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The Influence of NaCl Concentration of the Medium on the Potassium Content of *Aerobacter aerogenes* and on the Inter-relationships between Potassium, Magnesium and Ribonucleic Acid in the Growing Bacteria

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SUMMARY

Irrespective of the nature of the growth-limiting substance, the potassium content of *Aerobacter aerogenes* (growing in a chemostat at a fixed dilution rate) increased progressively from about 1·3 % to 4·2 % of the bacterial dry weight with changes in culture NaCl concentration from 0 to 40 g./l. Similarly, the potassium content of glycerol-limited *A. aerogenes* organisms, growing in a low ionic strength medium, varied with growth rate (from 0·9 % to 1·6 % of the bacterial dry weight with changes in dilution rate from 0·05 to 0·7 hr^{-1}); but this was not related to the small changes in culture NaCl content that occurred simultaneously. These observations are discussed with reference to conflicting views on the particular role of potassium in growing bacteria that accounts for its presence intracellularly in high concentration.

INTRODUCTION

Potassium is present in growing bacteria in concentrations that are substantially higher than those of other cations (Rouf, 1964; Tempest & Dicks, 1967; Tempest, Dicks & Ellwood, 1968) but the reason for a high intracellular potassium requirement has not been established unequivocally. Since the potassium concentration in growing bacteria usually is greater than 0·1 M (see the above references and Christian & Waltho, 1964), it seems unlikely to be required solely for purposes of enzyme activation. Dicks & Tempest (1966) concluded that the potassium content of *Aerobacter aerogenes* was determined largely by the bacterial RNA content and probably was required for maintenance of a functional ribosomal configuration, whereas Epstein & Schultz (1965) concluded that the bulk of the intracellular potassium was needed for osmoregulation since the potassium content of *Escherichia coli* varied with medium osmolarity. However, Epstein & Schultz did not equalize the growth rates of organisms in cultures of different salinity so that consequent differences in bacterial RNA and ribosome contents may have contributed to the observed differences in bacterial potassium content. On the other hand, the experiments reported by Dicks & Tempest (1966) took no account of the total salts concentration of the medium which was not controlled and probably varied; therefore, the parallel changes in cellular potassium and RNA contents that were observed when the growth rate was varied may have been fortuitous. To resolve these doubts it was necessary to establish whether the NaCl

concentration of the medium had an effect on the potassium content of bacteria growing at a fixed rate (in a chemostat) and, if so, whether changes in total salts concentration could have accounted for the observed changes in bacterial potassium content associated with changes in the growth rate.

METHODS

Organism. Aerobacter aerogenes NCTC 418 was maintained and subcultured monthly on tryptic meat-digest agar slopes.

Growth conditions. The organisms were grown in 0·5 l. Porton-type chemostats with automatic pH control (Herbert, Phipps & Tempest, 1965) in low ionic strength media of the following basal composition: Na_2HPO_4, 0·5 mM; $NH_4H_2PO_4$, 4·5 mM; $(NH_4)_2SO_4$, 2·5 mM; citric acid, 0·5 mM; $MgCl_2$, 0·75 mM; K_2SO_4, 3 mM and trace amounts of Fe^{3+}, Ca^{2+}, Mn^{2+}, Zn^{2+}, Cu^{2+}, Co^{2+}, Na_2MoO_4. For carbon-limitation, glycerol was added to a final concentration of 10 g./l.; for K^+-limitation, the glycerol concentration was increased to 30 g./l. and the K_2SO_4 concentration lowered to 0·5 mM; for Mg^{2+}-limitation, the glycerol concentration was 30 g./l. and the $MgCl_2$ concentration lowered to 0·25 mM. Each of these media contained barely sufficient NH_4^+ to provide the bacterial nitrogen requirement, but excess nitrogen was made available by using an ammonia solution (about 2 M) as the titrant for pH control. NaCl was added to the above 'low ionic strength' media as indicated in the Results section; however, some NaCl always was present in the culture since the reference electrode was connected to the culture through a 'bridge' tube containing a saturated NaCl solution. The flow rate of saturated NaCl into the growth vessel never exceeded 3 ml./day so that its concentration in the culture was insignificant at all but the lowest dilution rates (Table 2).

Analytical procedure. Culture bacterial dry weights and bacterial potassium, magnesium, phosphorus and RNA contents were determined by previously described methods (Tempest, Hunter & Sykes, 1965; Tempest, Dicks & Hunter, 1966).

RESULTS

When bacteria are limited in their growth by the availability of a particular element they may be expected to contain the minimum concentration of that element necessary to satisfy the structural and metabolic needs for growth at the imposed rate. Therefore, by growing organisms at a fixed rate in a series of K^+-limited environments, each containing graded amounts of NaCl, it should be possible to establish unequivocally whether there is a dependence of bacterial potassium content on NaCl concentration. Since in a K^+-limited culture nearly all the potassium is contained within the organisms, little being present in the extracellular fluid, any change in bacterial potassium content must produce a change in the steady-state concentration of bacteria in the culture. Clearly this occurred with K^+-limited chemostat cultures of *Aerobacter aerogenes* (growing at a fixed rate) when the medium NaCl concentration was increased progressively from almost zero to 40 g. NaCl/l. (Table 1). Analysis of the organisms separated from each culture confirmed that the steady-state bacterial potassium content had increased with NaCl concentration, but the bacterial magnesium, phosphorus and RNA contents did not vary proportionately (Table 1). Thus, whereas the

ratios of cell-bound magnesium:RNA were maintained constant the potassium:RNA ratios increased progressively with medium NaCl concentration (Fig. 1). Although the potassium content of K^+-limited *A. aerogenes* varied with medium NaCl concentration, the presence of 20 g. NaCl/l. in the growth environment did not annul the effect of growth rate on the bacterial potassium, magnesium, RNA and phosphate contents; each changed proportionately when the dilution rate was lowered from 0·3 to 0·1 hr^{-1} (Table 1). At the lower growth rate the concentration of potassium in *A. aerogenes* organisms was less than one-half that in organisms growing at the

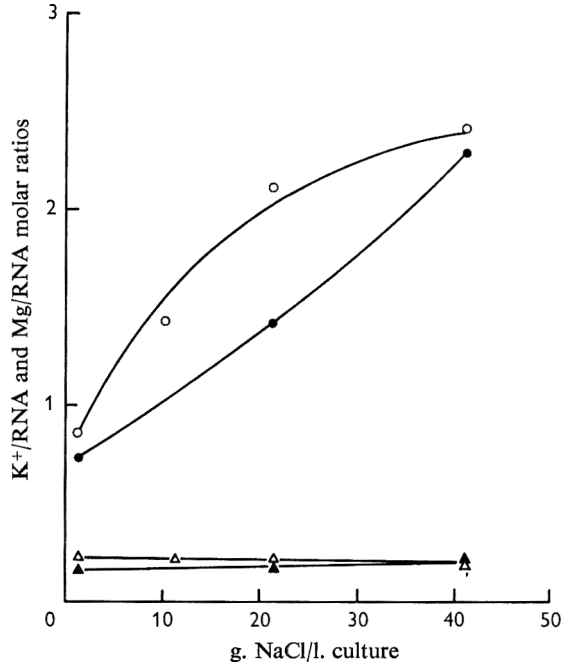

Fig. 1. Steady-state ratios of potassium/RNA (circles) and magnesium/RNA (triangles) in chemostat cultures of *Aerobacter aerogenes* which were K^+-limited (blocked symbols) and glycerol-limited (open symbols) and in which the medium NaCl content was progressively increased.

higher rate, even though 20 g. NaCl/l. was present in the culture throughout. Thus, changing the growth rate modified the bacterial requirement for potassium and, presumably, altered the osmotic differential between the extracellular and intracellular environments.

With chemostat cultures of *Aerobacter aerogenes* in which K^+ was present in excess of requirement and growth limited by the availability of another component of the medium, changes in the culture NaCl content again caused changes in bacterial potassium content (Table 1) similar to those detailed above. When the carbon and energy source (glycerol) limited growth, the steady-state culture bacterial concentration again varied with medium NaCl content indicating either a decreased efficiency of glycerol metabolism or, more likely, an increased maintenance energy requirement with the presence of NaCl in the culture. With Mg^{2+}-limited cultures of *A. aerogenes* addition of more than 10 g. NaCl/l. to the medium affected magnesium assimilation;

when the NaCl concentration was increased to 40 g./l. steady-state growth conditions no longer could be maintained.

The fact that medium NaCl content did affect the potassium content of bacteria growing in a chemostat raised the question of whether the changes in bacterial potassium content with dilution rate, reported by Tempest *et al.* (1966), resulted from associated changes in culture salts content rather than accompanying changes in bacterial RNA content. This possibility was difficult to check with cultures in which the carbon source was present in excess of requirement, since excreted metabolic end-products (e.g. acetate) accumulated in the environment and added to the osmolarity of the culture extracellular fluids. With glycerol-limited cultures of *Aerobacter aerogenes* no significant

Table 1. *The effect of NaCl on the magnesium, potassium, phosphorus and RNA contents of Aerobacter aerogenes, growing in a chemostat (35°, pH 6·5)*

The figures quoted in this table are average values from at least two samples collected, processed and analysed on separate days.

Diln. rate (hr^{-1})	NaCl concn. (g./l.)	Dry wt of organisms (g./l.)	g./100 g. dry bacteria			
			Magnesium	Potassium	Phosphorus	RNA
		Potassium-limited organisms				
0·3	Nil	3·09	0·194	1·30	2·14	15·5
0·3	10	2·13	0·202	1·88	2·35	—
0·3	20	1·62	0·210	2·49	2·44	15·2
0·3	40	0·82	0·225	4·20	2·50	15·7
0·1	20	2·54	0·124	1·52	1·50	9·5
		Glycerol-limited organisms				
0·3	Nil	5·16	0·203	1·30	2·37	13·3
0·3	10	4·57	0·204	2·14	2·34	13·0
0·3	20	4·20	0·200	3·28	2·33	13·3
0·3	40	3·08	0·208	4·15	2·68	15·3
		Magnesium-limited organisms				
0·3	Nil	2·96	0·196	1·35	2·13	—
0·3	10	2·94	0·194	2·38	2·01	—
0·3	20	2·32	0·205	3·25	2·20	—
0·3	40	Steady state not attained				

accumulation of extracellular metabolic end-products could be detected at growth rates less than 0·8 hr^{-1} (35°, pH 6·5) and because the glycerol concentration in the cultures also was extremely low, the medium osmolarity could be assessed approximately from the weight of total salts present in the extracellular fluids after drying (104°, 16 hr.) accurately measured volumes (usually 10·0 ml.). The results of an experiment in which the dilution rate was increased progressively from 0·05 to 0·7 hr^{-1} are shown in Table 2. Clearly, the extracellular salt concentration varied with dilution rate, but the cellular potassium content varied differently. As the dilution rate was raised progressively the bacterial potassium content increased, but this obviously was not due to changes in culture salinity which actually *decreased.* As reported previously with K^+-, Mg^{2+}- and PO_4^{3-}-limited cultures of *A. aerogenes* (Tempest *et al.* 1966; Dicks & Tempest, 1966), the bacterial magnesium, phosphorus and RNA contents of glycerol-limited *A. aerogenes* varied with dilution rate in a manner that paralleled the changes

in bacterial potassium content, and the molar proportions of magnesium:potassium: RNA:phosphorus were maintained constant at about 1:4:5:9 (Table 2).

Table 2. *The effect of dilution rate on the magnesium, potassium, phosphorus and ribonucleic acid contents of Aerobacter aerogenes, growing in a chemostat (35°, pH 6·5)*

The figures quoted in this table are average values from at least two samples that were collected and analysed on separate days. The weights of total salts in the extracellular fluids were obtained by drying known volumes of culture that had been freed from bacteria by centrifugation (3000 *g*, 10 min.)

Diln. rate (hr^{-1})	Extracellular salt concn. (g./l.)	Dry wt of organisms (g./l.)	g./100 g. dry bacteria				Molar ratios		
			Magnesium	Potassium	Phosphate	RNA	K/Mg.	P/Mg	RNA/Mg
0·05	3·1	4·90	0·124	0·92	1·50	7·9	4·5	9·4	4·5
0·1	2·0	5·00	0·168	1·07	1·95	10·9	3·9	9·0	4·6
0·3	1·4	5·15	0·206	1·37	2·43	14·3	4·1	9·1	4·9
0·7	1·0	5·05	0·260	1·62	2·90	17·2	3·9	8·6	4·7

DISCUSSION

The experiments reported in this paper show that both the rate at which *Aerobacter aerogenes* organisms were grown and the NaCl content of the medium in which they were grown influenced markedly their intracellular potassium content. But whether these two effects were related in a common cellular function (e.g. maintenance of ribosomal activity, osmoregulation, stimulation of respiration, etc.) cannot be decided from the available evidence. It seems unlikely that all the potassium contained in organisms growing in the presence of, say, 40 g. NaCl/l. was associated with the ribosomes, since the ratio of potassium to RNA-phosphate would then have been greater than 2, whereas it is likely always to be less than 1 since the net charge on the RNA-phosphate is unity. On the other hand, if the potassium content of *A. aerogenes* organisms was totally unrelated to their RNA content, then it is difficult to understand why both varied with growth rate (Table 2) and growth temperature (Dicks & Tempest, 1966) in a parallel manner and quite independent of small changes in culture osmolarity.

Bacterial respiration rate has been shown to vary with growth rate (Herbert, 1958; Tempest, *et al.* 1965) and possibly this could have accounted for the growth-rate-linked variation in potassium content if the two were related (as suggested by Eddy & Hinshelwood, 1951). In support of this hypothesis was the observation that addition of 20 g. NaCl/l. to the medium in which either glycerol-limited or K^+-limited *A. aerogenes* was being grown caused the steady-state respiration rate to be increased by about 25%. However, both glycerol-limited and K^+-limited *A. aerogenes* organisms contained the same amount of potassium when grown at identical rates in media of low ionic strength (Table 1), yet their steady-state respiration rates were markedly different (e.g. Q_{O_2}-values of 114 *versus* 383 when grown at a dilution rate of 0·3 hr^{-1}, 35° pH 6·5, in glycerol-limited and K^+-limited environments, respectively). Hence there can be no simple quantitative relationship between bacterial potassium content and respiration rate.

The simplest hypothesis that can be proposed to accommodate the effects of both

growth rate and medium NaCl content on the potassium content of bacteria assumes that they are not related to a single cellular function. Thus, we propose that the potassium content of an organism basically is prescribed by its intracellular polyanion content (largely RNA in the case of the above *A. aerogenes* organisms) and this concentration of potassium, together with that of other low molecular weight compounds, is sufficient to provide an adequate internal hydrostatic pressure when the organisms are suspended in environments of moderately low ionic strength (i.e. water activity $> 0{\cdot}99$). However, when exposed to environments of water activity $\ll 0{\cdot}99$, plasmolysis can be prevented only by a further uptake of potassium (plus an equal amount of low molecular weight anion, presumably), and this occurs. But since there is no corresponding change in the magnesium content of the organisms or, more particularly, in the RNA : Mg^{2+} ratio (Fig. 1), this 'extra' potassium must be compartmented in a way such that it does not affect the structural configuration and functioning of the ribosomes.

