTRODUCTION

Lipids form an essential part of the cell membrane of Mycoplasma organisms. The ability of the mycoplasmas to synthesize membrane lipids is restricted and several lipids and lipid precursors, essential for membrane structure and function, have to be supplied exogenously (Smith, 1964; Razin, 1967). In view of the importance of acetate as a lipid precursor it seemed worth while to study the conditions which govern its transport into the Mycoplasma organisms and its incorporation into cell lipids. There is clear evidence about the utilization of acetate for carotenoid synthesis in *M. laidlawii* (Smith, 1963). More recently, Smith & Henrikson (1965) showed the existence of a biosynthetic pathway leading from acetate to mevalonic acid in two Mycoplasma strains. Phosphorylation of acetate, a preliminary step in its utilization for lipid biosynthesis, has been shown to occur in several Mycoplasma strains (Castrejon-Diez, Fisher & Fisher, 1962).

METHODS

Organisms. Mycoplasma laidlawii (oral strain), serologically related to *M. laidlawii* strain A, was isolated in our laboratory from the human oral cavity. *M. gallisepticum* strain A 5969 was obtained from Dr M. E. Tourtellotte (Department of Animal Diseases,

University of Connecticut, Storrs). *M. mycoides* var. *mycoides* (PG 1) was provided by Dr D. G. ff. Edward (The Wellcome Research Laboratories, Beckenham, Kent) and *Mycoplasma* sp. strain 14 (goat strain) was originally supplied by Dr H. E. Adler (School of Veterinary Medicine, University of California, Davis, U.S.A.). *M. hominis* type 1, strain H34 (ATCC 15056), *M. fermentans* strain GII (ATCC 15474) and *M. orale* strain 823B (ATCC 15539) were obtained from the American Type Culture Collection (Rockville, Md., U.S.A.).

Growth conditions. Growth of organisms was usually done in 500 ml. volumes of a modified Edward medium (Razin, 1963) dispensed in 1 l. flasks. In some experiments uniformly labelled [^{14}C]sodium acetate (specific activity 15·7 mc./m-mole) or uniformly labelled [^{14}C]sodium pyruvate (specific activity 6·5 mc./m-mole) purchased from the Radiochemical Centre, Amersham, England, was added to the growth medium in amounts of 1 μc./100 ml. medium. Unlabelled acetate or pyruvate was added to the growth medium to a final concentration of 2×10^{-3}M. The organisms were harvested after incubation for 16–24 hr at 37° by centrifugation at 15,000 *g* for 15 min. The packed organisms were washed twice in 0·25 M-NaCl containing 0·01 M-$MgCl_2$. Cell membranes were separated from the 'soluble' fraction after osmotic lysis of the washed organisms (Razin & Cleverdon, 1965).

Extraction and fractionation of cell lipids. Washed packed organisms were extracted with chloroform + methanol (2 + 1, by vol.) at 50° for 30 min. The extract was separated from the insoluble residue by filtration through filter paper previously extracted with chloroform + methanol. The solvent was evaporated to dryness by heating under a stream of nitrogen, and the lipid residue was redissolved in 1–2 ml. chloroform. This solution was applied to a 7×95 mm. column of activated silicic acid (100 mesh, Mallinckrodt Chemical Works, St Louis, Mo., U.S.A.) pre-washed with chloroform. Neutral lipids were eluted from the column with 7 ml. chloroform, by the use of positive pressure. The polar lipids were then eluted with 7 ml. methanol (Ansell & Hawthorne, 1964). Methanolysis of lipids was done by the method of Kates (1964). The lipids extracted from the organisms according to Folch, Lees & Sloane-Stanley (1957) were hydrolyzed in methanolic hydrogen chloride solution, and the resulting fatty acid methyl esters were extracted with light petroleum.

Incorporation of acetate by washed organisms. Reaction mixtures (total vol. 1·0 ml.) contained 0·5 ml. of a suspension of washed organisms (1·0–1·2 mg. cell protein) in 0·4 M-sucrose containing 0·05 M-phosphate buffer (pH 8·0), 5 μmoles $MgCl_2$, 20 μmoles glucose and 5 μg. CoA. The reaction mixtures were incubated at 37° for 10 min. and 1 μmole of sodium acetate and 0·02 μc of [^{14}C]sodium acetate then added to each test-tube. After further incubation for 30 min. acetate incorporation was stopped by addition of 3 ml. of ice-cold sucrose + phosphate buffer and immediate centrifugation at 25,000 *g* for 10 min. The sedimented organisms were washed once with 4 ml. of sucrose phosphate buffer and the pellet lysed in 0·5 ml. of 0·02 M-sodium laurylsulphate. For the determination of 'free' and 'bound' acetate in the organisms 4 ml. ice-cold 12·5 % (w/v) trichloroacetic acid (TCA) were added to the reaction mixture at the end of the incubation period. The test-tubes were kept in an ice-bath for 30 min. and then centrifuged at 4°. The precipitates were washed with 5 ml. ice-cold 10 % (w/v) TCA and resuspended in 0·02 M-sodium laurylsulphate. Samples of the materials dissolved in sodium laurylsulphate were taken for radioactivity measurement.

Radioactivity measurement. Samples (0·25–0·30 ml.) of lysed organisms or membranes

were transferred to scintillation vials. Eight ml. of 0·7 % 2,5–diphenyloxazole (PPO, scintillation grade), 0·005 % 1,4-bis-2(5-phenyl-oxazolyl)-benzene (POPOP, scintillation grade, Packard Instrument Co., Inc., Illinois, U.S.A.) and 5 % (w/v) naphthalene in dioxane were added to each vial, followed by addition of 1·5 ml. 0·3 % PPO and 0·01 % POPOP in toluene. Lipid extracts were placed in the scintillation vials and evaporated by heating under a stream of air and scintillation liquid added as above. ^{14}C-counting was done in a Packard liquid scintillation counter.

Analytical methods. Protein in cell fractions was determined according to Lowry, Rosebrough, Farr & Randall (1951). Acetokinase activity in suspensions of disrupted organisms was determined according to Rose (1955) by measuring hydroxamic acid formed from acetate in the presence of hydroxylamine under acid conditions. Organisms were disrupted by treatment of their suspensions in the M.S.E. ultrasonic disintegrator (60 W., 20 kc.) for 2·5 min. Phosphotransacetylase in disrupted suspensions was measured according to Stadtman (1955).

Chemicals. Lauric acid (99 %+, pure), palmitic acid (99 %+, pure), stearic acid (99 %+, pure), oleic acid (99 %+, pure), and thiamine pyrophosphate were the products of Nutritional Biochemicals Corporation (Cleveland, Ohio, U.S.A.). The fatty acids were added to the growth medium in ethanolic solution. Coenzyme A, grade I was purchased from Sigma Chemical Co. (St Louis, Mo., U.S.A.).

RESULTS

Incorporation of acetate by various Mycoplasma strains

Of the seven Mycoplasma strains included in the present study only four incorporated acetate from the growth medium (Table 1). Acetate was incorporated mainly into lipids located in the cell membrane. The larger part of the incorporated acetate

Table 1. *Incorporation of* [^{14}C]*acetate by growing Mycoplasma organisms*

Strain	Yield of organisms (mg. protein)	Radioactivity (counts/min.) Whole organisms	Total lipid	Neutral lipid fraction	Polar lipid fraction
M. laidlawii	25·0	55,000	46,300	1,900	42,100
M. gallisepticum	13·7	8,850	7,246	870	5,860
M. hominis	4·2	2,480	2,180	1,255	864
M. orale	5·1	3,650	3,590	2,000	1,255
M. fermentans	7·5	204	126	11	37
M. mycoides var. *mycoides*	20·5	260	46	0	0
Mycoplasma sp. strain 14	25·0	290	34	0	0

Organisms were grown in 200 ml. Edward medium containing 2% (v/v) PPLO-serum fraction and 2 μc of [^{14}C]acetate.

was found in the polar lipids of *Mycoplasma laidlawii* and *M. gallisepticum* and in the neutral lipids of *M. hominis* and *M. orale*. Methanolysis of the polar lipids of *M. laidlawii* showed the label derived from radioactive acetate to be confined to the fatty acid methyl esters. *M. laidlawii* incorporated appreciably more acetate than the other strains. Therefore most of the subsequent experiments were done with this strain.

Conditions influencing acetate incorporation by washed organisms

Washed cells of *Mycoplasma laidlawii* incorporated acetate when suspended in buffer containing glucose, CoA and magnesium ions (Table 2). Glucose, apparently serving as an energy source, could not be replaced by pyruvate (20 μmoles/ml.) or by adenosine triphosphate (10 μmoles/ml.). The requirement for CoA was more pronounced with twice-washed organisms than with organisms washed only once (Table 2). Exogenous thiamine pyrophosphate was not required for acetate uptake.

Acetate incorporation by the organisms was temperature-dependent and pH-dependent. No incorporation took place below 15° or above 45°. Incorporation was best at 30–37° and at pH 8·3 (Fig. 1). The uptake of acetate was highest with organisms harvested at the early logarithmic phase of growth. At later growth phases the capacity of the cells to incorporate acetate declined steeply.

Table 2. *Requirement of glucose, coenzyme A and magnesium for acetate incorporation by washed Mycoplasma laidlawii*

The complete reaction mixture contained 0·5 ml washed organisms (equiv. 1·2 mg. cell protein) suspended in 0·4 M-sucrose containing 0·05 M-phosphate buffer (pH 8·0); 20 μmoles glucose, 5 μmoles $MgCl_2$; 5 μg. CoA; 1 μmole sodium acetate; 0·02 μc [^{14}C]acetate in total volume 1 ml.

Reaction mixture	Acetate incorporation (counts/min./mg. cell protein)
Complete	1020
Without glucose	40
Without CoA	264
	612*
Without magnesium chloride	480

* Organisms washed only once.

'Free' and 'bound' acetate

The acetate taken up by the washed organisms was rapidly incorporated into compounds non-extractable with cold 10 % (w/v) TCA ('bound acetate'). The amount of radioactivity extractable with cold TCA ('free acetate') did not exceed 10–20 % of the total radioactivity incorporated by the organisms throughout the reaction period, when the external concentration of acetate was 1×10^{-3} M. Under these conditions acetate incorporation reached completion after incubation for 25–30 min. (Fig. 2). The amount of acetate incorporated into the TCA-extractable fraction was dependent on the external concentration of acetate and followed a typical saturation curve (Fig. 3).

Effect of metabolic inhibitors on acetate uptake

Ethylenediaminetetraacetic (EDTA) acid (1×10^{-2} M) completely inhibited acetate incorporation by washed *Mycoplasma laidlawii* organisms, apparently by chelation of the magnesium ions required for the reaction. Thallous sulphate (2×10^{-3} M) inhibited the incorporation of acetate by about 40 %. Table 3 shows that acetate incorporation by *M. laidlawii* was strongly inhibited by sodium iodoacetate, at a concentration as low as 1×10^{-5} M. Sodium arsenate, sodium fluoride and *p*-chloromercuribenzoate were

also inhibitory, but to a smaller degree. Sodium azide (1×10^{-3}M), potassium cyanide (1×10^{-3}M), and ouabain (1×10^{-3}M) did not affect acetate uptake by washed *M. laidlawii* organisms.

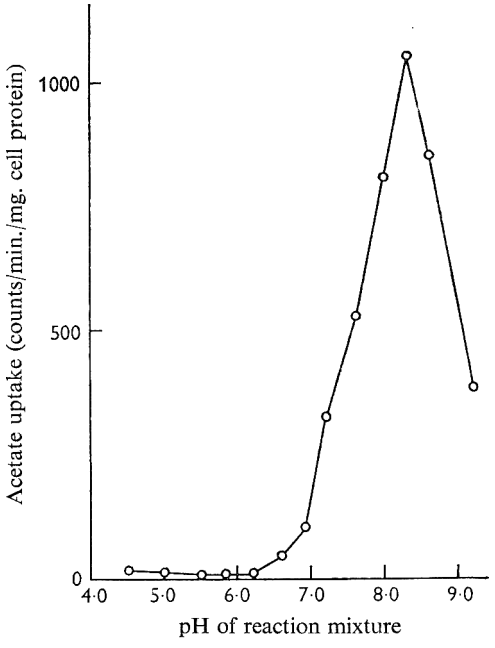

Fig. 1

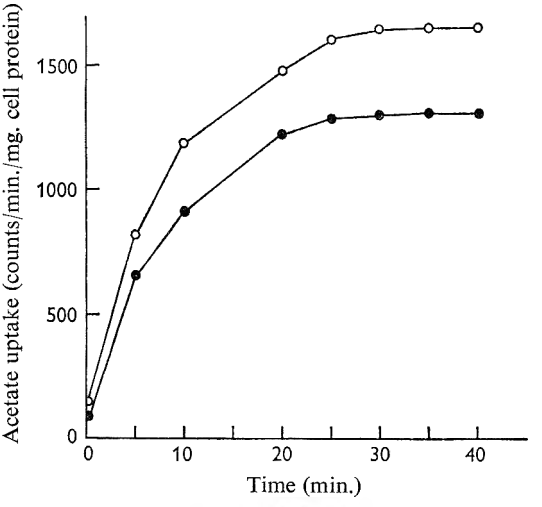

Fig. 2

Fig. 1. Effect of pH value on acetate uptake by washed *Mycoplasma laidlawii.*

Fig. 2. Uptake of [^{14}C]acetate by washed *Mycoplasma laidlawii.* ○, Total radioactivity in organisms; ●, radioactivity in the cold TCA-insoluble fraction of organisms.

Table 3. *Inhibitors of acetate uptake by washed Mycoplasma laidlawii*

The reaction mixtures were as in Table 2. The inhibitors were added to the reaction mixtures 20 min. before the addition of acetate.

Inhibitor	Concentration of inhibitor (M)	Acetate uptake: Counts/min./mg. cell protein	Acetate uptake: Inhibition* (%)
No inhibitor	—	1646	0
Sodium iodoacetate	1×10^{-4}	16	99
	1×10^{-5}	250	85
Sodium arsenate	1×10^{-2}	348	79
	1×10^{-3}	1450	12
	1×10^{-4}	1650	0
Sodium fluoride	1×10^{-3}	480	71
	1×10^{-4}	1160	30
	1×10^{-5}	1450	12
p-Chloromercuribenzoate	1×10^{-3}	49	97
	1×10^{-4}	1402	15

* Compared to control without inhibitor.

Effect of structurally related organic acids on acetate uptake

To test the specificity of the acetate incorporation system, several organic acids which structurally resemble acetate were added to the reaction mixtures at a concentration ten times greater than that of acetate. Sodium propionate and butyrate inhibited acetate uptake, whereas sodium pyruvate enhanced it (Table 4). Sodium formate, lactate and oxaloacetate were without effect on acetate incorporation. The

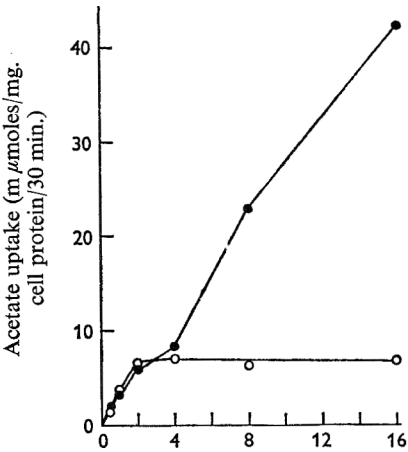

Fig. 3

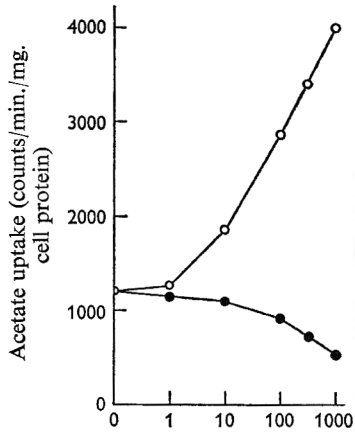

Fig. 4

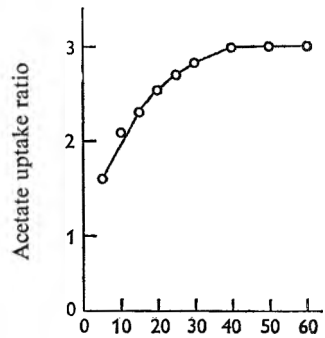

Fig. 5

Fig. 3. Distribution of acetate incorporated by *Mycoplasma laidlawii* between the cold TCA-soluble fraction (○) and cold TCA-insoluble fraction of organisms (●).

Fig. 4. Effect of sodium pyruvate (○) and sodium propionate (●) on the uptake of acetate by washed *Mycoplasma laidlawii*. The concentration of acetate in the reaction mixtures was 1×10^{-3} M.

Fig. 5. Enhancement of acetate uptake by pyruvate as a function of time. *Mycoplasma laidlawii* organisms were incubated with 1×10^{-3} M sodium acetate together with or without 1×10^{-2} M-sodium pyruvate. Results are expressed as the ratio between [^{14}C]acetate incorporated in the presence and in the absence of pyruvate.

Table 4. *Effect of structurally related organic acids on the uptake of [^{14}C]acetate by washed Mycoplasma laidlawii*

The reaction mixtures were as in Table 2.

Organic acid	Acetate incorporation (counts/min./mg. cell protein)	Incorporation* (%)
Control	2568	100
Sodium formate	2520	98
Sodium propionate	550	21
Sodium butyrate	1146	44
Sodium pyruvate	3750	145
Sodium lactate	2712	106
Sodium oxaloacetate	2680	104

* Compared to a control containing 1×10^{-3} M sodium acetate alone.

inhibitory effect of propionate as well as the stimulatory effect of pyruvate depended on the concentration of these acids (see Fig. 4).

The stimulatory effect of pyruvate was more pronounced when the organisms were incubated with it before the acetate was added. The degree of enhancement by pyruvate increased with time (Fig. 5). Fractionation of the organisms with cold 10 % (w/v) TCA showed that pyruvate increased the amount of acetate incorporated into the TCA-insoluble fraction, while it had no effect on the amount of 'free' acetate found in the TCA-soluble fraction (Table 5). Pyruvate did not replace glucose as an

Table 5. *Effect of pyruvate on the incorporation of acetate into TCA-insoluble and TCA-soluble fractions of washed Mycoplasma laidlawii*

The reaction mixtures were as in Table 2.

	mμmoles acetate incorporated/mg. cell protein/30 min.			
	TCA-insoluble fraction		TCA-soluble fraction	
Acetate (M)	No pyruvate	With pyruvate*	No pyruvate	With pyruvate*
5×10^{-5}	0·87	2·70	0·12	0·17
1×10^{-4}	1·92	4·95	0·22	0·22
2×10^{-4}	4·70	9·20	0·48	0·4[illegible]
4×10^{-4}	9·30	16·20	0·52	0·4[illegible]

* Pyruvate concentration: 1×10^{-2}M.

Table 6. *Incorporation of [^{14}C]acetate and [^{14}C]pyruvate by growing Mycoplasma laidlawii*

Organisms were grown in 200 ml. of Edward medium containing 1 % (v/v) PPLO-serum fraction and 2 μc. of [^{14}C]acetate or [^{14}C]pyruvate

		Radioactivity (counts/min.)		
			Lipid fraction	
Growth medium containing:	Yield of organisms (mg. protein)	Whole organisms	Polar lipid fraction	Neutral lipid fraction
[^{14}C]Sodium acetate	22·5	22,200	18,200	450
[^{14}C]Sodium pyruvate	25·7	4,200	3,480	40

energy source required for acetate incorporation by washed organisms, neither could it support the growth of *Mycoplasma laidlawii* when glucose was omitted from Edward medium. However, labelled pyruvate was incorporated by growing or washed *M. laidlawii* to 10–20% of that of acetate incorporated under the same conditions. Most of the radioactivity derived from pyruvate was detected in the polar lipids of the organisms (Table 6). Methanolysis of these lipids showed the label to be confined to the fatty acid methyl esters.

Repression of acetate incorporation by long-chain fatty acids

The finding that acetate was incorporated mainly into the polar lipids of *Mycoplasma laidlawii* indicated its use for the biosynthesis of long-chain fatty acids. Addition of palmitic and stearic acids to the growth medium almost completely inhibited acetate incorporation. Oleic acid had no effect on acetate uptake (Table 7)

Inhibition of acetokinase activity of Mycoplasma laidlawii by short-chain fatty acids

Acetokinase activity was observed in all the strains included in this study (Table 8), a finding which amplifies the previous reports of Castrejon-Diez *et al.* (1962) and Smith & Henrikson (1965). Acetokinase activity of *Mycoplasma laidlawii* was local-

Table 7. *Inhibition of acetate incorporation into lipids of Mycoplasma laidawii by long-chain fatty acids*

Organisms were grown in 100 ml. Edward medium containing 1 % (v/v) PPLO-serum fraction and 1 μc. of [^{14}C]acetate. The fatty acids were added to the medium to a final concentration of 5 μg./ml

Fatty acid added	Cell yield (mg. protein)	Radioactivity in lipid fraction (counts/min.)
None	11·0	12,300
Palmitic acid	11·6	3,820
Stearic acid	8·3	5,990
Stearic acid + palmitic acid	10·2	163
Oleic acid	11·3	12,560
Lauric acid	9·5	10,220

Table 8. *Acetokinase activity of several Mycoplasma strains*

Each tube contained 50 μmoles of Tris buffer (pH 7·4), 250 μmoles sodium acetate, 10 μmoles $MgCl_2$, 700 μmoles neutralized hydroxylamine, 10 μmoles ATP, equiv. 1 mg. cell protein, in a total volume of 1·0 ml. Reaction time was 2 min. at 37°.

Strain	Acetokinase activity (μmoles hydroxamic acid formed/mg. protein/min.)
M. laidlawii	2·8
M. gallisepticum	3·3
M. hominis	1·2
M. fermentans	0·9
M. mycoides var. *mycoides*	1·5
Mycoplasma sp. strain 14	1·6

Table 9. *Acetokinase activity of Mycoplasma laidlawii*

The reaction mixtures were as in Table 8, except for the fatty acid.

Substrate (m-mole)	Acetokinase activity (μmoles hydroxamic acid formed/mg. protein/min.)
Acetate (0·12)	2·35
Propionate (0·12)	0·00
Butyrate (0·12)	0·00
Pyruvate (0·12)	0·00
Acetate (0·12) + propionate (0·12)	2·15
+ propionate (0·60)	0·72
+ propionate (1·20)	0·34
+ butyrate (0·12)	2·00
+ butyrate (0·60)	0·86
+ pyruvate (0·60)	2·25

ized in the 'soluble' fraction of the cells. Propionate and butyrate did not replace acetate as substrates for this reaction. Furthermore, both acids markedly inhibited the phosphorylation of acetate. Pyruvate had no effect on this enzymic activity (Table 9). Propionate and butyrate did not inhibit phosphotransacetylase activity of *M. laidlawii*.

DISCUSSION

Only some of the Mycoplasma strains included in the present study were capable of acetate incorporation. In those strains which incorporated radioactive acetate the label was found almost entirely in the cell lipids. *Mycoplasma laidlawii* incorporated over 90% of the label into its polar lipids. Hence, incorporation into carotenoids represented only a minor fraction of its total acetate uptake. We have good reasons to believe that acetate is used by *M. laidlawii* mainly for the biosynthesis of saturated long-chain fatty acids. This assumption is based on previous nutritional findings that *M. laidlawii* does not require saturated fatty acids for growth (Razin & Rottem, 1963) and on the present finding that palmitic and stearic acids almost completely inhibit acetate uptake, apparently by a mechanism of end-product inhibition. The *Mycoplasma* strains which were found incapable of acetate incorporation, such as *M. mycoides* var. *mycoides* and the goat Mycoplasma are known to require both saturated and unsaturated fatty acids for growth, in addition to glycerol and cholesterol (Rodwell & Abbot, 1961; Rodwell, 1967). Nevertheless, these strains were found to possess acetokinase activity, suggesting a block in fatty acid and sterol synthesis further up in the biosynthetic pathways, or the absence of an acetate transport system.

Acetate uptake and utilization by the mycoplasmas decreased very steeply with the ageing of the cultures. The same was previously described in respect of the nucleolytic (Razin, Knyszynski & Lifshitz, 1964) lipolytic (Rottem & Razin, 1964) and adenosine triphosphatase (ATPase) activity (Rottem & Razin, 1966) of mycoplasmas. Leakage of enzymes and co-factors from ageing organisms seems to be the most likely explanation for the unusually rapid decline in enzymic activity. The marked tendency of mycoplasmas to swell and lyse in aged cultures (Freundt, 1958; Razin & Cosenza, 1966) supports this assumption.

Washed *Mycoplasma laidlawii* organisms required CoA for acetate incorporation. This requirement was more pronounced when the organisms were washed several times, demonstrating the marked tendency of mycoplasmas to lose co-factors on washing (Neimark & Pickett, 1960; Razin & Cohen, 1963). CoA was found to be required for growth of *M. laidlawii* in a defined medium (Tourtellotte, Morowitz & Kasimer, 1964).

The acetate taken up by the organisms was very rapidly incorporated into cold TCA-insoluble compounds. The portion of label derived from acetate in the cold TCA-extractable fraction was consistently small. Pyruvate considerably enhanced acetate incorporation into cell lipids without increasing the amount of 'free acetate' in the TCA-extractable fraction. No explanation for the pyruvate effect has yet been found. Pyruvate did not replace glucose as an energy source, either in the acetate incorporation system, or in the growth medium of *Mycoplasma laidlawii*. The observation that the stimulatory effect of pyruvate increased with time (Fig. 5) and was more pronounced when pyruvate was pre-incubated with the organisms can be explained by the time required for the organisms to transform pyruvate to an active precursor

for lipid biosynthesis. The possibility that this precursor is α-glycerophosphate was brought up when White & Klein (1966) showed this compound to stimulate acetate incorporation into fatty acids by yeast extracts. In our experiments, however, α-glycerophosphate did not enhance acetate uptake by washed *M. laidlawii*. Furthermore, the small amount of labelled pyruvate incorporated by the cells was not found in the glycerol moiety of the lipids.

Inhibition of acetate uptake by propionate and butyrate may be explained either by (*a*) competitive inhibition of the transport system responsible for the transfer of external acetate into the cells; or (*b*) inhibition of the utilization of acetate for lipid biosynthesis. The finding that both propionate and butyrate inhibited activation of acetate by acetokinase indicates that their inhibitory effect was at least partially attributable to the inhibition of acetate incorporation into the lipids. Similarly in brain cells propionate and butyrate have been found to block acetate metabolism to a greater extent than its transport (Quastel, 1965).

Inhibition of acetate uptake by iodoacetate may be simply explained by inhibition of glycolysis, thus cutting off the energy supply required for acetate incorporation. However, the very low concentrations at which this inhibitor was still effective point to a more specific effect, probably on the transport system of acetate.

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The Carbon Metabolism and Swelling of *Fusarium culmorum* Conidia

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SUMMARY

The substrate of basal respiration of conidia of *Fusarium culmorum* (W. G. Smith) Sacc. was found to be a mixture of triglycerides. The rate of utilization of this substrate increased when the conidia germinated. One of the main products of glucose metabolism in the germinating conidium was glucosamine which was a major constituent of the hyphal and conidial walls. The conidia fixed atmospheric carbon dioxide primarily into one compound, probably glutamine. Experiments on the conditions necessary for the swelling of the conidia indicated that metabolic pathways are involved, possibly terminating in the formation of glucosamine as the compound which initiates swelling. The use of tritiated water eliminated a theory of selective permeability as the mechanism for swelling; a theory based on a change in the elasticity of the conidial wall is now proposed.

INTRODUCTION

The macroconidia of *Fusarium culmorum* have been shown to require carbon and nitrogen sources for germination (Marchant & White, 1966) and under these conditions the conidia swell because of the uptake of water. The conidia are, however, capable of existing for quite long periods on their reserve carbon source, which they respire slowly. Since both germination and swelling require an external carbon source it was necessary to gain some knowledge of the carbon metabolism of the conidia. Studies on the carbon metabolism of fungi have confirmed that the basal metabolic pathways found in other organisms are operative in fungi (Moses, 1957; Gottlieb, 1963). It is obvious, however, that different fungi differ in detail and it is not possible to generalize about the carbon metabolism in specific organisms.

The germination of conidia of *Fusarium solani* f. *phaseoli* and certain aspects of their carbon metabolism were studied by Cochrane, Cochrane, Simon & Spaeth (1963), Cochrane, Cochrane, Collins & Serafin (1963), Cochrane, Berry, Simon, Cochrane, Collins, Levy & Holmes (1963) and Cochrane, Cochrane, Vogel & Coles (1963). It has been pointed out, however, that the germination of the conidia of *F. culmorum* differs in more than one respect from that of *F. solani* (Marchant & White, 1966). Lopez & Fergus (1966) examined the nutritional requirements of *F. roseum* and their results are in general agreement with the utilization of sugars already shown (Marchant & White, 1966) for the conidia of *F. culmorum*.

In the present work the products of glucose metabolism in conidia of *Fusarium culmorum* were studied by supplying [U-^{14}C]glucose, and examining the identity and distribution of the products. The substrate of basal respiration, which was identified

as a fat, was extracted and identified by thin-layer chromatography and gas chromatography. It was found that conidia were capable of fixing atmospheric carbon dioxide; therefore the effect of carbon dioxide concentration on the rate and products of fixation were examined.

That the spores of many fungi swell before germination has been noted many times. This swelling has been ascribed to the uptake of water, in many cases to a passive process in the absence of any nutrients. There are, however, many fungi which produce spores that are only capable of swelling in the presence of nutrients. Ekundayo & Carlile (1964) examined the swelling of sporangiospores of *Rhizopus arrhizus*, and showed that these required a utilizable carbon source in order to swell at all, and a nitrogen source and various ions to reach maximal swelling. A mechanism for the active swelling of *R. arrhizus* spores was postulated by Ekundayo (1966) which involves the enzyme, protein disulphide reductase (EC 1.8.4.1, Nickerson & Falcone, 1959; Hatch & Turner, 1960).

Marchant & White (1966) reported on the swelling of conidia of *Fusarium culmorum* which was associated with germination. The swelling of these conidia comprised two components, the expansion of the mucilaginous sheath (Marchant, 1966*a*; Marchant, 1966*b*) and the swelling of the conidium itself because of water uptake. The swelling of the conidium was shown to take place only in the presence of carbon and nitrogen sources. In the present work a more complete study of this process has been made.

METHODS

Organism. The strain of *Fusarium culmorum* used was obtained from the Commonwealth Mycological Institute (IMI 96283). The organism was grown and the conidia harvested as described previously (Marchant & White, 1966).

Extraction and characterization of storage lipid. The lipid was extracted from ungerminated and germinated conidia by the following method. The conidia (initial dry wt. 40 mg.) were centrifuged down from the suspending medium and killed by immersion in boiling water for 3 min. The conidia were then extracted with 2 ml. 95 % (v/v) methanol in water at 70° for 15 min. The methanol extraction was then repeated twice and the residue further extracted twice with 2 ml. diethyl ether at 35° for 15 min. The combined methanol extracts were partitioned against ether and water, and all the ether extracts then combined. Where dry weights were required the extract was dried to a constant weight at 50° and compared with the total dry wt. of a parallel sample of conidia. Dry weight and lipid determinations were made at least in duplicate. Some lipid determinations were done by Soxhlet extraction with ether and methanol.

The composition of the extracted lipid was determined by running thin-layer chromatograms. The glass plates used were 10 × 20 cm. and coated with a 250 μ thick layer of silica gel, activated at 80° for 20 min. The sample was applied to the plates in 0·1 ml. ether (60–80 μg./spot) and then developed for 10 cm. in a solvent of light petroleum (b.p. 40–60°)+ether+glacial acetic acid (90+10+1, by vol.). The chromogenic reagents used were 5 % phosphomolybdic acid in ethanol and heating to 80°, and the bromothymol blue reagent (Randerath, 1963). A standard of tripalmitin containing small quantities of the mono- and di-glycerides was used.

To identify the components of the major triglyceride spot the entire lipid extract from 40 mg. dry-wt. conidia was spread across the width of a plate. The triglyceride

band was identified by spraying one edge of the plate and the remainder of the band then scraped off and eluted. The triglyceride was deacylated by the method of Morgan, Hanahan & Ekholm (1963), with 0·5 N-NaOH in methanol. The products were partitioned between chloroform and water; the chloroform fraction, containing the methyl esters of fatty acids, was retained for gas chromatography. The water fraction was separated on a thin-layer plate by using *n*-butanol + acetic acid + water (5 + 1 + 2, by vol.) as solvent and ammoniacal silver nitrate as the chromogenic reagent. A standard was run to identify the glycerol component.

The methyl esters of the fatty acids were dried and taken up in light petroleum. A sample (0·5 μl.) was then run on a Pye panchromatogram through a polyethylene glycol adipate column (PEGA) and an Apiezon L column (APL). The percentage of each acid was calculated from the run on the PEGA column.

Utilization of storage lipid. The utilization of lipid through the glyoxylate pathway was investigated by assaying the isocitrate lyase activity in ungerminated and germinated conidia. The assay was done as described by Kornberg & Madsen (1958). The conidia were left intact for the assay but were frozen and then thawed to increase their permeability; the freezing procedure increased the observed activity by 50 %.

The effect of external carbon sources, glucose and glucose + ammonium sulphate on the oxidation of the lipid reserve material was investigated by using uniformly labelled [^{14}C]glucose. The experiment was done in Warburg flasks at 25°; conidia were either supplied with 150 μmoles [U-^{14}C]glucose or 150 μmoles [U-^{14}C]glucose + 30 μmoles ammonium sulphate, in 3 ml. 0·03 M-phosphate buffer (pH 6·5). Total oxygen uptake was measured and samples of the total $^{14}CO_2$ absorbed by the 0·2 ml. 10 % (w/v) KOH in the centre well were counted in a liquid scintillation counter with toluene scintillator liquid. After subtraction of oxygen uptake due to basal respiration the amount of oxygen taken up was compared with the theoretical amount calculated from the $^{14}CO_2$ evolved.

The fate of [^{14}C]*glucose in germinating conidia.* The experiment was done in Warburg flasks and the conidia were supplied with 100 μmoles [U-^{14}C]glucose (2 μc). One experiment was done in which the conidia were supplied with 100 μmoles ammonium sulphate and a duplicate without ammonium sulphate. The experiment was done in 3 ml. 0·03 M-phosphate buffer (pH 6·5) for 1·5 hr at 25°. At the end of the experiment the centre well contents (0·2 ml. 10 %, w/v, KOH) were washed out and made up to 5 ml. to give an estimate of $^{14}CO_2$ evolved from respiration. The conidia themselves were centrifuged down from the medium, washed once with ice-cold water, and the supernatant fluid + washings made up to 5 ml. The conidia were extracted twice with hot water (80°) and these extracts made up to 5 ml. The conidia were then extracted twice with ethanol at 50° and these extracts also made up to 5 ml. The residue was suspended in 5 ml. water. Portions (0·4 ml.) of the samples were counted in a liquid scintillation counter with toluene scintillator liquid. A sample of the initial glucose was also counted. Quenching errors were checked by adding 0·02 ml. (0·008 μc) of the standard glucose to the samples and noting the increase in count. It was found that quenching effects were negligible.