Although the magnesium content of *Aerobacter aerogenes* was unaffected by the medium NaCl content (Table 1), addition of NaCl to the growth environment affected the efficiency of magnesium uptake. Thus, steady-state conditions could not be obtained with Mg^{2+}-limited chemostat cultures of *A. aerogenes* containing 40 g. NaCl/l., and even in the presence of 20g. NaCl/l. less than 80% of the available magnesium was taken up by the organisms. Sodium can displace magnesium that is adsorbed on the surface of bacteria (Strange & Shon, 1964; Tempest & Strange, 1966) and the adsorption process may be a necessary stage in the assimilation of magnesium by bacteria (Tempest, Dicks & Meers, 1967). Hence, NaCl toxicity in general may be due, at least in part, to interference in the uptake of magnesium by the growing organisms.

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The Effect of Carbon Dioxide Concentration and Buffer System on Nitrate and Nitrite Assimilation by *Dunaliella tertiolecta*

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SUMMARY

Dunaliella tertiolecta required carbon dioxide in substrate concentrations (1·75 %, v/v) to assimilate either nitrate or nitrite at maximum rates in light. The addition of glucose, glycerol, acetate, pyruvate or α-ketoglutarate did not remove the requirement for carbon dioxide. The rates of nitrate and nitrite assimilation in light depended upon the buffer system used. The lowest rates of nitrate assimilation, 1·3 μmoles/hr/mg. chlorophyll, were observed in 0·05 M-phosphate buffer (pH 7·6) and the highest, 13·7 μmoles/hr/mg. chlorophyll, in 0·05 M-Tricine buffer (pH 7·6). Nitrite assimilation was lowest, 7·5 μmoles/hr/mg. chlorophyll, in 0·05 M-phosphate (pH 7·6) while the highest rates, 18·7 μmoles/hr/mg. chlorophyll, were observed in 0·05 M-Tricine (pH 8·6). The low rate of assimilation of nitrate and nitrite in 0·05 M-phosphate buffer (pH 7·6) was increased by diluting the buffer to 0·005 M, at this concentration the rate in phosphate buffer was comparable to that in tris or Tricine buffers at the same pH values. Buffer type had little effect on either nitrate or nitrite assimilation in the dark. There was no evidence with any buffer system used for the evolution of extra oxygen associated with nitrate or nitrite assimilation in the light. These results provide further evidence for the existence of two independent systems of nitrate reduction, one within and the other without the chloroplast. In addition, they indicate that in *Dunaliella tertiolecta* the enzyme system which fixes carbon dioxide is unlikely to be the rate-limiting step in photosynthesis.

INTRODUCTION

Some algae, Scenedesmus (Bongers, 1958), Chlorella (Davis, 1953) and Anabaena (Hattori, 1962) evolve oxygen at greater rates during nitrate and nitrite assimilation in light than under otherwise similar conditions when these anions are absent. The extra oxygen bears a stoichiometric relationship to the nitrate or nitrite assimilated. However, the green alga *Dunaliella tertiolecta*, although showing a large increase in both nitrate and nitrite assimilation in light, did not show the stoichiometric evolution of extra oxygen found in other algae (Grant, 1967). Furthermore, Dunaliella required carbon dioxide in order to show any increase in nitrate or nitrite assimilation in light. The present paper examines this requirement for carbon dioxide and explores the effect of different buffer systems on nitrate and nitrite assimilation and the oxygen evolution associated with it.

METHODS

Organism and culture methods. The strain of *Dunaliella tertiolecta* (Butcher) was the same as used previously (Grant, 1967), as were the culture media and methods, except that 48 hr cultures were used, not 72 hr cultures. The organisms from the 48 hr culture gave consistently higher rates of nitrate and nitrite assimilation in light, although the dark assimilation rates were either unchanged or only slightly lower.

Preparation of suspensions of organisms. Organisms were harvested and concentrated as previously (Grant, 1967) except that an artificial sea water instead of 3% NaCl was used to wash and resuspend organisms after centrifugation. The artificial sea water contained (g./l.): NaCl, 20·9; Na_2SO_4, 3·9; KCl, 0·7; NaBr, 0·1; H_3BO_3, 0·03.

Chlorophyll determination. Chlorophyll was extracted from organisms in a sample of suspension after centrifugation for 2 min. at 1000 ***g***. Extraction with 2 ml. portions of 100% acetone at room temperature was continued until no further pigment was released and the residue was colourless. The acetone extract was diluted with distilled water to 90% (v/v) immediately before determining the extinction at 665 and 645 mμ and the concentration of chlorophylls *a* and *b* was then calculated by using the equation of Humphrey & Jeffrey (in preparation). Where chlorophyll is referred to subsequently it means $a+b$. The use of 100% acetone, although less satisfactory than 90% acetone as an extracting solvent, prevented the action of chlorophyllase (Barrett & Jeffrey, 1964).

Packed cell volume was determined by centrifuging a sample of organism suspension in a haematocrit tube at 3000 ***g*** for 60 min.

Dry weight was determined by centrifuging a sample of organism suspension in a conical centrifuge tube for 5 min. at 1000 ***g***, and after carefully removing the supernatant fluid with a Pasteur pipette, resuspending the pellet in distilled water and transferring to a tared vial. The suspension was dried overnight at 45° in a vacuum oven and weighed after storage for 2 hr over P_2O_5 in a desiccator.

Numbers of organisms were determined by direct counting on a Petroff-Hauser counter.

Nitrate and nitrite were determined as in Grant (1967), except when phosphate was present. The phosphate was removed by adding to each ml. of supernatant fluid (containing ~ 50 μmoles phosphate) 100 μmoles magnesium sulphate and 200 μmoles ammonium hydroxide. The resulting precipitate was centrifuged down and a sample of the supernatant fluid passed through the cadmium reducer. Failure to remove phosphate resulted in rapid destruction of the cadmium+mercury reducer and erratic reduction of nitrate.

Oxygen evolution and uptake were measured in a respirometer at 20°, illuminated as required from below by incandescent lamps which gave at the lower surface of the vessel an energy of 27 mW/cm^2 between 400 and 750 nm, with a peak at 675 nm. This was above the intensity required to saturate photosynthesis under these conditions.

Where carbon dioxide was not provided by bicarbonate+carbonate mixtures as an internal pH and CO_2 buffer, it was supplied by adding carbonate+bicarbonate mixtures to the trough of the manometer flask as described by Warburg & Krippahl (1959, 1960). Carbonic anhydrase (1 μg./ml.) was added to both trough and vessel contents in experiments when carbon dioxide was maintained below a partial pressure

of 7·5 mm. mercury. Above this value carbonic anhydrase was not used because it did not increase the rate of oxygen evolution.

Buffers. Tris and Tricine buffers were prepared in distilled water and the pH value adjusted with HCl. Bicarbonate+carbonate buffer used internally was artificial sea water (composition given above) containing 0·035 M-K_2CO_3 and 0·065 M-$NaHCO_3$. The external carbon dioxide buffers were carbonate and bicarbonate mixtures prepared in distilled water as described by Warburg & Krippahl (1959).

RESULTS

Effect of culture age

In batch culture the rate at which *Dunaliella tertiolecta* assimilated nitrate in light and the rate at which oxygen was evolved decreased rapidly as the culture aged. (Fig. 1). This rapid decrease in activity was not due to exhaustion of nitrate in the medium since after 4 days one-third of the total nitrate supplied still remained. Some of the decrease may have been due to a decrease in chlorophyll content of the organisms (Madgwick, 1966). This rapid change in activity made comparison of assimilation rates in different species difficult. However, in the present experiments, when the cultures were used at exactly the same age and grown under identical conditions, organisms of uniform composition and similar nitrate and nitrite assimilating activity were obtained. These organisms had the following properties: cell weight 58 ± 8 $\mu\mu$g.; packed cell volume 218 ± 29 μ^3; chlorophyll content $1{\cdot}06 \pm 0{\cdot}13$ $\mu\mu$g.; total nitrogen content $4{\cdot}6 \pm 0{\cdot}1$ $\mu\mu$g

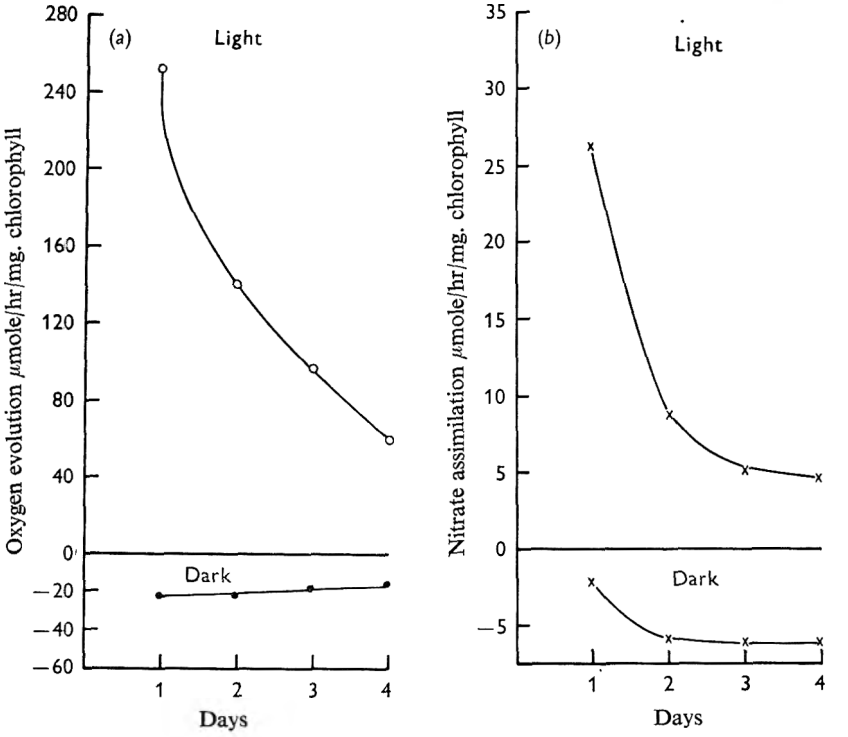

Fig. 1. Change in activity of cells with age.

Carbon dioxide requirement

When the carbon dioxide concentration in the gas phase was varied over a partial pressure range of 0 to 35 mm. mercury (0 to 4·5 %, v/v) the rates of nitrate and nitrite assimilation followed a parallel course to that of oxygen evolution (Fig. 2). At low partial pressures of carbon dioxide it was difficult to maintain constant rates of oxygen evolution for more than 20 min., even with low concentrations of organisms and carbonic anhydrase present. However, the results showed that nitrate and nitrite assimilation rates in the light required more than catalytic amounts of carbon dioxide to be present and that the concentrations required for maximum rates of oxygen evolution and those required for maximum assimilation in light of nitrate and nitrite were very similar. In the dark, variation in carbon dioxide concentration had no effect.

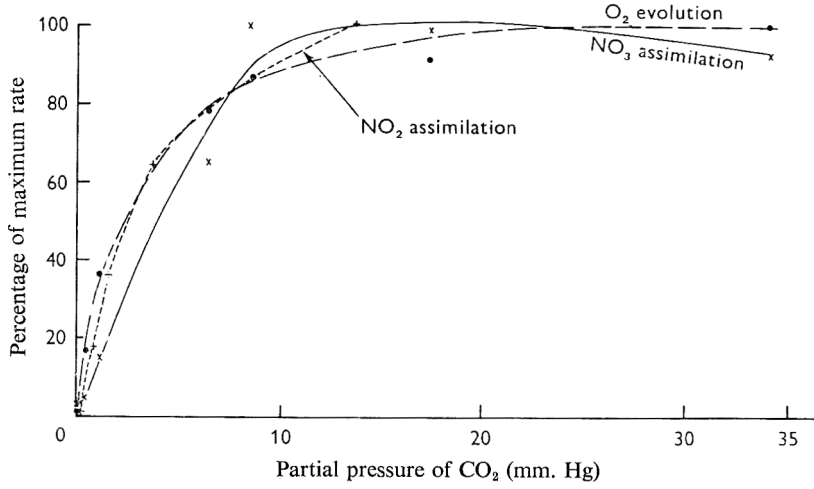

Fig. 2. NO_3 assimilation, NO_2 assimilation, and O_2 evolution as a function of CO_2 partial pressure. Maximum rates: 135 μmole O_2/hr/mg. chlorophyll, 13·0 μmole NO_3/hr/mg. chlorophyll, 12·5 μmole NO_2/hr/mg. chlorophyll.

Effect of added compounds

The addition of a variety of low molecular weight organic compounds did not increase the rate at which either nitrate or nitrite was assimilated in the light in absence of carbon dioxide (Table 1). Glycerol, acetate and α-ketoglutarate in fact decreased the nitrate assimilation rate below that observed in the control vessels to which no addition was made; glucose and glycerol had a similar effect on nitrite assimilation. In darkness glucose caused a small increase in the rate of assimilation of nitrate; the other compounds decreased the rate or had no effect. Where these substances were added in the presence of CO_2 no significant effect on nitrate or nitrite assimilation was noted. Experiments with [U-^{14}C]glucose (specific activity 3×10^4 dpm/μmole) and [U-^{14}C]-acetate (specific activity 1×10^6 dpm/μmole) showed that *Dunaliella tertiolecta* incorporated less than 12 $\mu\mu$mole glucose/hr/mg. chlorophyll but that it incorporated acetate at a rate almost 50 times as great.

Table 1. *Effect of organic compounds on the rate of nitrate and nitrite assimilation in the absence of carbon dioxide by Dunaliella tertiolecta*

Each vessel contained in a total of 2·0 ml.: tris + HCl buffer (pH 7·6) 100 μmole; KNO_3, 2·0 μmole; and organisms containing 0·2 mg. chlorophyll. Addition of substrate or bicarbonate where indicated was 50 μmole and, except in vessels containing bicarbonate, 0·1 ml. of 20 % KOH was added to the centre well of each vessel. All values are expressed in μmole/hr/mg. chlorophyll.

	Nitrate assimilation		Nitrite assimilation	
	Light	Dark	Light	Dark
No addition	1·1	1·3	2·0	1·0
D-Glucose	1·0	1·8	0·5	1·0
α-Ketoglutarate	0·4	0·7	2·0	1·0
Glycerol	0·0	0·6	0·0	1·0
Acetate	0·3	0·6	—	—
Pyruvate	1·2	0·8	—	—
Glutamate	1·2	0·6	—	—
Bicarbonate	14·6	1·3	23·0	1·0

Effect of pH value and buffer system

Table 2 shows the effect of four buffer systems at various pH values on nitrate assimilation, nitrite assimilation and oxygen evolution. In general nitrate and nitrite assimilation responded in the same way, but oxygen evolution did not always change similarly. Nitrite did not accumulate either in organisms or medium under any of the

Table 2. *Dunaliella tertiolecta: effect of buffer type on nitrate assimilation*

Conditions as described in text; all values in μmole/hr/mg. chlorophyll. Chlorophyll in each vessel was 0·05 to 0·1 mg. Final buffer concentration 0·05 M, and initial concentration of KNO_3 or KNO_2 was 10^{-3} M.

		Nitrate assimilation		
Buffer	pH value	Light	Dark	Oxygen evolution
Phosphate	7·6	1·3	1·7	262
Tris	7·6	10·3	0·9	305
Tricine	7·6	13·7	1·4	278
Tris	8·6	10·5	0·4	214
Tricine	8·6	11·1	0·3	231
Bicarbonate	8·6	8·7	0·3	181
		Nitrite assimilation		
		Light	Dark	
Phosphate	7·6	7·5	3·8	290
Tris	7·6	15·0	0·0	315
Tricine	7·6	15·0	3·4	276
Tris	8·6	13·3	0·6	260
Tricine	8·6	18·7	0·1	260
Bicarbonate	8·6	14·5	0·1	248

conditions tested, nor was free ammonia detected (lower limit of detection 2·5 μg. NH_3-N/ml.) following nitrate or nitrite assimilation. Therefore it was concluded that all nitrate or nitrite assimilated by *Dunaliella tertiolecta* was reduced to cell nitrogen. The maximum rates of nitrate and nitrite assimilation occurred in Tricine buffer at pH 7·6 and 8·6, respectively, and the minimum in phosphate buffer at pH 7·6. Values

over the range of pH 4·8 to 9·2 had little effect on assimilation rates, except in phosphate buffer where a sharp maximum at pH 6·5 was observed. This disappeared when the concentration of buffer was decreased from 0·05 M to 0·005 M. Oxygen evolution was not decreased in phosphate buffer, but both oxygen evolution and nitrate assimilation were decreased in bicarbonate buffer by 20 %.

The effect of nitrate and nitrite assimilation on oxygen evolution in different buffer systems

Although the buffer type and concentration affected the over-all rate of nitrate and nitrite assimilation there was no detectable increase in oxygen evolution during either nitrate or nitrite assimilation in any of the systems examined. Since the amount of O_2 evolved during the experiment did not exceed 20 μmole and since agreement between replicates was always better than 5 % for NO_3 or NO_2 estimation and for O_2 evolution, the extra oxygen production would have been detectable for any amount of nitrate assimilation in excess of 0·5 μmole and any nitrite assimilation in excess of 0·66 μmole, provided that the 2·0:1 and 1·5:1 ratios as predicted, held. In fact the differences observed in O_2 evolution between controls and vessels containing nitrate or nitrite were well within this 5 % value. (Table 3).

Table 3. *Dunaliella tertiolecta: effect of nitrate and nitrite on oxygen evolution in different buffer systems*

All values are in μmole and are those recorded after 1 hr. Nitrate and nitrite assimilation measurements differed in each experiment and batch of organisms. Each vessel contained ~ 0·075 mg. chlorophyll and initial concentration of nitrate or nitrite was 10^{-3} M. Final concentration of buffer was 0·05 M.

Buffer	pH value	Oxygen evolved		Nitrate assimilated	Oxygen evolved		Nitrite assimilated
		$+NO_3$	$-NO_3$		$+NO_2$	$-NO_2$	
Tris	8·6	18·3	18·3	0·9	20·4	21·0	1·43
Tricine	8·6	20·1	19·6	1·0	22·1	21·5	1·41
Bicarbonate	8·6	15·3	15·6	0·8	11·1	11·7	0·80
Tris	7·6	13·6	13·0	0·7	22·0	21·4	1·28
Tricine	7·6	12·4	12·0	1·0	19·9	19·6	1·73
Phosphate	7·6	12·9	13·2	0·7	6·7*	22·2	0·60
Phosphate	6·5	12·2	11·8	1·0	19·8	18·5	1·20
Phosphate	4·8	10·6	10·6	0·4	5·2*	17·6	0·70

* In these experiments oxygen evolution ceased after 10 min. but, in other experiments, when phosphate concentration was decreased to 0·01 M the O_2 evolution rate was restored to that of the $-NO_2$ control.

Formation of extra carbon dioxide during dark assimilation of nitrate and nitrite

Measurements of oxygen uptake and carbon dioxide evolution in the presence and absence of nitrate and nitrite were made in phosphate and Tris buffer only. Extra carbon dioxide was evolved during the assimilation of nitrate and nitrite and, with the exception of phosphate buffer at pH 4·8, the results agreed well with th 2·0:1 molar ratio for extra CO_2 evolved to nitrate assimilated, and the 1·5:1 ratio for nitrite found by Warburg & Negelein (1920) with Chlorella.

DISCUSSION

Since in these experiments nitrate and nitrite assimilation were affected in a similar manner, in the following discussion nitrate assimilation alone will be referred to and nitrite assimilation implied unless specifically mentioned. It is clear from these results that *Dunaliella tertiolecta* requires substrate amounts of CO_2 to exhibit an increase in NO_3 assimilation in light. The amounts needed are more than the catalytic requirement to allow maximum rates of phostosynthetic electron transport such as has been shown in chloroplasts isolated from higher plants (Good, 1963, 1965). Reduced carbon skeletons supplied exogenously do not substitute even in part for CO_2. The ineffectiveness of glucose in this respect might be due to the inability of *D. tertiolecta* to assimilate this common metabolite in light or dark, in which it differs from Chlorella (Rodriguez-Lopez, 1966; Hulsen & Prenzel, 1966) and Scenedesmus (Taylor, 1950). However, acetate is taken up by *D. tertiolecta* and the results of Craigie & McLachlan (1964) indicate that the organisms should be permeable to glycerol. The failure of these potential sources of endogenous CO_2 and skeletons for amination to replace externally supplied CO_2 indicates a close association or coupling of photosynthetic carbon reduction and photosynthetic nitrate reduction.

Nitrate assimilation in light was not accompanied by extra oxygen evolution, although the intensity of light supplied was well above that necessary to saturate photosynthesis. This held true over a range of pH 4·5 to 9·2 and in four different buffer systems. It seems, therefore, that it is a characteristic of *Dunaliella tertiolecta* and is not restricted to special experimental conditions. Previous work (Grant, 1967) showed some oxygen evolution during NO_3 assimilation although the amounts had no relationship to NO_3 assimilated. These experiments with younger organisms which assimilate NO_3 and NO_2 up to five times more rapidly confirm that the two processes of O_2 evolution and NO_3 assimilation are not closely related. In this respect *D. tertiolecta* differs from *Chlorella pyrenoidosa* (Van Niel, Allen & Wright, 1953) *Ankistrodesmus braunii* (Kessler, 1953, 1964) and *Anabaena cylindrica* (Hattori, 1962). In darkness, nitrate and nitrite assimilation by *D. tertiolecta* both give increased amounts of CO_2 evolution. The extra CO_2 evolved is in a 2·0:1·0 ratio to NO_3 assimilated and a 1·5:1·0 ratio to nitrite assimilated. Therefore in the dark, nitrate reduction resembles that reported in other species of algae (Warburg & Negelein, 1920; Cramer & Myers, 1949; Syrett, 1955).

The buffer system chosen for measurement of NO_3 or NO_2 assimilation had a significant effect on the rate observed, although pH alone did not. Phosphate buffer in particular depressed assimilation rates in light under conditions where either HPO_4^{2-} or $H_2PO_4^-$ ions were present in high concentration. Similar, although much smaller, effects were observed in tris and bicarbonate buffers. It was outside the scope of this work to investigate the cause of this effect, but when nitrate assimilation by different species of algae is to be compared, preliminary investigation of the buffer system effects should be made.

On the basis of studies with whole organisms of a few species of algae and higher plant chloroplasts, two theories for the action of light on nitrate assimilation have been proposed. One proposes that light and dark reduction proceed by the same mechanism, and the increased rate in light is due to more carbohydrate being available for breakdown, thus providing more reduced pyridine nucleotide. The other assumes

that the increase in pyridine nucleotide (or ferredoxin or flavoprotein) reduction is brought about directly by photosynthetic electron transport, and that this then reduces nitrate directly. Evidence for each of these theories and their shortcomings have been discussed by Kessler (1964). The results obtained with *Dunaliella tertiolecta* cannot be explained by either theory without modification. It is unlikely, in this organism, that the rate of pyridine nucleotide production by dark respiration could be increased 20-fold by an increase in carbohydrate supply, even though CO_2 is a pre-requisite for increased rates of nitrate assimilation in light. The difference observed in the sensitivity of light and dark assimilation to iodoacetate (Grant, 1967) is also an indication that different processes are involved. The photo-respiratory system operative in some higher plants (Forrester, Krotkov & Nelson, 1966) appears to have a maximum of three times the normal dark respiratory rate and therefore could not supply a 20-fold increase in assimilation rate. It is concluded from these results that the effect of light, even though dependent on CO_2, does not act by increasing pyridine nucleotide reduction by an increased rate of respiration.

The dependence on CO_2, the relatively low light intensity required to saturate both nitrate and nitrite assimilation in *Dunaliella tertiolecta* (Grant, 1967), and also in Anabaena (Hattori, 1962) and Scenedesmus (Bongers, 1958), and the absence of extra oxygen evolution during nitrate assimilation in light, show that the action of light on nitrate assimilation is more complex *in vivo* than the simple schemes proposed by Losada, Ramirez, Paneque & del Campo (1965) and by Joy & Hageman (1966) from their work with cell-free systems of higher plant chloroplasts. The magnitude of the increase in nitrate assimilation in light shows that the reduction is closely associated with photosynthetic electron flow. If there were a close association between the photosynthetic nitrate-reducing system and the photosynthetic carbon-reducing system, as would be predicted if both these processes took place wholly within the chloroplast, nitrate and nitrite reduction in light could be controlled by feedback inhibition of an intermediate further along in the reductive sequence. Since *D. tertiolecta* does not accumulate ammonia *in vivo* even under conditions of low pH value and low CO_2 partial pressure, it seems that such a control system does operate *in vivo*. The failure of acetate, α-ketoglutarate and glycerol to replace CO_2 may be due to failure to penetrate the chloroplast membrane. Therefore, the evidence from whole organisms of *D. tertiolecta* is consistent with the existence of two separate systems for nitrate assimilation. One system apparently is located completely within the chloroplast, is dependent on CO_2 and light, and is insensitive to respiratory inhibitors such as iodoacetate. This probably corresponds to the system investigated by Losada *et al* (1965) and by Joy & Hageman (1966) which depends on ferredoxin, for the nitrite reduction step. However, it seems that the final step in which the amino group is transferred to the carbon skeleton must also take place within the chloroplast, in which respect the Dunaliella differs from maize chloroplasts (Ritenour, Joy, Bunning & Hageman, 1967). The second system, which probably corresponds to that occurring in higher plant roots, is outside the chloroplast and depends on respiration for reduced pyridine nucleotide, and is thus sensitive to iodoacetate inhibition.

Because *Dunaliella tertiolecta* did not evolve extra oxygen when assimilating nitrate or nitrite in light, extra oxygen evolution is not an inescapable consequence of nitrate reduction by the light-activated system. This is additional evidence for the view put forward by Gibbs, Bamberger, Ellyard & Everson, (1967) that the carbon dioxide

fixation step is not invariably the rate limiting one in photosynthesis. The results of Homann & Schmid (1967), with chloroplasts isolated from mutant varieties of tobacco, provide further experimental evidence for this view. Therefore the evolution of extra O_2 during light-activated nitrate reduction will depend on the relative activities of the CO_2 fixing enzyme system and the photosynthetic electron transporting enzyme system. Scenedesmus (Bongers, 1958) resembles *D. tertiolecta* in this regard. Therefore there is no longer any basis for seeking for stoichiometric ratios between extra O_2 evolution and nitrate assimilation in systems assimilating either nitrate or nitrite in light.

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Regulation of the Methionine-specific Aspartokinase and Homoserine Dehydrogenase of *Salmonella typhimurium*

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SUMMARY

Salmonella typhimurium, like *Escherichia coli*, has a methionine-regulated β-aspartokinase and homoserine dehydrogenase in addition to threonine-sensitive and lysine-sensitive aspartokinase isoenzymes and a threonine-sensitive homoserine dehydrogenase. These methionine-regulated enzymes are not subject to feedback inhibition by methionine (or by methionine and S-adenosylmethionine), but the homoserine dehydrogenase is inhibited by the methionine precursor cysteine and to a lesser extent by homocysteine. Studies of enzyme concentrations in methionine analogue-resistant strains show that mutations which lead to de-repression of the later methionine-forming enzymes also result in de-repressed synthesis of the methionine-regulated β-aspartokinase and homoserine dehydrogenase, although the methionine-forming enzymes as a whole are not subject to co-ordinate control.

INTRODUCTION

In *Salmonella typhimurium* methionine arises from homoserine via *O*-succinyl-homoserine, cystathionine and homocysteine, and the enzymes which catalyse this pathway are subject to control by the end-product, such that the synthesis of all of them (homoserine-*O*-transsuccinylase, cystathionine synthetase, cystathionase and the homocysteine methylase complex) are repressed by methionine, and the activity of the first is inhibited by it (Rowbury, 1964). Homoserine arises from aspartate in bacteria by a series of reactions catalysed by the three enzymes β-aspartokinase, aspartate-β-semialdehydedehydrogenase and homoserine dehydrogenase. These three reactions are common to the formation of threonine and methionine, while the first two are also essential for lysine synthesis (Stadtman, 1963). Extensive studies have established that three distinct β-aspartokinase enzymes regulated respectively by lysine, methionine and threonine + isoleucine, and two homoserine dehydrogenase enzymes regulated, respectively, by threonine + isoleucine and by methionine, are formed by *Escherichia coli* (Stadtman, 1963; Patte, Le Bras, Loviny & Cohen, 1963; Patte, Le Bras & Cohen, 1967). The present work shows that *S. typhimurium* also has a methionine-regulated β-aspartokinase and homoserine dehydrogenase, together with enzymes regulated by lysine and threonine + isoleucine.

To study further the regulation of methionine synthesis in *Salmonella typhimurium*, mutants insensitive to the growth-inhibitory effects of methionine analogues have been

isolated (Lawrence, Smith & Rowbury, 1968). Most of these mutants excrete methionine and they fall into at least four phenotypically distinct groups: *metI*, *metJ metK* (all excreting), and *metK* (non-excreting). *MetI* strains are no longer subject to feedback control of the first methionine-forming enzyme, homoserine-*O*-transsuccinylase, (Rowbury, Ayling & Chater, unpublished observations), while *metJ* and excreting *metK* strains, all of which overproduce methionine, no longer show enzyme repression of cystathionine synthetase, cystathionase and the homocysteine methylase complex. Control of methionine synthesis is, however, normal in the non-excreting *metK* strains (Lawrence *et al.* 1968). The experimental results described in the present paper show that the effect of mutation to analogue-resistance upon the methionine-specific β-aspartokinase and homoserine dehydrogenase is similar to that upon the later methionine-forming enzymes.