The remainder of the water extract was evaporated to dryness and then taken up in 0·25 ml. 50 % (v/v) ethanol in water. The entire extract was spotted on 3 MM Whatman paper and developed in 75 % (w/w) phenol in water, then with butanol +

acetic acid + water (90 + 29 + 10, by vol.). The final chromatograms were put over X-ray film for 4 weeks before being sprayed with ninhydrin.

The remainders of the conidial residues were similarly collected and hydrolysed with 1 ml. N-H_2SO_4 for 3 hr at 100°. Two drops of bromothymol blue were then added to the hydrolysis mixture, followed by saturated barium hydroxide solution until the mixture was alkaline. Carbon dioxide was bubbled through to re-acidify the solution and then one drop of ammonium hydroxide (sp.gr. 0·88) was added. The precipitate was centrifuged down, washed and the supernatant fluid and washings combined. About 83 % of the total radioactivity from the residue was recovered by this method. The entire hydrolysis samples were spotted on 3 MM Whatman paper and developed for 40 hr in ethyl acetate + acetic acid + water (3 + 3 + 1, by vol.). One each of the duplicate samples had 150 μg. [U-^{14}C]glucose added to it as a marker, and in addition a marker containing 150 μg. each of glucose, fructose, galactose, arabinose and xylose was run. The chromatograms were put over X-ray film for 4 weeks before being sprayed with ammoniacal silver nitrate.

Carbon-dioxide fixation. Conidia were germinated in a medium containing the following: 3000 μmoles glucose, 1500 μmoles ammonium sulphate in 25 ml. 0·03 M-phosphate buffer (pH 6·5). The conidia were contained in a closed system of capacity 1100 ml. The whole system was flushed through with air from which the CO_2 had been removed with Carbosorb. The concentration of CO_2 was then returned to 0·04 % (v/v) by introducing 1 ml. 2 N-H_2SO_4 on to a calculated quantity of 0·1 M-KH $^{14}CO_3$. The system was shaken at 25° for 42 hr. Half-way through the experiment the system was flushed out and the $^{14}CO_2$ replenished. At the end of the experiment the germinated conidia were centrifuged down and resuspended in 5 ml. water; a sample was counted to give the total incorporation of $^{14}CO_2$. The remainder of the material was then extracted with hot water and ethanol, as described previously, to give the distribution of incorporated ^{14}C in the fractions of conidia.

The effect of CO_2 concentration on fixation by germinating conidia was examined as follows. Katz flasks (50 ml.) were used containing 1500 μmoles glucose, 750 μmoles ammonium sulphate in 12·5 ml 0·03 M-phosphate buffer (pH 6·5). In the centre well of each flask was placed a volume of $Na_2{}^{14}CO_3$ calculated to yield the following concentrations (%, v/v) of $^{14}CO_2$; 0·035, 0·07, 0·10, 0·50 and 0·83. The flasks were gassed with CO_2-free air and then 1 ml. 2 N-H_2SO_4 was injected into each flask through a vaccine stopper. The flasks were shaken for 22 hr at 25° before the conidia were killed by adding 1 ml. 20 % (w/v) trichloroacetic acid. After being centrifuged down and resuspended samples of the conidia were counted in the scintillation counter. A dry weight determination on a sample of the conidia and a count of the original $Na_2{}^{14}CO_3$ allowed the CO_2-fixation to be expressed as μg. $^{14}CO_2$ fixed/g. dry-wt conidia/hr.

The material left from the 0·035 % and 0·83 % $^{14}CO_2$ flasks was extracted with 80 % (v/v) ethanol in water and partitioned against light petroleum. The water extract was chromatographed in phenol, then butanol + acetic acid + water, as described previously, and the chromatogram put over X-ray film. The ether extract was run on a thin-layer chromatogram before also being put over X-ray film.

Conidial swelling. Conidial swelling was measured by using a micrometer eyepiece; a random sample of 100 conidia was counted in each case. From these measurements the mean maximum spore widths and the standard errors were calculated. The basal medium used for experiments consisted of: 500 μmoles glucose, 100 μmoles am-

monium sulphate in 10 ml. 0·03 M-phosphate buffer (pH 6·5). The nutrients were always used at these concentrations unless otherwise stated. Nitrate was supplied as potassium nitrate at 200 μmoles/flask, and glucosamine as glucosamine hydrochloride at 500 μmoles/flask. Sodium acetate was used in equivalent amounts to glucose (1000 μmoles/flask). The experiments were made in 50 ml. conical flasks at 25° on a reciprocal shaker.

Carbon dioxide concentrations, A concentration of 5 % (v/v) CO_2 in air was achieved by using gas, washed in water, from a cylinder containing 95 % (v/v) air + 5 % (v/v) CO_2. Conidia were maintained in a CO_2-free atmosphere by continually passing CO_2-free air through the flask.

Manometric methods. Oxygen uptake was measured in the usual Warburg apparatus at 25°, with direct absorption of CO_2 by 10 % (w/v) KOH in the centre well. The conidia were supplied with 150 μmoles glucose + 30 μmoles ammonium sulphate or 150 μmoles glucosamine hydrochloride in 3 ml. 0·03 M-phosphate buffer (pH 6·5).

Permeability of conidia to tritiated water. Conidia were shaken in 20 ml nutrient solution, containing either glucose + ammonium sulphate or glucose, for 4 hr at 25°. Four duplicate sets of 2 ml. samples were removed from each flask. One pair of duplicates from each flask was centrifuged, the deposit washed once with 2 ml. non-tritiated water and then resuspended in 2 ml. water. The centrifuged deposits of other sets of duplicates were similarly washed 2, 4 or 8 times. Portions (0·05 ml.) of these samples were then counted for radioactivity by using toluene scintillator liquid in a liquid scintillation counter.

RESULTS

Characterization and utilization of storage lipid

Thin-layer chromatography of the entire lipid extracts revealed three major spots only. The largest and rather elongated spot, with an R_F value of 0·4–0·55, corresponded to the triglyceride fraction of the tripalmitin standard. Two smaller spots with R_F values of 0·17 and 0·07 corresponded with the di- and mono-glyceride fractions of the standard. There were also faint spots close to the solvent front, probably representing long-chain hydrocarbon compounds (Malins & Mangold, 1960). Material left at the origin gave a positive yellow coloration with an ammonium vanadate + ammonium molybdate spray, indicating the presence of phospholipid. The elongated nature of the large triglyceride spot, irrespective of the loading of the plate, suggested that it represented a mixture of triglycerides.

The water fraction of the deacylated triglyceride yielded only glycerol, as would be expected if the extract contained only glycerides. The gas chromatography of the methyl esters of the fatty acids of the triglyceride fraction gave the results shown in Table 1, in which the percentage composition has been calculated and the probable identity of the acids given. These acids combined into triglyceride would give the large number of glycerides necessary to produce the characteristically shaped spot on the chromatograms.

The concentration of lipid in the conidia was examined before and after germination and also after shaking in the presence of a carbon source only. Conidia were shaken for 22 hr at 25° in media containing 1000 μmoles glucose ± 150 μmoles ammonium sulphate in 25 ml. 0·03 M-phosphate buffer (pH 6·5). The concentrations of lipid and percentages of the total dry weight are given in Table 2.

In the conidia shaken in glucose alone there was either no utilization of lipid or the net synthesis had not kept pace with the increase in dry weight, resulting in a decrease in the percentage of lipid. Glucose + ammonium sulphate treatment, however, resulted in a net increase of lipid; but once again a decrease in the percentage lipid of the total dry weight.

To see whether the utilization of lipid was affected by treatment with glucose + ammonium sulphate the isocitrate lyase activity in germinated and ungerminated conidia was examined. The conidia were germinated as for the lipid determinations for 20 hr. The mean isocitrate lyase activity for ungerminated conidia, obtained from duplicated experiments, was 3·5 μg. glyoxylate/mg. dry-wt conidia/hr; the corresponding value for germinated conidia was 4·5 μg. glyoxylate/mg. dry-wt conidia/hr.

Table 1 *Fatty acids obtained by the deacylation of the triglyceride fraction from Fusarium culmorum conidia*

Carbon chain length and degree of unsaturation	% of total	Probable identity
14:0	0·3	Myristic acid
16:0	24·0	Palmitic acid
16:1	0·5	Palmitoleic acid
18:0	11·0	Stearic acid
18:1	31·0	Oleic acid
18:2	33·2	Linoleic acid

Table 2. *Synthesis and utilization of storage lipid during germination of Fusarium culmorum conidia*

Treatment	Total dry wt (mg.)	Weight of lipid (mg.)	Lipid as % of total dry wt
Glucose + ammonium sulphate	88·4	31·8	36·0
Glucose	75·3	20·5	27·2
Untreated control	43·4	21·3	49·2

The difference, although small, appears to be a real one and therefore it would seem that lipid utilization did increase during germination. It is also probable that glyceride synthesis was increased by the presence of a nitrogen source.

The results of the isocitrate lyase assay were confirmed by the results of the ^{14}C labelling experiment. Comparisons of the actual and theoretical oxygen uptake values revealed that there was some stimulation of basal respiration in the presence of a nitrogen source. The percentage change in oxygen uptake due to basal respiration was very small when the conidia were supplied with glucose only (+4·5 %) but in the presence of glucose + ammonium sulphate the oxygen uptake due to basal respiration increased by 62·5 %. This stimulation is of the same order as that shown by the enzyme assay, which substantiates that the small difference recorded is in fact a real one.

The fate of [^{14}C]glucose in germinating conidia

The distribution of ^{14}C from glucose into the various fractions of the conidia is given in Table 3. The two treatments, with and without a nitrogen source, showed differences primarily in the amount of CO_2 produced and in the insoluble material left

after the extractions. The smaller amount of CO_2 evolved in the absence of a nitrogen source agreed with the manometric data given earlier (Marchant & White, 1966); the incorporation into insoluble material probably accounted for this difference. It has been shown that more glucose is assimilated in the absence of a nitrogen source (Marchant & White, 1966) and this assimilated carbon seems to appear in the final insoluble fraction.

The chromatograms of the water-extracts revealed a large range of weakly labelled amino acids. The autoradiograms also showed small amounts of label in the organic acids of the tricarboxylic acid cycle. There were, however, two large spots of radioactivity which were tentatively ascribed to glucose and glucosamine (Fink, Cline & Fink, 1963). The smaller of the two spots was subsequently cut out and eluted, then hydrolysed with sulphuric acid to confirm its identity as glucosamine. The products of hydrolysis were neutralized, as described above, with barium hydroxide and then

Table 3. *The effect of ammonium sulphate on the metabolism of* ^{14}C *glucose by Fusarium culmorum conidia*

The values given are mean values calculated from duplicate experiments.

Fraction	With ammonium sulphate (counts/100 sec.)	Without ammonium sulphate (counts/100 sec.)
$^{14}CO_2$ evolved	1682	1419
Water extract	6365	6920
Ethanol extract	596	565
Residual material	5964	6920

chromatographed in ethyl acetate + acetic acid + water (3 + 3 + 1, by vol.) with standards of [U-^{14}C]glucose and 'cold' glucosamine. The results indicated that the compound was indeed glucosamine. There were no detectable differences between the treatments with and without a nitrogen source.

The chromatograms of the hydrolysed residue showed most heavy labelling in glucose with small quantities in xylose and mannose, galactose and arabinose were only weakly labelled, if at all. The differences between the two treatments were again difficult to detect.

Carbon-dioxide fixation

The distribution of ^{14}C fixed from $^{14}CO_2$ is given in Table 4. The majority of the fixed $^{14}CO_2$ was in the insoluble fraction with only small quantities in the more soluble fractions. The amount in the water extract is almost certainly attributable to the soluble 'primary' products of fixation.

The effect of CO_2 concentration on fixation was marked, up to 0·1 % (v/v) $^{14}CO_2$, showing an almost linear increase in the rate of fixation. Above that concentration increasing concentrations of CO_2 produced a smaller increase in fixed $^{14}CO_2$. However, even at the maximum concentration of $^{14}CO_2$ used (0·83 %, v/v) the system was not saturated and increasing concentrations still resulted in increased fixation. Paper chromatography of the water extracts of these conidia produce unusual labelling patterns. The majority of the amino acids were unlabelled, with low degrees of labelling in the organic acids. Glutamic acid was fairly heavily labelled, but the largest

concentration of ^{14}C was in a compound tentatively identified as glutamine. The thin-layer chromatograms of the ether extracts showed a uniform pattern of labelling throughout the glycerides.

The effect of the nitrogen source on conidial swelling

Marchant & White (1966) showed that the ammonium ion was a suitable source of nitrogen for conidial swelling. To gain some idea of the part played by nitrogen in the swelling, potassium nitrate was substituted for ammonium sulphate as sole nitrogen source. The results showed that although the rate of swelling was initially

Table 4. *The distribution of incorporated ^{14}C from $^{14}CO_2$ fixed by conidia of Fusarium culmorum*

The values given are mean values calculated from duplicate experiments

Fraction	Counts/100 sec.
Water extract	674
Ethanol extract	117
Residual material	1255

Table 5. *The effect of acetate on the swelling of Fusarium culmorum conidia*

Treatment	Mean maximum conidial width (μ)	S.E.
Initial sample	5·51	±0·12
Glucose + ammonium sulphate, 4·5 hr	6·19	±0·10
Acetate + ammonium sulphate, 4·75 hr	5·07	±0·10

slower in nitrate the final degree of swelling after 5 hr was indistinguishable from that in ammonium sulphate. Ammonium sulphate supplied without glucose produced no effect on the size of the conidia. The result with nitrate suggested that nitrogen was probably involved metabolically in the swelling process, and that the lag period might represent a period of adaptation to nitrate as sole nitrogen source.

The effect of the concentration of ammonium sulphate on the rate of swelling was examined. Ammonium sulphate was supplied at 10, 100 and 1000 μmoles/flask. The results showed that there was little difference between the results with 100 and 1000 μmoles/flask; 10 μmoles/flask produced a decreased rate of swelling, but even this represented a substantial degree of swelling. These results suggest that the requirement for a nitrogen source was fairly small. The ability of sporangiospores of *Rhizopus arrhizus* to swell without an added nitrogen source may reflect a different nitrogen status in these sporangiospores.

The effect of acetate on the conidial swelling

Acetate does not support germination of conidia of *Fusarium culmorum* as sole carbon source (Marchant & White, 1966), although it stimulates oxygen uptake. It can be seen from Table 5 that acetate did not produce any swelling of conidia, even in the presence of ammonium sulphate. If acetate were being utilized through the tricarboxylic acid and glyoxylate cycles then the role of carbon metabolism in conidial swelling involves pathways between glucose and acetate.

The effect of temperature and carbon-dioxide concentration on conidial swelling

Examination was made of the effect of temperature on conidial swelling, to test that metabolic reactions were involved. It was possible that the effect on the conidia of glucose and ammonium sulphate was a direct one which allowed osmotic uptake of water. If this were so, lowering the temperature would not completely prevent conidial swelling. However, at 3° conidial swelling was completely inhibited (Table 6).

Increased CO_2 concentration inhibits the germination of conidia of *Fusarium culmorum* and the complete removal of CO_2 is also inhibitory (Marchant, unpublished). In view of the role of CO_2 fixation in the metabolism of the conidia it was thought that this system might play a part in conidial swelling. The experiment showed that over the range 0–5 % (v/v) CO_2 in air there was no effect on swelling. Although this does not preclude the possible involvement of CO_2 fixation in conidial swelling, it does mean that utilization of the products must be indirect.

Table 6. *The effect of temperature on the swelling of Fusarium culmorum conidia*

Treatment	Mean maximum conidial width (μ)	S.E.
Initial sample	5·49	±0·08
Glucose+ammonium sulphate, 25° for 4·5 hr	6·65	±0·12
Glucose+ammonium sulphate, 3° for 4·5 hr	5·40	±0·08

Pre-treatment and transfer experiments

Samples of conidia of *Fusarium culmorum* were shaken for 16 hr in a solution containing only glucose and were then transferred to solutions containing glucose, or ammonium sulphate or glucose+ammonium sulphate. The results indicated that while this treatment did not affect the ability of the conidia to swell when they were placed in glucose+ammonium sulphate, neither did it permit any swelling in ammonium sulphate only. It has already been shown (Marchant & White, 1966) that glucose under these conditions is assimilated, and therefore the products of this assimilation are not available to bring about swelling when ammonium sulphate is supplied.

A similar pretreatment of the conidia with ammonium sulphate only showed that partial swelling occurred on transfer to a solution containing glucose, although swelling was not so great as in glucose+ammonium sulphate. This pretreatment with a nitrogen source did not remove the stimulation of oxygen uptake by conidia with glucose as substrate, which was noted previously (Marchant & White, 1966), neither did it allow any germination when the conidia were suspended in glucose medium. It seems therefore that the role of ammonium sulphate in swelling and its role in the stimulation of oxygen uptake and germination can be separated.

When conidia were shaken for 1 hr in glucose+ammonium sulphate, subsequent transfer to glucose medium produced no retardation of swelling. When, however, the conidia were transferred to ammonium sulphate medium then the conidia returned to their original condition. This reversal of swelling can be achieved even after 3 hr in glucose+ammonium sulphate. All the results point to a product of metabolism as being the trigger responsible for conidial swelling. If this is so, then these results can be explained in terms of a product which is broken down in the presence

of ammonium sulphate but without glucose, but this product is relatively stable in the presence of glucose or in the absence of ammonium sulphate.

The effect of glucosamine on conidial swelling and oxygen uptake

The results presented indicate that the possible trigger for swelling is a product involved in both carbon and nitrogen metabolism. The inability of acetate to serve as the carbon source suggested that the part of carbon metabolism concerned was involved in the pathway from glucose to pyruvate. Similarly the requirement for a small quantity of ammonium sulphate indicated the involvement of a nitrogen-containing compound. The compound most likely to satisfy the requirements appears to be glucosamine, which is formed in the presence of glucose and which could not be formed rapidly in any quantity from acetate.

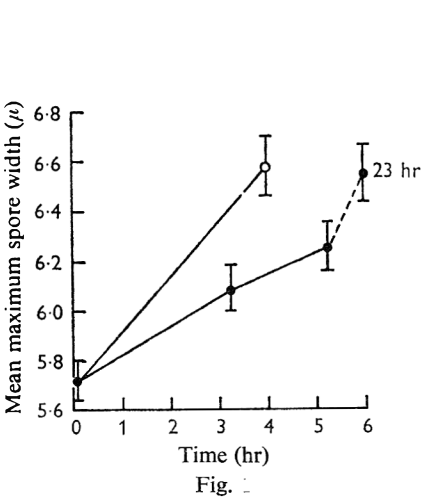

Fig. 1

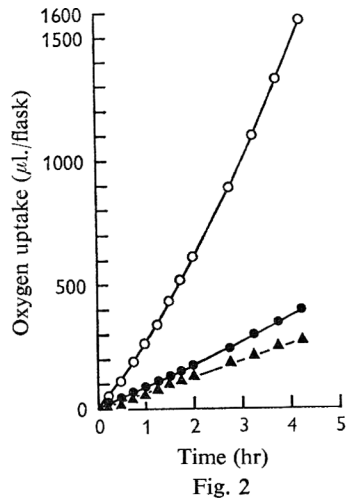

Fig. 2

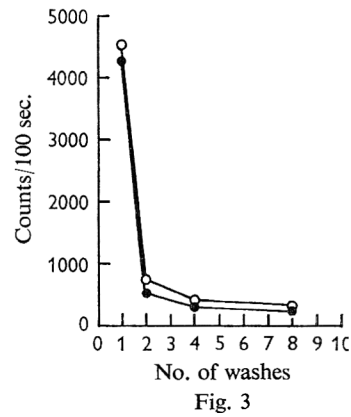

Fig. 3

Fig. 1. The effect of glucosamine on the swelling of the conidia of *Fusarium culmorum*. Standard errors are shown. ○—○, Glucose+ammonium sulphate; ●—●, glucosamine.

Fig. 2. Oxygen uptake by conidia of *Fusarium culmorum* supplied with: ●—●, glucosamine; ○—○, glucose+ammonium sulphate; ▲—▲, buffer (pH 6·5).

Fig. 3. The loss of tritiated water from conidia of *Fusarium culmorum* treated with: ○—○, glucose+ammonium sulphate; ●—●, glucose.

To test this, conidia were shaken in glucosamine hydrochloride (500 μmoles/flask) and their swelling measured (Fig. 1); oxygen uptake was also observed as a measure of how much glucosamine was being respired (Fig. 2). Although the swelling in glucosamine was slower than in glucose+ammonium sulphate the final degree of swelling was quite high; stimulation of oxygen uptake by glucosamine was negligible and there was no evidence of any germination of conidia. The rate of swelling was more than ten times faster than would have been predicted from the oxygen uptake data if glucosamine were being converted to glucose+ammonia. The absence of any germination also argues against utilization of glucosamine in this way.

Permeability of the conidia

The mode of action of the trigger compound was more difficult to test, but the loss of tritiated water from conidia was taken as a measure of their permeability, in one direction at least. The removal of tritiated water by successive washes with water from conidia shaken in glucose and in glucose+ammonium sulphate was measured (Fig. 3). The conidia swollen in glucose+ammonium sulphate had a higher initial radioactivity count, but the rate of loss of tritiated water was proportional to the initial value, indicating that there was no differential permeability.

DISCUSSION

To be able to discuss fully the implications of the physiological and fine structure studies of the conidia of *Fusarium culmorum* it is necessary to have some knowledge of their metabolism. Studies on the basal respiration (Marchant & White, 1966) and fine structure (Marchant, 1966*a*) of conidia of *F. culmorum* showed that there was a basal respiratory substrate, which was probably a fat. It has long been known (Foster, 1949) that Fusarium species are capable of fat production on a large scale and this material has been taken to be a reserve carbon source. Characterization of this lipid has now shown that it is composed of triglycerides, with smaller quantities of the mono- and di-glycerides; these may represent intermediate stages in the synthesis and degradation of the triglycerides. Examination of the quantities of lipid in conidia during germination showed that there was a net synthesis of lipid, although the proportion of lipid of the total dry weight declined. There was also evidence that during germination there was an increase in the rate of utilization of this lipid, but this only occurred in the presence of a nitrogen source.

The distribution of ^{14}C from glucose into the various fractions of the conidia in the presence and absence of ammonium sulphate as nitrogen source did not seem to affect the metabolism in a qualitative manner; the labelling patterns were essentially similar. The presence of large quantities of glucosamine in the water-soluble fraction of the conidia, and the fact that there was no measurable incorporation into chitin in the walls, is of interest in connexion with work on the walls of *Fusarium culmorum*. Marchant (1966*b*) postulated that the germ-tube wall initially contains no chitin, but that this is deposited at a later stage, some distance behind the growing point. The evidence that other sugars are deposited in wall material supports this hypothesis of the growth of the germ tube. Also of relevance to the cell wall hypothesis is the evidence that one of the main products of CO_2 fixation is glutamine; this compound, together with fructose-6-phosphate, forms the basis of glucosamine synthesis (Dixon & Webb, 1964). Carbon dioxide has an inhibitory effect on the germination of conidia of *F. culmorum* (Marchant, unpublished), and the control of chitin synthesis may be one way in which this is achieved.

Ekundayo (1966) proposed a theory for spore swelling in *Rhizopus arrhizus* which stated that the limiting factor was the availability of reduced nicotinamide nucleotide as a hydrogen donor for protein disulphide reductase. This enzyme is responsible for reducing disulphide linkages in the wall protein to sulphydryl groups, thereby increasing the plasticity of the wall. The main objection to this theory for *Fusarium culmorum* appears to be that there is no swelling of the conidia when they are placed

in a glucose medium lacking ammonium sulphate as a nitrogen source. Under these conditions glucose is respired and reduced nictotinamide nucleotides are unlikely to be a limiting factor.

The swelling and germination of the conidia of *Fusarium culmorum* have thus been separated and it appears that swelling involves only part of the metabolism of germination. The results with glucosamine strongly suggest that this compound may be important in the initiation of conidial swelling. The relative inefficiency of the glucosamine used may have been due to its inability to enter the cells rapidly or to the inability of the conidia to utilize it efficiently in that form. It seems fairly certain that the glucosamine was not re-utilized as glucose + ammonia but exerted an effect on the elasticity of the conidial walls without becoming incorporated. Frey-Wyssling (1957) suggested a method of measuring the tensile strength of cell walls, but the mathematical treatment depends on a spherical cell and could not be applied to *F. culmorum* conidia. The results with tritiated water agree with those of Ekundayo (1966), who found no change in the permeability of sporangiospores of *Rhizopus arrhizus* during germination.

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A New Genus of the Actinomycetales—*Intrasporangium* gen.nov.

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SUMMARY

A new species and genus of the Actinomycetales is described, for which the name *Intrasporangium calvum* is proposed. The organism is a typical actinomycete, producing branching mycelium 0·4–1·2 μ in diam. which has a definite tendency to fragment. It is characteristic for the organism to produce sporangia intercalary in the mycelial hyphae. The sporangiospores are non-motile. This new genus is proposed to be a member of the family Actinoplanaceae.

INTRODUCTION

Actinomycetes which form sporangia were first described in detail by Couch (1950, 1955, 1963). These are now widely recognized as a rather distinct group of the Actinomycetales (*Bergey's Manual*, 1957; Waksman, 1959; Kalakoutskii, 1965; Krassilnikov & Kalakoutskii, 1965; Lechevalier & Lechevalier, 1965). The Actinoplanaceae are at present subdivided according to whether or not the sporangiospores are motile, as well as by the shape of the sporangium. Sporangia are produced by the members of this family on the tips of the sporangiophores. The formation of sporangia in a strain of the genus *Actinoplanes* was studied in some detail by ultra-thin sections by Lechevalier & Holbert (1965).

The present paper describes a type of actinomycete which produces sporangia which are intercalary in the mycelial hyphae. The name proposed for the new genus is *Intrasporangium* and the type species *I. calvum*. One strain has so far been isolated. It is deposited as strain no. 7 KIP in the culture collection of the Institute of Microbiology, U.S.S.R. Academy of Science, Moscow, U.S.S.R., and in the culture collection of the All-Union Research Institute for Antibiotics, Moscow, U.S.S.R.

METHODS

Media. The composition of the media used which are not given here can be found in the following: Waksman (1961); *Biology of Individual Groups of Antibiotic-producing Actinomycetes* (1961); Lechevalier & Lechevalier (1957); Lechevalier, Solotorovsky & McDurmont (1961).

Wheat meal agar: wheat meal, 20 g.; dry baker's yeast, 50 mg.; tap water to 1 l.; pH 7·0.

Yeast peptone agar: baker's yeast, 5 g.; peptone 5 g.; glucose, 10 g.; tap water to 1 l.; pH 7·0.

Proflo glucose agar: Proflo (cottonseed meal, product of Traders Oil Mill Co., Fort Worth, Texas, U.S.A.), 5 g.; glucose, 10 g.; tap water to 1 l.; pH 7·0.

Proflo starch agar: as above with starch instead of glucose.

Photographs and microscopy. Micrographs were taken through a trinocular Soviet 'MBI–6' microscope with a 36 mm. photomicrography attachment. 'Micrat–200' high contrast film was used.

The undisturbed plate cultures were examined through × 20 or × 40 apochromatic objectives using a long focus 'tele-system' condenser.

For more precise examination of the mode of sporangial and spore formation, microcultures on thin agar layers sandwiched between a glass slide and a coverslip were used. In this case a water-immersion phase-contrast apochromatic objective × 70 (aperture 1·23) was used. Stained smears were examined through a × 90 apochromatic objective.

Disruption of sporangia and the release of spores were achieved by gently tapping and moving the coverslips of the microculture.

Motility was examined by putting drops of sterile tap water on undisturbed plate cultures and examining the colonies under a coverslip.

Electron micrographs were made by using the Elmi D–2 Zeiss electron microscope (original magnification × 8000) and collodion-covered grids. The material was not shadowed. For further details see Kalakoutskii & Kuznetsov (1964).

Staining when done was by the methods of Peshkoff (1955).

RESULTS

Description of Intrasporangium calvum *gen.nov. sp.nov.*

The organism was isolated on a meat-extract peptone agar plate from the air in a school dining room. Table 1 summarizes the results of attempts to cultivate it on other media. Meat-extract peptone agar or meat-extract peptone broth appeared to be most favourable for growth and development. These media were used throughout the work. The organism grew badly, if at all, on most of the media usually used for the cultivation of actinomycetes.

The organism grew slowly on meat-extract peptone broth or agar. The first signs of macroscopically visible growth appeared at 3–5 days of incubation at 28°. Microscopically, growth was evident in 1–2 days.

Fine branching mycelia 0·4–1·2 μ in diam. were produced. Fragmentation was not apparent during the *in situ* examination. But even very young mycelia were easily fragmented in the process of making smears, or when a drop of water was placed on a growing colony and the latter examined under coverslip. Mycelial filaments penetrated the agar and formed compact, small (1–5 mm. diam.) colonies. No signs of aerial mycelia were visible on any of the media used, even by microscopical examination.

The colonies on meat-extract peptone agar as well as on other media where growth was evident (Table 1) were round, glistening and whitish (old colonies cream-whitish). They were rather reminiscent of bacterial colonies and those of certain Nocardia and Mycobacterium species.

Sporangia were seen beginning from 5 or 6 days of incubation at 28°. They were abundant at 12 days and more (Pl. 1, fig. 1). Sporangia were mostly formed on the surface of agar cultures; they were less abundant in liquid cultures.

The sporangia were formed in a peculiar manner which was characteristic for the organism. They were formed not on the tips of sporangiophores, but intercalary in the mycelial hyphae (Pl. 1, fig. 2; Pl. 2, figs. 10, 11, 12). Sometimes the young sporangia were reminiscent of empty bags, and continuation of the mycelial hyphae through the sporangium could be seen, especially in damaged sporangia (Pl. 1, fig. 3). The content of young (4–6 days at 28°) sporangia was usually homogenous. Later on, the hyphae

Table 1. *Growth of Intrasporangium calvum on various media*

Growth examined at 2 and 4 weeks of incubation at 28°. In all cases growth was much worse than usual with the actinomycetes. Liquid media usually remained clear during growth of the organism, which formed a cottony sediment at the bottom.

	Media	Growth		Media	Growth
1	Meat-extract peptone broth	++	16	Meat-extract peptone+1% yeast extract agar (Difco)	++
2	Meat-extract peptone+0·5% glycerol broth	++	17	Meat-extract peptone+glucose +blood serum+yeast extract agar	++
3	Meat-extract peptone+0·1% yeast extract broth (Difco)	++	18	Meat-extract peptone diluted 1/10 with tap water agar	+
4	Meat-extract peptone+0·5% glucose broth	++	19	Malt agar+1% $CaCO_3$	—
5	Meat-extract peptone+0·5% soluble starch broth	++	20	Rice agar	+
6	Meat-extract peptone+0·01% Na thioglycollate broth	++	21	Oatmeal agar	+
7	Meat-extract peptone broth diluted 1/10 with tap water	+	22	Potato agar	—
8	Meat-extract peptone+10% (v/v) blood serum broth	++	23	Potato plug	—
9	Meat-extract peptone agar	++	24	Skimmed milk	—
10	Meat-extract peptone agar in an atmosphere containing 5% (v/v) CO_2	++	25	Yeast glucose agar	+
11	Meat-extract peptone agar in an atmosphere of 90% (w/v) H_2+10% (w/v) O_2	++	26	Wheat meal agar	+
12	Meat-extract peptone+0·5% glycerol	++	27	Cornsteep glucose agar	±
13	Meat–peptone+0·5% glucose agar	++	28	Yeast starch agar	±
14	Meat-peptone+0·5% soluble starch agar	++	29	Soil extract agar	—
15	Meat-extract peptone+10% (v/v) blood serum agar	++	30	Yeast peptone agar	++
			31	Proflo glucose agar	—
			32	Proflo starch agar	—
			33	Casein-hydrolysate yeast-extract agar	±
			34	Glutamic acid glucose agar	—
			35	Sauton medium agar	+
			36	Czapek medium agar	—
			37	Glucose asparagine agar	—
			38	Water agar	—

++, rather good growth; +, sparse growth; ±, some growth visible, at least microscopically; —, no growth.

inside a sporangium thickened (Pl. 2, figs. 10, 11) and accumulation and segregation of nuclear material occurred (Pl. 1, fig. 4). Then round or oval bodies 1–2 μ diam. were formed inside the sporangium (Pl. 1, fig. 5*a*, *b*). The exact sequence of formation of these bodies is difficult to follow by time-lapse photography because they are formed in different planes of focus. Mature sporangia were usually round or oval; very characteristic too were lemon-shaped sporangia. The sporangia were usually 5–15 μ diam. No significant differences were found in shape or size of sporangia that were formed intercalary or on the tips of hyphae (Pl. 1, fig. 6).

In mature sporangia one to more than 20 round or oval bodies, 1–2 μ diam., were

seen (Pl. 2, fig. 7). They are usually in a state of Brownian movement inside the sporangium, which, when mature, seems to be filled with a kind of sap. This also interferes with photography. We have never seen active release of these bodies from a sporangium, though sometimes 'empty bags' could be seen among old sporangia (Pl. 2, fig. 12). The round bodies from the sporangium can be released (Pl. 2, fig. 8). If a sporangium is disrupted on a fresh medium, the released bodies germinate usually by one or two germ tubes (Pl. 2, fig. 9). They could then serve the purpose of multiplication in this organism and be regarded as spores of dispersion. Attempts to find any signs of active motility in released spores were unsuccessful.

Staining properties. The mycelia were Gram-positive (in older cultures Gram-variable), not acid-fast. The sporangia and their contents were not stained by the Sudan Black stain for lipids. The maturing sporangia were strongly basophilic (methylene blue stain). Characteristic pictures were obtained with the HCl+Giemsa nuclear stain (Pl. 1, fig. 4).

Physiological properties of the organism. As can be seen from the data in Table 1, the organism was rather fastidious in its nutritional requirements. Its growth seemed to depend on some substances contained in the peptone used (fermentative peptone manufactured by the meat combine in Semipalatinsk). Incubation in an atmosphere of hydrogen or at increased CO_2 concentrations did not improve growth. Growth was prevented by strictly anaerobic conditions. Growth was possible between 28° and 37°; there was no growth at 45° in meat-extract peptone broth; and growth was faster at 37° than at 28°. Nitrate was reduced to nitrite when KNO_3 was added to meat-extract peptone broth. No liquefaction of gelatin occurs when the organism grew on meat-extract peptone gelatin.