METHODS

Organisms. The strains of *Salmonella typhimurium* LT-2 used in the present work were maintained on slopes of Oxoid nutrient agar at 2–5° and subcultured at monthly intervals. Strain HfrB2 and its analogue-resistant derivatives *metI706*, *metJ714*, *metK721* and *metK731*, which were all prototrophs, have been described previously (Lawrence, *et al.* 1968). Strain *metB23* responds to methionine, homocysteine or cystathionine, and is deficient in cystathionine synthetase (Smith & Childs, 1966).

Growth of organisms and preparation of extracts. Organisms were grown in the minimal medium of Davis & Mingioli (1950) at 25° on a reciprocating shaker (60 strokes/min.) for 16 to 18 hr. They were harvested, washed once with a culture volume of water and extracts prepared by ultrasonic disintegration (Rowbury & Woods, 1961).

Enzyme assays. Cystathionine synthetase and cystathionase were assayed as described by Rowbury & Woods (1966). β-Aspartokinase was assayed by measuring aspartylhydroxamate formation. Sodium aspartate (5 mM), ATP (5 mM), $MgCl_2$ (2 mM) and hydroxylamine (0·2 M) were incubated with ultrasonic extracts containing 2 to 4 mg. protein in a final volume of 2 ml. tris buffer (10 mM, pH 8·1) for 2 hr at 30°. Aspartyl hydroxamate was measured by adding 0·2 ml. 4 N-hydrochloric acid, 0·5 ml. 25% trichloracetic acid and 1 ml. of 5% ferric chloride in 0·1 N-hydrochloric acid. Extinctions of the contents of the reaction tubes were measured at 550 mμ in a Unicam SP 600 spectrophotometer. Homoserine dehydrogenase was assayed by following the reduction of NADP. Homoserine (50 mM) and NADP (0·3 mM) were incubated at 30° in 3 ml. tris buffer buffer (100 mM, pH 9·0) + potassium chloride (300 mM). Ultrasonic extract (2 to 5 mg. protein) was added at time zero and NADP reduction followed at 340 mμ in a Unicam SP 800 recording spectrophotometer.

RESULTS

β-Aspartokinase and homoserine dehydrogenase in Salmonella typhimurium

Organisms of the prototrophic HfrB2 strain of *Salmonella typhimurium* grown in minimal medium had readily measurable amounts of β-aspartokinase and homoserine dehydrogenase. Studies of the β-aspartokinase activity suggested the presence of three isoenzymes. Part of this activity (about 20%) was inhibited by threonine (6·6 mM) and another part (about 45%) was inhibited by lysine (6·6 mM). The presence of a threonine-

regulated isoenzyme was confirmed by the observation that organisms grown with threonine (2 mM) + isoleucine (2 mM) had decreased values of the threonine-inhibited activity, while a decrease in the lysine-inhibited portion elicited by the addition of lysine (2 mM) to the culture fluid reinforced the view that a lysine-controlled isoenzyme was formed by strain HfrB2. When organisms were grown with threonine (2 mM) + isoleucine (2 mM) + lysine (2 mM) the aspartokinase activity remaining was insensitive to threonine (6·6 mM) + lysine (6·6 mM) and this represented a methionine-regulated isoenzyme because its synthesis was repressed at least twofold by 10 mM methionine (Table 1).

Table 1. *The effect of methionine on the formation of β-aspartokinase and homoserine dehydrogenase in Salmonella typhimurium*

Organisms were grown at 25° in minimal medium supplemented with threonine + isoleucine + lysine (all at 2 mM), and with methionine (10 mM) added where stated. For strain *metB23* homocysteine (0·3 mM) was added when methionine was not present. Enzyme assays were as described in Methods but for β-aspartokinase threonine (6·6 mM) and lysine (6·6 mM) were added and for homoserine dehydrogenase threonine (6·6 mM) was added.

		β-aspartokinase (mμmoles/mg. protein/min.)		Homoserine dehydrogenase (mμmoles/mg. protein/min.)	
S. typhimurium	Strain properties	Grown without methionine	Grown with methionine	Grown without methionine	Grown with methionine
HfrB2	Prototrophic, analogue-sensitive	0·50	0·25	1·70	0·92
metB23	Methionine-requiring (cystathionine synthetase deficiency)	2·30	0·00	7·40	0·90
metI706	Resistant to α-methylmethionine	0·53	0·32	1·20	0·80
metJ714	Resistant to ethionine	2·93	2·06	6·40	5·90
metK721	Resistant to ethionine, norleucine, α-methylmethionine, excreting methionine	3·97	3·28	7·10	8·10
metK731	Resistant to ethionine, norleucine, α-methylmethionine, not excreting methionine	0·77	0·30	0·72	0·45

The major portion (about 60%) of the homoserine dehydrogenase activity of *Salmonella typhimurium* HfrB2 (grown in minimal medium) was inhibited by threonine (6·6 mM) and when organisms were grown in minimal medium + threonine (2 mM) + isoleucine (2 mM) this threonine-inhibited portion was decreased twofold. The residual homoserine dehydrogenase present in organisms grown in minimal medium + threonine + isoleucine + lysine (all at 2 mM) was not inhibited by methionine but was decreased twofold when methionine (10 mM) was also present in the culture medium (Table 1). To simplify study of the methionine-regulated enzymes in all subsequent experiments, organisms were grown in media containing threonine (2 mM) + isoleucine (2 mM) + lysine (2 mM), to repress as far as possible the synthesis of the lysine-sensitive and threonine-sensitive isoenzymes, and enzyme assays were made in the presence of threonine (6·6 mM) + lysine (6·6 mM), or of threonine alone in the case of homoserine dehydrogenase.

Methionine-regulated β-aspartokinase and homoserine dehydrogenase

For further study of the methionine-regulated β-aspartokinase and homoserine dehydrogenase the methionine-requiring strain *metB23* was used with which conditions of methionine limitation (and therefore enzyme derepression) could be achieved. Organisms were grown with 10 mM-methionine (excess methionine) or with 0·3 mM-homocysteine (which provided a growth-limiting amount of methionine). The amounts of β-aspartokinase and homoserine dehydrogenase were very low in the former culture (Table 1), but high activities were found in the methionine-limited organisms, these being four- to fivefold higher than those of strain HfrB2 grown in minimal medium (Table 1).

Methionine and some of its precursors and related compounds were tested for possible feedback effects on these methionine-repressed enzymes. S-Adenosylmethionine was included among the compounds tested because of its co-operative effect with methionine in the regulation of homoserine-*O*-transsuccinylase (Lee, Ravel & Shive, 1966). β-Aspartokinase was not inhibited by methionine (6·6 mM) either alone or in the presence of S-adenosylmethionine (6·6 mM); the methionine precursors cysteine, homoserine, cystathionine and homocysteine (all tested at 6·6 mM) were also without appreciable effect (Table 2). Methionine, S-adenosylmethionine and cystathionine also did not affect homoserine dehydrogenase, but cysteine and homocysteine markedly inhibited its activity (Table 2). The methionine-regulated homoserine dehydrogenase showed normal kinetics with respect to both homoserine and NADP; the K_m for homoserine was about 2·5 mM and for NADP about 0·1 mM.

Table 2. *The effect of methionine and related compounds on the activities of β-aspartokinase and homoserine dehydrogenase in Salmonella typhimurium*

Organisms of strain *metB23* were grown at 25° in minimal medium supplemented with threonine + isoleucine + lysine (all at 2 mM). Enzyme assays were as described in Methods with threonine (6·6 mM) and lysine (6·6 mM) added in the case of β-aspartokinase, and threonine (6·6 mM) alone added in the case of homoserine dehydrogenase. Other additions are as stated below.

Additions	β-Aspartokinase (mμmoles/mg. protein/min.)	Homoserine dehydrogenase (mμmoles/mg. protein/min.)
Expt. 1, none	2·27	7·4
Methionine	2·22	6·9
S-Adenosylmethionine	2·17	—
Methionine + S-adenosylmethionine	2·22	6·9
Expt. 2, none	3·85	7·2
Methionine	3·63	6·9
Cysteine	3·63	1·5
Homoserine	3·73	—
Cystathionine	3·43	7·2
Homocysteine	3·53	4·9

Enzyme levels in analogue-resistant strains

The methionine-regulated β-aspartokinase and homoserine dehydrogenase were present in low amounts (comparable with those in HfrB2) in *metJ706* and the non-excreting *metK731* and their activities were subject to repression by methionine (Table 1). *MetJ714* and the excreting *metK721*, however, had much higher amounts

of enzyme, comparable to those in *metB23* when grown under conditions of methionine limitation, and methionine repressed enzyme formation either only slightly in these strains or, with respect to the homoserine dehydrogenase of *metK721*, actually resulted in an increase in the amount of this enzyme (Table 1). Table 3 shows the amounts of two other methionine-forming enzymes in these strains for comparison with β-aspartokinase and homoserine dehydrogenase.

Table 3. *De-repression of methionine-forming enzymes in Salmonella typhimurium by mutation to analogue-resistance*

Organisms were grown at 25° in minimal medium with the supplements described in Table 2. β-Aspartokinase and homoserine dehydrogenase assays were conducted as described in Table 2, while the cystathionase and cystathionine synthetase activities were measured as described in Methods.

S. typhimurium	β-Aspartokinase	Homoserine dehydrogenase mμmoles/mg.	Cystathionine synthetase protein/min.	Cystathionase
HfrB2	0·50	1·70	0·30	0·95
metJ714	2·90	6·40	5·40	1·80
metK721	3·80	7·20	3·02	1·90

DISCUSSION

Salmonella typhimurium, like *Escherichia coli*, has methionine-regulated β-aspartokinase and homoserine dehydrogenase isoenzymes. It seems likely that endogenous methionine partially represses both enzymes in wild-type strains because limitation of methionine in strain *metB23* increased the enzyme levels four- to fivefold (Table 1). The methionine-regulated enzymes, unlike the other isoenzymes, were not subject to feedback inhibition of enzyme activity by end-product (Table 2); neither methionine, S-adenosylmethionine or methionine + S-adenosylmethionine affected either activity. Homoserine dehydrogenase was, however, strongly inhibited by cysteine, which provides the sulphur for methionine synthesis. Datta (1967) observed inhibition of homoserine dehydrogenase by cysteine in *E. coli*, although the methionine-regulated enzyme was not specifically studied. Datta proposed that the inhibition served a regulatory function to prevent methionine over-production during growth at high concentrations of cysteine. In view of the inhibition of the same enzyme by homocysteine (Table 2) it may be that the effect is a non-specific one akin to the inhibitory effects of cysteine and homocysteine on cystathionase (Wijesundera & Woods 1962).

The present work shows that the regulatory genes which control the amounts of the enzymes which catalyse methionine synthesis from homoserine also influence synthesis of the β-aspartokinase and homoserine dehydrogenase, in that the latter two enzymes are de-repressed in *metJ* and excreting *metK* strains. Control of the methionine-forming enzymes is not, however, co-ordinate since the extent of possible de-repression varies from enzyme to enzyme. Thus, mutation to analogue resistance leads to about six- to eightfold increase in β-aspartokinase, and a fourfold increase in homoserine dehydrogenase as compared with a 10- to 18-fold increase in cystathionine synthetase but only a twofold increase in cystathionase (Table 3).

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Studies on the Growth and Feeding of *Tetrahymena pyriformis* in Axenic and Monoxenic Culture

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SUMMARY

The specific growth and feeding rates of *Tetrahymena pyriformis* GL grown axenically in proteose-peptone yeast-extract medium and monoxenically in suspensions of the bacterium *Klebsiella aerogenes* were studied. The relation between the initial concentration of substrate, whether bacteria or soluble organic complexes, and the maximum yield of ciliates was linear, although some inhibition was noted at higher substrate concentrations. The effective yields of Tetrahymena are 9·1 % (carbon to carbon) in axenic cultures and 50 % (dry-weight bacteria to dry-weight ciliate) in monoxenic cultures. The maximum growth rates at 25° in axenic and monoxenic cultures were 0·20 and 0·22 hr^{-1}, respectively. Carbon balance studies on axenic cultures suggested that of the carbon utilized during growth 36·5 % was incorporated into the Tetrahymena and 69 % was respired. The removal rates of *K. aerogenes* from suspension by *T. pyriformis* were studied and there was evidence which suggested that the individual feeding rate of a ciliate was governed by the concentration of ciliates as well as the concentration of bacteria present. From these observations a model for ciliate feeding was derived.

INTRODUCTION

The rôle of the ciliated protozoa in the activated-sludge process of sewage treatment has been the centre of much discussion and speculation since the introduction of the process. Some workers, notably Pillai (1942) and Pillai & Subrahmanyan (1944), were of the opinion that ciliates play the major rôle in the purification of domestic wastes and said that the peritrich protozoan *Epistylis* sp. in pure culture was capable of purifying sewage to the same degree as healthy whole activated sludge. However, most other authors have suggested that ciliates play a secondary rôle in that they aid the removal of suspended particulate matter and bacteria. Curds (1963) showed that certain ciliates have the ability to flocculate suspended matter in pure culture, and Curds, Cockburn & Vandyke (1968) showed that the introduction of ciliates into activated sludges consisting only of bacteria, caused a highly significant improvement in the clarity and the general quality of the effluents. They suggested that one of the major rôles of the ciliates is the removal, by predation, of the large numbers of suspended bacteria which only become apparent when protozoa-free activated sludges are grown. The purpose of the work described here was to examine whether or not ciliates might ingest sufficient numbers of bacteria to account for the removal of bacteria noted in small-scale activated-sludge experiments.

It is surprising that, although bacteria are the natural food for ciliates, little informa-

tion about the predatory activities of ciliates on bacteria is available. Some qualitative experiments on the feeding of ciliates on bacteria have been described (Burbanck, 1942; Curds & Vandyke, 1966), but there are almost no quantitative data in the literature.

In some of our earlier experiments the two ciliates *Opercularia coarctata* and *Histriculus vorax* were used as experimental organisms, but these were not easily grown and in particular it proved very difficult to obtain reliable estimates of the dry-weight yields of these organisms since they could be grown only in the presence of bacteria. To overcome these difficulties the ciliate *Tetrahymena pyriformis* was chosen for experiment; although it is not a very typical activated-sludge ciliate it has the great advantage of being easily grown both in the presence and absence of bacteria. It is reasonable to expect *T. pyriformis* to behave like other free-living ciliates and that the results obtained with this organism may be used as a guide for further studies with ciliates more typical of those associated with activated sludge.

METHODS

Organisms. Tetrahymena pyriformis GL (from The Culture Collection of Algae and Protozoa, The Botany School, Downing Street, Cambridge, L 1630/1 GL) was maintained axenically in Oxoid 1% (w/v) proteose peptone + 0·25 % (w/v) yeast-extract medium (PPYE). (Whenever PPYE medium was used in different concentrations for experimental purposes the two components of the medium were always mixed in the weight ratio of 4:1, PP:YE.) The bacterium *Klebsiella aerogenes* (National Collection of Industrial Bacteria, NCIB 8017) was routinely maintained on nutrient agar slopes.

Enumeration of bacterial populations. Population densities of bacteria were measured with a Unicam Spectrophotometer at 600 μm in 4 cm. cells. Bacterial dry-weight estimates were obtained from an extinction/dry-wt bacteria calibration curve which was linear up to a bacterial dry wt of 120 μg./ml. Dry-wt measurements for bacteria and protozoa were made by filtering thoroughly washed suspensions of the organisms through pre-washed Oxoid membrane filters. The membrane filters were then dried for 1 hr at 105°.

Enumeration of ciliate populations. Numbers of protozoa were counted in two ways. In axenic cultures, ciliates were counted by using a Vickers J 12 Electronic Cell Counter (Vickers Instruments Ltd., Haxby Road, York). A 50% transmission neutral-density filter was inserted between the chamber and the cathode of the photomultiplier tube so that only those particles of about 30 μ and above were counted. The electronic counter could also be used for ciliates in monoxenic culture, provided that the bacteria were not present in flocculated clumps. When clumps of bacteria were present the ciliates were counted under a microscope in a 0·2 mm. deep haemocytometer. At least three counts were made and the mean taken. Ciliates were previously killed for direct microscope counts by adding 0·1 ml. Lugol's iodine to 1 ml. culture and the count was corrected to allow for this dilution.

Estimation of organic carbon. Organic carbon determinations were made with a Beckman Carbonaceous Analyser. Samples containing organic carbon in solution were prepared by acidifying (pH 2) with HCl and the CO_2 removed by bubbling with CO_2-free air for 5 min. Samples containing suspensions of organic carbon were

similarly acidified and then homogenized for 5–10 min.; 20 μl. volumes of the prepared samples were then injected into the catalytic combustion tube.

Culture medium for monoxenic cultivation of Tetrahymena pyriformis and Klebsiella aerogenes. The nature of the work made it imperative to formulate a culture medium which would satisfy the growth requirements of the prey *K. aerogenes*, but would not support the growth of *T. pyriformis* in the absence of bacteria. A carbon-limited chemically defined medium which fulfils these requirements is given in Table 1. Sucrose was chosen as sole carbon source since, although it is readily utilized by *K. aerogenes*, it is not utilized by, nor does it inhibit, the ciliate (Seaman, 1955). The salts and sucrose solutions were prepared, autoclaved, and stored separately as stock solutions (Table 1). Preparation of the final medium was as follows. The required volume of phosphate buffer solution was made up in de-ionized water and autoclaved; after cooling, appropriate volumes of sterile stock salts and stock sucrose solutions were added aseptically. A linear relationship between the initial sucrose concentration of the medium and the effective yield of *K. aerogenes* was established; this made it possible to grow predetermined concentrations of bacteria by adding the appropriate quantity of sucrose to the medium.

Estimation of feeding rates. Flasks (100 ml.) of sterile culture medium containing known concentrations of sucrose were inoculated with *Klebsiella aerogenes* and shaken at 25° for 2 days. At the end of this time the bacterial population had reached its maximum and had just entered the stationary period. Each flask was then inoculated with a large number of well-washed *Tetrahymena pyriformis* organisms which had been grown axenically. The flasks were then shaken at 25° for 5 hr. Both the ciliate and bacterial populations were measured at zero time and after a 5 hr feeding period; during this time the number of ciliates increased, while the bacterial numbers decreased because of predation. Control flasks containing bacteria without protozoa never exhibited any measurable decrease in extinction during the 5 hr period. To define the effects of different numbers of ciliates and of bacteria on the feeding rate, it was necessary to take account of the changes in numbers during the 5 hr feeding period. Equations derived from simple considerations of growth kinetics were applied to compensate for non-linear changes in the populations, by averaging the integrated population curves over the time taken for the test. The population ratios calculated in this way were not significantly different from those obtained when the simple arithmetic means of the initial and final populations were used; these were therefore taken as being adequate for the purpose.

RESULTS

Yield of Tetrahymena pyriformis grown axenically

The relation between the initial concentration of PPYE medium and the maximum yield (usually after 60 hr) of *Tetrahymena pyriformis* in terms of numbers and dry weight is illustrated in Fig. 1. The relationship was linear in lower concentrations of medium, but some inhibition was shown in higher concentrations. The dry weight of a single *T. pyriformis* organism was determined to be $1{\cdot}39 \pm 0{\cdot}26 \times 10^{-9}$ g. (mean of 20 determinations) when grown axenically and the organic carbon content of the organism was estimated to be $55{\cdot}40 \pm 1{\cdot}28$ % (mean of 21 determinations). Figure 1 also illustrates the relation between the initial concentration of organic carbon in PPYE

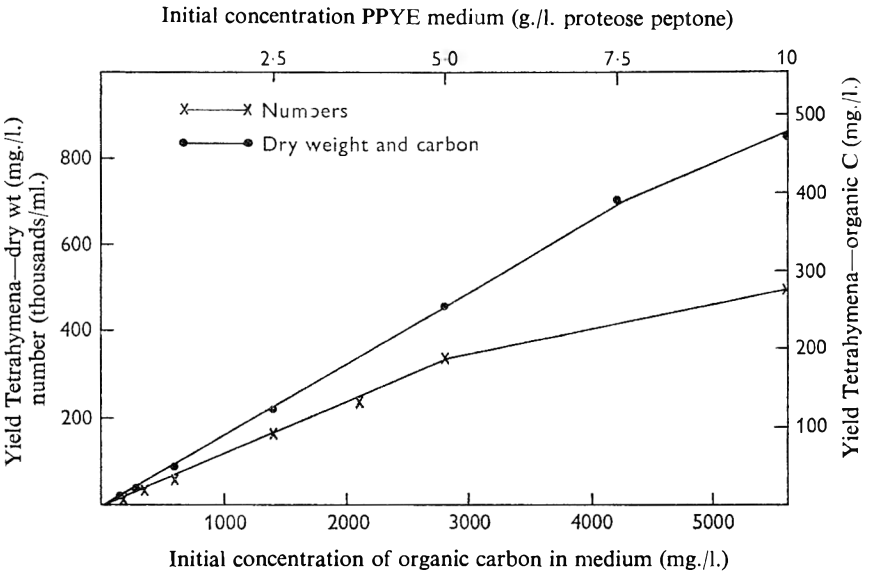

Fig. 1. Effect of substrate (PPYE and organic carbon) concentration on yield of *Tetrahymena pyriformis*.

Table 1. *Components of carbon-limited medium for monoxenic cultivation of Tetrahymena pyriformis and Klebsiella aerogenes*

	Concentration (g./l.)	
	Stock solution	Final medium
A. Salts solution		
KCl	7·48	0·037
NaCl	23·40	0·117
$MgSO_4.7H_2O$	26·64	0·123
$CaCl_2$	2·78	0·014
$FeSO_4.7H_2O$	2·78	0·014
$MnCl_4.4H_2O$	0·40	0·002
NH_4Cl	38·22	0·191
EDTA	10·00	0·050
5 ml. of solution A was added to 1 l. of solution B		
B. Sorensen phosphate buffer (pH 7·3)		
		Concentration (g./l.) in final medium
Na_2HPO_4		0·682
KH_2PO_4		0·254
C. 10 % (w/v) sucrose stock solution		

medium and the yield of ciliate carbon. It will be seen that the effective yield calculated from the linear regions of the graphs:

$$Ye = \frac{\text{dry wt ciliates formed}}{\text{dry wt PPYE in medium}} \times \frac{100}{1}$$

was 9·0 % and in terms of carbon:

$$Ye = \frac{\text{carbon in ciliates formed}}{\text{carbon in PPYE medium}} \times \frac{100}{1}$$

was 9·1 %. These effective yields appeared to be rather low, but it was possible that the medium contained a high proportion of organic matter that was not utilized by the ciliate.

Carbon balance for Tetrahymena pyriformis growing axenically

A series of flasks containing sterile 0·5 % (w/v) PPYE medium were each inoculated with *Tetrahymena pyriformis* and shaken at 25° until maximum growth had been attained (usually about 60 hr). The organic carbon contents of the initial PPYE medium, the medium containing ciliates after maximum growth and the medium after removal of the ciliates by centrifugation were determined. The difference between the initial organic carbon content of the medium and the carbon content of medium + ciliates after growth was assumed to be completely due to the loss of carbon dioxide by ciliate respiration.

Richman (1958) discussed several equations used by various authors to express the transformation of energy within an organism, two of which are

$$\text{input}_{(i)} = \text{growth}_{(g)} + \text{respiration}_{(r)} + \text{egestion}_{(e)} \quad (1)$$

and

$$\text{assimilation}_{(a)} = \text{growth}_{(g)} + \text{respiration}_{(r)}. \quad (2)$$

Expressed in terms of carbon flow these may be written:

$$C_i = C_g + C_r + C_e \quad \text{and} \quad C_a = C_g + C_r.$$

In the case of *T. pyriformis* utilizing a soluble substrate the term C_e in equation (1) includes not only the ciliate secretions (if any) but also the soluble carbon remaining in the medium which is not utilized by the ciliate during growth.

Substituting the results obtained, as percentages, in equation (1)

C_i	=	C_g	+	$C_r(CO_2)$	+	C_e
100 %		8·26 ± 0·8 %		15·6 ± 0·9 %		77·4 ± 1·3 %

(means and standard deviations of six determinations)

Whereas there was a close agreement between the C_g value (8·26 ± 0·8 %) obtained in the carbon balance and the effective carbon yield value (9·1 %) obtained above, the C_e value was rather high (77·4 %) which suggested that the PPYE medium contained a large proportion of carbon not utilized during growth. Assuming that ciliate secretions were insignificant, then only 22·6 % of the carbon present in the initial medium was assimilated during growth. This was not entirely surprising because the medium was chosen for its convenience, without regard to the efficient use of its nutrient content.

Thus, substituting the results in equation (2):

C_a	=	C_g	+	C_r
(carbon utilized)		36·5 %		69·0 %

That is to say, 36·5 % of the carbon utilized during growth (or the yield constant Y = wt of ciliate C formed/wt of substrate C utilized = 0·365) was incorporated into ciliate cytoplasm and 69 % was oxidized to carbon dioxide by their respiratory activities. These values approach those obtained when bacteria are grown on soluble substrates.

Growth rates of Tetrahymena pyriformis growing axenically

The growth rates of *Tetrahymena pyriformis* growing axenically in various concentrations of PPYE at 25° were determined. A reciprocal (Lineweaver–Burke) plot of

the results is presented in Fig. 2*a*. The maximum growth rate ($\mu_{max.}$) obtained from this graph (i.e. where the curve intersects the *y* axis) was 0·2 hr^{-1}, which was equivalent to a doubling time of 3·46 hr (where $t_D = \log_e 2/\mu_{max.}$). The Michaelis coefficient K_s, that is the substrate concentration at which the growth rate is equal to half its maximum, was also obtained from this graph and estimated to be 0·24 % (w/v), PPYE providing 0·13 % (w/v) carbon.

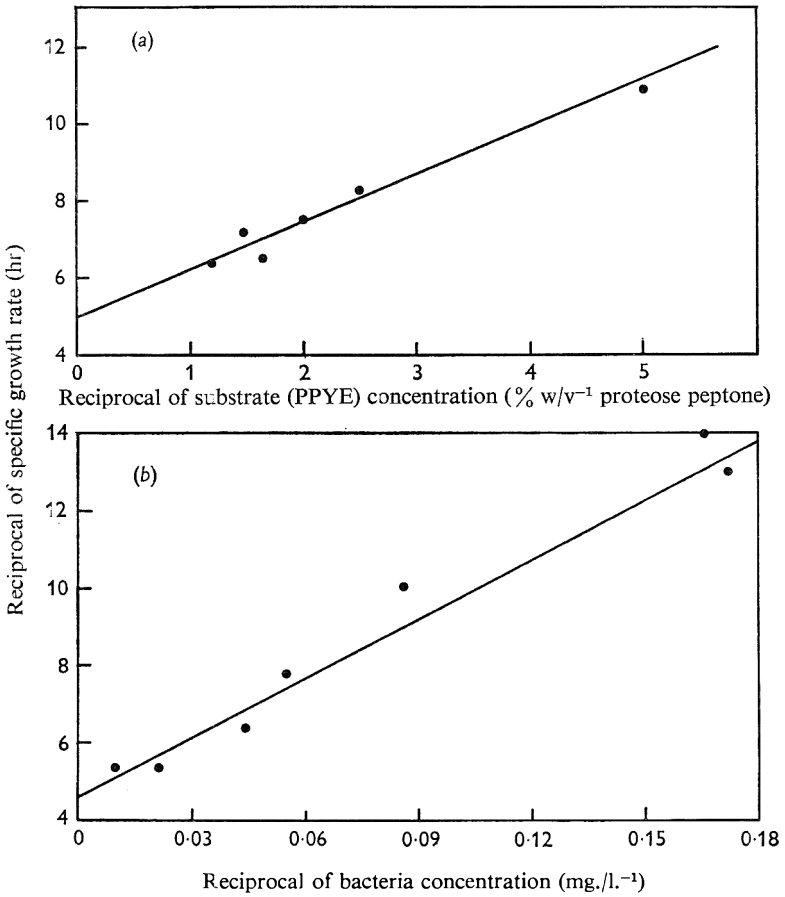

Fig. 2. Lineweaver–Burke plots of effect of substrate concentration on specific growth rate of *Tetrahymena pyriformis*. (*a*) Axenic culture: $\mu_{max.} = 0{\cdot}2\ hr^{-1}$ ($\equiv t_D = 3{\cdot}5$ hr); $K_s = 0{\cdot}24$ % (w/v) PPYE. (*b*) Monoxenic culture: $\mu_{max.} = 0{\cdot}22\ hr^{-1}$ ($\equiv t_D = 3{\cdot}15$ hr); $K_s = 11{\cdot}6$ mg./l. dry wt bacteria.

Yield of Tetrahymena pyriformis growing monoxenically on Klebsiella aerogenes

There was a linear relation between initial concentration of sucrose in the culture medium and the yield of *Klebsiella aerogenes*, the effective yield (*Ye* = dry wt bacteria formed/initial concentration of sucrose in medium) being 46·1 %. Similarly, from bacterial concentrations below 140 mg. dry wt/l., there was a linear relation between the concentration of *K. aerogenes* and the yield of *Tetrahymena pyriformis*, as shown in the results presented in Fig. 3. The effective yield of *T. pyriformis* feeding on *K. aerogenes* was 50 %. This was a considerably higher yield than the value obtained

for *T. pyriformis* growing in PPYE medium, but this might be expected since the food for the ciliate in the natural environment would be bacteria rather than soluble organic matter. (The yield of *T. pyriformis* decreased at higher bacterial concentrations.)