Tests for antibiotic properties of the organism. No antibiotic activity was found by using the agar block method, with *Sarcina lutea*, *Staphylococcus aureus*, *Escherichia coli*, *Bacillus subtilis*, *Candida albicans*, *Mycobacterium sp.* v-5 as test organisms.

DISCUSSION

The mode of formation of sporangia in *Intrasporangium calvum* here reported clearly distinguishes this organism from other members of the family Actinoplanaceae. It seems reasonable to place it in the following tentative scheme for the subdivision of Actinoplanaceae:

Actinomycetes forming sporangia:

Family Actinoplanaceae: Couch, 1950

I. Sporangia formed intercalary in the mycelial hypha. Sporangiospores non-motile
   Genus: *Intrasporangium* Kalakoutskii, Kirillova et Krassilnikov, 1966

II. Sporangia formed on the tips of sporangiophores
   A. Sporangiospores motile
      (*a*) Sporangia spheroid or irregular. Aerial mycelium usually lacking*
      Genus: *Actinoplanes*, Couch, 1950
      (*b*) Sporangia cylindrical, bottle-shaped. Aerial mycelium usually lacking
      Genus: *Ampulariella*, Couch, 1963
      (*c*) Sporangia spherical. Aerial mycelium present
      Genus: *Spirillospora*, Couch, 1963

B. Sporangiospores non-motile

(*a*) Spheroid sporangia. Aerial mycelium present. Spores spheroid or short rods
Genus: *Streptosporangium* Couch, 1955

(*b*) Sporangia irregularly shaped. Aerial mycelium usually lacking. Spores short rods.
Genus: *Amorphosporangium*, Couch, 1963

(*c*) Club-shaped sporangia formed both on the substrate mycelium and the air mycelium. Each sporangium contains only one chain of spores
Genus: *Microellobosporia*,† Cross, Lechevalier & Lechevalier, 1963

* A species of *Actinoplanes* (*A. armeniacus*) has been reported in which aerial conidia were produced as motile peritrichously flagellate sporangiospores (Kalakoutskii & Kuznetsov, 1964).

† It seems that more study is required to differentiate clearly the mode of spores formation in this genus from that in certain species of *Actinomyces* (*Streptomyces*).

In the above system, developed mainly by Couch (1963), actinomycetes with motile or non-motile sporangiospores are included. This distinction seems sharp enough to substantiate further subdivision of the family.

It has been mentioned that in colonial form and morphology *Intrasporangium calvum* resembles certain Nocardia species. The ability to produce motile spores by peculiar intercalary septation of the mycelium is characteristic of species of the genus *Dermatophilus*. In the latter case, however, no signs of a common sporangial envelope are usually visible.

A study of spore formation in sporangia of *Intrasporangium calvum* by electron microscopy of thin sections should be interesting. Our preliminary observations by phase-contrast microscopy suggest that it may be different from that already described in a strain of *Actinoplanes* by Lechevalier & Holbert (1965). The value of examining not only the structure of reproductive structures in actinomycetes but also the manner in which they are formed has been recently discussed with special reference to the systematics of these organisms (Kalakoutskii, 1965). Attention was also recently drawn to the fact that these structures in actinomycetes closely follow those in fungi (Krassilnikov & Kalakoutskii, 1965).

The formation of peculiar vesicles at a subterminal or terminal position on the mycelium of actinomycetes and Nocardia was mentioned by several authors (Lieske, 1921; Krassilnikov, 1938; Solovieva, Taig, Singal & Rudaja, 1964; Mariat, 1965). Lieske described one kind of these vesicles as involution forms ('Teratologishe Wuchsformen'). Krassilnikov mentioned formation of chlamydospores. However, the most definite reference to intercalary sporangia in actinomycetes was made by Shchepkina (1940; see especially footnote 2 on p. 645), who distinguished them from chlamydospores.

The consistency and regularity of sporangial formation in *Intrasporangium calvum*, the rather regular shape and size of sporangia, as well as the formation of special spores inside the sporangia make it unlikely that we were dealing with involution forms. Such structures have not been described in any other genus of the Actinomycetes so far.

The authors are indebted to Mr A. A. Sokolov for help in the preparation of the electron micrographs.

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EXPLANATION OF PLATES

PLATE 1

Fig. 1. Colonies of *Intrasporangium calvum* growing on meat-extract peptone glycerol agar; 18 days at 28°. Note abundant sporangia on agar surface. × 300.

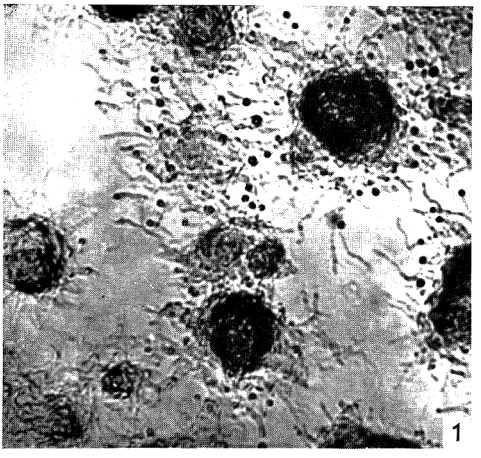
Fig. 2. Six-day culture of *I. calvum* in a microculture on meat-extract peptone agar. Note the intercalary mode of sporangia formation. Phase contrast; × 2000.

Fig. 3. A young sporangium with disrupted sporangial wall. Note the continuation of the hypha through the sporangium. Twelve days on meat-extract peptone agar; microculture; phase contrast; × 2000.

Fig. 4. Formation of a sporangium. HCl+Giemsa nuclear stain. Note the segregation of the nuclear material inside sporangium. Eighteen days on meat extract peptone agar; × 2000.

Fig. 5. Maturing sporangium; 14 days on meat-extract peptone agar. Microculture; phase contrast; × 2000. Same sporangium is photographed at different focus planes: (*a*) to stress the intercalary position of the sporangium; (*b*) to stress the formation of spores inside sporangium.

Fig. 6. Fourteen days on meat-extract peptone agar. Note the relative size of a sporangium formed intercalary (to the left) and the one formed on the tip of a hypha (to the right). Microculture; phase contrast; × 2000.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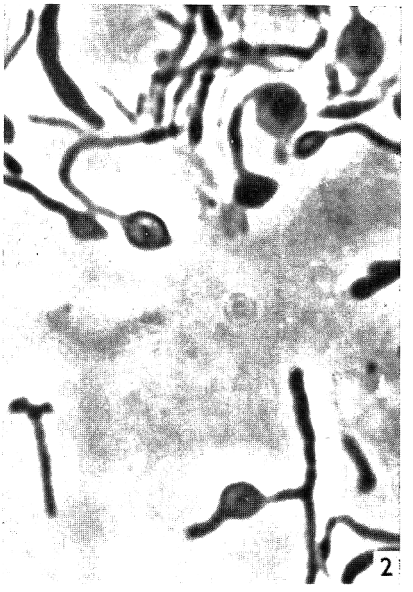
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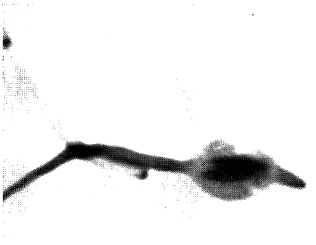
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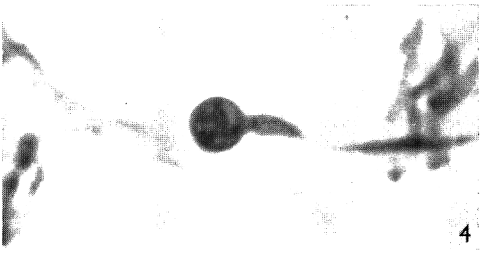
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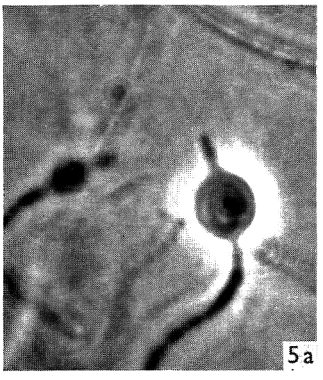
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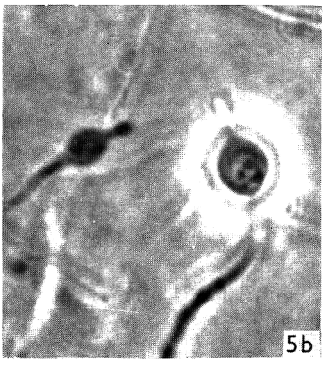
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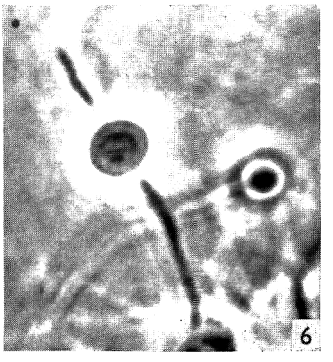
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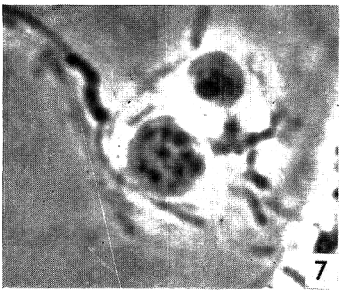
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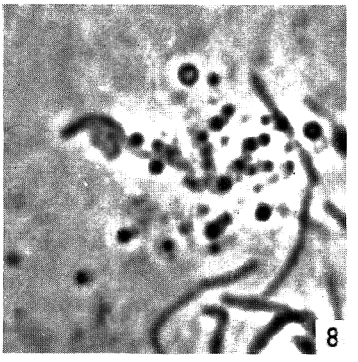
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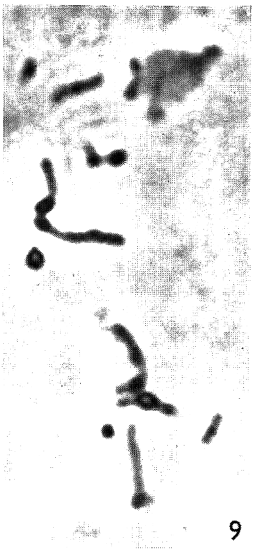
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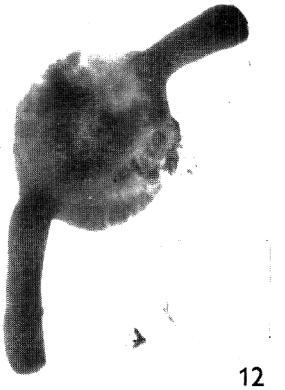
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PLATE 2

Fig. 7. Mature sporangium on meat extract peptone agar. At least 10 spores are visible in a sporangium. Microculture; 38-day culture; phase contrast; ×2000.

Fig. 8. A sporangium from 15-day culture on meat-extract peptone agar was disrupted and is seen to the left. Note the uneven size of the released spores. Microculture; phase contrast; ×2000.

Fig. 9. Mature sporangium from a 41-day culture was disrupted (visible at the top) on a fresh meat-extract peptone agar. Two days of incubation on the same agar. Note the germination of the released spores. Microculture; phase contrast; ×2000.

Electron micrographs showing sporangia formation of *Intrasporangium calvum*. Magnification: ×16,000.

Fig. 10. A hypha from a 8-day culture on meat extract peptone agar.

Fig. 11. A hypha from a 25-day culture on meat extract peptone glycerol agar.

Fig. 12. A mature disrupted sporangium from a 35-day culture on meat extract glycerol agar.

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Some Properties of a Mutant Strain of *Escherichia coli* which Requires Lysine and Methionine or Lipoic Acid for Growth

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SUMMARY

The requirement of a mutant strain of *Escherichia coli* for lysine + methionine was due to its inability to make lipoic acid. Aerobic growth of the mutant in minimal medium + lipoic acid was equal to that of the wild-type organism. The factor was replaceable by acetate + succinate. When grown without lipoic acid, suspensions of this organism did not oxidize pyruvate but did so upon addition of the factor; they also accumulated pyruvate from glucose. Extracts from deficient organisms did not oxidize α-ketoglutarate with 3-acetyl-NAD as acceptor. The growth requirements were only exhibited aerobically when provision of acetate + succinate required the operation of the lipoic-dependent pyruvate and α-ketoglutarate oxidase systems, respectively. Anaerobically, these metabolites were formed by lipoic-independent mechanisms, such as fumarate reductase which is repressed by oxygen.

INTRODUCTION

Mutant strains of *Escherichia coli* have been described which respond to mixtures of methionine and lysine (Back & Westaway, 1962; Taylor & Thoman, 1964). One of these strains has been examined in the present work; it responded to lipoic acid in place of lysine + methionine and it appeared that the primary lesion was an inability to synthesize lipoic acid, which led to secondary effects on the metabolism of the organism. Lipoic acid is a growth factor for several micro-organisms and its function has been established as an electron acceptor in the oxidative decarboxylation of pyruvate and α-ketoglutarate (Reed, 1960). It has, therefore, an important role in the metabolism of substrates by the tricarboxylic acid cycle. In the present work the mutant was used to study the effect of deficiency of lipoic acid on the growth requirements of the organism under aerobic and anaerobic conditions, and to examine at the enzymic level the reasons for the observed growth responses.

METHODS

Organisms. Escherichia coli strain 15 was derived from *E. coli* strain 513 (Back & Westaway, 1962) and both strains were maintained by monthly subculture on Oxoid nutrient agar slopes, incubated for 18 hr at 37° and stored at 4°.

Growth tests. Minimal medium C containing inorganic salts with 0·2 % (w/v) glucose was the basal medium (Roberts *et al.* 1957). The inoculum (equivalent 0·25 μg.

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dry-wt organism/ml.) was prepared from washed organisms grown overnight on nutrient agar slopes. Anaerobic incubation was in completely filled tubes sealed with a rubber bung; aerobic cultures were grown in 5 ml. volumes in 15 mm. diam. tubes shaken on a reciprocating shaker at 120 oscillations/min. Growth was measured after incubation for 24 hr at 37° with an EEL colorimeter (Evans Electroselenium Ltd., Harlow, Essex) and is recorded as equiv. mg. dry-wt organism/ml. (instrument reading 10 = 0·33 mg./ml.).

Experiments with suspensions of organisms. Organisms were grown at 37° in medium C, supplemented as required. The inoculum was derived from nutrient agar slopes and was equivalent to about 2 μg. dry-wt organism/ml. Aerobic cultures were in 300 ml. volumes in 2 l. flasks, incubated with shaking at 300 oscillations/min.; anaerobic growth was in completely filled flasks. The cultures were harvested by centrifugation after incubation for 10 hr while still in the logarithmic phase of growth. The organisms were washed in 0·04 M-phosphate buffer (pH 7·0) and resuspended in the same buffer to a concentration equiv. 10–15 mg. dry-wt. organism/ml.

Respiratory activity was determined manometrically at 37°. Formation of products from glucose utilization was determined with organisms incubated aerobically or anaerobically in reaction mixtures containing per ml.; organisms, equiv. 20 mg. dry-wt; potassium phosphate buffer (pH 7·0), 100 μmoles; glucose, 20 μmoles. Aerobic incubation was in 4 ml. volumes in 50 ml. flasks shaken at 300 oscillations/min. and anaerobic incubation was in tubes under an atmosphere of nitrogen. After incubation for 3 hr at 37° the suspensions were centrifuged at 0° and the supernatant fluids analysed for products.

Analytical methods. Glucose was determined by the method of Huggett & Nixon (1957). Pyruvate was estimated as the 2,4-dinitrophenylhydrazone (Friedemann & Haugen, 1943). Acetate was measured by the method of Rose (1955) which involves its conversion to acetyl phosphate with acetokinase and measurement of the acetyl phosphate by the hydroxamate method of Lipmann & Tuttle (1945). A crude extract of aerobically grown *Escherichia coli* 518 was used as the source of acetokinase.

Preparation of cell-free extracts. Suspensions of organisms (equiv. 20 mg. dry-wt/ml. in 0·04 M-phosphate buffer, pH 7·0) were disrupted by ultrasonic treatment for 4 min. with a Mullard E 7590B generator operating at 3·5 A. and producing 25 kc./sec. The preparations were centrifuged at 18,000 *g* for 15 min. at 4° and the supernatant fluids used for the determination of enzymic activities. Protein was measured spectrophotometrically (Warburg & Christian, 1941).

Assays of enzyme activity. α-Ketoglutarate dehydrogenase was measured spectrophotometrically by the method of Amarasingham & Davis (1965), in which 3-acetyl-NAD is the electron acceptor; it was also assayed with ferricyanide as acceptor in the system of Hager & Kornberg (1961), measurement being made at 420 mμ. Lipoic acid dehydrogenase was measured by the method of Hager & Gunsalus (1953), except that disappearance of sulphydryl groups was estimated by the procedure of Ellman (1959). Reduced lipoic acid, required for this assay, was prepared by the method of Wagner *et al.* (1956). Fumarate reductase was assayed anaerobically by following the rate of oxidation of reduced benzylviologen in the presence of fumarate; measurement was made with the EEL colorimeter, using a 625 filter (Wyn-Jones & Lascelles, 1967).

Special chemicals. DL-lipoic acid (L. Light and Co. Ltd., Colnbrook, Bucks., England) was used. 3-Acetyl-NAD was from Calbiochem (California, U.S.A.); 5,5′-

dithio-bis(2-nitrobenzoic acid), for the determination of sulphydryl groups, was from the Aldrich Chemical Co. Inc. (Milwaukee, Wisconsin, U.S.A.); benzylviologen was from British Drug Houses Ltd.

RESULTS

Growth requirements under aerobic and anaerobic conditions

Under aerobic conditions, growth of the mutant strain 15 of *Escherichia coli* was dependent on lipoic acid, and with this supplement it grew as well as the parent organism in the unsupplemented medium (Table 1). Lipoic acid was replaced by a mixture of acetate + succinate, though neither substance alone was effective and growth did not attain the degree given with lipoic acid. The low concentration of lipoic acid required for maximum growth (about 5 mμM) was of the same order as that required by other exacting micro-organisms. The requirement of acetate + succinate was higher by several orders of magnitude, which was to be expected if lipoic acid functioned catalytically in their synthesis. Anaerobically, the mutant grew on the unsupplemented minimal medium to the same extent as the parent, and the addition of lipoic acid or of acetate + succinate had no effect (Table 1).

Table 1. *Growth responses of Escherichia coli strains 15 and 518*

Cultures were grown as described in the Methods in medium C supplemented as shown.

	Supplement to minimal medium (final conc.)			Growth in 24 hr (equiv. mg. dry-wt organism/ml.)	
Strain	Acetate (mM)	Succinate (mM)	DL-lipoic acid (mμM)	Aerobic	Anaerobic
518	—	—	—	0·71	0·34
15	—	—	—	0	0·31
	1	—	—	0	0·26
	—	1	—	0	0·28
	1	1	—	0·35	0·31
	—	—	10	0·71	0·31

Utilization of glucose and pyruvate by suspensions of organisms

The difference in behaviour under aerobic and anaerobic conditions suggested that the mutant could make acetate + succinate anaerobically by lipoic-independent mechanisms, whereas aerobically these metabolites were provided by the lipoic-dependent oxidation of pyruvate and α-ketoglutarate. This was partially confirmed by experiments with suspensions of the mutant and parent strains grown with and without lipoic acid.

Suspensions of the mutant grown aerobically without lipoic acid (with acetate + succinate present) did not oxidize pyruvate but did so upon addition of the factor (Fig. 1). Suspensions of such deficient organisms accumulated pyruvate when incubated aerobically with glucose, in contrast to organisms grown with lipoic acid which behaved like the parent strain (Table 2). Surprisingly, acetate was also found as a product of glucose utilization by lipoic-acid-deficient organisms. This acetate might have been formed via the pyruvate oxidase system which is induced by pyruvate and which is formed in the later stages of growth of *Escherichia coli* (Gounaris & Hager, 1961). The reaction does not appear to be quantitatively significant for the growing

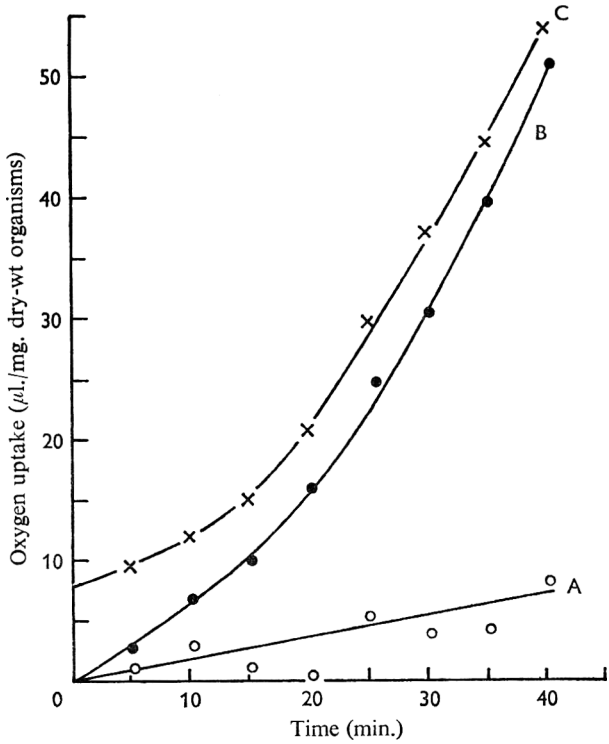

Fig. 1. Oxidation of pyruvate by suspension of *Escherichia coli* strain 15. Each manometer vessel contained in 2·5 ml.: organisms (equiv. 10 mg. dry-wt) suspended in 50 mM-phosphate buffer (pH 7·0) with sodium pyruvate (20 μmoles) added at zero time from the side arm; lipoic acid (1 μmole) was present in B. The centre well contained 5 N-NaOH. Incubation was at 37° in air. The endogenous respiration (not more than 12 μl./mg. dry-wt organism/hr) has been subtracted. Curves A and B contained lipoic-deficient organisms, grown with mM-acetate+succinate; curve C contained organisms grown with 10 mμM-lipoic acid.

Table 2. *Formation of acetate and pyruvate from glucose by suspensions of Escherichia coli strains 15 and 518*

Organisms were grown in the basal medium supplemented as indicated, and the washed suspensions incubated aerobically or anaerobically in 4·0 ml. mixture containing: organisms, equiv. 80 mg. dry-wt; potassium phosphate buffer (pH 7·0), 400 μmoles; glucose, 80 μmoles. Incubation was for 3 hr, by which time the glucose was completely utilized. After removal of the organisms by centrifugation acetate and pyruvate were determined in the supernatant fluids (see Methods).

		Products found			
		Aerobically		Anaerobically	
Strain	Growth conditions	Acetate	Pyruvate	Acetate	Pyruvate
		(μmoles/ml.)			
15	Aerobic with 10 mμM-lipoic acid	2·0	1·6	11	0·7
	Aerobic with mM acetate+succinate	7	8	14	0·7
	Anaerobic without supplement	14	0·4	8	1·3
518	Aerobic without supplement	3	0·6	13	0·5
	Anaerobic without supplement	15	2·2	13	0·4

organism since the mutant requires exogenous acetate when grown aerobically without lipoic acid.

Under anaerobic conditions, suspensions of the lipoic acid deficient mutant behaved like the parent strain; only small amounts of pyruvate were detected and there was considerable accumulation of acetate (Table 2). These observations confirmed the existence of a lipoic-independent mechanism for acetate formation, which operated under anaerobic conditions.

Enzymic activities

Lipoic dehydrogenase. The activity of this enzyme was examined in extracts of the mutant and parent strain grown under various conditions (Table 3). The enzyme was present in the mutant grown either with or without lipoic acid and was, indeed, significantly higher in organisms grown aerobically with acetate and succinate in place of the factor. The level of activity in both parent and mutant organisms grown anaerobically was about one third of that found in aerobic organisms.

Table 3. *Enzymic activities in extracts of Escherichia coli strains 15 and 518*

Organisms were grown in the salts medium supplemented as shown. Activities were determined in extracts of the organisms as described in the Methods.

Strain	Growth conditions	Lipoic dehydrogenase	α-Ketoglutarate dehydrogenase		Fumarate reductase
			NAD*	Ferricyanide	
		(μmoles substrate or product/hr/mg. protein)			
15	Aerobic with 10 mμM-lipoic acid	1·4	0·07	1·7	0·85
	Aerobic with mM acetate + succinate	2·4	0	1·26	0·85
	Anaerobic without supplement	0·4	0	0	7·8
518	Aerobic without supplement	1·4	0·06	1·26	0·45
	Anaerobic without supplement	0·6	0	0	4·3

* 3-acetyl-NAD.

Enzymes concerned in succinate formation. The dependence of the mutant upon succinate for aerobic growth without lipoic acid could be attributed to its inability to form succinate from α-ketoglutarate via the α-ketoglutarate oxidase system. Extracts of the mutant grown aerobically without lipoic acid contained no detectable α-ketoglutarate dehydrogenase activity with 3-acetyl-NAD as electron acceptor, but the activity was found with ferricyanide, which bypasses lipoic acid as electron acceptor. Preparations from the mutant grown aerobically with lipoic acid had both activities at degrees similar to that found in the parent (Table 3). α-Ketoglutarate dehydrogenase with 3-acetyl-NAD or ferricyanide as acceptor was detectable only in organisms grown aerobically; this applied to both parent and mutant strains and confirmed recent observations of Amarasingham & Davis (1965).

The ability of the mutant to grow anaerobically without added succinate could be attributed to the synthesis of succinate by fumarate reductase. This enzyme catalyses fumarate reduction more rapidly than succinate oxidation, whereas succinic dehydrogenase favours the formation of fumarate from succinate (Hirsch, Rasminsky, Davis & Lin, 1963). Fumarate reductase was high in mutant and parent organisms grown anaerobically (Table 3). In both strains it was repressed by oxygen, and in the case of the mutant the degree of activity was low in organisms grown with or without lipoic

acid. Repression of this enzyme by oxygen accounts for the dependence of the mutant on added succinate when the normal mechanism for its aerobic formation from α-ketoglutarate is prevented by deficiency of lipoic acid.

DISCUSSION

The mutant strain 15 of *Escherichia coli* is apparently blocked at a stage in the synthesis of lipoic acid; there is no knowledge of this biosynthetic pathway. The consequences of lipoic acid deficiency only become evident when the cultures are grown aerobically and are dependent upon the operation of the tricarboxylic acid cycle for the biosynthesis of intermediates. Under these conditions the organism apparently forms acetate+succinate predominantly by the lipoic-mediated pyruvate and α-ketoglutarate oxidase complexes. Other enzymic mechanisms which provide these metabolites by lipoic-independent steps are repressed or inhibited by oxygen, whereas they can function anaerobically. Consequently, the mutant grows anaerobically without supplementation.

Acetate can be formed anaerobically by *Escherichia coli* by the phosphoroclastic reaction, which is not detectable in organisms grown aerobically (Henning, 1963). The pyruvate-induced pyruvate oxidase described by Gounaris & Hager (1961) does not apparently provide sufficient acetate to support growth of the mutant. The alternative anaerobic mechanism for succinate formation is by the fumarate reductase enzyme, which is repressed by oxygen in both mutant and parent strain. Repression of this enzyme by aerobiosis was previously found by Hirsch *et al.* (1963).

The original observations of the response of the *Escherichia coli* mutant 15 to lysine + methionine may be partly explained as follows. Succinylation reactions occur in the biosynthesis of both amino acids (Davis *et al.* 1959; Rowbury & Woods, 1964) and their addition to the medium would therefore spare the requirement for succinate. However, the fact that acetate is no longer necessary for growth when these amino acids are present is more difficult to understand. Possibly, lipoic-independent reactions for acetate formation, such as the system described by Gounaris & Hager, (1961) may provide sufficient amounts of acetate when other growth requirements have been met. A similar pattern of growth responses has been observed with a mutant strain of *E. coli* which requires 4-hydroxybenzoic acid, a precursor of quinones (Wyn-Jones & Lascelles, 1967). Aerobic growth occurs without the factor in the presence of methionine+lysine or succinate; under anaerobic conditions, however, the organism grows without supplementation. Thus, with both mutant strains, a deficiency in the aerobic electron transport chain results in an impairment in the tricarboxylic acid cycle as a source of intermediates.

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The Antigens of *Pseudomonas aeruginosa* Studied by the Ouchterlony Technique and Immuno-electrophoresis

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SUMMARY

Preliminary agglutination and precipitin tests to which 60 newly isolated strains of *Pseudomonas aeruginosa* were submitted confirmed the existence of at least five serological groups. Ouchterlony precipitin tests and immuno-electrophoresis experiments, made with the concentrated trichloroacetic acid extracts of eight strains representative of these groups, revealed the antigenic heterogeneity of this species while the presence of at least 12 antigens was demonstrated. One of these was common to seven of the eight strains. Each of five strains, representative of five different serological groups, possessed at least one antigen peculiar to itself. The remaining ones appeared to be distributed at random among the strains. At least three different antigens migrated towards the positive electrode, of which two appeared to be electrophoretically inhomogeneous.

INTRODUCTION

Various attempts have been made to differentiate between members of *Pseudomonas aeruginosa* by determining intraspecific variations (Seleen & Stark, 1943; Holloway, 1960; and others). Grouping of strains of this species on the basis of antigenic structure has also attracted much attention (Aoki, 1926; Sandiford, 1937; Gaby, 1946; Mayr-Harting, 1948; Christie, 1948; Munoz, Scherago & Weaver, 1949; Homma, Sagehashi & Hosoya, 1951; Verder & Watt, 1953; Gould & McLeod, 1960) and might be expected to provide a means for detecting minor differences between strains. Agglutination tests, however, have given rather disappointing results, partly because of difficulties in distinguishing flagellar from somatic antigens (see for example Mayr-Harting (1948) and some experiments briefly reported in the present paper). Precipitin tests made with bacterial extracts (van den Ende, 1952; Köhler, 1957), though useful and more precise, cannot be regarded as providing a perfect criterion for comparison of strains because they fail to reveal the number of antigens forming the precipitate of which the nature can only be elucidated by comprehensive absorption experiments.

The present paper describes the application of the gel precipitin test (Ouchterlony, 1949, 1953) to this problem. As the interpretation of Ouchterlony experiments is not always straightforward, confirmation of the results was sought by immuno-electrophoresis (Grabar & Williams, 1955; Cleve & Schwick, 1957; Hirschfeld, 1959, 1960; Lapresle, Kaminsky & Tanner, 1959). Though used to examine only a few strains of

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Pseudomonas aeruginosa, the methods gave results which seem to explain some of the difficulties previously encountered in the serological classification of this organism and provide some additional information about the antigens.

METHODS

Collection and preservation of strains. Sixty strains of *Pseudomonas aeruginosa* (labelled E1 to E60) were isolated from specimens, all of human origin, submitted to the Department of Pathology for bacteriological examination.

A pure culture of each strain was maintained on Hartley's digest agar (HA) slopes. In addition, each strain was preserved in the dry state (Stamp, 1947). Since *Pseudomonas aeruginosa* is a proteolytic organism, the desiccator was kept in a refrigerator until the nutrient gelatin had dried to thin pellets. A fresh subculture was prepared for each experiment by inoculating a dried pellet into Difco Nutrient Broth (DNB).

Preliminary agglutination and precipitin tests. In the first series of experiments all the strains were tested against immune sera prepared by van den Ende (1952) which had been stored at $-20°$. Bacterial suspensions for flagellar (H) and somatic (O) agglutination, and trichloroacetic acid (TCA) extracts for precipitin tests were prepared, and the experiments were made as described by van den Ende (1952).

For precipitin tests the extracts were diluted 1/2 in 0·87 % (w/v) NaCl solution and the antisera 1/10 in 0·87 % (w/v) NaCl containing 5 % (w/v) sucrose. Every 5 min., for 2 hr, the tubes were examined for rings of precipitate at the interface and, after being left overnight at room temperature, for precipitates which had settled.

In the second series of experiments cross-agglutination tests, both H and O, were made with H and O suspensions of the homologous strains and the new antisera (see next section). Antiserum dilutions of 1/100 were used. The newly prepared H antisera and TCA extracts of the homologous strains were used in cross-precipitin tests. Antiserum dilutions of 1/10 were used against 1/2 dilutions of the extracts.

Preparation of immune sera. H and O immune sera were prepared from 14 strains, thought on the basis of preliminary tests to include at least one representative of the groups of van den Ende (1952). H antisera were prepared against formalinized whole organisms and O antisera against organisms which had been heated and treated with ethanol as described by van den Ende (1952), one rabbit being used for each suspension. The sera were sterilized by filtration, preserved with thiomersalate (0·01 %, w/v) and tested by agglutination of homologous H and O bacterial suspensions.

Preparation of TCA extracts. Preliminary experiments showed that the antigen concentration in extracts prepared as described by van den Ende (1952) was inadequate for gel precipitin tests. Larger quantities of culture were therefore used for these experiments and the antigen extracts were concentrated before being tested. The selected strains were grown in oxygenated H.I.D. medium (Mead & van den Ende, 1953), at 37° for 15 hr in 700 ml. volumes in 1 l. bottles; tributyl phosphate was used as antifoam. The bacteria were collected by centrifugation at 5000 rev./min. for 30 min. in a refrigerated angle-head centrifuge, washed twice in ice-cold distilled water and resuspended in 10 ml. ice-cold 0·25 N-trichloroacetic acid/g. wet wt bacteria (Boivin & Mesrobeanu, 1933). The mixture was stirred at 0–4° for 3 hr and then centrifuged in a refrigerated centrifuge at 3000 rev./min. for 30 min. or until the supernatant fluid was

clear; this was then treated with 0·5 vol. pre-cooled 0·5 M-Na_2HPO_4 and adjusted to pH 7·2 with 0·25 N-NaOH.

The extracts were dialysed for 2 days against four changes of distilled water containing 0·01 % (w/v) thiomersalate, concentrated by pervaporation to about 5 ml. and freeze-dried. For Ouchterlony experiments the dried extract from 2 g. wet bacteria was redissolved in 0·2 ml. complement fixation titration (c.f.t.) saline (Mayer, Osler, Bier & Heidelberger, 1946).

Preparation of a concentrated multivalent antiserum and concentration of individual antisera. To aid detection by gel precipitin tests of as many antigens as possible, a mixture was prepared of 1 ml. of each of six H antisera, each representative of one of the serological groups of van den Ende (1952). The antisera selected were those against the strains: E 52 (group I), E 8 (group II), E 55 (group III), E 5 (group IV), E 1 (Group V), E 58 (group VI). The globulin fraction was precipitated from the mixed sera by adding 0·5 vol. saturated $(NH_4)_2SO_4$, the mixture being left at room temperature for 3 hr. The precipitate was collected by centrifugation at 6000 rev./min. for 30 min. and redissolved in 1.0 ml. distilled water. The solution was dialysed in a refrigerator against 500 ml. 0·87 % (w/v) NaCl containing 0·01 %(w/v) thiomersalate for 3 days, the saline being changed at the onset of the second and third days. Finally, the solution (vol. 4·5 ml.) was dialysed for 1 day against saline, diluted 1/10, and freeze-dried. The residue was redissolved in 0·5 ml. distilled water containing 0·01 % (w/v) thiomersalate as preservative. Each of the six antiserum samples was therefore twice concentrated. Each of the six individual antisera was also concentrated twice by freeze-drying and redissolving in half the original volume of water containing 0·01 %(w/v) thiomersalate.

Micro-Ouchterlony experiments. These were carried out in a gel of 1 % (w/v) washed agar (Dulbecco & Vogt, 1954) in 0·87 % (w/v) NaCl on 9 × 9 cm. glass plates. The plates, laid on a level surface, were completely covered with a 1 % (w/v) solution of washed agar in distilled water which was allowed to dry to a thin film at 37° overnight. The surface of each plate was then sprinkled with a few granules of thiomersalate powder and carefully covered with 17·0 ml. of a hot solution of 1 % washed agar in 0·87 % (w/v) NaCl to give a layer 2 mm. thick. Holes of 2 mm. diameter were cut in the agar with a small cutter. Sets of six holes were arranged hexagonally with a seventh in the centre. The distance between the centres of each pair of holes was 5 mm. The holes on the outside were filled with solutions of the antigens under test and the centre one with concentrated immune serum. The plates were kept in a moist chamber and examined every day with a magnifying lens, for lines of precipitate which were recorded on a drawing.

After complete development of the lines, the plates were washed with 0·87 % (w/v) NaCl containing 0·01 % (w/v) thiomersalate for 2 days and stained for 24 hr with 0·002 % (w/v) nigrosine in 2 % (v/v) acetic acid in water. Finally, the plates were washed in 2 % acetic acid for 6 hr and the lines again recorded by drawing as well as photographing.

Immuno-electrophoresis. The method used was based on that of Grabar & Williams (1955). A thin glass plate, 7·7 cm. × 25·5 cm., was covered with hot 1 % (w/v) aqueous agar (washed agar) which was allowed to dry at 37° overnight. The plate was then covered with enough hot 1·5 % (w/v) solution of washed agar in sodium diethylbarbiturate HCl buffer (pH 8·2, ionic strength 0·05; Grabar & Williams, 1955) to give a layer about 1 mm. thick. When the gel had set, five holes, 1 mm. in diameter and

11 mm. apart, were punched in the agar in a straight transverse line near the centre of the plate. Each hole was filled with the concentrated TCA extract of one *Pseudomonas aeruginosa* strain prepared as previously described. The plate was placed on the water-cooled floor of an enclosed Perspex apparatus resembling that of Paigen (1956). The ends of the agar were connected by several thicknesses of filter paper to large electrode vessels containing sodium diethylbarbiturate HCl buffer (pH 8·2, ionic strength 0·1) to which thiomersalate, 0·01 % (w/v), had been added. With cooling water at 5–10° flowing through the floor of the covered apparatus, a direct current of 14 mA., giving a gradient of 7·5 V/cm. in the gel, was applied for 1·5 hr. The plate was then removed and four grooves, 1 mm. wide, were cut in the agar parallel to the long axis between and equidistant from each pair of holes. Each groove was filled with a different twice-concentrated H antiserum. The plate was kept in a moist chamber and examined daily for lines of precipitate, which were recorded. After a week the plate was washed and stained and the lines recorded, the procedure in each case being the same as that described for Ouchterlony experiments.

RESULTS

Agglutination and precipitin tests

In the first series with the sera of van den Ende (1952) many cross-reactions were observed, though in most cases these involved only trace reactions. From the results as a whole, 37 of 60 strains were shown to fall into one or another of the six van den Ende serological groups; for the rest a definite grouping was impossible.

In the cross-agglutination and precipitin experiments with the 14 newly prepared antisera, only the homologous strains were used. Because of many cross-reactions a clear-cut delimitation into groups was impossible in the case of H agglutination. The results of O agglutination suggested a definite grouping into at least five of the serological groups. Notwithstanding more abundant cross-reactions, in most cases only traces, the results of the precipitin reactions agreed well with those of O agglutination.

Ouchterlony precipitin experiments

For these experiments eight strains, thought to be representative of five serological groups, were selected and concentrated TCA extracts were prepared. These strains were: E52 (group I), E55 (group III), E5, E16 and E56 (group IV), E1 (group V), E36 and E58 (group VI).

It was frequently observed that lines which were single when first formed split to form double lines within periods varying between 7 hr and 3 days after the plates had been put up. Furthermore, on keeping the plates for several days, some of the lines which were definitely seen after 1 or 2 days either disappeared or were in the process of fading.

Precipitin tests with multivalent antiserum

In the first experiment (Fig. 1) the TCA extracts of strains E1 and E5 were allowed to react with the multivalent antiserum (MS). In the first plate every alternate well was filled with extract of E1, the first with concentrated, the third with a 1/2 and the fifth with a 1/4 dilution of the extract. On the second plate the same procedure was followed with the extract of E5, while on the third plate the extracts of both strains were put up.

With the extracts of E1 and E5 on separate plates the former formed a maximum of five and the latter three lines. On the third plate the extract of E1 produced four lines and that of E5 produced two. One of the latter two lines appeared to join up with one line of E1 while the second line crossed all the lines of E1. These results suggest the presence of at least five antigens in E1 and three in E5 of which one is common.

In performing experiments with the eight selected strains, the design was such that each extract was in turn next to each of the other extracts. The extracts of all the strains, with the exception of E58, each produced a line near the antigen well which eventually

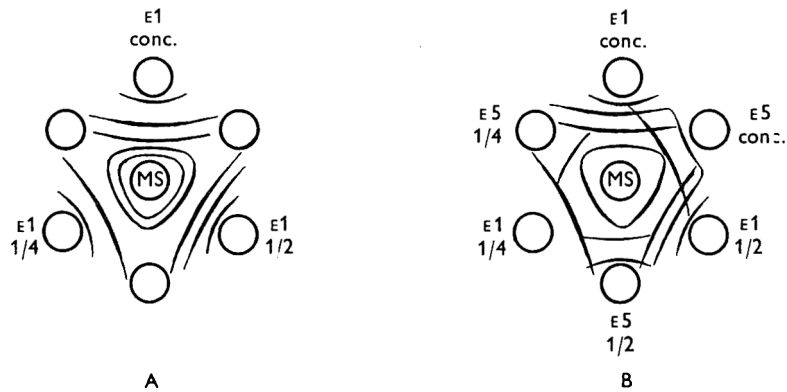

Fig. 1. Results of Ouchterlony precipitin tests with concentrated (conc.) and serial two-fold dilutions (1/2 and 1/4) of the trichloroacetic acid-extracts of *Pseudomonas aeruginosa* strains E1 and E5 against the concentrated multivalent antiserum (MS). A, Plate with concentrated and diluted extracts of E1 only. B, Concentrated and diluted extracts of both strains on the same plate.

split up into two. For each extract these lines joined up with those of every other extract except for strain E5 where identity with the lines of strains E36, E52 and E55 was uncertain. For the latter three strains, however, like that of E5, the lines joined up with the corresponding lines of all the other strains. Furthermore, against the anti-E1 serum (see next section) all the extracts, except that of E58, produced two similar lines which clearly joined up with one another.

In addition to the above-mentioned line the extracts of the various strains produced lines as follows:

TCA extracts of E1 and E36. Each produced one line which joined up with one another.

TCA extracts of E5 and E16. One exclusive line was produced by each.

TCA extract of E52. Two additional exclusive lines were produced.

TCA extract of E58. Altogether three exclusive lines were produced.

The results of these tests are summarized in Table 1.

Precipitin tests with concentrated individual antisera

The eight extracts described above were also used in Ouchterlony tests against each of the six individual H antisera, which had been included in the multivalent antiserum.

Against its homologous antiserum, the extract of E1 produced three lines. These appear to be different from those which were observed against the multivalent antiserum; their separation was incomplete and they presumably obscured the double

line which, apparently, is common to all the strains except E58. Against this antiserum the extracts of the other strains, with the exception of E58, each produced a double line near the antigen well and in each case it clearly joined up with those of every other extract. The following additional lines were produced against anti-E1 serum by the various strains: one line by and common to E5 and E16 but not identical to any of the lines formed by E1; one by and common to E52 and E1; a double line by E36. Apparently it was the latter line which appeared as a single line against the multivalent antiserum. No lines were produced by the extract of E58. All the antigens which were demonstrated against this antiserum must be present in E1 otherwise the antiserum could not have showed them up.

The results produced against the remaining five antisera, as well as those described above, are summarized in Table 2.

Table 1. *Summary of results of Ouchterlony experiments in which the TCA extracts of Pseudomonas aeruginosa strains were tested against the multivalent antiserum*

Grouping of strains was based on precipitin tests with the antisera of van den Ende (1952)

Strain	Group	Total no. of lines	No. of exclusive lines	No. of non-exclusive lines	Strains with which non-exclusive lines are shared	Strains with which no lines are shared
E52	I	3	2	1*	E36, E16, E1, E56 and E55. E5 uncertain	E58
E55	III	1	—	1*	E1, E56, E16, E52 and E36	E58
E5	IV	2	1	1*	E1, E16 and E56. E36, E52 and E55 uncertain	E58
E16	IV	2	1	1*	E1, E5, E52, E36, E56 and E55	E58
E56	IV	1	—	1*	E1, E5, E16, E36, E52 and E55	E58
E1	V	2	—	1*	E5, E16, E36, E52, E55 and E56	E58
				1	E36	
E36	VI	2	—	1*	E1, E16, E52, E55 and E56	E58
				1	E1	
E58	VI	3	3	—	—	All other strains

*Double lines were observed so two distinct antigens may be present.

Immuno-electrophoresis

In these experiments the TCA extracts of strains E1, E5, E16, E36, E55 and E58 were used. The concentrated H antisera were those prepared against strains E1, E8, E5, E52, E55 and E58 thought to be representative of different serological groups.

TCA extract of E1. Four lines, *a*, *b*, *c* and *d*, were produced against the homologous antiserum. Two, *a* and *b*, were located towards the positive electorde, *c* close to the starting-point and *d* towards the negative electrode. Line *a*, although continuous, produced two peaks, one at the starting-point and the other markedly towards the positive electrode, suggesting the presence of two electrophoretic components with the same immunological determinant groups.

Against anti-E8 serum lines identical to *a*, *b* and *c* were produced. Each of the antisera against E5, E52 and E55 produced one line apparently corresponding to *a*.

Thus at least four antigens were revealed in E1 of which at least one is common to E5, E52 and E55 and three to E8 since it was shown up by their homologous antisera.

TCA extract of E5. Against the homologous antiserum two lines, *e* and *f*, were produced which behaved similarly to *a* and *c* respectively of E1. Identical lines were formed with anti-E1 serum. At least two antigens have, therefore, been shown to be common to E1 and E5.

Table 2. *Summary of the results of Ouchterlony experiments with concentrated individual antisera to strains of Pseudomonas aeruginosa*

Antisera for	Antigens demonstrated
E1	One* antigen common to E5, E16, E52, E55, E56 and E36 One antigen common to E1 and E52 One antigen common to E5 and E16 Two* antigens in E36 (one* apparently identical to those shared by all the strains except E58) Two antigens in E1 only Presumably all these must be present in E1
E5	One antigen in homologous strain only
E8	One antigen in each of E1 and E36
E52	Two antigens in homolous strain only
E55	Two antigens in homologous strain only
E58	Two antigens in homologous strain: one exclusive and one shared with E36

*Double lines were observed so two distinct antigens may be present.

Table 3. *Summary of antigens in strains of Pseudomonas aeruginosa demonstrated by immuno-electrophoresis experiments*

Strain	No. of antigens demonstrated	Antigens common to other strains
E1	4	Two with E5 One with each of E16, E36, E52 and E55
E5	3	Three with E16 Two with E1
E16	3	Three with E5 One with each of E1 and E52
E36	2	Two with E5 One each with E1 and E58
E55	2	One with E1
E58	3	One with E36

TCA extract of E16. With the anti-E5 serum three lines, *g*, *h* and *i*, were produced. Both *g* and *i* showed two peaks but the former was located towards the positive and the latter towards the negative electrodes. Line *h* was situated towards the positive electrode. The antisera against E1 and E52 each produced one line; for the former similar to *g* and for the latter to *h*.

This strain must contain at least three antigens, all of which are common to E5

since they were detected by using anti-E5 serum. One of these is shared by E1 and another by E52.

TCA extract of E36. Both the antisera against E5 and E8 produced two lines, *j* and *k*, in similar positions; the former towards the positive electrode and the latter at the starting point. The antisera against E1 and E58 each produced a line similar to *j*. The presence of two antigens, both common to E5, were, therefore, revealed. It also shares one antigen with each of E1 and E58.

TCA extract of E55. Two lines, *l* and *m*, were produced against the homologous serum only. The former, which showed evidence of having two peaks, was located towards the positive electrode.

TCA extract of E58. Only the homologous antiserum reacted to produce a broad, diffuse line running from the origin to a point about 1·4 cm. towards the negative electrode. At this point it split up into at least two lines, *n* and *o*, both of which were convex towards the serum groove. A third line, *p*, distinct but broad with diffuse edges, was formed still further away on the cathode side.

The results of these experiments are summarized in Table 3.

DISCUSSION

Since *Pseudomonas aeruginosa* is liable to variation on repeated cultivation in the laboratory (Christie, 1948), serial subculture was avoided. Tests were restricted to those strains which survived drying and a fresh subculture from a dried pellet was used for every experiment. Other difficulties were:

(i) Variation in the number and nature of the antigens produced by one strain under different cultural conditions, sometimes but not always due to selection of mutants (see for example Pirt, Thackeray & Harris-Smith, 1961). Although the conditions used by me were probably uniform, a complete investigation might call for the use of a variety of culture techniques.

(ii) The limits of concentration at which the antigen could be detected; the more concentrated the bacterial extract (and antiserum) used for the test, the more numerous the lines which appeared.

(iii) The choice of extractant for the antigens. Köhler (1957) preferred the Fuller formamide method to the Lancefield method because it extracted the minimum number of antigens needed to give clear-cut results in his investigation. Boivin & Mesrobeanu's (1937) trichloroacetic acid method was used for the present work because of the experience already gained with it in this laboratory.

Agglutination and precipitin tests. In contrast to the results recorded by van den Ende (1952), the present precipitin tests with TCA extracts did not give such a clear picture. If these tests alone are taken as the basis for grouping, only 37 of 60 strains can be placed into groups. When these extracts were later submitted to the Ouchterlony technique, it appeared that their antigen contents were rather low. It is possible that precipitin tests with more concentrated extracts might have allowed more of the new strains to be grouped.

Although the percentage of strains which could be grouped was much lower than van den Ende's (1952), these results confirm his finding that at least six groups can be distinguished on the basis of precipitin tests with TCA extracts. The agglutination and precipitin tests with the 14 selected strains confirmed the existence of at least

five groups, but it proved impossible to differentiate between strains E1, E6, E8 and E42.

Ouchterlony diffusion tests. Two of the more unfortunate phenomena found in Ouchterlony diffusion tests occurred with my Pseudomonas reagents: (*a*) the disappearance of lines of precipitate was occasionally observed; such lines gradually faded; (*b*) the splitting of lines of precipitate was frequently observed. Initially single lines subsequently split to form double lines. Wilson & Pringle (1954, 1955) observed the formation of multiple lines with advancing development and at very high relative concentrations of one of the reagents. Korngold & van Leewen (quoted from Kabat & Mayer, 1961, p. 89) attribute this effect to diffusion of excess antigen beyond the original line of specific precipitate to broaden the band or to form a second band. In my plates the strength and distance from the antigen well of many of the lines concerned is suggestive of a low antigen concentration rather than antigen excess. In these experiments mixtures of antigens were examined and it is not unlikely that two antigens having almost the same diffusion coefficient may, initially, form lines in the same position but that, given sufficient time, they may move further apart. Preference is therefore given to the interpretation that, in these experiments, double lines imply the presence of two antigens. In view of the possibility, however, that double lines may indicate the presence of one antigen only, the minimum numbers of antigens which were observed, and their distribution, in the eight strains tested are shown in Table 4.

Table 4. *Distribution of twelve trichloroacetic acid-extractable antigens in eight strains of Pseudomonas aeruginosa*

Grouping of strains, according to van den Ende, is based on the preliminary agglutination and precipitin tests.

Strains . . .	E1	E5	E16	E36	E52	E55	E58	E56
Groups . . .	V	IV	IV	VI	I	III	VI	IV
				Antigens demonstrated				
	A*	A	A	A	A	A	—	A
	B	B	B	—	—	—	—	—
	C*	—	—	C	—	—	—	—
	D	—	—	—	D	—	—	—
	E	—	—	—	—	—	—	—
	F	—	—	—	—	—	—	—
	—	G*	—	—	—	—	—	—
	—	—	—	H	—	—	H	—
	—	—	—	—	I	—	—	—
	—	—	—	—	—	J	—	—
	—	—	—	—	—	—	K	—
	—	—	—	—	—	—	L	—

*Double lines were observed so two distinct antigens may be present.

Strains E1, E5, E16, E36 and E52 possess common antigens, as was suggested by the preliminary precipitin tests. It is also evident that E1 is very different from the others. Unfortunately E8 was lost during these experiments, but the results with anti-E8 serum suggest a close similarity between E1 and E8. The results confirm the close relationship, if not identity, between E5 and E16 suggested by preliminary precipitin tests. Differences between E56 and E5 and E16 and between E36 and E58 are shown up.

Immuno-electrophoresis. These results confirmed those of the Ouchterlony experi-

ments in showing that the strains investigated yielded many antigens of which several were common to more than one strain. For example, each of the strains, with the exception of E58, shared at least one antigen with E1.

Conclusions

Ouchterlony and immuno-electrophoresis experiments confirmed the multiplicity of antigens in *Pseudomonas aeruginosa* and the existence of five out of the six serological groups of van den Ende (1952).

Unless double lines in the Ouchterlony experiments indicate two antigens, 12 different TCA extracted antigens were demonstrated. Of these, seven were peculiar to certain strains, each thought to be representative of a different serological group, namely 2 to E1, 1 to E5, 1 each to E52 and E55 and 2 to E58. Immuno-electrophoresis also indicated the presence of at least one antigen peculiar to each of strains E1, E16, E55 and E58.

The cross-reactions which were observed in the preliminary precipitin tests are explicable in terms of the common antigens. An investigation of more strains out of each serological group may provide evidence that those antigens found to be peculiar to certain strains are the ones by virtue of which grouping is possible. These results extend those of van den Ende (1952) and emphasize the limitations of simple precipitin tests for demonstrating serological relationships.

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The Identity of the Gram-negative Bacterium NCIB 8250 ('Vibrio 01')

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SUMMARY

The original strain of Vibrio 01 (Happold & Key, 1932) appears to have been lost. The Bacterium NCIB 8250, which is frequently referred to as 'Vibrio 01', has different properties from those of the original isolate and is a Gram-negative non-motile oxidase-negative coccobacillus.

INTRODUCTION

The Bacterium Vibrio 01 (Happold & Key, 1932) has been quite widely used in studies on the metabolism of aromatic compounds (e.g. Evans, 1947; Dagley, Fewster & Happold, 1952; Cain, 1961). In the course of experiments designed to delineate the metabolic versatility of this organism (Fewson, 1967) a culture was obtained from the National Collection of Industrial Bacteria (NCIB, Torry Research Station, Aberdeen, Scotland). It soon became apparent that the specificity of growth of this strain, NCIB 8250, was different from that originally described for Vibrio 01 by Evans (1947). In subsequent work, which is described in this paper, it also became apparent that the Bacterium NCIB 8250 was basically different from the bacterium isolated by Happold & Key (1932).

METHODS

Stock cultures of the Bacterium NCIB 8250 were maintained in Oxoid cooked-meat medium stored at 4°. Subcultures were made into Oxoid nutrient broth at intervals of approximately 1 month and were also kept at 4°. Inocula required for the various tests were produced by a further subculture into nutrient broth and incubated for 17–24 hr at 30° immediately before use.

Oxidation-fermentation reactions were measured as described by Hugh & Leifson (1953) and Sellers (1964). All other tests were done according to the methods described by Cowan & Steel (1965).

Oxoid media were used for testing growth on nutrient agar (CM 3), MacConkey agar (CM 7) and Koser's citrate medium (CM 65) and also for following acid production from sugars (Andrade Peptone Water, CM 61). Other reagents were the best quality available from British Drug Houses Ltd., Poole, Dorset.

Incubation was at 30° in all cases.

RESULTS

The Bacterium NCIB 8250 appeared as Gram-negative non-motile coccobacilli about 1 μ wide, frequently arranged in pairs and occasionally as short chains, but never as tetrads. No capsule was detected. The cell size of the organism was apparently related

to the conditions of growth: in general the faster the growth the larger were the organisms.

Numerous experiments failed to demonstrate oxidase activity by Kovacs test. No urease was detected with either Christensen's or SSR media although the Bacterium NCIB 8250 was shown to grow on urea as sole source of nitrogen (Fewson, 1967) and can therefore presumably form urease under some conditions. Nitrate reduction was not detected in the standard assay but the production of nitrite from nitrate was shown when nitrate was the sole source of nitrogen in a succinate-salts medium (Fewson, 1967). There was vigorous catalase activity.

No acid was produced from glucose, sucrose, lactose or dulcitol and the Hugh–Leifson and the Sellers media showed no action on glucose, either aerobically or anaerobically

There was good growth on nutrient agar, MacConkey agar and Koser's citrate medium. Peptone water cultures gave diffuse growth with a thin pellicle.

Tests in appropriate media for the production of indole, acetoin and hydrogen sulphide, for methyl red reaction and for the utilization of malonate were all negative. There was no liquefaction of gelatin. Some ammonia was formed in peptone medium.

DISCUSSION

Vibrio 01 was isolated by Happold & Key in 1932. It was stated to be a "gram negative vibrio" with an oxidase system, without action on any of the common sugars and not liquefying gelatin. Presumably the organism was motile, in view of the designation 'vibrio' and the fact that it was stated to resemble *Vibrio tyrosinatica* (Happold & Key, 1932) and *V. cuneatus* (Evans, 1947; Kilby, 1951). There seems little doubt, therefore, that Vibrio 01 was a member of the Pseudomonas–Vibrio group, although a precise classification cannot be arrived at from the information available. Early experiments on the metabolism of aromatic compounds were done with this organism (Evans & Happold, 1939; Evans, 1947; Prof. W. C. Evans, personal communication) but the original strain appears to have been lost. Most subsequent work apparently done with Vibrio 01 has, in fact, been done with the Bacterium NCIB 8250, which is clearly a different organism. Bacterium NCIB 8250 is neither like the original Vibrio 01 nor characteristic of the genus *Vibrio* since, amongst other things, it is a non-motile oxidase-negative coccobacillus. Evans (1947) found that Vibrio 01 grew on *m*-hydroxybenzoate but not on *o*-hydroxybenzoate: Bacterium NCIB 8250 shows the opposite pattern (Fewson, 1967). It is also noteworthy that Dagley *et al.* (1952) found that 'Vibrio 01 originally isolated by Happold & Key' grew on phenylalanine as carbon source, whereas later it was reported (Chapman & Dagley, 1960; 1962; Fewson, 1967; Prof. S. Dagley, personal communication) that Bacterium NCIB 8250 showed little or no growth with phenylalanine as carbon source. Bacterium NCIB 8250 does, however, resemble the original strain of Vibrio 01 in its failure to grow on the common sugars and in its general ability to metabolize aromatic compounds. Previous reports describing experiments carried out with Vibrio 01 should obviously be treated with care unless it can be clearly established whether the original Vibrio 01 or the Bacterium NCIB 8250 was used.

Davis & Park (1962) placed Bacterium NCIB 8250 in the genus *Comomonas* because one of the authors observed 'a few motile organisms' and 'occasional organisms bearing

one or two flagella'. Motility was never observed in the present work and this has been confirmed (W. Hodgkiss, personal communication). Davis & Park (1962) also gave conflicting results for the oxidase activity of Bacterium NCIB 8250 but it is of interest that the report of motility was made on a culture which was stated to be, by Kovacs test, oxidase-positive and to show no growth on pyruvate or lactate, which are compounds supporting good growth of Bacterium NCIB 8250 (Fewson, 1967). Bacterium NCIB 8250 is now listed by the National Collection of Industrial Bacteria (1964) as an *Achromobacter* sp. but cannot be retained in this genus if the proposals of Brisou & Prévot (1954) and Steel & Cowan (1964) are followed. Sebald & Véron (1963) identified Bacterium NCIB 8250 as *Moraxella lwoffi* (*Acinetobacter lwoffii*, Steel & Cowan, 1964), apparently very largely on the basis of the G+C content of the DNA of the organism. G+C base composition, although a useful tool in classification, cannot be the sole criterion of taxonomy since, in view of the small range of G+C contents possible, it is inevitable that quite different organisms will be found to have similar gross DNA analyses (De Ley, 1964). It seems likely, however, that Sebald & Véron (1963) were substantially correct, since the characteristics of the Bacterium NCIB 8250 reported in the present paper are typical of those of the Acinetobacter–Moraxella group of bacteria (Steel & Cowan, 1964; Henderson, 1965). Doctors Stanier & Doudoroff and their co-workers have come to similar conclusions (Dr R. Y. Stanier, personal communication). Work will have to be done on a large number of strains before the question of nomenclature can be finally settled since it has been found by the present author that the Bacterium NCIB 8250 differs from *Acinetobacter lwoffii* NCTC 5866 and from *Acinetobacter anitratus* NCTC 7844 in its ability to grow on a number of aliphatic and aromatic compounds.

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Identification of Mycobacteria by Overall Similarity Analysis

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SUMMARY

Morphological, physiological, growth and chemosusceptibility characters were determined for 80 'atypical' acid-fast and 3 non acid-fast strains isolated from tuberculous, or presumed tuberculous, patients, and for 24 control strains belonging to various mycobacterial species. Overall similarity analysis was used for establishing the relationships upon the basis of 60 characters codified by 153 features.

The overall analysis differentiated five clusters having mean matching indices above 80 % (80 phenon = 80P), namely: 80P–I (human and bovine mycobacteria); 80P–II ('atypical' mycobacteria belonging to the Ist, IInd and IIIrd Runyon groups); 80P–III (mycobacteria related to *Mycobacterium fortuitum*); 80P–IV (scotochromogenic mycobacteria with greater metabolic abilities); 80P–V (*M. smegmatis*). Nine strains (among which were three non acid-fast bacilli) were not included in these clusters.

The 'atypical' mycobacteria cluster (80P–II) was formed by three clearly delimited subgroups, which broadly corresponded to Runyon's classification.

A more detailed analysis of certain groups, and the determination of the characters of some 'hypothetical average organisms', allowed the further differentiation of some subgroups and aberrant strains.

The need to standardize the coding methods is stressed. Because of the value of overall similarity in such a variable genus, an international system to classify and identify strains upon a phenetic basis is suggested.

INTRODUCTION

Classification on a phenetic basis (overall similarity analysis, numerical taxonomy, Adansonian classification), using the method suggested by Sneath (1957) and Sneath & Cowan (1958), has found followers in various branches of microbiology: Hill (1959); Gilardi, Hill, Turri & Silvestri (1960); Talbot & Sneath (1960); Hill, Turri, Gilardi & Silvestri (1961); Colwell & Liston (1961); Beers & Lockhart (1962); Beers, Fisher, Megraw & Lockhart (1962); Silvestri, Turri, Hill & Gilardi (1962); Liston, Wiebe & Colwell (1963); Proctor & Kendrick (1963); Colwell & Mancel (1964); Kendrick & Proctor (1964). It was debated at the 17th International Congress of Microbiology at Montreal, at a symposium of the Systematics Association (Heywood & McNeill, 1964), at the Conference at Quebec in 1964; it has been the object of theoretical discussions, e.g. Lysenko & Sneath (1959), Floodgate (1962*a*, *b*), Sattler (1963), Reynolds (1965), and a book has been published on this subject by Sokal & Sneath (1963).

This shows the interest roused by a method that offers a chance to end much doubt that exists in the systematics of micro-organisms. The correlations between DNA

relationships (Marmur, Falkow & Mandel, 1963) and those obtained by phenetic analysis (Colwell & Mandel, 1964, 1965; Silvestri & Hill, 1965) emphasize the value of the method.

Concerning the mycobacteria, precise studies of the relationship between known species have been carried out with immunological techniques (Siebermann & Barbara, 1964; Koyama, 1964; Kassirskaia & Mencikov, 1965), or with bacteriophages (Manion, Bradley, Zinneman & Hall, 1964). Numerical taxonomy was applied to the study of mycobacteria mainly by Mexican research workers: Bojalil & Cerbón (1961); Cerbón & Bojalil (1961); Bojalil, Cerbón & Trujillo (1962), as well as by Wayne & Doubek (1963), enabling them to systematize the material and to describe new species.

In the pathological material examined in our laboratories mycobacteria of a type other than human or bovine were frequently found (Nasta & Bogdanescu-Cioclov, 1959; Bogdanescu, Bungetianu & Racotta, 1965). Since classification based upon some of the simple characters (Runyon, 1959; Collins, 1962; Marks & Richards, 1962; Buraczewska, 1963; Kagramanov, 1963) was not completely satisfactory, we have tried to classify the strains on a phenetic basis.

Methods

Strains. Twenty-four previously identified strains (Table 1) and 78 strains isolated from 78 tuberculous, or presumed tuberculous, patients (mainly from sputa) were examined. Variants with different cultural characters, developed spontaneously or obtained by passage through mice from 5 of the 78 strains, have been included as separate strains.

Tests. We studied 60 characters that may be subdivided into 9 groups:

(1) Morphological characters of growth (smooth or rough, eugonic or dysgonic) on Löwenstein-Jensen medium (LJm), after 30 days incubation (examined with a hand lens.)

(2) Texture characters of growth after 8–15 incubation days on LJm (Marks & Richards, 1962): adherence to the medium, viscosity, texture (granular or butyrous), emulsibility in distilled water.

(3) Microscopic characters after 30 days on LJm: true branching (slide culture in Youman medium), cording, shape (bacilli, coccobacilli), length, thickness (measured with the ocular micrometer), percentage of acid-fast micro-organisms, depth of staining.

(4) Ability and rate of growth on two media (LJm and 7 % glycerol agar) at three different temperatures (25°, 37° and 45°) after loop inoculation (3 mm diam.) of a 10^{-2} mg./ml. bacillary dilution.

(5) Pigmentation in light and dark (Juhlin, 1960).

(6) Drug resistance (percentage method of Canetti, Rist & Grosset, 1963): INH (0·2, 1 and 100 μg./ml.); PAS-acid (1 and 10 μg./ml.); ethionamide (20 μg./ml.); cycloserine (25 μg./ml.); thiosemicarbazone (10 and 40 μg./ml.).

(7) Presence of certain enzymes and vitamins: catalase (Peizer & Widelock, 1955); nicotinamide (Konno, 1960); nitrate reductase (Virtanen, 1960); acetamidase, benzamidase, urease, nicotinamidase, salicilamidase, succinamidase (after 24 hr, Bönicke, 1960).

(8) Ability and rate of utilization of certain amino acids and organic salts: DL-

alanine (Käppler & Janowiec, 1963); sodium acetate, benzoate, citrate, lactate, oxalate, pyruvate, propionate, succinate and tartrate (Gordon & Smith, 1953), after incubation of 0·2 ml. from a 1 mg./ml. bacillary dilution.

Table 1. *List of strains from culture collections or previously identified*

No. and name	Source, etc.	Symbol in diagrams
1 H37 RV	Human virulent collection strain*	H–HS
2 H37 RV resistant to 10 μg. INH/ml.	Human collection strain grown *in vitro* on INH media*	H–HR
3 Strain 1020	Chemosusceptible strain with characters of human tubercle bacilli, isolated from a phthisical patient	H–1020
4 Strain 1325	INH-resistant strain with characters of human tubercle bacilli, isolated from a phthisical patient	H–1325
5 BCG	Bovine attenuated collection strain*	B–BCG
6 Vallée	Bovine virulent collection strain*	B–V
7 Strain B–7	Strain isolated from tuberculous cow with characters of bovine tubercle bacilli†	B–7
8 *M. avium*	Avian collection strain*	A
9 Strain A–1	Strain isolated from tuberculous fowl†	A–1
10 Strain A–2	As above	A–2
11 Strain A–3	As above	A–3
12 AF526/59	Pathogenic strains, isolated in England from cattle and classified in the Ist group of Marks & Richards†	2 M
13 AF281/60		6 M
14 AF1445/61		5 M
15 AF226/62		7 M
16 AF179/63	As above, classified in the IVth group of Marks & Richards	1 M
17 AF283/63	As above, classified in the Vth group of Marks & Richards	8 M
18 AF1400/61	As above, classified in the VIth group of Marks & Richards	4 M
19 AF1156/62		3 M
20 *M. fortuitum*	Collection strain§	MF
21 *M. phlei*	As above	MP
22 *M. smegmatis* (Washington)		MSW
23 *M. smegmatis* (607)	As above	MS–607
24 Tuberkelähnlicher Bacillen of Rabinowitsch (1897)	As above	MR

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(9) Ability and rate of fermentation of certain sugars and higher alcohols (Gordon & Smith, 1953): arabinose, galactose, glucose, lactose, maltose, mannose, raffinose, rhamnose, trehalose, xylose, dulcitol, inositol, mannitol and sorbitol (incubation as for 8).

In order to estimate growth rate, the importance of which in the genus *Mycobacterium* was emphasized by Wayne (1964), we recorded the time growth became visible in the 4th group of tests (2–28 days incubation), and the time the medium changed colour in the 8th and 9th groups of tests (up to 60 days incubation).

Coding methods. Methods very similar to those suggested by Silvestri *et al.* (1962) were used as follows:

(1) Quantitative data:

(*a*) Silvestri *et al.* 1962:

Degree of the quantitative character	Computed features		
	A	B	C
0	−	−	−
1	+	−	−
2	+	+	−
3	+	+	+

i.e. 'additive coding' (Sneath, 1962). In this way we codified, for example, the percentage of acid-fast organisms or the drug-resistance.

(*b*) For the growth rate and growth ability the following coding variant was used (see Discussion):

		A	B	C	D
0	No growth in 28 days on X medium	−	−	NC	NC
1	Growth in 15–28 days	+	−	−	−
2	Growth in 8–14 days	+	+	−	−
3	Growth in 4–7 days	+	+	+	−
4	Growth in <3 days	+	+	+	+

NC = no comparison.

(2) Characters subordinated to other characters (Silvestri *et al.* 1962), for instance: emulsibility 'yes' or 'no'; if 'yes', high or low:

	A	B
Yes, high	+	+
Yes, low	+	−
No	−	NC

(3) For pigmentation in the dark we used a special coding:

	Colour of the culture	Presence of pigment (A)	Red pigment (B)	Bright pigment (C)
1	White-grey	−	−	−
2	Yellowish	+	−	−
3	Lemon-yellow	+	−	+
4	Dull-orange	+	+	−
5	Bright-orange	+	+	+

Table 2 shows the characters examined, the number of features given for each character and the coding method (according to the system used above).