Growth rates of Tetrahymena pyriformis feeding on Klebsiella aerogenes

The growth rates of *Tetrahymena pyriformis* growing monoxenically with various concentrations of *Klebsiella aerogenes* were determined. The different concentrations of the bacterium were achieved by incorporating an appropriate concentration of sucrose in the medium, inoculating with *K. aerogenes*, and allowing a period of 48 hr

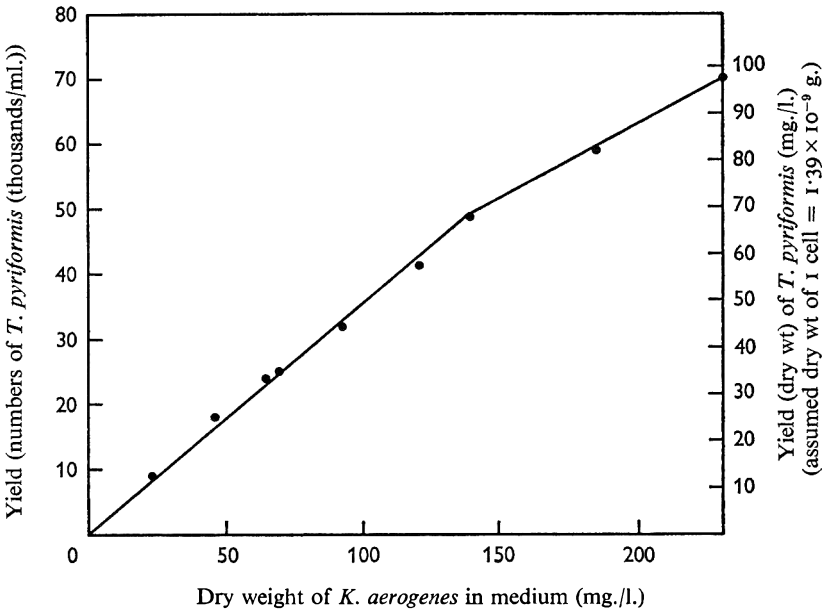

Fig. 3. Effect of substrate (bacteria) concentration on yield (number and dry wt) of *Tetrahymena pyriformis*.

for maximum bacterial growth to be attained. Ciliates were then added to the bacterial suspension and the ciliate growth rate during the first 10 hr measured. The results obtained are given in Fig. 2*b* in the form of a Lineweaver–Burk plot. In monoxenic culture the maximum growth rate at 25° was 0·22 hr^{-1} (a doubling time of 3·15 hr) which corresponded approximately to that obtained in axenic culture. However, the Michaelis coefficient substrate concentration was only 11·6 mg. dry wt bacteria/ml. (providing about 5·5 mg. carbon/l.).

Feeding rate of Tetrahymena pyriformis on Klebsiella aerogenes

Initially the feeding rates of various concentrations of *Tetrahymena pyriformis* in various concentrations of *Klebsiella aerogenes* were estimated. When the observed average individual ciliate feeding rates (f) were plotted against the mean concentration of bacteria ($\bar{b}$) present during the 5 hr feeding period, a wide scatter of points was obtained (Fig. 4). However, when the individual feeding rate was plotted against the

ratio $\bar{b}/\bar{n}$, the weight of bacteria available to each ciliate (Fig. 5), the scatter was considerably decreased. This suggested that both the concentration of predator (ciliate) and prey (bacteria) played important rôles in controlling the feeding rate of the ciliate. As the ratio of bacteria to ciliates increased the individual ciliate feeding rate increased until a plateau, the maximum individual feeding rate, was reached. Beyond this point bacteria were always in excess and so there was no increase in feeding rate. However, from Fig. 5, there appears to be a peak in feeding rate before the plateau is reached but the significance of these points was not established. However, subsequent continuous-culture studies, still in progress, have not shown a similar peak, but the maximum feeding rate determined by this method was somewhat higher than recorded in this work. Furthermore, continuous-culture has shown that there is a

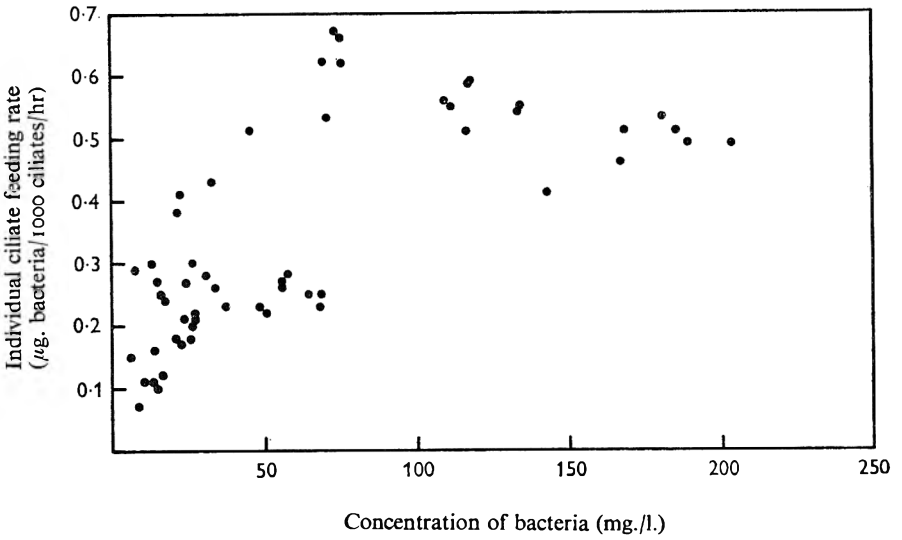

Fig. 4. Effect of substrate concentration (bacteria) on individual feeding rate (f) of *Tetrahymena pyriformis*.

relation between the growth and feeding rates of *T. pyriformis*. Therefore, at maximum feeding rate the growth rate was also at its maximum, so that the yield constant (Y = wt ciliate formed/wt bacteria consumed) could be calculated from the following equation:

$$Y = \frac{W}{f_{\max.} \times t_{D\min.}}. \tag{3}$$

When the observed data were substituted into this equation (i.e. W = 1·39 μg., $f_{\max.}$ = 0·49 μg., and $t_{D\min.}$ = 3·15 hr) an absurdly high yield was obtained. However, it was found that the minimum doubling-time calculated previously was far smaller than that actually obtained during the 5 hr feeding period. Presumably, the method of preparation of the ciliate inoculum was sufficiently disturbing to induce a lag in the ciliate growth and feeding activities. The mean minimum doubling time actually observed, when bacteria were in excess during the feeding period, was calculated to be 6·8 hr and when this value was substituted in equation (3) a yield constant of 0·414

(41·4%) was obtained. This agreed reasonably well with the 50% effective yield obtained previously.

It may be seen from Fig. 4 that despite the wide scatter of points there was a general tendency for the feeding rate of *Tetrahymena pyriformis* organisms to increase with increasing concentrations of bacteria until a plateau was reached. This indicated that the kinetics of monoxenic feeding of *T. pyriformis* were analogous to the axenic growth kinetics of bacteria, as described by Monod's (1942) equation. This seems plausible when the growth rate of a ciliate is directly proportional to its feeding rate. The original form of Monod's equation was

$$\mu = \frac{\mu_{\max.} S}{K_s + s}, \tag{4}$$

where s = substrate concentration and μ = growth rate coefficient. When considering the rate of feeding of *T. pyriformis* on *Klebsiella aerogenes*, by analogy:

$$f = \frac{f_{\max.} \bar{b}}{K_s + \bar{b}}, \tag{5}$$

where $\bar{b}$ = mean bacterial concentration and f = feeding-rate coefficient. However, equation (5) did not fit precisely since it did not take into account the predator population effect which has been indicated (Fig. 5). Contois (1959) found that when *Aerobacter aerogenes* was grown in a chemostat, the specific growth rate of this bacterium was dependent not only on the concentration of the limiting nutrient but also on the population density of the growing bacteria; he therefore modified Monod's equation (4) as follows:

$$\mu = \frac{\mu_{\max.} S}{CP + s} \tag{6}$$

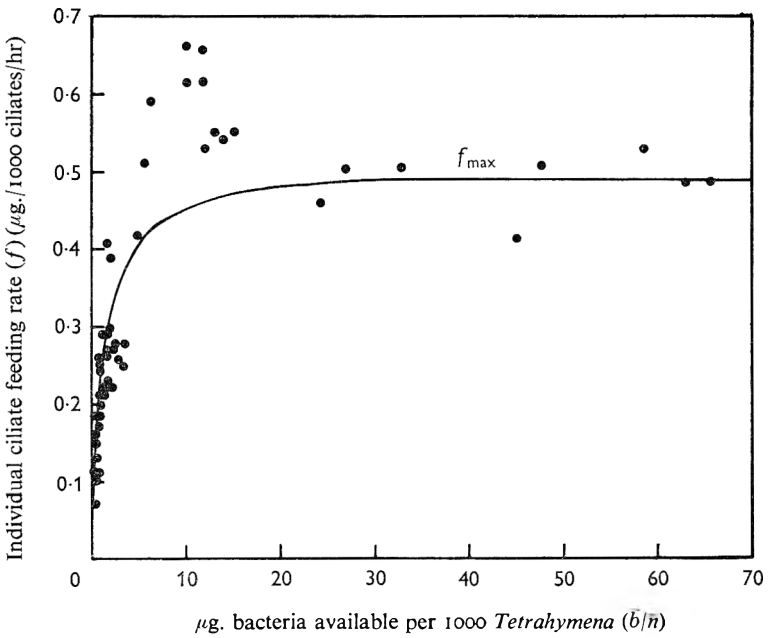

Fig. 5. Effect of substrate concentration (dry wt bacteria available per 1000 ciliates) on individual feeding rate of *Tetrahymena pyriformis*.

where C 'is a growth parameter which is constant under defined conditions' and P is the bacterial population density.

When equation (6) is expressed in terms of ciliate feeding then:

$$f = \frac{f_{\max.}\bar{b}}{C\bar{n}+\bar{b}}, \tag{7}$$

where $\bar{n}$ is the mean number of ciliates.

Rewriting $$\frac{\bar{b}}{f} = \frac{\bar{b}+C\bar{n}}{f_{\max.}} \quad \text{or} \quad \frac{\bar{b}}{f} = \frac{\bar{b}}{f_{\max.}} + \frac{C\bar{n}}{f_{\max.}}.$$

Whence $$\frac{\bar{b}}{\bar{n}f} = \frac{\bar{b}}{\bar{n}}\frac{1}{f_{\max.}} + \frac{C}{f_{\max.}}. \tag{8}$$

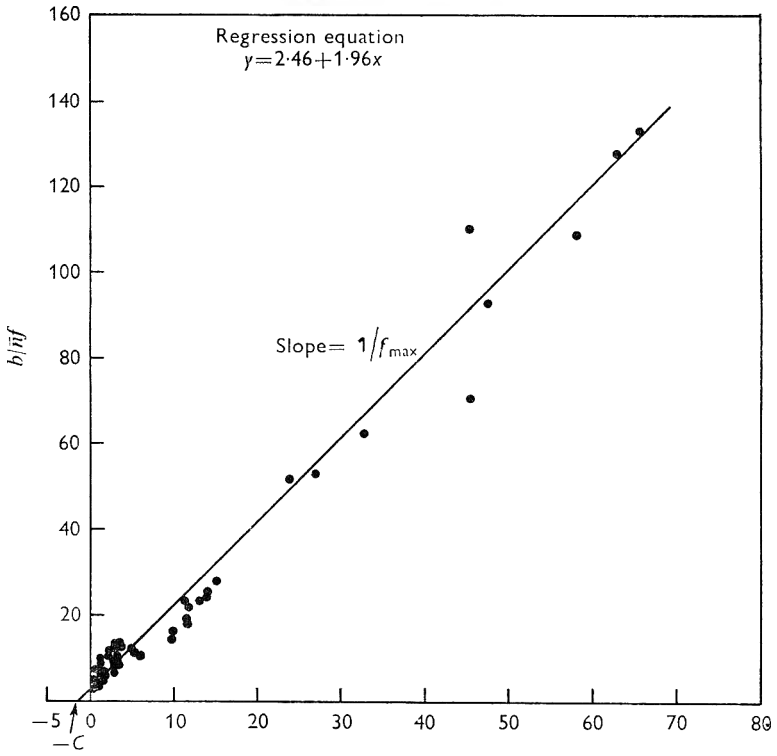

Fig. 6. Linear form of feeding data illustrated in Fig. 5. Line of best fit obtained by regression analysis (Regression equation $y = 2{\cdot}46 + 1{\cdot}96x$.).

Therefore from equation (8) when $\bar{b}/\bar{n}f$ is plotted against $\bar{b}/\bar{n}$ a straight line will be obtained if the data fit equation (7). Furthermore, the slope of this line will be equal to $1/f_{\max.}$ and will intercept the abscissa at a value equal to the constant C. Figure 6 is such a plot and illustrates that there was indeed a linear relationship. The line of best fit was obtained by regression analysis and the values of $f_{\max.}$ and C were calculated to be 0·49 and 1·26, respectively. On inserting these values into equation (7) the relation between bacterial concentration, ciliate population and individual feeding rate may be described mathematically by the equation

$$f = \frac{0{\cdot}49\bar{b}}{1{\cdot}26\bar{n}+\bar{b}}, \tag{9}$$

and since the gross feeding rate of the total ciliate population (F) is equal to $\bar{n}f$ then

$$F = \bar{n}\frac{0{\cdot}49\bar{b}}{1{\cdot}26\bar{n}+\bar{b}}. \tag{10}$$

Using equation (9) the effects of various ciliate populations on the individual ciliate feeding rate in four fixed concentrations of bacteria were predicted. The four predicted curves are included in Fig. 7 for comparison with the experimental points obtained by presenting various ciliate populations with the four concentrations of bacteria. It will be seen that the correlation was reasonable, considering the experi-

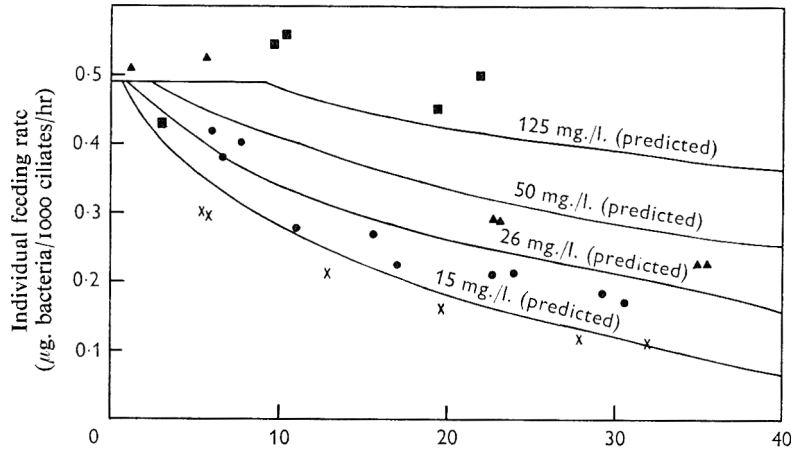

Fig. 7. Predicted and observed effect of ciliate population density on individual feeding rate of *Tetrahymena pyriformis* at four substrate (bacteria) concentrations. ×, Observed at $\bar{b}$ = 15 mg./l.; ●, observed at $\bar{b}$ = 26 mg./l.; ▲, observed at $\bar{b}$ = 50 mg./l.; ■, observed at $\bar{b}$ = 125 mg./l. Predicted lines calculated from equation (9).

mental errors that must be involved when determining feeding rates. The individual feeding rate of the ciliates diminished with increasing ciliate concentration throughout the range of bacterial concentrations tested, especially at the lowest bacterial concentrations. Presumably there was mutual interference between the feeding currents of individual ciliates which was more serious when the bacteria were fewer in number. It can be seen that, for any given concentration of ciliates, the feeding rate diminished as the bacterial concentration decreased.