The formation of clusters. The comparison of the strains was made without a computer, determining the matching index between each couple of strains according to:

$$\% M = n_c/(n_c+n_d)\times 100,$$

where n_c = the number of concordances (between + and +, and − and − features) and n_d = the number of discordances (between + and − features) (Silvestri *et al.* 1962). For grouping the strains we used a modified Single Link Method (Sneath,

1962). Each new strain joined one or other end of the nucleus according to its higher $\%M$ with one or other half-cluster. In order to make clustering cleaner it has been necessary to change the position of some strains in the alignment (Sneath & Cowan, 1958). We also calculated the arithmetic means of the indices within and between the groups.

Table 2. *Coding of the characters analysed*

Group of characters	Character no.	Characters	Nos. of features	Coding method
I	1	Smooth or rough culture	1^{+}	.
	2	Eugonic or dysgonic growth	1^{+}	.
II	3	Adherence to the medium	2	1 *a*
	4	Viscosity of the culture	1^{+}	.
	5	Texture of the culture (granular or butyrous)	2	2
	6	Emulsibility in distilled water	2	2
III	7	True branching	1^{+}	.
	8	Cording	2	2
	9	Shape and length of the bacilli	3	1 *a*
	10	Thickness of the bacilli	1^{+}	.
	11	Percentage of acid-fast organisms	3	1 *a*
	12	Depth of staining	1^{+}	.
IV	13	Growth rate on LJ medium at 37°	3^{++}	1 *b*
	14–18	Growth rate on LJ medium at 25° and 45° and on glycerol agar medium at 25°, 37°, and 45°	$4(\times 5) = 20$	1 *b*
V	19	Pigmentation in the dark	3	3
	20	Pigmentation in the light	1^{+}	.
VI	21–23	INH-, PAS-, and TB_1-resistance	$3(\times 3) = 9$	1 *a*
VI	24–26	Streptomycin-, cycloserine-, and ethionamide-resistance	$2(\times 3) = 6$	1 *a*
VII	27	Catalase activity	3	1 *a*
VII	28–35	Nicotinamide presence; nitrate reductase and 6 amidase activities	$2(\times 8) = 16$	1 *a*
VIII	36–46	Rate of utilization of alanine and 9 organic salts	$3(\times 10) = 30$	1 *b*
IX	47–60	Rate of fermentation of 14 sugars and higher alcohols	$3(\times 14) = 42$	1 *b*
Total	60 characters		153 features	

$^{+}$ Coded (+) or (−).

$^{++}$ The feature 'growth or no growth' was eliminated since all the strains grew on LJ medium at 37°.

Cluster analysis. From clusters having more than 15 strains and showing a $\%\ \overline{M} \geqslant 80$ we eliminated the features common to all the strains and proceeded to make a new comparison of the strains based on the remaining features (Hill *et al.* 1961).

RESULTS

General Survey

The grouping of the strains in accordance with the M indices is shown in Fig. 1. Five clusters were recognized at the 80-phenon level:

(I) A cluster of 19 strains (80P-I), including the human and bovine control strains (1–7 of Table 1) and 12 strains having some atypical 'key characters' (negative to the

Konno test, PAS-resistance, dysgonic growth, INH-resistance associated with positive catalase). The mean intragroup index, $\overline{M}$ %, was 85·7.

(II) A cluster of 45 strains (80P–II) with very inhomogenous pigmentation, including 12 control strains (8–19 of Table 1), i.e. 4 avian strains and 8 strains which were classified in England as belonging to the Ist, IVth, Vth and VIth groups of Marks & Richards (1962), and 33 newly isolated strains, 3 non-photochromogens and 30 scotochromogens. $\overline{M}$ % = 84·5.

In this last cluster there were 3 subgroups and 1 aberrant strain (174):

80P–IIA: 4 strains received from England included in the Ist group of Marks & Richards, with $\overline{M}$ % = 91·8;

80P–IIB: 29 newly isolated strains with orange-red pigmentation ($\overline{M}$ % = 88·2);

80P–IIC: 4 avian control strains, 4 strains received from England included in the IVth, Vth and VIth groups of Marks & Richards, and 3 newly isolated strains ($\overline{M}$ % = 85·8).

The strain 174, not comprised in these subgroups, is a scotochromogen too, but has some peculiar characters.

(III) A cluster of 28 strains (80P–III), including one control strain, *Mycobacterium fortuitum* (strain 20 of Table 1). The majority of the strains are without pigment and are rapid growers ($\overline{M}$ % = 80·1).

(IV) A cluster (80P–IV) of 3 newly isolated scotochromogenic strains, with are $\overline{M}$ % = 81·0.

(V) A cluster (80P–V) of 3 control strains belonging to *Mycobacterium smegmatis* (strains 22–24 of Table 1); $\overline{M}$ % = 90·3.

The five clusters comprise 98 strains. Nine strains could not be included: one control strain (*Mycobacterium phlei*—strain 21 of Table 1, MP in Fig. 1); 5 newly isolated acid-fast strains and 3 non-acid-fast strains.

The table below shows intra- and inter-cluster mean $\overline{M}$ % values:

		II						
	I	A	B	C	III	IV	*M. phlei*	V
I	**85·7**	.	.	.	.	.	.	.
IIA	79·5	**91·8**	.	.	.	.	.	.
IIB	72·7	80·5	**88·2**	.	.	.	.	.
IIC	73·4	78·6	82·5	**85·8**	.	.	.	.
III	56·9	62·1	65·9	67·7	**80·1**	.	.	.
IV	53·8	63·4	64·7	63·3	67·6	**81·0**	.	.
M. phlei	50·2	58·0	62·6	63·5	71·5	69·7	**100·0**	.
V	32·9	35·9	37·7	39·5	59·8	57·1	71·0	**90·3**

Thus 80P–I and 80P-II are representing together a 70–phenon, while *Mycobacterium fortuitum* group (80P-III) as well as 80P-IV and *M. phlei* are obviously different. The *M. smegmatis* group (80P-V) showed a very low similarity with the human, bovine, atypical and avian mycobacteria and a higher one with 80P-III and 80P-IV.

Further analysis of the clusters

The analysis was carried out as mentioned (see Methods), by recomparing the strains after removing the common features within each group and, when it was possible, by comparing them with the 'hypothetical average organism' (HAO) of the cluster. For rearrangements of the strains according to their similarities, Sneath's

technique (Sneath, 1962) was completed with Silvestri's (Silvestri *et al.* 1962). The characters of a cluster HAO were simply established by allotting to each feature the majority sign (+ or −) in all the strains of that cluster.

The removal of the common features has differentially reduced the number of variable features within each group. The decreasing of n_c (in numerators and denominators) lowers the value of M, and hence the differences between the strains are accentuated mainly for low values of M. At the same time, the percentage of common features is an indication of the homogeneity of the group.

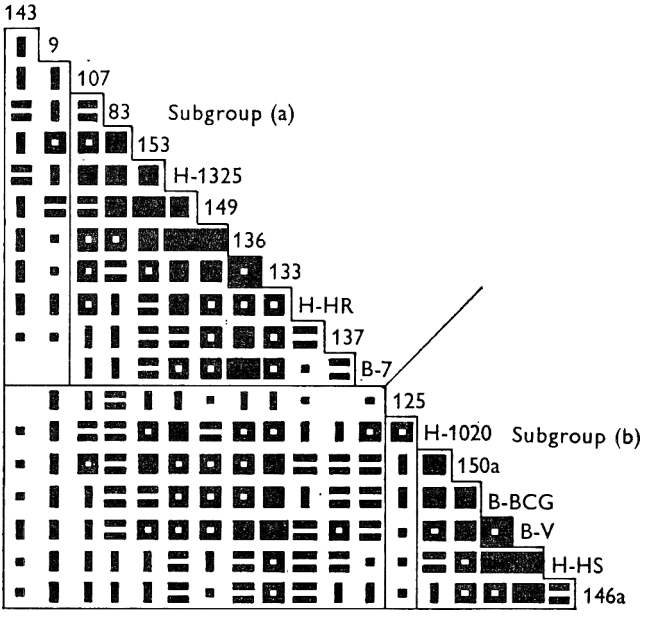

Fig. 2. % M value diagram of 80P–I group after removing the 95 common features, showing two subgroups and three 'aberrant' strains, nos. 143, 9 and 125. % M: ■, 95–100; ◘, 90–94·9; ▣, 85–89·9; ▣, 80–84·9; ▣, 75–79·9; ▣, 70–74·9; ◫, 60–69·9; ◘, 50–59·9; □, 0–49·9.

80P-I includes 19 strains, drug-sensitive or with acquired chemoresistance, grouped together with control strains of *Mycobacterium tuberculosis* of human and bovine type.

By comparing the 58 variable features, left after removing the common ones, two 'nuclei' became evident: (1) the strains 133 and 136; (2) the BCG and Vallée strains. By grouping the other strains around them, two subgroups ensued (Fig. 2). These subgroups do not differ from one another on account of their type (human or bovine) but mainly owing to differences in chemo-susceptibility. Indeed, subgroup *b* includes only strains susceptible to all the tested drugs, while in subgroup *a*, excepting strains 133 and *B*-7, all the others show different chemoresistance extents to one or another drug. The subgroups do not differ only in chemoresistance: in contrast with the strains of subgroup *b*, most of those belonging to *a* show dysgonic growth, a less granular texture and better emulsibility, a very slow growth rate on LJm, resistance to 10 μg. INH and they do not utilize acetate and do not ferment glucose.

80P-II. The obvious differences in the three subgroups (Fig. 1) require separate analyses.

In *80P-IIA*, after the elimination of the common features, only 19 variable features (out of 153) were left. The homogeneity of the 4 strains confirmed both their classification in group I of Marks & Richards (1962) and the species rank accorded to this group (*Mycobacterium kansasii*).

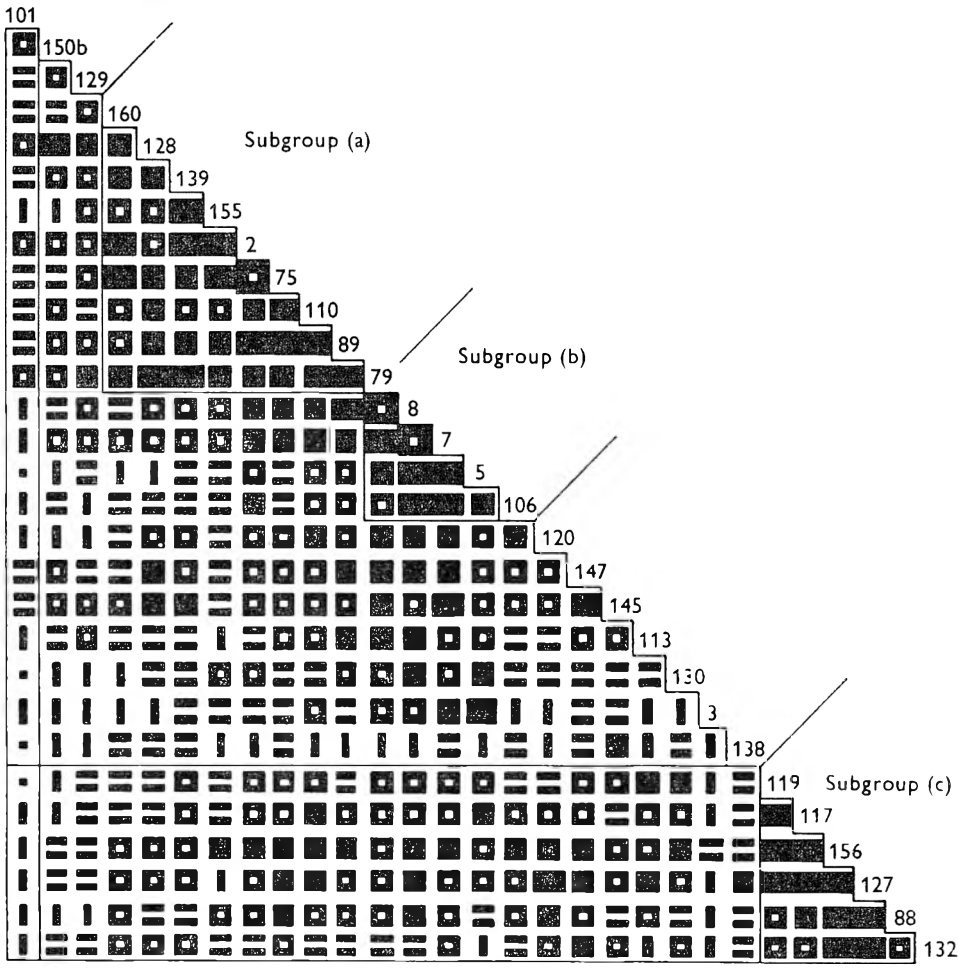

Fig. 3. % *M* value diagram of 80P–II B group after removing the 90 common features, showing three subgroups and an 'aberrant' strain, no. 101 (see Table 3). % *M*: ■, 95–100; ◘, 90–94·9; ◙, 85–89·9; ◙, 80–84·9; ◙, 75–79·9; ◙, 70–74·9; ◫, 60–69·9; ◫, 50–59·9; □, 0–49·9.

80P-IIB (29 newly isolated scotochromogenic strains). The review of similarities, based on 63 remaining variable features, has identified a subgroup of 6 strains (subgroup *c*). The remaining 23 strains make two other subgroups (subgroups *a* and *b* in Fig. 3).

The differences between the three subgroups (statistically estimated upon a majority of characters) appear in the extent of streptomycin, PAS and ethionamide resistance, in urease activity, in acetate utilization rate, in lactate utilization and maltose fermentation. The presence of a bright-orange pigment is a very constant character.

We established the characters of the HAO for the 80P-IIB group and compared them with each strain of the group (Table 3). Only strain 101 showed an *M* index of less than 75 % with the HAO. Thus, the group is homogenous, with a relatively low variability.

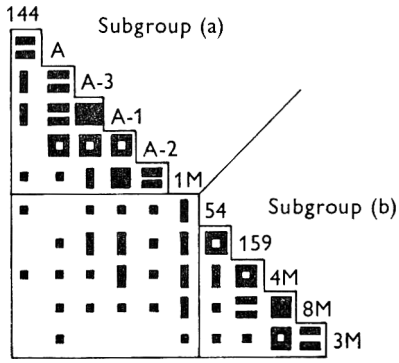

Fig. 4. % *M* value diagram of 80P–IIC group after removing the 106 common features, showing two subgroups. % *M*: ■, 95–100; ◘, 90–94·9; ▣, 85–89·9; ▣, 80–84·9; ▣, 75–79·9; ▣, 70–74·9; ▣, 60–69·9; ▣, 50–59·9; □, 0–49·9.

Table 3. *The % M values of the strains grouped in 80P-IIB with the 'hypothetical average organism' of the same group*

Strain	% *M*	Strain	% *M*
110	92	5	84
7	92	119	84
106	90	88	84
2	89	147	82
75	89	113	81
89	89	130	81
79	89	160	80
8	89	128	80
156	89	150*b*	79
139	87	3	79
120	87	132	79
117	87	138	76
127	86	129	75
155	84	101	69
145	84		

80P-IIC (11 strains). After the elimination of the common features, 47 variable features remained, and the comparison based upon them differentiated two subgroups (Fig. 4): one, *a*, included all the avian strains, the control strain 1 M (belonging to Marks & Richards IVth group, considered by these authors as including avian type strains) and one newly isolated strain; the other, *b*, included three control strains classified in the Vth and VIth Marks & Richards groups and two newly isolated ones. Only four strains in *a* and two strains in *b* grew on LJm at 45°.

The characters which clearly differentiated subgroup *a* are: a relatively slow growth (at least 15 days on LJm at 25° and 7 days at 37°), Th- and TB_1-resistance, a positive nitrate reductase test, and a more rapid lactate utilization (in most cases in 20 days).

80P-III (28 strains, including the control strain *Mycobacterium fortuitum*) showed

a greater variability. Six strains originated from three dissociated strains by mouse passage (39*a*, *b*; 114*a*, *b*; 148*a*, *b*). Only 53 features were common to all the strains of the group.

A comparison based upon the 100 variable features gave two subgroups differentiated by culture consistency and adherence to the medium, by the appearance or

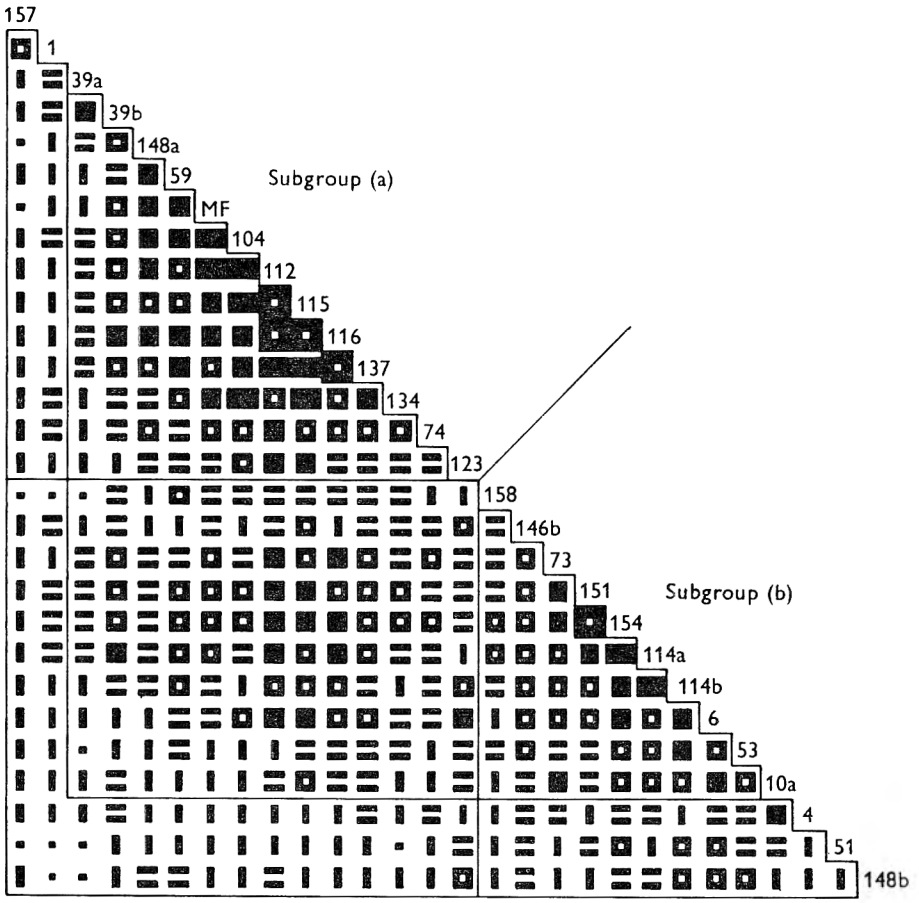

Fig. 5. % *M* value diagram of 80P–III group after removing the 53 common features, showing two subgroups and five 'aberrant' strains, nos. 157, 1, 4, 51 and 148*b* (see Table 4). % *M*: ■, 95–100; ■, 90–94·9; ■, 85–89·9; ■, 80–84·9; ▣, 75–79·9; ▣, 70–74·9; ▣, 60–69·9; ▣, 50–59·9; □, 0–49·9.

not of a faint yellowish pigmentation, by the growth rate at 25°, by ethionamide- and TB_1-resistance, by acetate and succinate utilization rate, and by glucose, mannose and trehalose fermentation rates (Fig. 5).

Some strains of the group are slow growers (157, 1, 39*a*, 39*b*, 10*a*, 4), and others did not grow on glycerol agar at 37° (1, 114*a*, 6). Strain 74 grew on LJm at 45°.

The peripheral strains of the subgroups showed relatively low similarities with the nuclei. The overall similarity analysis between the strains and the HAO of the group showed the deviation of these strains from this mean value (Table 4). If we consider

as a limit of the 'species' a 75 % *M* value against the HAO, the strains 157, 1, 4, 148*b* and 51 would not be included in the group.

80P-IV included only three newly isolated scotochromogenic strains, dull-orange pigmented (excepting the bright-orange pigmented strain 81), having short rods (less than 1 μ), intermediate growth rates, and greater metabolic abilities. Their number does not allow a more detailed statistical analysis.

80P-V is composed of three *Mycobacterium smegmatis* control strains with no newly isolated strains. Because of the low number of strains and the high $\overline{M}$ of the group, no statistical problems are raised.

Table 4. *The % M values of the strains grouped in 80P-III with the 'hypothetical average organism' of the same group*

Strain	% *M*	Strain	% *M*
112	91	74	81
151	91	148*a*	80
154	91	146*b*	80
115	89	10*a*	80
114*a*	89	39*a*	79
116	88	MF	79
39*b*	87	158	79
137	85	123	77
73	85	53	77
114*b*	84	1	74
104	83	4	74
134	83	148*b*	74
6	83	51	71
59	81	157	69

DISCUSSION

In order to achieve objectivity regarding numerical taxonomy, two conditions are supposed to be fulfilled: (1) the analysis of as many characters as possible, and (2) their comparison based upon a uniform coding method.

It is probable that, in our study, the addition of new mycobacterial characters would define more accurately the relative position of the strains. For instance, the impossibility of distinguishing by the tests used, in the presence of acquired chemoresistance, between the *Mycobacterium tuberculosis* strains and the *M. bovis* strains might raise discussion. Nevertheless, some classifications include both human and bovine tubercle bacilli in one species, *M. tuberculosis* (*Topley and Wilson's Principles*, 1964).

Since the 80P-I group, which includes weakly metabolizing and slowly growing strains, is shown mainly on the basis of negative features, further study using more characters is indicated.

As to the second condition, there have been several suggestions. Thus, the inclusion of negative matches as similarities (Hill *et al.* 1961; Silvestri *et al.* 1962) is considered by Sneath (1962) as distorting the results if the organisms are very diverse. Of course, if the inclusion of negative features increases the similarity percentages, then excluding them will decrease the number of comparisons— a great statistical disadvantage. Moreover, as Beers & Lockhart (1962) showed, the negative score (−) has in many cases a conventional value, not meaning the lack of a definite character but the opposite of a character marked with (+) (for instance, drug resistance *versus* susceptibility).

Sneath (1962) suggests care when comparing negative features, since, for instance, the inability of a given strain to ferment glucose will bring about its inability to ferment any other sugar. This was seen in most cases in our data, but it has underlined the similarity between strains with extremely weak metabolic capacity, increasing the human and bovine group up to 80P value, i.e. to a comparable similarity level with the other groups. It does not seem logical to consider the *Mycobacterium smegmatis* group have a higher similarity level than the *M. tuberculosis* group solely because the comparisons consider only the features marked (+). Moreover, further analysis of the group, involving the elimination of the common features, removes this 'similarity excess'. It must be mentioned that an attempt made to compare the 80P-I strains solely on the basis of the (+) features did not resolve the problems discussed above.

Inclusion of the (−) features leads to an alignment with all the samples of the genus *Mycobacterium* we had at our disposal arranged along a gradient of fermentative ability, beginning with *M. tuberculosis* (human and bovine types) and ending with *M. smegmatis*.

Finally, using the same argument as Beers & Lockhart (1962), if one considers that inability to ferment sugars is not due to the lack of genes but to the activity of repressors, theoretically the fermentation inability might be marked with (+), i.e. repressor activity, and the reverse with (−), repressor inactivity.

In subdividing the characters into features we preferred the 'additive codification' (Silvestri *et al.* 1962). Sneath (1962) shows that the non-additive method does not afford any information concerning the degree of difference between different characters. With regard to the genus *Mycobacterium*, not only the metabolic abilities scored as 'yes' or 'no', but also the rate of utilization of the substrate plays a prominent part in differentiation (Wayne, 1964).

We must still give the reasons that led us to use for some characters the coding method we have marked 1 *b* (Methods). It was of the following type:

	A	B	C	D
0 No growth in 28 days on X medium	−	−	NC	NC
1 Growth in 15–28 days	+	−	−	−
2 Growth in 8–14 days	+	+	−	−
3 Growth in 4–7 days	+	+	+	−
4 Growth in 1–3 days	+	+	+	+

Thus, the additive method was used only in comparing variants 1–4. In this way the matching indices (M) between the *0* variant and the variants *2–4* are equal ($M = 0\,\%$). On the other hand we did not score total incompatibility between variants *0* and *1*, since a strain that did not grow in 28 days is potentially still able to grow. Therefore, between a strain with behaviour *0* and a strain with behaviour *1*, M will be 50 %. $M_{0,1}$ will be hence equal to 50 % as well as $M_{1,3}$ or $M_{2,4}$, but having a lower 'weight', since $M_{0,1}$ is codified only by two features while $M_{1,3}$ or $M_{2,4}$ are codified by 4 features.

'Character weight' also introduces a subjective element in weighting of the characters. For instance, we were subjective in giving only one feature to photochromogenicity but three for the scotochromogenicity; we gave two features for streptomycin-, ethionamide-, and cycloserine-resistance but three for INH-, PAS-, and TB-resistance etc. (Table 2).

The pathogenicity of *Mycobacterium tuberculosis* led to the neglect for a long time

of the related species, thus distorting the objective biological study of the whole genus (Wayne, 1964). The increasingly frequent isolation of 'atypical' strains showed the need for more knowledge. Runyon's classification, based upon a few characters, was extremely useful for a rapid and rough identification. Attempts to improve it (Collins, 1962) or to achieve classification according to practical (medical) criteria (Kagramanov, 1963; Buraczewska, 1963) have been made. The tentative classification of Marks & Richards (1962) also aims at a practical purpose, in studying only potentially pathogenic strains. They emphasized that the Vth, VIth and VIIth groups were provisional, and should be studied by overall similarity analysis. The studies of Bönicke (1960), Cerbón & Trujillo (1963), Morellini *et al.* (1964) tried to cover the whole genus, but the differentiation of species was based on key characters.

By using overall similarity analysis the groups of strains fell into a definite order. The extreme position of *Mycobacterium smegmatis* was also pointed out by Bojalil *et al.* (1962) using the same method. *M. tuberculosis*, usually placed in the centre of the genus, becomes, when one considers interspecific relationships, a peripheral species which has peculiar characters because of its specialization.

We have already shown that the two subgroups in 80P-I are differentiated to a great extent by the chemoresistance of the strains, with which other differences are also associated. It seems logical to ask whether difference in pathogenicity, which is the main distinction between the two 'species' *Mycobacterium tuberculosis* and *M. bovis*, is any more important that the character of INH-resistance. The recent research of Cattaneo, De Ritis & Lucchesi (1965) has shown differences between the fatty-acid content of the INH-susceptible and INH-resistant tubercle bacilli.

As to *Mycobacterium kansasii*, the key character of photopigmentation might seem well chosen, but according to Bojalil *et al.* (1962). *M. marinum* also has this character and it is also found in *M. balnei*, *M. scrofulaceum*, *M. marianum*, and sometimes in *M. aquae* type I and *M. battaglini* (Morellini *et al.* 1964). Hauduroy, Hovanessian & Roussianos (1965) show that even in *M. kansasii* photochromogenicity is a secondary and unstable character, which should only be used for a first orientation. Consequently, a photochromogen does not necessarily belong to *M. kansasii* and a strain of this species is not necessarily a photochromogen. The high similarity values between 80P-IIA and 80P-I support the opinion of Weissfeiler, Karassova & Holland (1964) that *M. kansasii* has been derived by mutation from *M. tuberculosis*.

The 80P-IIB subgroups (Fig. 3) were distinguished neither by their amidase activities nor by their capacity to ferment mannose, by which Bojalil *et al.* (1962) distinguished *Mycobacterium marianum* from *M. gordonae*.

The probability that the Battey strains belong to *Mycobacterium avium* has been emphasized many times. Wayne & Doubek (1963), using overall similarity analysis, differentiated three subgroups in the IIIrd Runyon group, only one of which was potentially pathogenic and which perhaps belonged to the Battey-avian–porcine complex. Since the tests used by them are not the same as ours, we cannot compare them further. On our data the 80P–IIC group, although numerically scanty, could be split into two subgroups, but the key character of growth at 45° seems of little use for differentiating this complex of strains. Not all the strains in the 80P-III group possessed the key characters commonly used for the identification of Runyon's IVth group (rapid growth, growth on glycerol agar at 37°).

There is great similarity between the 80P-II subgroups (A, photochromogens

B, scotochromogens; C, avian complex of strains). The scotochromogenic mycobacteria are generally not pathogenic, but their isolation from human pathological products is very frequent, in contrast with the saprophytic mycobacteria (*Mycobacterium phlei*, *M. smegmatis*). Their relationship with the pathogenic groups (pointed out in recent work: Siebenmann & Barbara, 1964; Koyama, 1964; Kassirskaia & Mencikov, 1965) raises the question of close genetic connexions which would deserve more thorough examination. The scotochromogenic strains in the 80P-IV group could not be identified as belonging to any of the species of Bojalil *et al.* (1962) or Morellini *et al.* (1964). We do not wish to propose a new species for these strains. However our failure to identify them with any of Bojalil's or Morellini's species emphasizes that it is sometimes impossible to identify mycobacteria by key characters.

The strains which could not be grouped raise a similar question. Most likely they would represent unique strains belonging to certain definite groups (species), but they cannot be identified further in the present state of our knowledge. There are also strains that were initially clustered but which were subsequently eliminated by the second analysis. These aberrant strains might be considered either as variants arising as an effect of unknown agents, or as mixtures (either mixed cultures or genetic hybrids) belonging to different groups. The latter explanation is possible since we have five strains which dissociated on culture. These five strains raise genetic problems. Most of them are on the periphery of the group to which they belong: strains 146*a* (Fig. 2); 150*b* (Fig. 3); 39*a*, 39*b*, 146*b*, 10*a*, 148*b* (Fig. 5). They even occur outside any group (strain 10*b*, Fig. 1). The pairs with very similar partners, such as 39*a* and *b*, 114*a* and *b* (Fig. 5), differ by one or two obvious characters (pigmentation, consistency), and thus may give clues about the first steps in the variation of mycobacteria.

In conclusion, from the results obtained both by us and others, overall similarity analysis seems to be the best method, for the time being, for systematizing this group. By this method, both Bojalil *et al.* (1962) and ourselves were able to group mycobacteria according to their relative affinity. This work revealed a great similarity between the slowly growing 'atypical' mycobacteria, and moreover the present study has shown the close relationship between them and *Mycobacterium tuberculosis*. Our work also shows, however, the considerable variability of strains isolated from human pathological material and the difficulty of identifying them.

To produce an adequate taxonomic revision of this genus would require a comprehensive overall similarity analysis of a large number of pure strains (both from collections and newly isolated) from all over the world, and from all the environments where mycobacteria live. Standardized tests and coding methods would be needed. An international reference centre could establish on this basis the groups (phenons, species) and subgroups (phenons, varieties) as well as the characters of the corresponding 'hypothetical average organism' or 'hypothetical median organism' (Liston *et al.* 1963). When comparing an unknown strain with these 'hypothetical organisms' it would be sufficient to use only those characters which proved to be significant as a result of a statistical estimation (Beers & Lockhart, 1962). Thus, newly isolated strains from various countries would be correctly placed by such comparisons. This would solve both the present points of controversy—the theoretical one regarding the classification and the practical one regarding the identification of mycobacterial strains.

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The Photo-assimilation of Acetate by *Pyrobotrys* (*Chlamydobotrys*) *stellata*

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SUMMARY

Alcohols, amino acids, organic acids and sugars were tested as carbon sources for the growth of *Pyrobotrys* (*Chlamydobotrys*) *stellata* in the light and dark. Growth was only recorded with acetate in the light. A carbon balance sheet of ^{14}C-acetate assimilation showed a greater incorporation of ^{14}C into polysaccharide and less released as $^{14}CO_2$ in the light, compared with the dark. The primary products of ^{14}C-acetate assimilation into the soluble fraction of the organisms were identified; after 10 sec. 40 % of the total ^{14}C present in this fraction was in succinic acid, 15% in citric acid and 16 % in malic acid. The percentage of total ^{14}C in this fraction present in succinic and malic acids decreased consistently with time, while that in citric acid initially increased before decreasing. After 10 sec. the specific activity of succinic acid was more than twice that of citric acid. 4×10^{-3} M-monofluoroacetate (MFA) effectively inhibited the incorporation of ^{14}C-acetate into tricarboxylic acid cycle intermediates and related compounds, and markedly inhibited $^{14}CO_2$ evolution. 10^{-6} M-N^1-(3-4,dichlorophenyl)-*N*,*N*-dimethyl urea (DCMU) did not significantly inhibit ^{14}C-metabolism. The key enzymes of the glyoxylate cycle, isocitrate lyase (E.C.4.1.3.1) and malate synthetase (E.C.4.1.3.2) were found to be present in *P. stellata*, and did not disappear in the absence of acetate, but even so growth was not recorded on acetate in the dark.

INTRODUCTION

Many algae will utilize acetate as a sole source of carbon for growth (Pringsheim, 1946). The most specialized type of algal acetate nutrition appears to be that shown by *Pyrobotrys* (*Chlamydobotrys*) *stellata*, which can only grow on acetate in the light and was unable to utilize any other carbon source for growth tested by Pringsheim & Wiessner (1960). The growth of *P. stellata* on acetate appears to differ in several important respects from the growth on acetate of other algae investigated, particularly *Chlorella pyrenoidosa* (Syrett, Merrett & Bocks, 1963; Syrett, Bocks & Merrett, 1964; Goulding & Merrett, 1966; Merrett & Goulding, 1967). *Chlorella pyrenoidosa* can assimilate and grow on acetate in the dark, growth being dependent on the induction of glyoxylate cycle enzymes (Syrett *et al.* 1963, 1964). Growth in the light is not dependent on the presence of glyoxylate cycle enzymes (Syrett *et al.* 1963; Goulding & Merrett, 1966). Unlike *C. pyrenoidosa* the glyoxylate cycle is implicated in the growth of *P. stellata* on acetate in the light (Wiessner & Kuhl, 1962), but *P. stellata* will not grow on acetate in the dark. Thus, the regulation of the glyoxylate cycle enzymes, isocitrate lyase (E.C.4.1.3.1) and malate synthetase (E.C.4.1.3.2) may be different in *P. stellata* from that in *C. pyrenoidosa*. The aerobic assimilation of

acetate in the light by *P. stellata* was unaffected by N^1-(3,4-dichlorophenyl)-N,N-dimethyl urea (DCMU) (Wiessner & Gaffron, 1964) a specific inhibitor of non-cyclic photophosphorylation (Losada & Arnon, 1963). In *C. pyrenoidosa* DCMU induced a pattern of acetate assimilation in the light similar to that in the dark (Goulding & Merrett, 1966). In the presence of DCMU, in the light, less acetate carbon was incorporated into the organisms, particularly into the lipids, polysaccharide and protein, and more was released as carbon dioxide, while isocitrate lyase and malate synthetase, normally repressed in the light, were induced (Goulding & Merrett, 1966). It would appear that these two algae differ in their enzyme complements and in their dependence on the light reactions of photosynthesis for acetate photo-assimilation. This being so we decided to investigate the products of aerobic acetate photo-assimilation in *P. stellata* although these have already been described for *C. pyrenoidosa* (Merrett & Goulding, 1967).

METHODS

Organism

Pyrobotrys stellata Korschikoff (L 10–1 e) (= *Chlamydobotrys stellata*) was obtained from the Algensammlung Pflanzenphysiolisches Institut, Göttingen, Germany. The organism was grown in the culture medium of Wiessner (1962), stock cultures being maintained in 20 ml. medium contained in cotton-wool plugged tubes kept at room temperature (20°) with a light intensity of 200 lumens/ft.^{2}.

Growth. Organisms for experimental use were grown in 125 ml. of medium in Dreschel bottles. They were inoculated with organisms from 2-day stock cultures to give a population density of 500-organisms/mm.^{3}. The cultures were grown at 25° ± 0·2° at a light intensity of 300 lumens/ft.^{2} and air containing 5 % (v/v) carbon dioxide was bubbled through the cultures at a rate of 4 l./hr. After 3 days organisms were harvested by centrifugation at 500 *g* for 10 min., washed twice and resuspended in 0·067 M-phosphate buffer (pH 6·7) to give a concentration equivalent to 8 mg. dry-wt organism/ml., the concentration being estimated turbidimetrically by the use of an appropriate calibration curve. For kinetic experiments organisms were washed and resuspended in distilled water to give a concentration equivalent of 4 mg. dry-wt organism/ml.

Pyrobotrys stellata normally utilizes acetate as the carbon source for growth in the light; other carbon compounds were tested to see whether they supported growth. Samples of growth medium (50 ml.) lacking acetate were dispensed into 250 ml. conical flasks and supplemented with carbohydrate to a final concentration 1 % (w/v) or alcohols, organic acids or amino acids to a final concentration 0·2 % (w/v). The media were adjusted to pH 6·5. The flasks were aseptically inoculated with organisms from stock cultures, the dry weight of the inoculum being estimated directly on separate samples. Cultures were kept in the light (intensity 200 lumens/ft.^{2}) or in the dark at 25° for 2 weeks, then the dry weight of organism estimated directly, and the viability determined by inoculating stock culture tubes.

Radiochemical experiments

Acetate assimilation by *Pyrobotrys stellata* was followed by using ^{14}C-acetate. A suspension of *P. stellata* was equilibrated in the light or dark for 1 hr in the main compartment of large photochemical Warburg Flasks (light intensity 1200 lumens/ft.^{2};

temperature 25°). ^{14}C-acetate was then tipped from the side arm. After 1 hr the organisms were killed either by the addition of 10 N-sulphuric acid through the side arm, or samples of suspension were placed in ice-cold centrifuge tubes which were rapidly centrifuged and the deposited organisms killed by immersion of the tubes in boiling water for 2 min. The preparation of fractions from these organisms and the determination of ^{14}C in the fractions was as described previously (Goulding & Merrett, 1966). $^{14}CO_2$ evolved was absorbed by hydroxide of Hyamine (Packard Inc., Wembley) in detachable centre wells. These were transferred to glass counting-vials containing 10 ml. of 0·5 % (w/v) 2,5-diphenyloxazole (PPO) + 0·03 % (w/v) 1,4-bis-2-(4-methyl-5-phenyloxazolyl)-benzene (POPOP) in toluene, mixed well and counted in a Tricarb Liquid Scintillation Spectrometer with automatic background subtraction, corrections being made for quenching. The amount of ^{14}C-acetate added in experiments was determined by persulphate combustion (Katz, Abraham & Baker, 1954).

In kinetic experiments 20 ml. of suspension containing organism equivalent 80 mg. dry-wt in distilled water were transferred to rapid sampling apparatus (Syrett *et al.* 1964) and maintained at 25° at a light intensity of 1200 lumens/ft.^{2}. After equilibration 30 μmoles sodium acetate were added, followed at zero time by 10 μmoles sodium-^{14}C-acetate containing 50 μc. Organisms were sampled at intervals, extracted with ethanol and water, labelled compounds separated by two-dimensional chromatography, located by radio-autography, compounds identified, and ^{14}C in each compound determined, all as described previously (Merrett & Goulding, 1967).

Preparation of cell-free extracts

Organisms were washed and resuspended in ice-cold 0·1 M-phosphate buffer pH 7·5 and passed through an ice-cold French pressure cell at 15,000 lb./in.^{2} (Milner, Lawrence & French, 1950). The extract was centrifuged at 500 *g* at 2° for 5 min., followed at 13,000 *g* for 20 min., and the supernatant fluid used for enzyme assays. The protein content was estimated by the method of Lowry, Rosebrough, Farr & Randall (1951), with bovine albumin as a standard.

Enzyme assays

Isocitrate lyase activity was estimated by the method of Kornberg & Madsen (1958) usually on cell-free enzyme preparations, but occasionally by using frozen whole-organism suspensions (Syrett *et al.* 1963).

Malate synthetase was determined by the method of Syrett *et al.* (1963).

Organic acids

Organic acids were eluted from chromatograms and estimated by fluorescence of their resorcinol derivative by the method of Frohman & Orten (1953). Fluorescence was measured with a Locarte Fluorimeter with LF/3 blue filter, standard calibration curves being prepared for each acid estimated.

RESULTS

Utilization of carbon compounds for growth

A wide range of carbon compounds were tested to see whether they supported growth of *Pyrobotrys stellata*; alcohols, organic acids, amino acids and carbohydrates

were tested. Of all the compounds tested only acetate supported significant growth in the light. Carbon dioxide and compounds closely related to acetate, such as ethanol, did not support growth. The mean generation time for *P. stellata* growing on acetate in the light at 25° was 12·3 hr, while the final yield of organism was 27,750/mm.^{3}.

The assimilation of ^{14}C-labelled acetate

Organisms were equilibrated in Warburg flasks in light or dark, then 10 μmoles ^{14}C-acetate containing 2 μc were added from the side arm. After metabolism of acetate had ceased (this was longer in the dark than in the light) organisms were sampled and distribution of ^{14}C in the various fractions determined (Table 1). When the organisms metabolized acetate in the dark far more acetate ^{14}C was released as CO_2 as compared with metabolism in the light (Table 1). The increased yield of $^{14}CO_2$ in the dark was correlated with the incorporation of less acetate into the acid hydrolysate (polysaccharide + nucleic acids) fraction (Table 1). Although there was some increase in ^{14}C incorporation into the ethanol fraction in the light (Table 1), it was not as great as with *Chlorella pyrenoidosa*, where the bulk of the acetate carbon is incorporated into lipid in the light (Syrett *et al.* 1964; Goulding & Merrett, 1966).

Table 1. *The recovery of ^{14}C added as ^{14}C-1-acetate or ^{14}C-2-acetate to Pyrobotrys stellata in the light and in dark*

For experimental details see materials and methods and text

	mμc carbon-14/8 mg. dry-wt cells			
	Light		Dark	
	^{14}C-1-acetate	^{14}C-2-acetate	^{14}C-1-acetate	^{14}C-2-acetate
$^{14}CO_2$	246·3 ± 7·9*	189·7 ± 2·1	1175·2 ± 10·2	930·4 ± 7·1
Supernatant	22·1 ± 1·2	21·0 ± 1·0	21·0 ± 0·2	25·0 ± 1·7
Water soluble	225·9 ± 8·9	352·0 ± 2·3	249·7 ± 3·7	423·5 ± 8·3
Ethanol soluble	279·6 ± 8·7	290·1 ± 6·1	117·2 ± 2·9	150·6 ± 7·1
Acid hydrolysate	841·9 ± 21·2	892·9 ± 11·7	123·4 ± 3·1	206·0 + 0·9
Residue	31·8 ± 2·7	48·6 ± 2·7	39·6 ± 2·8	53·2 ± 3·1
Total recovered	1747·6 ± 50·6	1793·4 ± 25·9	1726·1 ± 22·9	1788·7 ± 28·2
^{14}C-added in acetate (determined by combustion)	1902·8	1931·2	1902·8	1931·2
% Recovery	91·8	92·9	90·5	92·7

*Figures are the means of duplicate flasks.

Short-term products of ^{14}C-2-acetate assimilation

The results of an experiment investigating ^{14}C-2-acetate assimilation in the light are given in Fig. 1. In the earliest sample (10 sec.) 40 % of the ^{14}C-2-acetate assimilated into compounds present on the chromatogram was in succinic acid, 15 % in citric acid and 16 % in malic acid. The percentage of total ^{14}C on the chromatogram in succinic and malic acids decreased consistently with time, while there was a slight increase in the percentage of the total ^{14}C present in citric acid before a decrease (Fig. 1). The total counts/min. present in succinic acid was greater than that in citric acid and so was the specific activity (Table 2). Glutamic acid rapidly incorporated ^{14}C from ^{14}C-2-acetate, while aspartic acid, serine, glycine, alanine, glutamine and

threonine became labelled in later samples but not to the same extent as glutamic acid (Fig. 1).

A compound found in an investigation of ^{14}C-acetate assimilation by *Chlorella pyrenoidosa*, at present unidentified and recorded as compound *X* by Merrett & Goulding (1967), was also found in *Pyrobotrys stellata*, where it accounted for 8·4 % of total ^{14}C assimilated after 1800 sec. Glucose was the major product of acetate assimilation after 1800 sec. and contained 19 % of the total ^{14}C (Fig. 1).

Table 2. *The specific activity of acids of the tricarboxylic cycle during ^{14}C-2-acetate assimilation by Pyrobotrys stellata in the light*

For experimental conditions see legend to Fig. 1.

Time of sampling from addition of ^{14}C-acetate (sec.)	Counts/min incorporated			Concentration of acid mμmole/7·2 mg. dry-wt			Specific activity mμc/μmole acid		
	Succinic	Citric	Malic	Succinic	Citric	Malic	Succinic	Citric	Malic
10	1927	721	.	71·2	63·5	.	148·9	62·9	.
30	2398	1136	1002	72·9	67·7	62·9	181·0	91·5	87·4
60	2460	1544	1254	73·7	65·6	62·9	183·1	129·5	109·6
120	2508	1747	1270	76·3	68·8	64·5	180·8	139·5	108·5
300	2980	2593	1527	83·1	70·3	66·1	197·3	201·9	127·0
1800	2879	2625	3386	86·4	71·9	66·1	182·8	200·2	281·3

Table 3. *The effect of monofluoroacetate (MFA) on ^{14}C-acetate metabolism by Pyrobotrys stellata in the light*

Organisms were incubated in the light for 60 min., some in the presence of 4×10^{-3} M-MFA: 80 μmoles ^{14}C-1-acetate containing 2 μc were added and after 1 hr organisms were sampled and fractionated as in Methods. Values given are means of duplicate flasks.

	mμc ^{14}C/8 mg. dry-wt organisms	
	Control	4×10^{-3} M-MFA
$^{14}CO_2$ evolved	42·1	2·4
Supernatant fluid	7·8	11·3
Water soluble	14·1	4·3
Ethanol soluble	8·1	2·9
Acid hydrolysate	16·8	2·4
Residue	1·6	0·6
Total	90·5	23·9

The effect of inhibitors on the photoassimilation of acetate

The effect of monofluoroacetate (MFA). The addition of MFA may result in the accumulation of fluorocitrate and the inhibition of aconitase (Treble, Lamport & Peters, 1962) and also the inhibition of malate synthetase (Dixon, Kornberg & Lund, 1960). Thus, MFA inhibits respiration proceeding by the tricarboxylic acid cycle and the glyoxylate by-pass. The effect of MFA on acetate metabolism by *Pyrobotrys stellata* was investigated by incubating organisms with MFA for 60 min., then adding ^{14}C-acetate and sampling the organisms after a further 60 min. (Table 3). Inhibition of ^{14}C-2-acetate incorporation into the ethanol, water-soluble fraction and acid-hydrolysate fraction was particularly marked, and $^{14}CO_2$ evolution was decreased to a very low value.

The effect of MFA was also investigated by determining the rate of incorporation of ^{14}C-2-acetate into the soluble fraction of the organisms. After 10 sec. the pattern of acetate assimilation was similar to that found in the absence of the inhibitor except that glutamic acid was not labelled; after 10 sec. the total counts incorporated increased only slightly throughout the rest of the experiment (Fig. 2). The incorporation of ^{14}C into succinic, citric and malic acids was strongly inhibited, while other compounds labelled in the absence of the inhibitor (Fig. 1) did not incorporate ^{14}C in detectable amounts (Fig. 2).

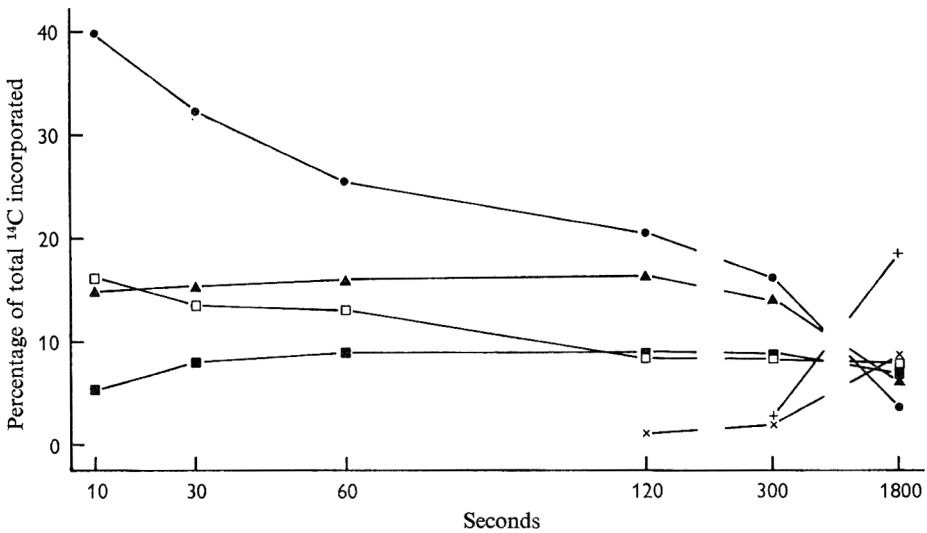

Fig. 1. Short-term products of ^{14}C-2-acetate assimilation in the light by *Pyrobotrys stellata*. Twenty ml. of organism suspension (equivalent 4 mg. dry-wt/ml.) were transferred to sampling apparatus, aerated and allowed to equilibrate in the light for 1 hr: 30 μmoles sodium acetate were added 10 min. before zero time, followed at zero time by 10 μmoles ^{14}C-2-acetate containing 50 μc. Organisms sampled, extracted and chromatographed and ^{14}C determined as in methods. Percentage ^{14}C in any one compound expressed as percentage of total ^{14}C present on the chromatogram. ●, Succinate; □, malate; ▲, citrate; ■, glutamate; ×, compound *X*; +, glucose.

Effect of N^1-(3,4-dichlorophenyl)-N,N-dimethyl urea (DCMU). DCMU is a powerful inhibitor of photosynthesis, inhibiting the generation of $NADPH_2$ and ATP by non-cyclic photo-phosphorylation (Losada & Arnon, 1963). Wiessner & Gaffron (1964) found that DCMU was without effect on aerobic acetate assimilation in the light by *Pyrobotrys stellata*, while Goulding & Merrett (1966) found that DCMU induced a dark pattern of acetate metabolism in the light in *Chlorella pyrenoidosa*, resulting in decreased lipid and polysaccharide formation and increased release of $^{14}CO_2$. The effect of DCMU on ^{14}C-2-acetate incorporation into various cell fractions of *P. stellata* (Table 4) confirmed the observation of Wiessner & Gaffron (1964) that acetate assimilation was unaffected by DCMU, and also showed that ^{14}C-2-acetate incorporation into other cell fractions was also unaffected by DCMU (Table 4).

Glyoxylate cycle enzymes in Pyrobotrys stellata

The nature of control of glyoxylate cycle enzymes in *Pyrobotrys stellata* has not hitherto been investigated. *P. stellata* organisms grown on acetate in the light were centrifuged down aseptically, resuspended in fresh medium and aerated in the presence of acetate in the dark, and in the light without acetate. The presence or absence of

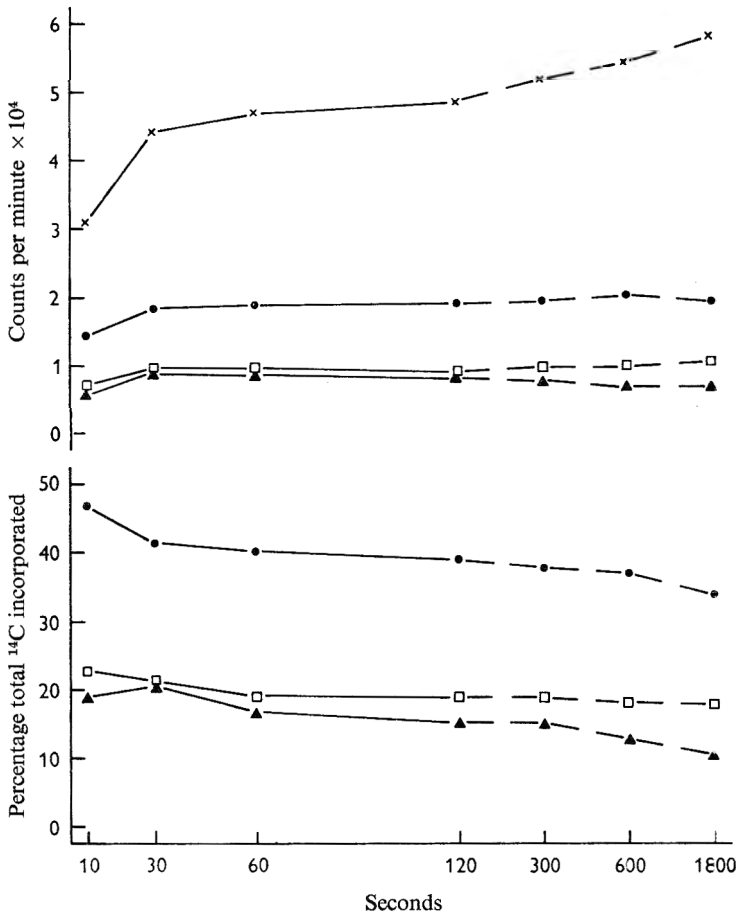

Fig. 2. Short-term products of ^{14}C-2-acetate assimilation by *Pyrobotrys stellata* in the light in the presence of 4×10^{-3} M-MFA. Twenty ml. of organism suspension (equiv. 4 mg. dry-wt/ml.) were transferred to sampling apparatus, aerated and allowed to equilibrate in the light for 1 hr in the presence of MFA to give final concentration 4×10^{-3} M: 30 μmoles sodium acetate were added 10 min. before zero time, followed at zero time by 10 μmoles ^{14}C-2-acetate containing 50 μc. Organisms sampled, extracted and chromatographed and ^{14}C determined as in methods. (*a*) Total counts min. per compound: × — ×, total counts/min. incorporated. (*b*) Percentage ^{14}C in any one compound expressed as percentage of total ^{14}C present on the chromatogram. ●, Succinate; ▲, citrate; □, malate.

CO_2 had no effect on the amounts of enzyme in the organisms. The results of these experiments (Fig. 3) suggest that malate synthetase and isocitrate lyase are constitutive in *P. stellata* but we were unable to test this experimentally because the organism will

not grow on any carbon source except acetate. Despite this, growth on acetate in the dark did not take place (as it does in *Chlorella pyrenoidosa* where these enzymes are induced when organisms are incubated with acetate in the dark; Syrett *et al.* 1963).

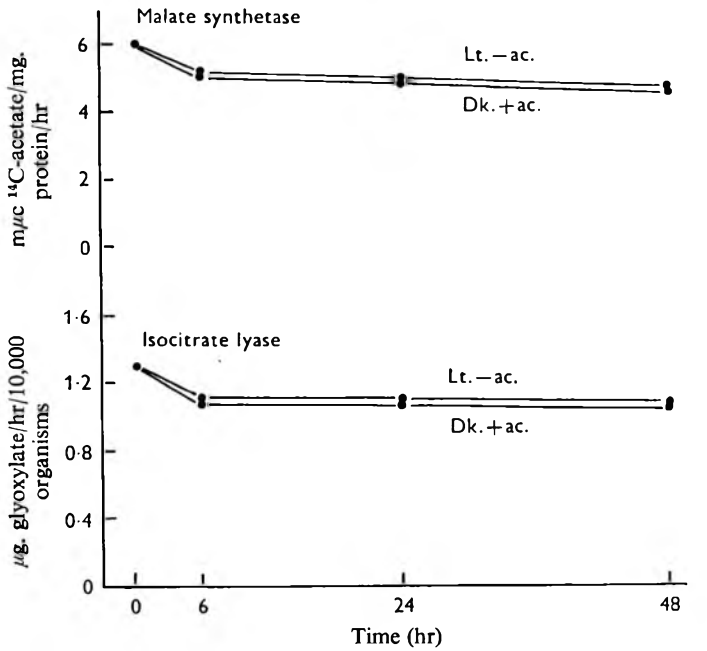

Fig. 3. Malate synthetase and isocitrate lyase in *Probotrys stellata*. Organisms were grown on acetate in the light, harvested aseptically and resuspended. (*a*) In acetate-free medium and incubated in the light; (*b*) in acetate-containing medium and incubated in the dark. Samples of organisms were removed at zero time and after 6, 24 and 48 hr incubation and assayed for enzyme activity.

Table 4. *The effect of 10^{-6} M-DCMU on ^{14}C-1-acetate assimilation by Pyrobotrys stellata in the light*

Organisms were allowed to equilibrate in the light in presence or absence of DCMU at time 0, 80 μmoles ^{14}C-1-acetate containing 2 μc added. Organisms sampled after 1 hr and fractionated, as in Methods.

	mμc ^{14}C/8 mg. dry wt. organisms	
	Control	10^{-6} M-DCMU
$^{14}CO_2$ evolved	54·7	69·6
Supernatant	5·8	4·1
Water extract	14·8	13·5
Ethanol extract	8·6	7·3
Acid hydrolysate	11·0	10·8
Cell residue	0·6	1·0
Total	95·5	106·3

DISCUSSION

The incorporation of ^{14}C-acetate into the soluble fraction of the cells of *Pyrobotrys stellata* resembles ^{14}C-acetate assimilation in *Chlorella pyrenoidosa* in that succinate becomes rapidly labelled with ^{14}C (Merrett & Goulding, 1967). After 10 sec. over 40 %

of the ^{14}C present in the *P. stellata* organisms was in succinic acid while only 15 % is in citric acid (Fig. 1). This result cannot be explained on the basis of a larger pool size of succinic acid trapping more ^{14}C passing through the tricarboxylic acid cycle, since initially the specific activity of succinic acid was greater than that of citric acid (Table 2). Moreover, in a kinetic-type experiment (as in Fig. 1) initially the greatest percentage of the total ^{14}C assimilated was in the primary products, and this decreased with time as ^{14}C enters other compounds. In several experiments we always obtained the result recorded in Fig. 1, where the slope of the graph for succinic and malic acids is decreasing while that of citric acid is increasing in the early samples. Cell-free extracts of *P. stellata* showed isocitrate lyase and malate synthetase activity and, assuming that these enzymes were functioning in the intact organism in the light, succinic acid could be formed from the breakdown of isocitric acid by isocitrate lyase. However, if succinic acid is formed in this manner it is difficult to see how the specific activity of succinate can be greater than that of citrate from which it was derived via isocitrate. The rapid increase in the specific activity of malic acid (Table 2) supports the view that malate synthetase was active in the intact *P. stellata* organisms, and malic acid was being formed from acetyl-CoA and glyoxylate. There was no evidence of malic formation from carboxylation reactions to this extent; $^{14}CO_2$ incorporation into the organisms was only 10 % of carbon-14 incorporation from ^{14}C-acetate. It also seems unlikely that succinate could be formed via malic acid, because initially the specific activity of succinate was greater than that of malic acid (Table 2). Monofluoroacetate effectively inhibited the incorporation of ^{14}C-acetate into tricarboxylic acid intermediates (Fig. 2) and blocked $^{14}CO_2$ evolution (Table 3). Furthermore, the total counts of radioactivity incorporated did not increase greatly during the experiment. Citrate did not accumulate and the formation of succinate was inhibited (Fig. 2). This was not so in *C. pyrenoidosa* when metabolizing ^{14}C-2-acetate in the light in the presence of MFA, where citrate did accumulate (Merrett & Goulding, 1967), and succinate formation was unaffected. It would appear that monofluoroacetate inhibits the uptake of acetate by *P. stellata*, which may indicate that this reaction is dependent on ATP formation resulting from respiration of some of the added acetate.

The effect of DCMU upon acetate assimilation in *Pyrobotrys stellata* was insignificant (Table 4) as compared with its effect on acetate assimilation in *Chlorella pyrenoidosa* where it stimulates $^{14}CO_2$ evolution sixfold (Goulding & Merrett, 1966). Thus, it appears that the role of light in acetate assimilation by *P. stellata* and by *C. pyrenoidosa* is somewhat different. Recent work on the two light reactions of photosynthesis has led to the majority view that they function in series in which the shorter wave system (system II) forms a strong oxidant leading to O_2 evolution, while the far red system (system I) forms a strong reductant that reduces NADP (Duysens, Amesz & Kamp, 1961; Hill & Bendall, 1960; Witt, Muller & Rumberg, 1961; Kok 1961). The photoassimilation of acetate by *C. pyrenoidosa* is dependent on system II and if this is blocked by DCMU, reversion to a dark metabolism of acetate occurs (Goulding & Merrett, 1966). Thus, it seems that an assimilation in light dependent on system I is not possible. *P. stellata* is a weak evolver of oxygen during photosynthesis and it seems probable that system II is deficient in it, while inhibitor experiments with DCMU confirm that acetate assimilation is dependent on system I. In contrast, under anaerobic conditions Wiessner & Gaffron (1964) found DCMU inhibits the photo-

assimilation of acetate in *P. stellata*, implying that under these conditions system II is functioning and may not be completely deficient. Wiessner (1965) showed the quantum requirement for acetate assimilation in far red light by *P. stellata* to be 8. System I is concerned with CO_2 reduction in higher plant photosynthesis, and it seems probable that *P. stellata* is unable to use this system for CO_2 reduction because of the absence of ribulose-1-5-diphosphate carboxylase from the organism (Merrett, 1967).

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Regulation of Amidase Synthesis by *Pseudomonas aeruginosa* 8602 in Continuous Culture

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SUMMARY

Pseudomonas aeruginosa 8602 was grown in continuous culture in minimal salts medium with 10 mM-succinate as carbon source. Changes in bacterial concentration and amidase activity were measured when the carbon source in the ingoing medium was changed to 10 mM-succinate plus 10 mM-acetamide. At low dilution rates ($D = 0{\cdot}2\ hr^{-1}$) amidase synthesis occurred rapidly with little or no lag. At higher dilution rates the lag increased until at the highest dilution rate tested ($D = 0{\cdot}76\ hr^{-1}$) almost no amidase had been synthesized 4 hr after the change of medium. It was concluded that at the highest dilution rates amidase synthesis was subject to catabolite repression by high intracellular concentrations of succinate and its metabolites. Severe repression of amidase synthesis was accompanied by incomplete utilization of acetamide for growth.

Periodic oscillations were observed in amidase activity and in bacterial concentration both during the transition period after the change of medium and when the culture had reached the new steady state. These oscillations occurred at all dilution rates and oscillations in amidase activity were also found when the non-substrate inducer *N*-acetylacetamide was supplied with 20 mM-succinate.

INTRODUCTION

Pseudomonas aeruginosa 8602 produces an aliphatic amidase (acylamide amidohydrolase E.C. 3.5.1.4) which is regulated by induction and catabolite repression. Cultures growing exponentially in succinate medium synthesize amidase at a constant differential rate after a lag of about one generation from the time of addition of substrate and non-substrate inducers. Amidase synthesis in pyruvate medium starts after a lag of only 0·25 of a generation and reaches a higher specific activity. The addition of 10 mM-succinate to exponentially growing cultures in pyruvate medium, induced by the non-substrate inducer *N*-acetylacetamide, results in almost complete repression of amidase synthesis (Brammar & Clarke, 1964; Brammar, Clarke & Skinner, 1967). Amidase induction by acetamide or *N*-acetylacetamide in carbon-starved bacteria is also repressed by succinate and the extent of repression is dependent on the relative concentrations of succinate and inducer. The effect does not appear to be due to direct competition between inducer and co-repressor of the type found

for repression of amidase synthesis by amide analogues such as cyanoacetamide (Clarke & Brammar, 1964).

The study of enzyme regulation during growth has been carried out almost entirely with organisms growing exponentially in batch culture. However, Gorini (1960) examined repression of the enzymes of the arginine biosynthetic pathway in continuous culture and showed that at certain dilution rates glutamic acid could relieve the repression, by arginine, of ornithine transcarbamylase synthesis (Gorini, 1963). McFall & Mandelstam (1963) studied catabolite repression of three inducible enzymes produced by *Escherichia coli*, β-galactosidase, serine deaminase and tryptophanase in both batch and continuous culture. In a different type of study, Tempest & Herbert (1965) examined the effect of dilution rate and carbon substrate on the metabolic activity of *Torula utilis*.

In batch culture, glucose represses the synthesis of certain enzymes so that the organisms do not utilize those substrates for growth until the glucose has almost disappeared from the medium. *Klebsiella aerogenes* exhibits typical diauxic growth in batch cultures containing glucose + maltose or glucose + lactose. The 'glucose effect' can also be observed in continuous culture in media containing these pairs of sugars. Growth, and the extent of utilization of the sugars, is related to the culture dilution rate (Baidya, Webb & Lilly, 1966; Harte, 1965). We considered that these results could be interpreted in terms of catabolite repression by glucose of the enzymes required to utilize the second sugar, and that this technique of growth on two different carbon compounds in continuous culture could be used to study enzyme regulation in other systems.

Pseudomonas aeruginosa has been grown in continuous culture in succinate medium at various dilution rates, and the rates of growth and amidase synthesis have been measured following a change of medium to succinate + acetamide. A brief report of part of this work has appeared previously (Boddy, Clarke & Lilly, 1966).

METHODS

Organism. The strain used was *Pseudomonas aeruginosa* 8602 (Brammar *et al.* 1967). It was maintained on slopes of lemco agar, subcultured weekly and stored at 4°.

Media. The minimal salts medium contained (g./l.): K_2HPO_4, 12·5; KH_2PO_4, 3·8; $(NH_4)_2SO_4$, 1·0; $MgSO_4.7H_2O$, 0·1; trace element solution, 5 ml. (Kelly & Clarke, 1962). Succinate (to give a final concentration of either 10 or 20 mM) was added to the medium in 20 l. Pyrex glass aspirators before autoclaving at 121° for 2 hr.

Acetamide and *N*-acetylacetamide were sterilized by Seitz filtration and added aseptically to the minimal salts + succinate media to give final concentrations of 20 mM and 10 mM respectively. The continuous culture experiments were all carried out under conditions in which growth was limited by the carbon source.

Culture equipment. An inoculum (70 ml.), grown on minimal salts + succinate medium, was blown from the seed vessel into a 5 l. vessel containing 3 l. of growth medium at a controlled temperature of 37°. The pH value of the medium was controlled at 7·2 by the addition of N-HCl. Air was supplied through the bottom of the vessel at 1 l./min. The carbon dioxide content of the effluent gas was measured by passing it through an infra-red analyser (type SBK, Infra-red Development Co. Ltd.). Foaming was prevented by the addition of polypropylene glycol (100 p.p.m.) to the medium.

During continuous operation the growth medium was fed from the 20 l. aspirator into the 5 l. vessel by a peristaltic pump constructed in this laboratory. Medium left the vessel by overflowing down a standpipe inserted through the bottom of the vessel. Samples (about 5 ml.) were withdrawn from the medium through a narrow-bore tube leading to a collecting bottle. The sample was diluted, chloramphenicol added, and then part used for determination of the bacterial concentration. The remainder was cooled rapidly in an ice-bath and assayed for amidase within a few minutes.

Dry weight of bacteria. The optical extinction of diluted bacterial suspensions were measured at 670 mμ with a Unicam SP 600 spectrophotometer. A standard curve was used to convert the readings to dry weights (mg./ml.).

Enzyme assays. Amidase was measured using the hydroxamate method for transferase activity described by Brammar & Clarke (1964). For samples with high enzyme activity, 0·1 ml. of the diluted bacterial suspension was used in each assay mixture which was incubated at 37° for 5 min. The amidase specific activities were calculated as μmoles acethydroxamate formed/mg. dry weight of bacteria/min.

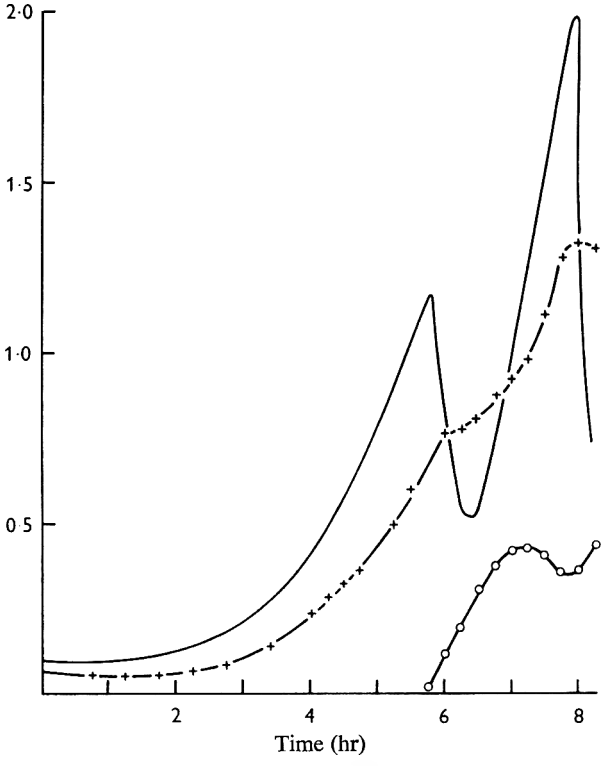

Fig. 1. Growth of *Pseudomonas aeruginosa* 8602 with 10 mM-succinate: +, bacterial concentration ($E_{670\,m\mu}$); ○, Amidase specific activity ($\times 10^{-1}$); —, carbon dioxide in exit gas (%).

RESULTS

Growth on succinate and acetamide in batch culture

Before starting the continuous culture experiments the growth of *Pseudomonas aeruginosa* 8602 in the 5 l. vessel was examined in batch culture in a medium containing 10 mM-succinate + 20 mM-acetamide (Fig. 1). The growth followed a typical diauxie

with succinate being consumed in the first growth phase. The presence of a diauxic growth lag was confirmed by the bimodal curve for the carbon dioxide in the effluent gas. Amidase synthesis began during the diauxic lag and was followed soon afterwards by renewed bacterial growth on the acetate which had been formed. At the end of the second growth phase the amidase specific activity had reached about 5.