Equation (10) was used to predict the gross feeding rate of the whole ciliate population at different concentrations. Whereas the individual ciliate feeding rate decreased with increasing ciliate population densities, the gross feeding rate of the total ciliate population increased with increasing ciliate concentrations (Fig. 8). It therefore seemed likely that predation continued until very high concentrations of *Tetrahymena pyriformis* and very low concentrations of bacteria were reached. Such was the finding when attempts were made to grow continuous cultures of bacteria and ciliates together; no steady state was established (Curds, 1967). The ciliates multiplied until the bacterial population was decreased to a very low value. Presumably because of loss of metabolic activity of *T. pyriformis* in the absence of an adequate supply of bacteria, there was then a phase of recovery in bacterial population density. This was eventually

succeeded by a resurgence of ciliate domination, and the cycle then repeated indefinitely. However, the results presented in Fig. 10 emphasize that competition between ciliates had less effect on feeding-rate at high bacterial concentrations.

The feeding rates of individuals in particular ciliate populations at various concentrations of bacteria were also calculated from equation (9); some are illustrated in Fig. 9. This shows that lower populations of ciliates reached the plateau or maximum

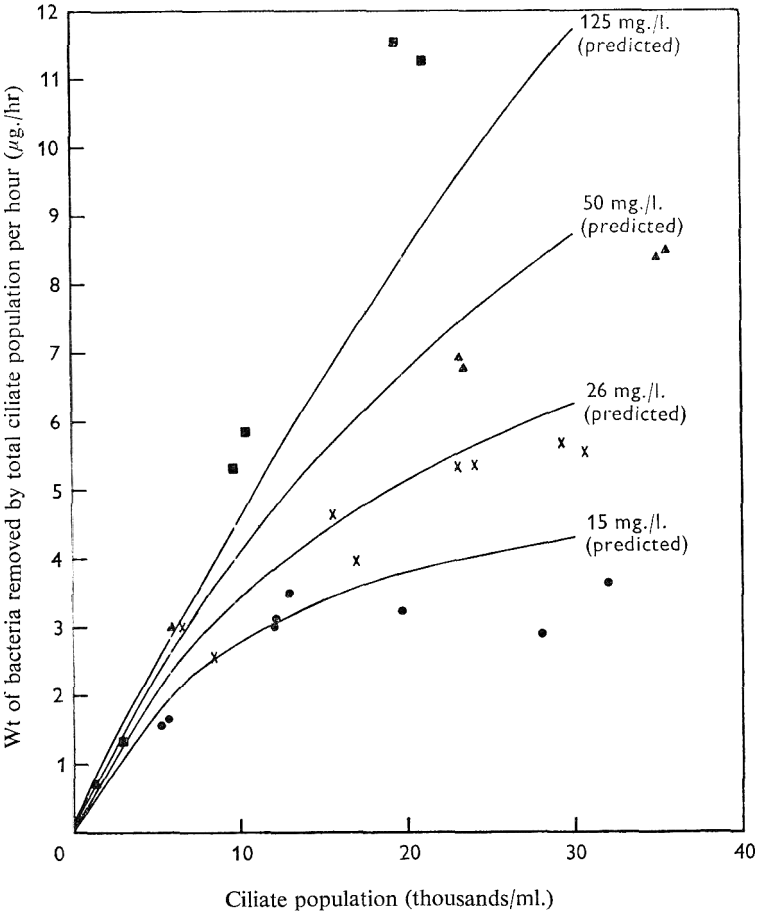

Fig. 8. Predicted and observed effect of ciliate population density on overall feeding rate of *Tetrahymena pyriformis* at four substrate (bacteria) concentrations. ×, Observed at $b = 15$ mg./l.; ●, observed at $b = 26$ mg./l.; ▲, observed at $b = 50$ mg./l.; ■, observed at $b = 125$ mg./l. Predicted lines calculated from equation (10).

individual feeding rate in lower concentrations of bacteria than did the higher ciliate concentrations. It was found, therefore, that the individual feeding rate of *Tetrahymena pyriformis* was dependent on two variables, populations of both ciliates and bacteria being important. Thus equation (9) describes a three-dimensional model. Figures 7 and 9 are cross-sections of this model, which is shown isometrically in Fig. 10.

The only quantitative data about the feeding of *Tetrahymena pyriformis* have been reported in a manner different from those expressed here. Seaman (1961) and Cox (1967) were more concerned with the volume of liquid cleared of particulate matter

by a ciliate population in a given time, than with the quantity of particles ingested. However, the data obtained from the feeding studies described here have been re-calculated to be able to compare them with those of Seaman (1961) and Cox (1967). There was considerable disagreement between the estimates of the volume of medium filtered by 10^6 ciliates given by Seaman (1961) who measured the uptake of trypan blue

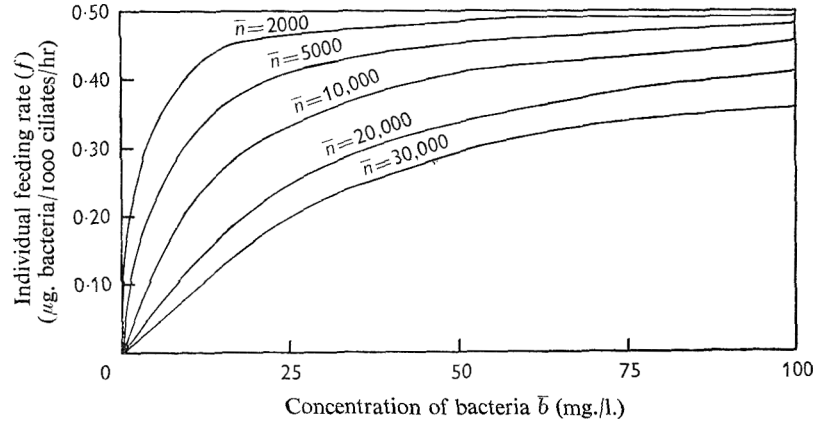

Fig. 9. Effect of substrate (bacteria) concentration on individual feeding rate of *Tetrahymena pyriformis* at five ciliate population densities predicted from equation (9).

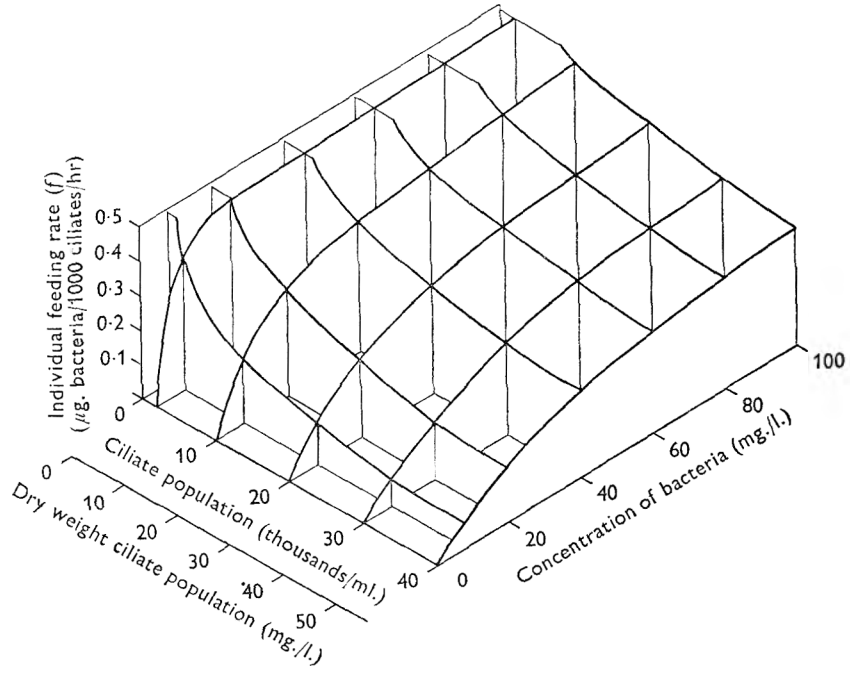

Fig. 10. Three-dimensional model of effect of substrate (bacteria) concentration and ciliate population density on individual feeding rate of *Tetrahymena pyriformis*.

particles, and Cox (1967) who used carbon particles. Seaman (1961) calculated that 10^6 ciliates cleared 0·034 to 0·042 ml./hr, whereas Cox (1967) observed that 5·3 to

10·3 ml./hr were cleared by a similar ciliate concentration. The results obtained in the present work with bacteria as the particulate matter, agree quite favourably (3 to 15 ml./10^6 ciliates hr) with those obtained by Cox (1967) and support his view that *T. pyriformis* is capable of removing much more particulate material from the surrounding medium than had been previously believed.

DISCUSSION

Very large populations of protozoa are frequently encountered in activated sludge and the rôle that these organisms might play in the trophic structure of the process has been considered by a number of workers. Curds & Vandyke (1966) showed that ciliate species isolated from activated sludge could grow for long periods on certain bacteria which had been reported to occur in sewage-treatment processes. Curds *et al.* (1968) showed that when sludges were artificially kept free from protozoa, very large numbers of bacteria appeared in the effluent, but that these virtually disappeared when ciliates were subsequently added. The quantitative feeding studies reported here have demonstrated that in pure culture *Tetrahymena pyriformis* can remove appreciable quantities of bacteria by predation; the quantity of food ingested by a ciliate is generally somewhat related to the size of the organism and the larger the ciliate the greater is the quantity of bacteria consumed by the individual per unit time (Curds, 1967). *Tetrahymena pyriformis* is a small organism in comparison with the ciliates generally found dominant in activated-sludge plants, and will therefore presumably consume fewer bacteria. However, even if the feeding rates of *T. pyriformis* are assumed, calculations indicate that the ciliate population densities normally encountered could easily account for the removal by predation alone of those quantities of bacteria noted by Curds *et al.* (1968). This would suggest that protozoan-induced flocculation (Curds, 1963) may play only a secondary rôle in the removal of bacteria in the activated-sludge process.

Several interesting facts about the growth and feeding of protozoa on bacteria have emerged in this work. The yield constant of 0·41 seems remarkably high for a protozoan, although yields similar to this are common for bacteria when growing aerobically (Hadjipetrou, Gerrits, Teulings & Stouthamer, 1964; Herbert, Elsworth & Telling, 1956). However, the yield constant estimated here lies between two of the three protozoan yields published in the literature. Coleman (1964) used *Escherichia coli* labelled with ^{14}C to feed the rumen ciliate Entodinium and calculated that 50% of the bacterial-carbon ingested was retained in the ciliate cytoplasm. Heal (1967*a*) who used techniques very much like those used in the present work, calculated that the soil amoeba Acanthamoeba has a yield constant of 0·37 when fed on *Saccharomyces cerevisiae*. The general conclusions of Proper & Garver (1966), who investigated the growth of the ciliate *Colpoda steinii* when fed on *E. coli*, were similar to those found here, but they calculated an effective yield of 78% (dry wt bacteria: dry wt of ciliate) which seems particularly high. Even when the yield given by Proper & Garver (1966) is ignored, the range of 0·37–0·50 is much higher than is generally recorded for metazoan invertebrates; and Heal (1967*b*) suggested that the differences between the micro-organisms (i.e. protozoa and bacteria) and the metazoan invertebrates are that: (i) the micro-organisms grow at relatively high rates, and (ii) they do not possess a stage in their life-cycle equivalent to the adult metazoan. Micro-organisms generally grow to a certain size, divide and proceed to grow again, whereas the macro-

invertebrates generally pass through a series of larval forms before becoming adults which only grow in the sense that tissues such as reproductive cells, etc. are produced. Engelmann (1961) showed that slow-growing or non-reproducing organisms tended to convert less food to protoplasm than did the fast-growing animals; MacFadyen (1963) stated that macro-invertebrates used about 90% of their assimilated energy in respiration over their complete life-cycle.

Cutler & Crump (1924) noted that the ratio of bacteria:ciliates rather than simply the bacterial concentration influenced the growth rate of ciliates and it would appear from the results in Figs. 6 and 7 that this ratio is important in determining the feeding-rate of the individual ciliate. Heal (1967*a*) also used this ratio in his studies on the feeding of Acanthamoeba, but several other workers concerned with the feeding activities of small crustaceans did not take this ratio into account. Furthermore, any influence that the predator population density might have had in those studies would not have been shown since those workers used a constant inoculum of predator organisms. McMahon & Rigler (1965), for example, used a constant inoculum of 100 *Daphnia magna* and varied only the concentration of available food organisms. Thus those authors found that, at this crustacean population density, as the food organism concentration was increased the feeding rate increased until a plateau was reached when food was in excess. It would have been of great interest to have known what the feeding rates would have been if both the predator and prey concentrations had been varied.

This sort of criticism can also be applied to the monoxenic growth rate experiments reported in the present work and in the work of Proper & Garver (1966) since Cutler & Crump (1924) gave evidence to suggest that the growth rate depended on both the ciliate and the bacterial populations. If this be so then presumably the Michaelis coefficient will also depend upon the ratio of prey:predator, so that as the predator population increases the Michaelis coefficient increases. If the yield of protozoa feeding on other micro-organisms is about 40% as seems to be indicated then, if one is studying the ecology of a particular environment (a lake, activated sludge, or the soil), if the doubling time and the biomass of the organisms in that environment are determined, then a reasonable assessment of the part played by these organisms in the trophic structure of the ecosystem may be obtained.

MATHEMATICAL SYMBOLS

$\bar{b}$	mean concentration of bacteria (μg./ml.)
f	feeding rate of ciliate individual (μg. bacteria/1000 ciliates/hr)
$f_{max.}$	maximum feeding rate of ciliate individual (μg. bacteria/1000 ciliates/hr)
F	feeding rate of total ciliate population (μg. bacteria/hr)
K_s	Michaelis coefficient
$\bar{n}$	mean concentration of ciliates (thousands/ml.)
t_D	doubling time (hr)
$t_{D\min.}$	minimum doubling time (hr)
$\mu_{max.}$	maximum growth rate (hr^{-1})
W	dry weight of 1000 ciliates (μg.)
Y	yield constant
Ye	effective yield

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Ultrastructure of Flagellated Lambda Symbionts in *Paramecium aurelia*

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SUMMARY

The symbionts called 'lambda' present in the cytoplasm of killer stocks 239 (syngen 4) and 299 (syngen 8) of *Paramecium aurelia* have been investigated. Observations with the electron microscope of ultrathin sections and of negatively stained material revealed that these symbionts have peritrichous flagellation. The ultrastructures of symbionts from both stocks were identical and were those of flagellated bacteria.