Growth on succinate and acetamide in continuous culture

Growth on succinate and succinate + acetamide in continuous culture was examined at various dilution rates. Initially, the culture was allowed to reach a steady state on a medium containing 10 mM-succinate. The incoming medium then was changed to one containing 10 mM-succinate + 20 mM-acetamide which would allow the bacterial concentration to double if completely utilized. The synthesis of amidase and the change in bacterial concentration were followed during the shift to the new steady-state condition. The results of an experiment carried out at a low dilution rate (0·22 hr^{-1}) are shown in Fig. 2. At this dilution rate, amidase synthesis started very soon after the change to the succinate + acetamide medium. The amidase specific activity continued to increase over several hours but with peaks of higher activity at more or less regular intervals of 45 to 50 min. Smaller but nevertheless significant fluctuations were observed in the bacterial concentration as it rose towards its new steady state. Three identical experiments were done at this dilution rate and in each case the curves for amidase and bacterial concentration were similar. Further measurements were made on the following day, by which time both the amidase and the bacterial concentrations had reached new mean steady-state values and it was found that regular oscillations were still occurring. The maximum amidase specific activity was about 100.

The results of a similar experiment done at a higher dilution rate (0·48 hr^{-1}) are shown in Fig. 3. Again, oscillations were observed in both amidase and bacterial concentration and these continued unabated on the following day. The periodicity appeared to be about the same as at the lower dilution rate but the increase in amidase specific activity was much slower and the maximum value reached was only about 50 in the new steady state compared to the value of about 100 obtained at the lower dilution rate.

The effect of dilution rate on the rate of amidase synthesis during the transition period following the change to a medium containing succinate + acetamide is shown more clearly in Fig. 4. This gives the results for similar experiments done at four different dilution rates and it can be seen that amidase synthesis is severely repressed at the higher dilution rates. At the highest dilution rate investigated (0·76 hr^{-1}) the amidase activity was only very slightly above the basal level on succinate alone. It can be seen from Fig. 4 that oscillations in amidase activity occur at all these dilution rates although the specific activities differ widely.

Amidase induction by N-acetylacetamide in continuous culture

Comparable experiments to those already described were carried out using the non-substrate inducer, *N*-acetylacetamide. After allowing the culture to reach a steady state on a medium containing 10 mM-succinate the medium was changed to one containing 20 mM-succinate + 10 mM-*N*-acetylacetamide. The changes in amidase specific activities at various dilution rates are shown in Fig. 5. Once again it was found that amidase synthesis was repressed at the higher dilution rates. The specific activities

obtained with this non-substrate inducer were much lower than with acetamide. Periodic oscillations in amidase activity were less apparent but could certainly be detected in the curves for the two lower dilution rates (0·17 and 0·51 hr^{-1}).

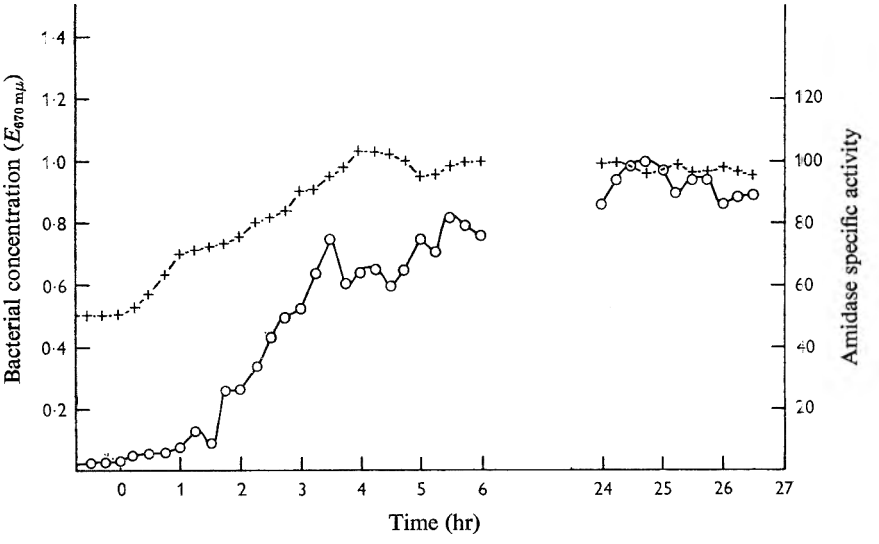

Fig. 2. Amidase synthesis by *Pseudomonas aeruginosa* 8602 in continuous culture (D = 0·22 hr^{-1}): +, bacterial concentration ($E_{670\,m\mu}$); ○, amidase specific activity. At zero time the ingoing medium was changed from 10 mM-succinate to 10 mM-succinate+20 mM-acetamide.

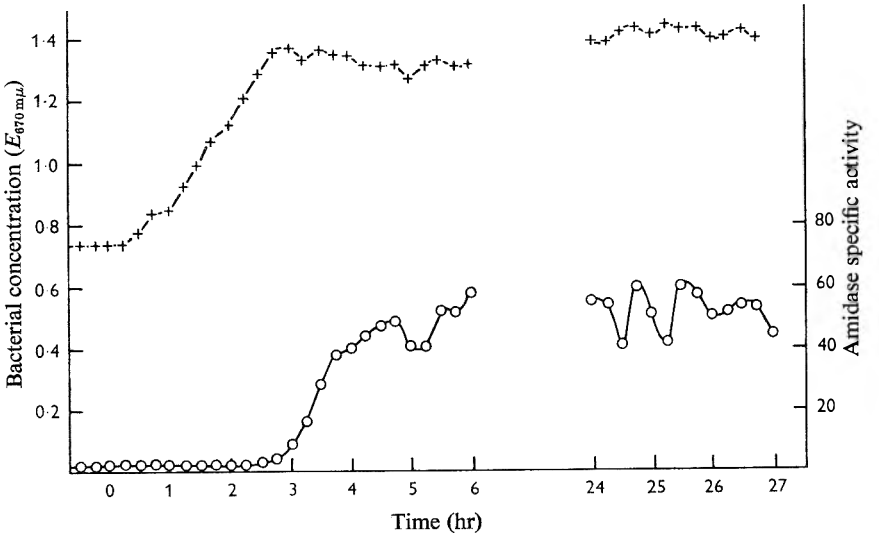

Fig. 3. Amidase synthesis by *Pseudomonas aeruginosa* 8602 in continuous culture (D = 0·48 hr^{-1}): +, bacterial concentration ($E_{670\,m\mu}$); ○, amidase specific activity. At zero time the ingoing medium was changed from 10 mM-succinate to 10 mM-succinate+20 mM-acetamide.

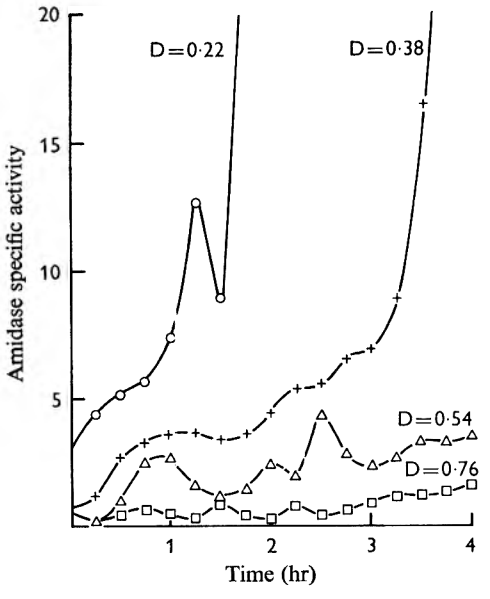

Fig. 4

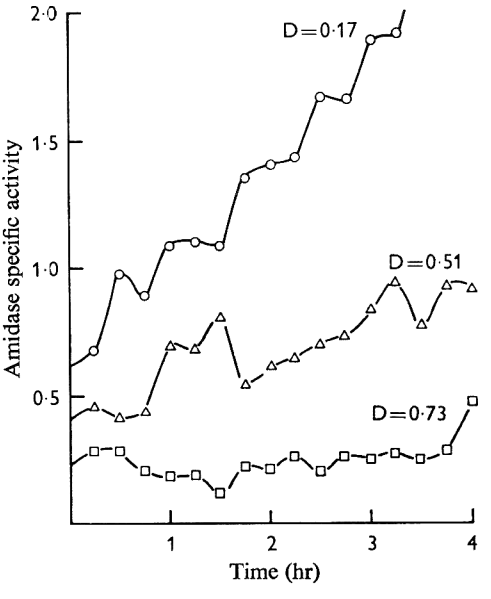

Fig. 5

Fig. 4. Effect of dilution rate, D (hr^{-1}), on amidase synthesis by *Pseudomonas aeruginosa* growing with succinate + acetamide. At zero time the ingoing medium was changed from 10 mM-succinate to 10 mM-succinate + 20 mM-acetamide.

Fig. 5. Effect of dilution rate, D (hr^{-1}), on amidase synthesis by *Pseudomonas aeruginosa* growing with succinate in the presence of the non-substrate inducer, *N*-acetylacetamide. At zero time the ingoing medium was changed from 10 mM-succinate to 20 mM-succinate + 10 mM-*N*-acetylacetamide.

DISCUSSION

The results in this study confirm that amidase induction in *Pseudomonas aeruginosa* 8602 is repressed by metabolites formed during succinate metabolism. In the batch-culture experiment amidase synthesis was completely repressed until the succinate had disappeared from the medium, and even then the maximum activity reached was not very high. This was partly because the inducer acetamide is rapidly hydrolysed by the newly formed enzyme and disappears from the medium. It had been shown previously that when acetamide is added to an exponentially growing culture amidase induction falls off as the acetamide is hydrolysed. It is also probable that amidase synthesis is repressed by intermediates of acetate metabolism, since it is known that acetate represses amidase synthesis in exponentially growing cultures induced with either acetamide or *N*-acetylacetamide (Brammar & Clarke, 1964). It is nevertheless possible to induce amidase synthesis under conditions in which succinate is still present in the medium as the major carbon source. The rates of amidase synthesis and the specific activities reached are much less in succinate medium than in pyruvate medium, both for induced and constitutive strains (Brammar *et al.* 1967). This suggests that succinate is a good source of the effective repressing metabolite and is similar to the observation that β-galactosidase in *Escherichia coli* is more readily induced in glycerol than in glucose medium (Loomis & Magasanik, 1964). Clarke & Brammar (1964) found that amidase could be induced in carbon-starved organisms from batch

culture by much lower concentrations of acetamide and *N*-acetylacetamide, with little or no lag. Under these conditions the intracellular concentration of intermediary metabolites would be expected to be minimal.

In continuous culture at low dilution rates the growth rate of the bacteria is correspondingly low. Growth is carbon-limited, intermediary metabolites are fully utilized and the internal carbon-pool depleted. When acetamide is introduced into the medium the conditions resemble those in carbon-starved batch culture, the conditions are favourable for amidase induction and the rate of synthesis will be regulated by the supply of inducer. The high amidase specific activity obtained under these conditions is similar to that obtained in batch culture in pyruvate medium. The specific activity of the pure enzyme is about 2000 (P. R. Brown & P. H. Clarke, unpublished) and at the low dilution rates on succinate + acetamide medium the amidase constitutes about 5 % of the dry weight of the bacterial cells.

At higher dilution rates and correspondingly higher growth rates, the pool of intermediary metabolites is less depleted. The balance between inducer and catabolite co-repressor is shifted in favour of repression, until at the highest dilution rate examined amidase synthesis is almost completely repressed. Under these severely repressing conditions the amount of amidase formed is insufficient to hydrolyze all the incoming acetamide, so that it cannot be fully utilized for bacterial growth. The catabolite repression by succinate appears to be partially overcome after a certain period of time. During the change from one steady state on succinate to the new steady state on succinate + acetamide the bacterial growth rate increases, resulting in a higher level of catabolite co-repressor and a low rate of amidase synthesis. As the new steady state is approached the growth rate gradually returns to its original level with a corresponding reduction in the concentration of intermediary metabolites and a decrease in repression of amidase synthesis. It is therefore a period in which there is an adjustment in the relative concentrations of intermediary metabolites. It has been suggested by Moses & Prevost (1966) that metabolic rearrangements occur during the transient catabolite repression of β-galactosidase synthesis in *Escherichia coli*.

In batch culture, acetamide is less effective as an inducer than *N*-acetylacetamide because it is consumed as soon as an appreciable amount of amidase has been formed. In carbon-starved bacteria from batch culture, and in continuous culture where the organisms are being continually presented with new acetamide, the reverse is the case. When amidase synthesis in continuous culture was examined under conditions of gratuity with *N*-acetylacetamide as non-substrate inducer, although the inducer concentration was rising faster at the higher dilution rates, the increase in the concentration of intermediary metabolites prevented induction. It was not possible to do prolonged experiments in continuous culture with *N*-acetylacetamide, as this compound is not sufficiently stable, so all these experiments were terminated after 4 hr.

A further effect observed at all dilution rates was the periodic oscillation of amidase activity in the culture accompanied by a less marked oscillation in bacterial concentration. Although the possibility that these oscillations were caused by physical factors cannot be entirely ruled out, we have carried out exhaustive tests on such parameters as mixing, pH and temperature control and are satisfied that these oscillations are not artifacts of the culture system. Certainly, in other series of experiments in the same type of culture vessel, we have not observed fluctuations in bacterial concentration to the extent found in these experiments.

These fluctuations suggest that there may be some degree of cell synchrony in these experiments. It is difficult to measure accurately the periodicity of the oscillations in bacterial concentration but it appears to be slightly longer than that for amidase concentration. We believe that this effect on bacterial concentration is a reflexion of oscillations in the concentration of a growth-limiting enzyme or enzyme systems. The oscillations in amidase concentration should only reflect changes in the concentration of the inducer or catabolite co-repressor and need not be in phase with or have the same periodicity as the changes in bacterial concentration. With acetamide as the inducer it was not possible to decide whether the changes in enzyme concentration were the result of variations in the level of the inducer or the catabolite co-repressor or both. These oscillations were still present when the non-substrate inducer *N*-acetylacetamide was used, so that it is unlikely that the oscillations observed with acetamide are the result of changes in the concentration of the substrate inducer caused by varying rates of acetamide hydrolysis.

It was quite possible that the oscillations in amidase concentration observed during the transition to the new steady state were caused by the sudden change in the incoming medium. However, these oscillations were still occurring to about the same extent on the following day, indicating that the system was probably undamped and likely to continue indefinitely.

This periodic oscillation of amidase activity is of considerable interest in view of the theoretical predictions by Goodwin (1963, 1966) that oscillations in enzyme synthesis could occur in systems controlled by feedback mechanisms and might be found in continuous culture. Oscillations in the extracellular pyruvate concentration have been observed by Sikyta & Slezak (1965) for continuous cultures of *E. coli* growing on lactate and this is probably a reflexion of changes in enzyme levels in the cells. Our results demonstrate (in continuous culture) the dynamic situation which exists between the induction of an enzyme by its substrate analogues or substrate and its repression by related metabolites. It seems likely that the observed oscillations are a further expression of the catabolite repression which exists in this system. The problem as to why there should be this metabolic synchrony in a bacterial population remains to be solved.

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Effect of Dodine Acetate on the Electrophoretic Mobility of *Neurospora crassa* Conidia

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SUMMARY

The electrokinetic behaviour of intact conidia and cell walls of *Neurospora crassa* was studied using a micro-electrophoresis technique. By chemical and enzyme treatments it has been established that amino, carboxyl and phosphate groups are integral components of the spore surface; acid phosphate groups, however, were not found on the surface of washed cell walls. The fungicide dodine acetate reduced the negative charge on conidia to zero and, with increasing concentration, gave a positive charge to the spores: at lower fungicide concentrations the negative charge on the surface of cell walls and stabilized protoplasts was also neutralized. These results are consistent with an ionic reaction between the dodine cation and the carboxyl and phosphate groups of the cell. There was no evidence that the toxic reaction between dodine acetate and *N. crassa* conidia is located on the spore surface—the spores were completely killed before there was a perceptible reduction in electrophoretic mobility.

INTRODUCTION

Although the electrophoretic properties of bacteria and erythrocytes have been extensively investigated, little attention has been given to fungi, except for yeasts. The only studies on fungal spores are those of Douglas, Collins & Parkinson (1959) and Hannan (1961). Their work has shown that spores, in common with other micro-organisms, bear a negative charge over a wide pH range of suspending medium, the nature of the ionogenic groups on the spore surface varying with the fungal species. The electrokinetic behaviour of fungal spores deserves study not only to determine the nature of the ionized, or ionizable, surface groups but also because it is with these groups that the initial reaction with a fungicide—which may be an ionic species—occur.

The surface-active agents toxic to micro-organisms are those which are ionic in character and their reaction at the cell surface can be followed very conveniently by micro-electrophoretic techniques—as has been shown by work on bacteria (James, 1965). Dodine acetate (*n*-dodecylguanidine acetate) is a well-established agricultural fungicide: chemically, it has the structure of a cationic surface-active agent and its accumulation by fungal conidia follows, in general, an ionic bonding pattern (Somers & Pring, 1966). The interaction between dodine acetate and the spore surface has been investigated by the micro-electrophoresis of the intact conidia, protoplasts, and cell walls of *Neurospora crassa*. Particular attention has been given to the nature of the charged groups present on the spore surface with which dodine acetate reacts.

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METHODS

Fungus

Conidia from 7-day cultures of *Neurospora crassa*, macroconidial wild-type Em 5297a, were washed and harvested as previously described (Richmond & Somers, 1962). Cell walls were prepared by shaking dense spore suspensions with an equal volume of ballotini (no. 12) in a Mickle disintegrator, kept at 4°, for 10 min. The centrifuged walls, free of whole cells, were washed 10 times with 10 % (w/v) sucrose, 5 times with 0·9 % (w/v) NaCl, and 5 times with water, following the technique of Dyke (1964). After disruption an appreciable proportion of the cell walls still retained the almost complete shape of the intact conidia and these walls were used for micro-electrophoresis. Total lipid was determined by solvent extraction of dried cell walls (*in vacuo* over phosphorus pentoxide) with hot chloroform+methanol (2+1, v/v) followed by hot ether.

Protoplasts were prepared by incubating young hyphae of *Neurospora crassa* with a *Helix pomatia* digestive-juice extract (L'industrie Biologique Française, Gennevilliers, France) as in the method of Kinsky (1962). The only amendment was that the final protoplast suspension was washed and stabilized in 0·58 M-sucrose maintained at pH 5·6 with sodium acetate buffer of ionic strength 0·05: at 4° the protoplasts were stable for up to 2 days in this medium.

Dodine acetate

A sample, m.p. 134–135°, was prepared by ethanol–ether recrystallization from technical-grade material. Stock solutions made up in ethanol were diluted in the experimental aqueous solutions so that the final ethanol concentration was less than 2 % (v/v). Radioactive dodine acetate, of specific activity 6 μC/mg., labelled in the guanidine carbon with ^{14}C, was generously supplied by American Cyanamid Co. Chemical analysis and radioassay of the dodine cation were carried out as before (Somers & Pring, 1966).

Micro-electrophoresis

The electrophoretic mobilities of conidia, cell walls, and protoplasts—all usually at a concentration of 1 million/ml.—were measured in a rectangular closed cell, air-thermostatted at 25·0 ± 0·2°, whose construction and operation is described by Gittens & James (1960). The cell was mounted in the lateral position of Hartman, Bateman & Lauffer (1952) and the symmetry checked. All subsequent measurements were made at the nearer stationary layer: human erythrocytes in 0·067 M-phosphate buffer (pH 7·35) were used to calibrate the cell (Gittens & James, 1960). The conductivity of the buffered suspensions was measured at 25°, on a Wayne-Kerr B221 bridge. Movement was usually timed over 180 μ in both directions (current reversal) and each mean mobility was obtained from at least 20 observations: the standard error of mean was less than 4 %.

Conidia, cell walls, and protoplasts were washed twice with the appropriate buffer before adding to the electrophoresis cell. All buffer solutions were used at a final ionic strength (I) of 0·05. The following buffer solutions were used for pH/mobility curves (Gittens & James, 1963): below pH 2·6, HCl and NaCl; pH 2·6–9·6, NaCl+sodium acetate+sodium barbiturate+HCl; above pH 9·6, NaCl+sodium acetate+

sodium barbiturate + NaOH. All experiments with dodine acetate were carried out in sodium acetate buffer, pH 5·6: dodine acetate was added to the buffered suspensions and electrophoretic observations made within 5 min.

Treatments to modify the surface groups of Neurospora crassa

Phosphatase. Washed conidia and cell walls were suspended in barbiturate buffer (pH 7·9, *I* 0·02) containing 0·0005 % (w/v) alkaline phosphatase (E.C. 3.1.3.1, British Drug Houses Ltd.) at 37° for 1 hr (Hill, James & Maxted, 1963), then centrifuged and washed twice in the final buffer solution.

1-Fluoro-2,4-dinitrobenzene (FDNB). Conidia were washed 3 times in Sorensen phosphate buffer (pH 7·0, *I* 0·05), then suspended in a 0·1 % (v/v) ethanolic FDNB solution containing 0·9 % (w/v) sodium bicarbonate (Gittens & James, 1963). The suspension was agitated for 5 hr at 25° and the conidia subsequently washed 5 times with ethanol before washing with the final buffer solution. The acid hydrolysis, with constant-boiling HCl, of the FDNB-treated conidia and subsequent paper chromatography of the hydrolysates followed the methods of Ingram & Salton (1957).

Diazomethane. Washed cell walls were dried *in vacuo* over phosphorous pentoxide and methylated with diazomethane as described by Best & Durham (1965): diazomethane was prepared from *N*-methyl-*N*-nitroso-*p*-toluenesulphonamide (De Boer & Backer, 1954). Methylated and control cell walls were incorporated into KBr discs and the infra-red spectra recorded on a Perkin Elmer 237 spectrophotometer.

Fungitoxicity of dodine acetate

Fungicidal activity was determined by the method previously described (Richmond & Somers, 1962). The spores were incubated with various concentrations of dodine acetate for 30 min. at 25°, washed twice, then diluted to 50,000/ml. in 0·02 % (w/v) sucrose, and germination counts made after 18 hr incubation in a moist chamber at 25°.

RESULTS

Nature of the surface charges on Neurospora crassa conidia

Many workers have shown that the changes in electrophoretic mobility of microorganisms with pH, measured in buffers of constant ionic strength, can be interpreted in terms of the ionogenic groups present at the cell surface (see James, 1965). In the light of these earlier investigations the form of the mobility–pH curve for *Neurospora crassa* conidia (Fig. 1) suggests the presence of strongly acidic groups and free amino groups (which give rise to the point of inflection at pH 10). Phosphate groups are indicated by a pK as low as 2·6 and confirmed by the displacement of the mobility–pH curve when conidia are treated with alkaline phosphatase (Fig. 1). The remaining negative groups after this treatment (pK 4) are probably carboxyl.

There was no evidence, however, that phosphate groups of pK 2–3 were present on the surface of washed cell walls (Fig. 2) and treatment of the walls with alkaline phosphatase did not alter the pK from 4. The mobility–pH plot in Fig. 2 shows that the amino groups—presumably from surface protein—were unaffected by the washing treatments. The total lipid content of the cell walls was 14 %, on a dry weight basis. Electrophoretic measurements on cell walls in the presence of the anionic sodium

dodecyl sulphate (SDS) showed that some of this lipid was situated on the cell surface. In acetate buffer (pH 5·6, I 0·05) the mobility of cell walls increased from −0·69 μ/sec./V./cm. to −1·23, −1·59 and −3·64 μ/sec./V./cm. in the presence of 10^{-5}, 10^{-4} and 10^{-3} SDS, respectively.

The mobility towards the anode of *Neurospora crassa* conidia increased after treatment with FDNB, which is consistent with the removal of $-NH_3^+$ groups (Fig. 1). The hydrolysis of FDNB-treated conidia followed by paper chromatographic analysis to identify the N-terminal groups showed only two spots, corresponding to the DNP-derivatives of ϵ-lysine and histidine.

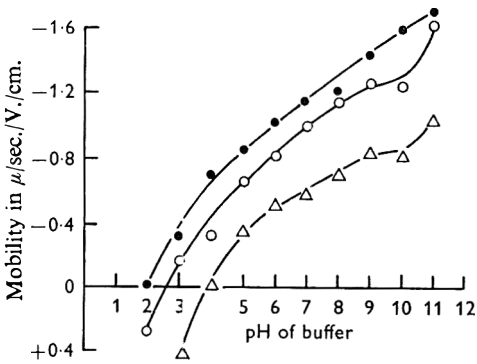

Fig. 1

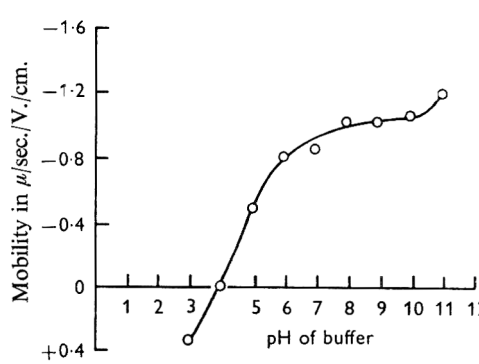

Fig. 2

Fig. 1. Electrophoretic mobility–pH curves for *Neurospora crassa* conidia. ○, Normal conidia; △, conidia treated with phosphatase; ●, conidia treated with FDNB.

Fig. 2. Electrophoretic mobility–pH curve for *Neurospora crassa* cell walls.

Ionized carboxyl groups commonly make a major contribution to the negative electrophoretic mobility of micro-organisms (Douglas, 1959; James, 1965) and their presence at the surface of intact conidia and isolated cell walls has already been presumed from the results following phosphatase treatment. Confirmation has come from the infra-red spectrum of cell walls esterified with diazomethane. Figure 3 shows a pronounced shoulder at 1740 cm.$^{-1}$ for treated walls (curve A) due to the carbonyl stretch of the ester: no such absorption was shown by cell walls subjected to the same preparative treatment, i.e. stirred in methanol–ether for 3 hr, but omitting diazomethane (curve B). Similar results—although with a less pronounced shoulder—have been reported for the bacteria *Aerobacter aerogenes* (Gittens & James, 1963) and *Bacillus subtilis* cell walls (Best & Durham, 1965).

Effect of dodine acetate on Neurospora crassa mobility

Figure 4 shows the changes in electrophoretic mobility of intact conidia and cell walls of *Neurospora crassa* on treatment with various concentrations of dodine acetate at pH 5·6: the dry weight of cell walls, 8 μg./ml., corresponded to 1 million conidia/ml. The negative charge borne by the spores and cell walls was gradually reduced by the dodine cation until at 580 μM for spores and 210 μM for walls it was completely neutralized. At higher dodine acetate concentrations the spores became positively charged. Determinations of the fungicidal activity of dodine acetate showed

that spores, at 1 million/ml., were completely killed after 30 min. immersion in solutions of concentration above 17 μM, i.e. where no effect on the mobility of the spores was apparent; the ED50 was at 5 μM. The uptake of dodine acetate by *N. crassa* spores is known to be very rapid (Somers & Pring, 1966), so that the differences in duration between the fungicidal tests (i.e. 30 min.) and the electrophoretic observations (i.e. up to 5 min.) will not be significant.

When dodine acetate was added to *Neurospora crassa* protoplasts, stabilized in sucrose-acetate medium, there was a rapid reduction in electrophoretic mobility but no evidence of charge reversal (Fig. 5). Protoplasts are obviously much more sensitive to the fungicide than are conidia, for when conidia at the same concentration, i.e. 0·2 million/ml., were incubated with 0·4 μM-dodine acetate the mobility of the conidia was unaffected; in fact even at 4 μM no significant reduction in conidial mobility occurred. The fungicidal ED50 for spores at 0·2 million/ml. was 0·7 μM-dodine acetate.

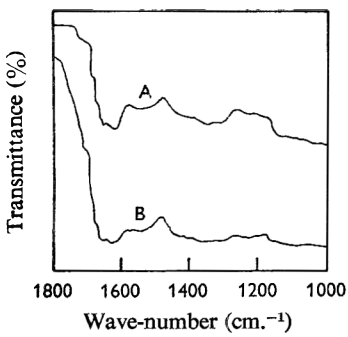

Fig. 3

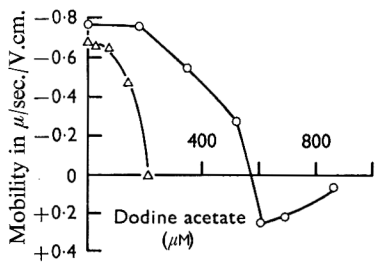

Fig. 4

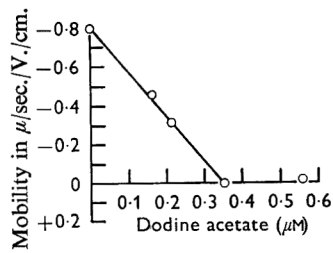

Fig. 5

Fig. 3. The infra-red spectra of *Neurospora crassa* cell walls. A, methylated with diazomethane; B, walls subjected to the same solvent and drying treatment as in A but diazomethane omitted.

Fig. 4. Effect of dodine acetate on the electrophoretic mobility of *Neurospora crassa* conidia and cell walls in acetate buffer (pH 5·6, I 0·05). ○, Conidia (1 million/ml.); △, cell walls (8 μg/ml.).

Fig. 5. Effect of dodine acetate on the electrophoretic mobility of *Neurospora crassa* protoplasts (0·2 million/ml.) in 0·58 M-sucrose buffered with acetate to pH 5·6 (I, 0·05).

In the absence of dodine acetate the mobility of protoplasts in the sucrose-acetate medium was $-0{\cdot}80\,\mu$/sec./V./cm., which corresponds to a mobility of $-1{\cdot}6\,\mu$/sec./V./cm. in buffer alone, correcting for the dielectric constant and assuming a simple inverse relationship between mobility and viscosity with other factors such as particle charge and size remaining constant. The protoplasts are, of course, instantly lysed in the absence of sucrose but this rough calculation indicates that protoplasts carry a greater negative charge than conidia at the same pH, i.e. $-0{\cdot}75\,\mu$/sec./V./cm. for conidia (Fig. 1). Preferential adsorption of sucrose on to the protoplasts, which could decrease the ionogenic area, does not seem likely for there was little difference between the mobilities of protoplasts determined in sucrose-acetate in which the sucrose concentration ranged from 0·3 to 1·2 M.

Excess dodine acetate was incubated with protoplasts in the sucrose-acetate medium and, after washing, the protoplasts were completely lysed by transferring to distilled

water. Table 1 shows that 30% of the accumulated dodine cation remained firmly bound to the protoplast membrane. It proved impossible to recover from the protoplast lysate more than a small fraction of the theoretical dodine cation content, presumably due to adsorption of the ion on the walls of the glass apparatus. Dodine cations accumulated by *Neurospora crassa* conidia have been found to be associated with cytoplasmic fractions of the cells (Somers & Pring, 1966) and it is probable that the cell fraction sedimented at 10,000 *g*—which had an appreciable dodine-binding capacity—contained cytoplasmic membrane fragments.

Table 1. *Binding of the dodine cation by Neurospora crassa protoplasts*

Protoplasts, at 7·2 million/ml., were incubated with 10 μM-^{14}C-labelled dodine acetate in 0·58 M-sucrose-acetate buffer (pH 5·6, I 0·05) for 30 min. at 25°, then centrifuged out at 1500 *g* and washed twice in sucrose acetate. The washed protoplasts were suspended in distilled water and the membranes separated by centrifugation at 15,000 *g*. The membranes were washed twice in water before radioassay.

Fraction	Dodine cation content (μmole/10^3 million protoplasts)
Washed intact protoplasts	0·17
Protoplast membranes	0·05

DISCUSSION

In principle the origin of surface charge on *Neurospora crassa* conidia may be due to fixed charges on the cell surface or to adsorbed ions. However, the chemical and enzymic treatments have established that amino, carboxyl, and phosphate groups are integral components of the spore surface and all contribute to the electrokinetic behaviour of the conidia. The surface amino groups have been identified as those of ϵ-lysine and histidine and presumably form part of the protein complexes found in fungal cell wall preparations (Aronson, 1965). Carboxyl groups could originate from the polysaccharide matrix common to the walls of all micro-organisms whilst phosphate groups may be associated with the lipid and nucleic acid components of the *N. crassa* spore wall. It is noteworthy, however, that no acid phosphate groups were found on the surface of purified cell wall preparations, suggesting that the phosphate groups on the surface of intact conidia are removed by the intensive washing used to prepare clean walls. Eddy & Rudin (1958), using a micro-electrophoresis technique, have found phosphate groups on the surface of both intact yeast cells and isolated walls, although it may be relevant that they washed cell walls with distilled water alone rather than with sucrose and NaCl as in the method of Dyke (1964). Certainly Harold & Miller (1961) have shown that the association of polyphosphate with the cell wall of *N. crassa* mycelium is an artifact of the fractionation procedure.

Dyar & Ordal (1946) and McQuillen (1950) have shown that cationic surface-active agents reduce the negative charge on bacteria and, with increasing concentration, completely neutralize the charge, finally reversing mobility to give a stabilized positive charge. Essentially the same pattern was observed for the interaction of dodine acetate with *Neurospora crassa* conidia. At pH 5·6 dodine acetate exists in solution as the positively charged dodine cation and this rapidly reacts with the surface carboxyl and phosphate groups of the spores: previous work has shown that the uptake of dodine cation by spores increases with increasing ionization of these groups (Somers & Pring, 1966). The charge on the spores is probably not completely neutral-

ized until they are saturated with the fungicide. After neutralization dodine cations can be held by van der Waals forces to surface lipid or the hydrocarbon chains of fixed dodine molecules so that a positive charge builds up on the spores. The decrease in positive charge at dodine acetate concentrations above 650 μM (Fig. 4) supports a suggestion of McQuillen (1950) that concentric rings of surface-active agent are built up round the cell with polar groups facing alternately inward and outward.

The saturation level of dodine acetate bound by cell walls of *Neurospora crassa* has been shown to be some 16 % of that retained by intact conidia (Somers & Pring, 1966), hence the charges on cell walls are neutralized by much lower supernatant concentrations than are required for the conidia (Fig. 4). This earlier work also showed the fungicide to be strongly bound to cell walls and it is possible that the dodine molecules are incorporated, via their hydrocarbon group, within the lipid matrix of the cell wall. A wall lipid content of 14 % is fairly high compared with the published values for yeasts and fungi (Phaff, 1963; Aronson, 1965) and the unwettable nature of *N. crassa* conidia suggests that some of the lipid is on the cell surface. Further confirmation of surface lipid has been given by the increased negative mobilities of walls treated with SDS: a result of reaction between surface lipid and the hydrophobic residues of the anionic agent which leaves the polar groups oriented towards the aqueous phase (James, 1965).

The protoplast membrane is apparently similar to other cytoplasmic membranes in consisting of a bimolecular leaflet of phospholipid coated with protein (Villanueva, 1966). The anionic carboxyl and phosphate groups at or near the cytoplasmic membrane of a fungal cell are considered to be binding sites for cationic fungicides (Somers, 1966) and it is probable that the same groups are present on the surface of a protoplast, especially in view of the high negative charge borne by protoplasts. The anionic charges on the protoplast surfaces are neutralized by much lower concentrations of dodine acetate than are required to kill conidia, which is consistent with the suggestion that reaction at the cell wall serves to detoxify some of the accumulated fungicide (Somers & Pring, 1966).

The electrokinetic results presented provide no evidence that the toxic reaction between dodine acetate and *Neurospora crassa* conidia is located on the spore surface —the spores were completely killed before there was a perceptible reduction in mobility. The cytoplasmic membrane may be expected to be extremely vulnerable to the fungicide but toxicity cannot be adequately explained on the basis of physical disorganization of this membrane (Somers & Pring, 1966). Enzyme inhibition at the cytoplasmic membrane or intracellular reaction still appear to be the most probable hypotheses to explain the fungitoxicity of dodine acetate.

We are indebted to Miss C. H. Slowley for providing fungal cultures and to Dr A. M. James for helpful discussions.

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Inequality of Mean Interdivision Time and Doubling Time

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If bacteria are watched from the time they are formed until they divide their interdivision times τ can be measured, and the distribution of these times will be given by the frequency function $f(\tau)$. The mean interdivision time $\bar{\tau}$ is then

$$\int_0^\infty \tau f(\tau)\, d\tau.$$

Powell (1956) pointed out that the equation

$$\bar{\tau} = \ln 2/k,$$

in which the specific growth rate is denoted by k and $\ln 2/k$ is the doubling time of the population, is usually false. However, several subsequent investigators have continued to assume this specious equality. In this note we prove the inequality

$$\bar{\tau} > \ln 2/k \tag{1}$$

and derive the approximation formula

$$\bar{\tau} \approx \ln 2/k + k\sigma^2/2, \tag{2}$$

in which σ is the standard deviation of $f(\tau)$. We make a few applications of (1) and (2) to the analysis of experimental and theoretical results.

If the interdivision times of individual bacteria are independent, it may be shown from renewal theory (Feller, 1941) that $2e^{-k\tau}f(\tau)$ is also a frequency function (Powell, 1956; Harris, 1959;); i.e.

$$\int_0^\infty 2e^{-k\tau} f(\tau)\, d\tau = 1. \tag{3}$$

Interdivision times are known not to be independent. The interdivision times of sisters are positively correlated (Powell, 1955; Schaechter, Williamson, Hood & Koch, 1962; Kubitschek, 1962), and the interdivision times of mother and daughter are negatively correlated (Powell, 1955). However, equation (3) can be derived without the assumption of independence.

If a bacterial population consisting of $N(0)$ cells is in balanced growth, and if $N(0)$ is sufficiently large so that the number of viable cells $N(t)$ at some time, t, can be written as

$$N(t) = N(0)\, e^{kt}$$

with negligible error, and if $f(\tau)$ is independent of time, one can account for all of the cells present at time T. One portion, R, consists of cells present at time 0 which have not yet divided; the remainder are new cells produced by divisions in the interval

[0, T]. At any intermediate time t ($0 \leqslant t \leqslant T$) the rate of formation of cells that will be extant at time T is

$$2kN(0)\, e^{kt} \int_{T-t}^{\infty} f(\tau)\, d\tau.$$

The number of new cells formed in the interval [0, T] which are extant at time T is

$$\int_0^T \int_{T-t}^{\infty} 2kN(0)\, e^{kt} f(\tau)\, d\tau\, dt.$$

Substituting $t = T-x$ gives

$$\int_0^T \int_x^{\infty} 2kN(0)\, e^{k(T-x)} f(\tau)\, d\tau\, dx.$$

Consequently, at time T the total number of cells is

$$N(0)\, e^{kT} = R + N(0)\, e^{kT} \int_0^T \int_x^{\infty} 2ke^{-kx} f(\tau)\, d\tau\, dx$$

or

$$\int_0^T \int_x^{\infty} 2k\, e^{-kx} f(\tau)\, d\tau\, dx = 1 - [R/N(0)]\, e^{-kT}.$$

As T approaches infinity, the term $[R/N(0)]\, e^{-kT}$ approaches zero giving

$$\int_0^{\infty} \int_x^{\infty} 2k\, e^{-kx} f(\tau)\, d\tau\, dx = 1.$$

Interchanging the order of integration gives

$$\int_0^{\infty} \int_0^{\tau} 2k\, e^{-kx} f(\tau)\, dx\, d\tau = 1,$$

which, upon performing the integration with respect to x, gives equation (3).

With the assumptions and approach used in this derivation it may be demonstrated that the frequency function of interdivision times of dividing cells is $2e^{-k\tau}f(\tau)$ (Powell's (1956) carrier distribution, $C(\tau)$). It may also be shown that the frequency function of interdivision times of all extant cells is $2(1-e^{-kt})\, f(\tau)$ (Powell's (1964) $P(\tau)$).

Factoring $2e^{-k\bar{\tau}}$ from equation (3) gives

$$2e^{-k\bar{\tau}} \int_0^{\infty} e^{-k(\tau-\bar{\tau})} f(\tau)\, d\tau. \tag{4}$$

The inequality,

$$1 - x < e^{-x}$$

holds for all $x \neq 0$. Since analysis and experiment demonstrate some dispersion of interdivision time (Powell, 1955; Kubitschek, 1962), we write the inequality

$$2e^{-k\bar{\tau}} \int_0^{\infty} [1 - k(\tau - \bar{\tau})]\, f(\tau)\, d\tau < 1.$$

Using the definition of $\bar{\tau}$, we have by integration

$$2e^{-k\bar{\tau}} < 1$$

and, hence,

$$\bar{\tau} > \ln 2/k. \tag{1}$$

Formula (2) can be derived by expressing the integral in equation (4) as

$$\int_0^{\infty} e^{-k(\tau-\bar{\tau})} f(\tau)\, d\tau = 1 + \sum_{i=1}^{\infty} \mu_i (-k)^i / i! \tag{5}$$

in which μ_i is the ith central moment of $f(\tau)$. Equation (5) is valid if all of the moments exist and the series converges. For many types of functions the terms of the series in (5) rapidly go to zero. If all terms beyond the second are ignored and the substitution is made for the integral in (4), we obtain

$$2e^{-k\bar{\tau}}(1-\mu_1 k+\mu_2 k^2/2) \approx 1.$$

Since $\mu_1 = 0$ and $\mu_2 = \sigma^2$, we have

$$1+\sigma^2 k^2/2 \approx e^{k\bar{\tau}}/2. \tag{6}$$

If the quantity $\sigma^2 k^2/2$ is much less than 1,

$$\ln(1+\sigma^2 k^2/2) \approx \sigma^2 k^2/2,$$

since $$\ln(1+x) = \sum_{n=1}^{\infty}(-1)^{n+1} x^n/n \quad \text{for} \quad |x| < 1.$$

Taking logarithms of equation (6) and substituting the approximation above gives

$$\sigma^2 k^2/2 \approx k\bar{\tau} - \ln 2$$

or

$$\bar{\tau} \approx \ln 2/k + k\sigma^2/2. \tag{2}$$

Formula (2) has been derived for the special case that $f(\tau)$ is a uniform distribution (Engleberg, 1964) or a Gaussian distribution (Barrett, 1966). If some information is given about $f(\tau)$, it is often practicable to use the extended law of the mean to establish a limit to the error in (2).

The approximation by formula (2) was tested by a numerical method using a digital computer. Powell (1955) has found a good fit of experimental measurements of $f(\tau)$ to a Pearson Type 3 distribution. The frequency function of this distribution with a mean of 1 was computed for various standard deviations. For each distribution the value of k was computed by successive approximation using equation (3) and integrating by Simpson's rule. The values of k are accurate to $\pm 0{\cdot}00001$. For the special case that $f(\tau)$ is a Pearson Type 3 distribution, Powell (1956) has shown that

$$k = (2^{1/g}-1)/m,$$

where $\tau = mg$ and $\sigma^2 = m^2 g$. The stated accuracy of the general numerical approximation of k was verified by direct solution. The values of $\bar{\tau}$ were estimated by formula (2).

Table 1 shows that the approximation $\bar{\tau} = \ln 2/k$ is good only for small values of $\sigma/\bar{\tau}$. When $\sigma/\bar{\tau} = 0{\cdot}2$, the error is less than 2 %; but when $\sigma/\bar{\tau} = 0{\cdot}5$, the error is nearly 10 %. The error in estimating τ by formula (2) increases as σ increases. The error of estimation at large σ results from a significant contribution by higher moments of the τ-distribution. At $\sigma = 0{\cdot}5$, the error is approximately 1 %. Since the coefficient of variation of interdivision time $(\sigma/\bar{\tau})$ rarely exceeds 0·5, the approximation by formula (2) is sufficiently accurate for most purposes. A better approximation can be made by including terms which correspond to the third and fourth moments, but the formula becomes quite complicated.

Table 2 compares the estimates of $\bar{\tau}$ with experimental results of Powell (1955). The agreement is satisfactory. To our knowledge, no other measurements have been made with sufficient precision to establish the discrepancy between mean interdivision time and doubling time.

Relation (1) can be used to analyse parts of Powell's (1964) note on Koch and Schaechter's hypothesis. By assuming exponential growth of individuals and equal

Table 1. *Estimation of mean interdivision time from specific growth rate and standard deviation of $f(\tau)$*

Standard deviation (σ)	Specific growth rate (k)*	Doubling time (ln 2/k)	Estimated mean interdivision time ($\bar{\tau}$)†
0·00	—	1·00000	1·00000
0·05	0·69375	0·99914	1·00001
0·06	0·69401	0·99876	1·00001
0·07	0·69433	0·99831	1·00001
0·08	0·69469	0·99778	1·00000
0·09	0·69510	0·99719	1·00001
0·10	0·69556	0·99654	1·00002
0·20	0·70285	0·98620	1·00026
0·30	0·71522	0·96914	1·00135
0·40	0·73304	0·94558	1·00422
0·50	0·75682	0·91787	1·01047

* Computed from $\int_0^\infty 2e^{-kt}f(\tau)d\tau = 1$ assuming τ is distributed as a Pearson Type 3 distribution with standard deviation = σ, and mean = 1·00000.

† Estimated by formula (2).

Table 2. *Comparison of estimates of mean interdivision time with the experimental results of Powell (1955)*

Organism	Standard deviation, σ (min.)	Specific growth rate, k (min.$^{-1}$)	ln 2/k (min.)	$\bar{\tau}$, observed (min.)	$\bar{\tau}$, estimated (min.)
Pseudomonas aeruginosa	5·37	0·01831*	37·85	38·1	38·11
Bacillus megaterium	7·76	0·03259†	21·27	22·3	22·25
Bacillus mycoides	14·2	0·02637*	26·29	28·7	28·95

* Calculated by Powell (1956) by fitting a Pearson Type 3 distribution to the data and numerically solving $\int_0^\infty 2^{-k\tau}f(\tau)d\tau = 1$ for k.

† Calculated by Powell (1956) by plotting the logarithm of number of extant cells versus time.

division, he derives an expression (his equation [4]) for $f(\tau)$ that is symmetrical about ln 2/k. But this can be true only if $\bar{\tau} = \ln 2/k$, contradicting (1). His error is the assumption that the frequency function of the size of dividing cells is the same as the frequency function of the size at division of sample of newly formed cells. These functions describe different populations.

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THE SOCIETY FOR GENERAL MICROBIOLOGY

The Society for General Microbiology held its forty-ninth General Meeting at Imperial College, London, on Monday, Tuesday and Wednesday, 3, 4 *and* 5 *April* 1967. *The following communications were made:*

ORIGINAL PAPERS

Chlorohydrin Formation during Epoxide Sterilization of Culture Media. BY T. J. WESTON (*Imperial Tobacco Co. (of Great Britain and Ireland), Ltd., Research Department, Raleigh Road, Bristol* 3)

Propylene oxide and ethylene oxide can be used for the cold sterilization of instruments, culture media and tissue slices (Hansen, H. & Snyder, W. C. (1947), *Phytopathology*, **37**, 369; Goss, R. C. & Marr, J. L. (1963), *Proc. Iowa Acad. Sci.* **70**, 125). These epoxides react with chlorides to form persistent toxic chlorohydrins in dried foodstuffs, such as flour and pepper (Wesley, F., Rourke, B. & Darbishire, O. (1965), *J. Food Sci.* **30**, 1037). Using a gas chromatograph, incorporating a halogen detector, chlorohydrins were found in agar media and potato tuber slices after fumigation with propylene oxide. The amount of chlorohydrins formed in the agar was dependent upon the amount of Cl^- present, fumigation time, and propylene oxide concentration.

The growth and morphology of fungi subsequently grown on the agar were affected for as long as the chlorohydrins remained. The chlorohydrins were not persistent in agar and potato slices for more than 1–2 weeks, being eliminated by hydrolysis during storage.

Haemadsorption and Haemagglutination by Mycoplasmas. By R. J. MANCHEE and D. TAYLOR-ROBINSON (*Common Cold Research Unit, Harvard Hospital, Salisbury*)

Del Guidice & Pavia ((1964), *Bact. Proc.* p. 70) reported that erythrocytes from several species adsorbed to colonies of *Mycoplasma pneumoniae*. In the present study, the factors concerned in demonstrating haemadsorption and its occurrence among different mycoplasmas were investigated. Haemadsorption occurred best to colonies which had recently developed on agar at pH 6·5. Crowding of colonies could completely abolish haemadsorption. The phenomenon usually occurred with erythrocytes from a wide range of species. Mycoplasmas isolated from various bird and animal sources haemadsorbed, e.g. *M. gallisepticum*, *M. agalactiae*, *M. bovigenitalium*, *M. pulmonis*, but not all strains within a serotype did so. Thus the 'Negroni' strain of *M. pulmonis* did not haemadsorb. Generally antiserum titres obtained by haemadsorption inhibition (HAdI) were low in comparison with those obtained by metabolic inhibition, and HAdI was not useful as a routine serological technique.

Mycoplasma haemagglutination was first reported in 1945 (van Herick, W. & Eaton, M. D. (1945), *J. Bact.* **50**, 47). In the present study haemagglutination occurred best at pH 6·5–7·0 in U-shaped cups at 37 °C. Mycoplasmas isolated from birds, cattle, goats, man, mice, and pigs, and grown in liquid medium haemagglutinated, generally at low titre. Inhibition of haemagglutination by specific antisera was demonstrated. There was lack of correlation between haemadsorption and haemagglutination; both these phenomena were exhibited by some mycoplasmas while others haemadsorbed only, and others haemagglutinated only.

Effects of R Factors on UV-Susceptibility of *Escherichia coli* K12. By A. G. SICCARDI (*Department of Medical Microbiology, Stanford University, Stanford, California U.S.A.*)

R factors are plasmids (transmissible extra-chromosomal DNA structures) which determine resistance to various anti-bacterial drugs, and sometimes further properties such as phage restriction and modification. Some R factors, like some colicine factors, confer partial protection against the bactericidal effect of ultraviolet irradiation. Of 28 R factors (a few of them associated with colicine factors) tested in *E. coli*, 13 gave protection (either causing a 'shoulder' on the dose/log-survival curve or decreasing its slope), 10 had little or no effect and 5 caused increased UV susceptibility, in both lysogenic and non-lysogenic K12 hosts. The protecting or sensitizing effect of representative R factors (and of several protecting *colIb* factors) was qualitatively the same in K12 of wild-type UV-sensitivity and in UV-sensitive mutants (*uvrA*, *uvrB*, *uvrC* and *rec*$^-$) believed to have defects in reactions, presumably enzymic, needed for repair of UV-damaged DNA. UV killing of a multiple auxotroph was, as expected, much diminished if the irradiated cells were 'starved' for 2 hr in amino acid-deficient medium before plating on nutrient agar. In K12 of wild-type UV-sensitivity (but not in UV- sensitive mutants) the effect of protecting R factors was reversed (i.e. they caused decreased survival) if the irradiated cells were 'starved' before plating—but not otherwise. Some R factors decreased the ability of K12, wild-type or UV-sensitive, to effect host-cell-reactivation of irradiated phage T1.

Perhaps plasmids which change the UV-susceptibility and host-cell-reactivating ability of their bacterial hosts do so by determining production of endodeoxyribonuclease(s) active on DNA strands which, because of UV damage, are not readily susceptible to host endonuclease(s) used in the repair of UV-damaged DNA. Such additional endonuclease activity might in some circumstances permit repair of otherwise irreparably damaged DNA regions—and in other circumstances effect DNA degradation exceeding the capacity of the repair mechanisms of the host.

Transduction of *try* Genes by Phages ϕ 80 *pt* in *Escherichia coli*. By J. P. GRATIA (*Laboratory of Microbiology and Hygiene, University of Liège. Belgium*)

Among the transducing particles of Bacteriophage ϕ 80, some transduce different segments of the tryptophan (*try*) operon and are still able to form plaques: ϕ 80 pt_0 transduces *try* O_T-*E-D* (Sato, K. & Matsushiro, A. (1965), *J. Mol. Biol.* **14**, 608), ϕ 80 pt_1 (same reference) and ϕ 80 ptd_{28} (produced by a defective lysogen; Gratia, J. P. (1967), *Life Sci.* **6**, 209) transduce *try C-B-A*. Such phages have been used to transduce *try* genes to *Escherichia coli* K12 strains carrying either point-mutations or deletions extending from *try A* to *try C* or further.

When point-mutants are infected by complementary ϕ 80 *pt* particles, i.e. *try* E^- or *try* B^- mutants by ϕ 80 pt_0 or by ϕ 80 pt_1 (or-ptd_{28}) respectively, most transductants are non-lysogenic haploïd recombinants. When deletion mutants are infected by ϕ 80 pt_1 or by ϕ 80 ptd_{28} (host-range derivatives have been used), transductants also appear, on minimal medium if the deletion does not overreach *try C* and on medium supplemented with indole if the deletion is further extended and not entirely complemented. But these transductants are partial diploïds, sometimes very stable, segregating very few *try*$^-$ cured cells, or sometimes quite unstable according to the recipient used. When the deletion strains carry a prophage in att_{80}, the transduction of *try C-B-A* always results in the formation of stable diploïds. Indole-requiring transductants, having inherited the T_1-receptor through ϕ 80 pt_1, have been superinfected by ϕ 80 pt_0 which is carrying the complementary genes, giving rise to 'supertransduced' *try*$^+$ *ind*$^+$ cells. These are stable triploïds carrying overlapping segments of the *try* operon each linked to a prophage. With ϕ 80 pt_1-transductants of a double-mutant marked by a *try C-B-A* deletion and in addition by a mutation in *try E*, and thus anthranilate-requiring, 'supertransduction' of anthranilate-independence by ϕ 80 pt_0 has also been achieved.

When recipients are carrying a prophage in att_{80}, transductants appear at a lower frequency. The reduction is particularly pronounced with ϕ 80 pt_1 or -ptd_{28} transducing immune bacteria.

Inducible Cephalosporinase in *Enterobacter cloacae*. By T. D. HENNESSEY (*Department of Bacteriology, Royal Postgraduate Medical School, London, W.* 12

The terms 'penicillinase' and 'cephalosporinase' are descriptive for β-lactamase, they denote the class of substrate for which the relative specificity of the enzyme is greatest. Distribution of cephalosporinase among Gram-negative bacteria, and the significance of the enzyme in terms of contribution to cephalosporin resistance, have been the subjects of much work. Presence of inducible β-lactamase in *Pseudomonas aeruginosa* was clearly shown by Sabath *et al.* ((1965), *Biochem. J.* **96**, 739), but demonstration of inducible enzyme in other Gram-negative bacteria has not been as well defined.

Three strains of *Enterobacter cloacae* (P99, 214, and 256) have been investigated with respect to inducibility of cephalosporinase. Their levels of resistance to cephaloridine were all about the same, but their activity in hydrolysing the drug, measured iodometrically in disrupted, log-phase cultures, was 1300, 200 and 2 enzyme units per mg. dry wt, respectively It was argued that if production of the enzyme in strain 256 contributed to cephaloridine resistance to the same extent as in P99 and 214, the enzyme was likely to be inducible. To test this hypothesis strain 256 was grown in the presence of penicillin, in concentrations of 10μg.–12 mg./ml. Cells were harvested and hydrolysis of cephaloridine by disrupted cells was compared with untreated controls. It was found that induction of cephalosporinase took place maximally when the penicillin concentration was 500 μg./ml., and that the ratio of induced: uninduced activities was 117. Using the same penicillin concentration, cephalosporinase activity of strain 214 was increased 5 times but there was no increase with P99 under similar conditions.

Ampicillin, cephaloridine and methicillin were also tested as inducers of cephalosporinase in strain 256. With a concentration of 500 μg./ml., both ampicillin and cephaloridine increased whilst methicillin decreased the rate of hydrolysis of cephaloridine.

Some Properties of a Phenol Oxidase Isolated from *Aspergillus nidulans*. By B. L. A. CARTER and A. T. BULL (*Department of Microbiology, Queen Elizabeth College, London, W.* 8

The melanin deposited in the hyphal walls of *Aspergillus nidulans* is a heterophenolic polymer composed, in part, of indolic residues (Bull, A. T. (1966), *Abstr.*, IX*th Int. Congr. Microbiol.*, p. 201, Moscow). This report details some properties of the phenol oxidase concerned in melanogenesis. Low and poor reproducibility of enzyme activity in cell-free extracts lead to a search for endogenous inhibitors, possibly released on cell disruption (^{14}C-pigment precursors are readily incorporated into melanin by whole mycelia (Bull, A. T., *loc. cit.*)). Products of phenol oxidase activity were examined as potential causes of the low enzyme levels but experiments designed to remove such phenolic/quinonoid metabolites produced on mycelial disruption did not enhance enzyme recovery. Acetone powders of cell-free extracts occasionally had activity suggesting that protein separation techniques might yield active material. The phenol oxidase was resolved eventually by DEAE-cellulose chromatography, being strongly adsorbed and only eluted with a buffer system containing 1·0 M-NaCl, while the inhibitor, also a protein, was eluted at low molarities. A reversed pattern of elution resulted when cationic exchangers were used.

A variety of phenol oxidase assay systems have been examined but we have found only those based upon coupled oxidation-reduction and oxygen uptake will allow accurate determination of initial rates and be unaffected by secondary reactions. The phenol oxidase confirmed to be of the tyrosinase type, has a high specific activity and its capacity for *o*-hydroxylation appears to be much higher than that reported for other sources of tyrosinase. Investigations of the kinetics, electrophoresis and cellular location will be considered together with the enzyme's behaviour on dextran gels. The activity peak (E^1) from DEAE-cellulose columns is a protein of high M.W. (*ca.* 500,000) which dissociates into a polypeptide (E^2) of one-quarter this size. Kinetic data on these two components will be discussed. Tyrosinase production and regulation in *A. nidulans* and other aspects of metabolism are being studied under conditions of continuous culture; preliminary results of this work will be presented.

Isolation of DNA from Kinetoplasts of *Crithidia fasciculata*. By B. A. NEWTON* (*Sub-Department of Chemical Microbiology, Department of Biochemistry, University of Cambridge*)

The kinetoplast is located at the proximal end of the flagellum in members of the protozoan family Trypanosomatidae. This organelle has long been known to contain Feulgen-positive material and is now thought to resemble a mitochondrion in some respects. DNA extracted from trypanosomid flagellates can be separated into a major and a minor component by equilibrium centrifugation (Schildkraut *et al.* (1962), *Nature, Lond.* no. 196, 795). This communication will describe the isolation and some of the characteristics of the minor DNA component from *Crithidia fasciculata* and provide evidence that it originates from the kinetoplast.

Organisms were grown in a chemically defined medium (Kidder & Dutta (1958), *J. gen. Microbiol.* **18**, 621), washed, suspended in 0·25 M sucrose containing 0·01 M sodium citrate and broken by shaking with glass beads. A fraction (P) containing cell pellicles, kinetoplasts and damaged nuclei was obtained by centrifugation at 3000*g* for 10 min.; RNA was removed from this fraction by RNase and DNA extracted by the method of Marmur ((1961), *J. Mol. Biol.* **3**, 208). This DNA banded as a single component (ρ = 1·716 g./c.c.) in a caesium chloride gradient. Treatment of P with pronase before DNA extraction yields material which can be separated into a major (ρ = 1·716 g./c.c.) and a minor (ρ = 1·688 g./c.c.) component by equilibrium centrifugation. The major component can be removed selectively by controlled DNase digestion of P before treatment with pronase; acridine orange staining has shown that this digestion does not remove kinetoplasts from the preparation.

The results indicate that the minor DNA component is largely associated with the kinetoplast but the possibility that small amounts exist in the nucleus cannot yet be eliminated. The minor component forms a very compact band in caesium chloride which reaches equilibrium after 2 hr centrifugation at 44,000 r.p.m., whereas the major component and most other types of DNA require 15–17 hr centrifugation at this speed to reach equilibrium. A similar rapidly banding DNA has recently been obtained from *Leishmania enriettii* (Du Buy, Mattern & Riley (1966), *Biochem. Biophys. Acta* **123**, 298).

An Infrared Study of the Effects of Partial Desiccation and Radiation on Nucleic Acids. By S. J. WEBB and M. D. DUMASIA (*Department of Bacteriology, University of Saskatchewan, Saskatoon, Saskatchewan*)

A study has been made of changes in the infrared (IR) spectra of RNA and DNA due to desiccation and irradiation and the modifying effects of myo-inositol on these changes.

Judging from changes in the depth and shifts in the various IR absorption bands, the desiccation of DNA between 75 and 55 % relative humidity (RH) removed water from –N, —NH, —NH_2, and —OH groups and between 55 and 10 % RH from the C—O, and P=O groups. These observations agree with those of Bradbury Price & Wilkinson ((1961), *J. Mol. Biol.* **3**, 301), Falk Hartman & Lord ((1963), *J. Am. Chem. Soc.* **85**, 387). However, the DNA of cells grown in a minimal salt medium was less hydrated than that of cells grown in an enriched medium and changes in its IR spectrum due to desiccation were less pronounced. Changes in the spectra of polyuridylic and polycytidylic acids were small compared to those of polyinosinic and polyadenylic acids. Severe alterations in the IR spectrum of DNA occurred as a result of irradiation with ultraviolet light and most of these changes appeared to be due to the loss of hydration sites.

All of the spectral changes observed were completely or partially prevented by myo-inositol but this protection was inhibited by the presence of NaCl. In addition, inositol was less able to prevent changes in polyuridylic and polycytidylic acids than it was changed in polyadenylic and polyinosinic acids. The results of this study add strong support to the

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concepts (Webb, S. J. (1965), *Bound Water in Biological Integrity*, Charles C. Thomas, publishers) that (*a*) cells grown in a minimal medium are better able to resist desiccation and radiation damage because their DNA is less hydrated; (*b*) DNA is more susceptible to damage due to the loss and reorientation of bound water than is RNA; and (*c*) compounds such as inositol bind with DNA in place of water and stabilize its structure.

Endogenous Metabolism and Survival of *Azotobacter insigne* VJ5. By H. STOCKDALE, E. A. DAWES and D. W. RIBBONS* (*Department of Biochemistry, The University of Hull*)

As a result of a survey of poly-β-hydroxybutyrate (PHB) in the *Azotobacteriaceae* (Stockdale, H., Ribbons, D. W. & Dawes, E. A. (1965), *J. gen. Microbiol.* **41**, xviii), *Azotobacter insigne* strain VJ5, a non-encysting organism of low carbohydrate content, was selected for further investigation. Its survival is influenced by the nutritional status of the cell and the conditions of starvation. Cells grown on a modified Norris's nitrogen-free medium containing 2 % glucose died at a rate of about 1·5 % of the initial viable population per hr when suspended in a basal salts medium with aeration. During this period the PHB content of the cells falls from 10 % to < 1 % and the endogenous Q_{O_2} declines from 8 to less than unity. The initial R.Q. is 0·89 ± 0·025. After 18–20 hr starvation, the rate of viability loss is accelerated to 4 % of the initial viable population per hr and only 5 % of the initially viable cells survive at 45–50 hr.

No protective effect could be shown when cells were suspended in sterile starvation medium from which cells had been removed by Millipore filtration, or in the supernatant of carbon-limited growth medium.

Rapid death (50 % decline in viability in 40 min.) occurs when cells are suspended in distilled water. The effect is not mitigated by the presence of a carbon source (glucose) or nitrogen source (ammonium nitrate). Sodium chloride of the same ionic strength as basal medium decreases the death rate (18 % decline in viability in 40 min.). The presence of a dialysed supernatant fraction of a cell sonicate in distilled water produces a death curve similar to that observed in basal medium. No such effect was seen with bovine serum albumin (Sigma Chemical Co.).

These observations substantiate the findings of Sobek, Charba & Foust ((1966), *J. Bact.* **92**, 687).

Inhibition of Growth of *Azotobacter* by Oxygen. By H. DALTON and J. R. POSTGATE (*A.R.C. Unit of Nitrogen Fixation, University of Sussex, Brighton, Sussex*)

High oxygen tensions inhibit or delay growth of many aerobic and facultatively anaerobic bacteria (e.g. Moore, B. & Williams, R. S. (1911), *Biochem. J.* **5**, 181) including *Azotobacter* spp. (Tschapek, M. & Giambiagi, N. (1955), *Arch. Mikrobiol.* **21**, 376). Inhibition or delay of growth of aerobes sometimes occurs when cultures are aerated very efficiently with normal air; it is generally attributed to depletion of CO_2 known to be necessary for the initiation of growth of many aerobes (Walker, H. H. (1932), *Science*, **76**, 602; Gladstone, G. P., Fildes, P. & Richardson, G. D. (1935), *Br. J. Exp. Path.* 16, 335). We report here a case in which inhibition of growth by efficient aeration seems attributable to oxygen.

Azotobacter chroococcum (NCIB 8003) was grown in continuous culture ($D = 0{\cdot}25\ hr^{-1}$) in a nitrogen-free mannitol medium. Batch cultures (200 ml.) of a similar medium were inoculated with about 60 μg. dry wt organisms and stirred rapidly (about 2500 r.p.m.: O_2 solution rate about 42 m-mole/l./hr) or slowly (about 300 r.p.m.; O_2 solution rate about 3·2 m-mole/l./hr) under 400 ml./min. of CO_2-free air. Growth was always delayed, and usually prevented absolutely, at the high stirring rate; it proceeded normally at the lower rate. Addition of 2 % CO_2 to the atmosphere did not prevent inhibition by rapid stirring. A similar effect was observed using air enriched with oxygen to 0·4 atm.

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When grown with fixed nitrogen (4 mM-NH_4Cl) under argon and oxygen, inhibition by rapid stirring was not detected at pO_2 0·2 atm. but could be observed at pO_2 0·6 atm.; it was not reversed by CO_2.

Azotobacters have the highest recorded Q_{O_2} values (Williams, A. M. & Wilson, P. W. (1954), *J. Bact.* **67**, 353) and sensitivity to oxygen would not be expected *a priori*. However, their nitrogenase system is very readily damaged by oxygen when resolved into soluble components (Bulen & Le Compte (1966), *Proc. Nat. Acad. Sci., Wash.* **56**, 979) and this sensitivity may provide an explanation of oxygen toxicity in these organisms.

The Repair of DNA in *Micrococcus radiodurans* following Ultra-violet Irradiation. By B. E. B. MOSELEY (*Molteno Institute, University of Cambridge*)

Micrococcus radiodurans is extremely resistant to ultraviolet (UV) and ionizing radiation. This resistance is caused by its ability enzymically to remove potentially lethal irradiation products from its DNA. For example, adjacent thymine bases in its DNA, dimerized during UV irradiation, are lethal unless removed and replaced by new bases.

Two radiation sensitive mutants of *M. radiodurans* have been isolated and their excision and repair processes following UV irradiation compared with those of the wild-type. The UV radiation dose used was one which reduced the viability of the mutants by a factor exceeding 10^5 though not affecting the viability of the wild-type.

Excision was studied by labelling cellular DNA with H^3-thymidine, irradiating the cells and measuring the loss of label from DNA and its appearance in the incubation medium. In all three strains 0·6 % of the thymine was dimerized and the dimer excised during the first hour of incubation. In the mutants, however, the excision rate of labelled thymine was five times faster than in the wild-type, after 4 hr incubation more than 90 % of the DNA label being in the medium. In the wild-type about 20 % of the DNA label was lost during this period.

Repair was studied by irradiating unlabelled cells, incubating them in a medium containing ^{3}H-thymidine and measuring incorporation of label into DNA. All three strains began to incorporate label but whereas in the wild type incorporation kept pace with excision, in the mutants the process stopped after less than 30 min.

It is concluded that the mutants, though they can recognize defects in DNA and excise them, are sensitive to radiation because the rate of excision of bases grossly exceeds the rate of re-incorporation and the DNA molecule disintegrates.

Chlortetracycline and Polyribosomes. By E. CUNDLIFFE (*Sub-Department of Chemical Microbiology, Department of Biochemistry, University of Cambridge*)

Bacillus megaterium KM was steady-state labelled with ^{32}P and then converted to protoplasts which were incubated until the optical density began to increase exponentially; chlortetracycline (CTC) was then added and the incubation continued. Samples were then taken into chilled glass vials containing Triton X 100, a neutral detergent, and deoxyribonuclease, and the resulting lysates were layered directly on to sucrose density gradients (15–40 % sucrose). Polyribosome profiles were visualized by counting the radioactivity in successive gradient fractions after centrifugation. With low concentrations of CTC (5 μg./ml.) a rapid breakdown of polyribosomes occurred, at higher concentrations there was less breakdown. It seems probable that CTC acts by interfering with the binding of amino-acyl-transfer RNA to the ribosome-messenger RNA complex. It is suggested that this has the effect of fixing an affected ribosome *in situ* on the messenger (or at least of inhibiting its movement temporarily) thereby also hindering the movement of those ribosomes further towards the 5′ end of the messenger. Those ribosomes to the 3′ side of the blockage are still able to proceed and to be released. Raising the concentration of CTC would be expected to affect more ribosomes per polyribosome and would therefore give less release.

With low concentrations of CTC, breakdown of polyribosomes was followed by a slower re-aggregation process which resulted in much ribosomal material being pelleted in the gradient tubes. The re-aggregation was sensitive to actinomycin D but was not inhibited by

concentrations of CTC sufficient to prevent all protein synthesis. The re-aggregated material contained many of the 70s ribosomes originally present as was shown by ribonuclease treatment of lysates. The re-aggregation process and the nature of its product are not clearly understood, although some species of RNA other than that in mature 70s ribosomes would appear to be necessary for the formation of the aggregates.

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