INTRODUCTION

Four stocks of *Paramecium aurelia* bear cytoplasmic symbionts known as lambda: stock 239 (syngen 4), stocks, 216, 229 and 299 (syngen 8). They were described by Schneller (1957, 1961, 1962), Schneller, Sonneborn & Mueller (1959) and Sonneborn (1950, 1959, 1965). These stocks are referred to as rapid lysis killers, since sensitive paramecia are lysed within 20 min. after being exposed to them. Paramecia of syngen 3 are especially sensitive to the action of all lambda-bearing killers. Lambda particles are much larger than most of the other symbionts which have been described for *P. aurelia*. Van Wagtendonk, Clark & Godoy (1963) reported that it was possible to grow lambda particles in cell-free cultures in an axenic medium. There has been little electron microscopy of lambda reported. Sonneborn (1965) published one micrograph of this symbiont. The present paper reports investigations on lambda of *P. aurelia* stocks 239 and 299 by use of phase-contrast and electron microscopy.

METHODS

Both stocks of Paramecium were grown in lettuce medium previously inoculated with *Aerobacter aerogenes* (Sonneborn, 1950). Lambda particles outside the host cytoplasm were observed with a bright phase-contrast microscope (American Optical Co.) in animals freshly crushed between a slide and coverslip. To observe the symbionts *in situ* two methods were used. In the first, whole paramecia were observed with a dark phase-contrast microscope (Carl Zeiss), using paramecia treated with lacto-orcein according to the method of Beale & Jurand (1966). In the second, 1 μ Araldite sections were mounted on slides, stained with toluidine blue and observed with ordinary bright-field microscopy.

The ultrastructure of lambda symbionts was investigated by examination of ultrathin sections and of negatively stained isolated symbionts with the A.E.I. 6 electron microscope. For ultrathin sectioning mass cultures of each stock were fixed in one of two ways. In the first method the paramecia were concentrated by centrifugation,

and the supernatant culture medium replaced with a modified Palade (1952) fixative containing 15 mg. sucrose and 0·1 mg. calcium chloride/ml. fixative. Fixation was for 30 min. at room temperature (20°) or at 37·5°, and during this time the suspension of paramecia was gently stirred every few minutes. After fixation the paramecia were concentrated by centrifugation and dehydrated with aqueous 35, 70, 90, and 100 % ethanol, and embedded in Araldite on a slow rotary shaker (Jurand & Ireland, 1965) for 1 hr. Some preparations were made by using 1,2-epoxy-propane + Araldite before embedding in Araldite.

The second method of fixation used a 30 % (w/v) solution of osmium tetroxide in carbon tetrachloride (Afzelius, 1962). The osmium tetroxide solution was prepared just before use, and 0·3 ml. was added to a concentrated suspension of paramecia in the culture medium (0·6 to 0·8 ml.). The mixture was left for 20 min. at room temperature and the test-tube gently shaken frequently. Carbon tetrachloride and the culture medium do not mix, and fixation takes place in the culture medium into which osmium tetroxide penetrates very readily from the carbon tetrachloride layer. After fixation, 70 % (v/v) ethanol in water was slowly added and the mixture gently shaken until the two layers united. The mixture was then centrifuged, and the supernatant fluid discarded and replaced by 70 % (v/v) ethanol in water. Further dehydration and embedding was the same as in the first method. This second method of fixation is particularly suitable for preservation of various cytoplasmic symbionts in different stocks of *Paramecium aurelia*. For example, it was found to prevent shrinkage and preserve ultrastructure of refractile bodies and virus-like particles in kappa of stocks 7 and 562 (Preer & Jurand, 1968). On the other hand, the host cell organelles are usually well preserved with the modified Palade fixative (1952).

Partially purified lambda symbionts were prepared for negative staining as follows. A homogenate of 0·3 ml. of paramecia of stock 239 in 10 ml. of Dryl (1959) solution was diluted with an equal volume of 0·01 M-sodium phosphate buffer (pH 7) and passed through a cellulose column 12 mm. in diameter and 50 mm. high. The column was prepared by suspending 2 g. Whatman Ashless Filter Paper Pulp in distilled water and then washing with phosphate buffer. Examination with the bright phase-contrast microscope indicated that the turbid wash from the column was free from trichocysts and fragments of the cell body wall. The large lambda particles were separated from the lipid droplets, mitochondria and fragments of cilia in the turbid wash by a light centrifugation of 1000 *g* for 5 min. About 90 % of the particles in the pellet were lambda, with a few bacteria and mitochondria. A drop of the preparation was mixed with a drop of 5 % (w/v) phosphotungstic acid (pH 7) and examined with the electron microscope. Bacterial contaminants of two kinds were present. Their shape and small size made it possible to distinguish them readily from the numerous and much larger lambda particles.

To decrease to a minimum the number of bacteria present in the final preparation of isolated lambda particles and to aid the identification of the few bacteria which were present, a modified procedure was followed for the isolation of stock 299 lambda particles. The culture of paramecia was concentrated by centrifugation and the supernatant fluid which contained the bacteria was retained for later use as described below. The precipitate of packed paramecia was resuspended in 25 % (w/v) sterile yeast extract (Difco) at a concentration of about 0·1 ml. packed organisms in 100 ml. yeast extract solution. The paramecia were allowed to remain at room temperature

for 30 to 60 min., then filtered through cheesecloth and centrifuged. This process was repeated twice. Food vacuoles of paramecia treated in this way were seen to have no bacteria in them. These paramecia were then treated according to the procedure that was used to isolate lambda particles from stock 239. The lambda particles of stock 299 collected in the turbid wash from the cellulose column had very few contaminating bacteria among them. This preparation was examined with bright phase-contrast and electron microscopy. To establish a clear picture of the bacterial contaminants present, the original culture medium in which the paramecia had been grown, and from which they had been removed, was centrifuged at 8000 *g* for 5 min. The concentrated bacteria of the pellet were examined in bright phase-contrast and electron microscopes. This made possible the recognition of any bacteria, however few, which were in the preparations of isolated lambda particles.

RESULTS

Lambda symbionts in *Paramecium aurelia* stocks 239 and 299 were distributed throughout the host cytoplasm (Pl. 1, fig. 1 to 4). Usually there were more lambda particles present in stock 239 organisms than in stock 299 organisms grown under the same conditions. The symbionts were Gram negative bacterium-like particles, 2 to 4 μ long and about 0·5 μ wide, and were very similar in the two stocks. Lambda of crushed paramecia of stock 299 are shown as seen in the bright phase-contrast microscope in Pl. 1, fig. 5. A partially-purified preparation of lambda particles of stock 239 obtained from the cellulose column is shown in Pl. 1, fig. 6.

Examination of ultrathin sections of paramecia with the electron microscope revealed that lambda symbionts in both stocks were flagellated. The flagella were fairly evenly distributed over the entire surface of the symbiont, and the general ultrastructure of the flagella in both stocks was identical. *In situ*, the lambda particles, together with flagella, were usually enclosed within elongated vacuoles delimited from the host cytoplasm by an interface boundary. Two or three symbionts might be enclosed in a single vacuole (Pl. 1, fig. 7, 8).

The presence of numerous flagella was confirmed by examining with the electron microscope negatively stained preparations of partially purified lambda (Pl. 2, fig. 9, 10). It is difficult to give an approximate figure for the number of flagella present on one symbiont. In sectioned material the flagella were visible only as fragments, and in negatively stained preparations it was quite possible that some of the flagella may have been lost because of mechanical breakage during purification. It was estimated that there might be at least 100 flagella on the entire surface of one lambda symbiont. This estimate was made from Pl. 2, fig. 11, where there are a number of flagella sectioned transversely.

In ultra-thin sections the diameter of the flagella appeared to be about 170 Å. The ultrastructure was much less granular than in negatively stained preparations and occasionally showed transverse striations. In negatively stained preparations the measurements are uncertain because of the flattening of flagella during desiccation and because it is difficult to estimate the extent to which the negative stain penetrated the flagella. In these preparations the flagella very frequently assumed a characteristic sinusoidal form and were up to 5 μ long (Pl. 3, fig. 12). Their ultrastructure in negatively stained preparations was granular and appeared as two rows of linearly arranged

electron-dense subunits extending along the length of the flagellum symmetrically on both sides at about one quarter of its diameter (Pl. 3, fig. 13).

The lambda symbionts were enclosed in a cell wall consisting of two unit membranes. Inside the symbionts there were tightly-packed dense granules about 120 Å in diameter. The distribution of these granules, although dense, was not very uniform. There were less dense areas without granules occupied by elongated fibrillar structures about 500 Å long and 200 Å wide (Pl. 3, fig. 14).

To investigate whether flagella confer mobility on the lambda particles, paramecia were crushed between a slide and coverslip and observed with a bright phase-contrast microscope. In these conditions the lambda particles often showed a limited 'wiggling' motion, quite different from the Brownian movement. However, no clear swimming movement was observed.

DISCUSSION

Cytoplasmic symbionts in paramecia have long been regarded as bacterium-like. This idea is further supported by the present work which shows that lambda particles have several features which are characteristic of bacteria. First, the filamentous appendages of lambda resemble bacterial flagella. They are more like flagella than other similar bacterial structures such as pili (fimbriae). This conclusion is reached from a consideration of the length, diameter, surface distribution and especially the sinusoidal form of these appendages in negatively stained preparations. The length and diameter are within the ranges for these structures in bacteria (Newton & Kerridge, 1965). The more or less uniform distribution over the surface of the lambda particle is peritrichous flagellation, which is very common in bacteria (Leifson, 1960). Moreover, after drying the sinusoidal form is typical of bacterial flagella (Stocker, 1956), whereas pili are always straight (Brinton, 1965). The flagella of lambda particles are strikingly similar in shape to most of the flagella of bacteria shown in the paper of Houwink & Iterson (1950), which is a most informative work on this subject. The ultrastructure of lambda flagella is virtually identical with that of bacterial flagella, e.g. in *Proteus vulgaris* (Abram, Koffler & Vatter, 1965). Secondly, lambda particles give a negative reaction with the Gram reagents and the ultrastructure of the wall is very like that of Gram-negative bacteria such as *Escherichia coli*, Spirillum species and others (Murray, Steed & Elson, 1965; van Iterson, 1965). The two thin double-unit membranes observed in lambda are characteristic of Gram-negative bacteria. Thirdly, the ultrastructure of the intracellular content of lambda particles is similar to that of bacterial cytoplasm. The tightly packed dense granules in lambda particles resemble bacterial ribosomes, and the elongated fibrillar structures present in the less dense areas are similar to bacterial nucleoids (van Iterson, 1965; Fuhs, 1965). Altogether, filamentous appendages which resemble bacterial flagella, the ultrastructure of the cell wall which is very similar to that of Gram-negative bacteria, and the ultrastructure of the cytoplasm which is similar to the cytoplasm of bacteria, all make it evident that a lambda particle may well be regarded as a prokaryotic unicellular organism, a Gram-negative bacterium.

ADDENDUM

After this paper was sent for publication we obtained the two remaining stocks known to contain lambda particles, stocks 216 and 229 (syngen 8), and also stock 114

(syngen 2) which contains sigma particles (Sonneborn, Mueller & Schneller, 1959). These three stocks, like stocks 239 and 299, are rapid lysis killers.

The lambda particles of stock 216 and 229, and sigma of stock 114 were investigated by using the same methods as described in the main part of this paper. The lambda particles of stocks 216 and 229 appeared to be very similar to those of stocks 239 and 299. The measurements of the lambda particles, the peritrichous flagellation and the ultrastructure of the flagella in all four lambda stocks were virtually identical. Two differences were noted: in stock 229 the diameter of the flagella was the greatest of all of the lambda particles, measuring about 240 Å, whereas in stock 216 it was 200 Å, in both cases measured in sections; secondly, in stock 216, at least in the culture investigated, lambda particles were often enclosed in larger vacuoles containing several (up to 12) particles, whereas in the other stocks the symbionts usually occurred singly, or in pairs, in one vacuole. Sigma particles of stock 114 were of the same diameter as lambda particles but were 10 to 15 μ long and spirally curved. Flagella of sigma particles were very similar in ultrastructure and in their peritrichous distribution to those of lambda particles. Sigma particles were enclosed in vacuoles singly or in pairs.

In summary, all of the stocks known to kill by rapid lysis, i.e., the lambda-bearing stocks 216, 229, 239 and 299 and the sigma-bearing stock 114, contain particles which are morphologically similar. Observations with the electron microscope indicate that lambda and sigma particles are flagellated bacteria.

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EXPLANATION OF PLATES

Figs. 1 to 6 are optical micrographs. Figs. 7 to 14 are electron micrographs. Scale marks correspond to 1 μ unless otherwise indicated.

PLATE 1

Fig. 1. Section (1 μ) of *Paramecium aurelia*, stock 239, embedded in Araldite and stained with toluidine blue. × 675.

Fig. 2. Section (1 μ) of *P. aurelia*, stock 299, embedded and stained as in fig. 1. × 675.

Fig. 3. Whole mount, *P. aurelia*, stock 239, fixed with osmium tetroxide and treated with lacto-orcein. × 370.

Fig. 4. Whole mount, *P. aurelia*, stock 299, fixed and treated as fig. 3. × 370.

Fig. 5. Lambda symbionts of crushed *P. aurelia*, stock 299 (bright phase-contrast). × 2250.

Fig. 6. Partially purified symbionts of *P. aurelia*, stock 239 (bright phase-contrast). × 2250.

Fig. 7. Lambda symbionts, *P. aurelia*, stock 239. Note sections of flagella (arrow). × 15,000.

Fig. 8. Three lambda symbionts, *P. aurelia*, stock 299, enclosed in cytoplasmic vacuole. Note sections of flagella (arrow). × 15,000.

PLATE 2

Fig. 9. Negatively stained lambda symbiont, *P. aurelia*, stock 239. × 19,200.

Fig. 10. Negatively stained lambda symbiont, *P. aurelia*, stock 299. × 15,600.

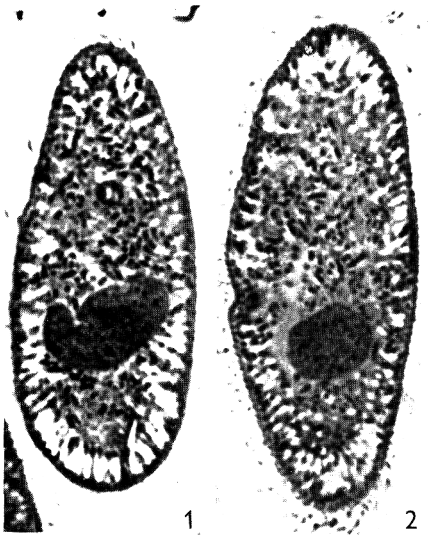
Fig. 11. Oblique section of *P. aurelia*, stock 299 lambda symbiont, showing cross-sections through flagella (arrow). × 36,000.

PLATE 3

Fig. 12. Negatively stained flagella of lambda symbiont, *P. aurelia*, stock 239. × 28,000.

Fig. 13. High power electron micrograph of negatively stained flagella, *P. aurelia*, stock 239. × 160,000.

Fig. 14. Longitudinal section of lambda symbiont, *P. aurelia*, stock 239. Note the ultrastructure of the cell wall (*cw*), granular content (*g*) and less dense areas (*l*). × 28,000.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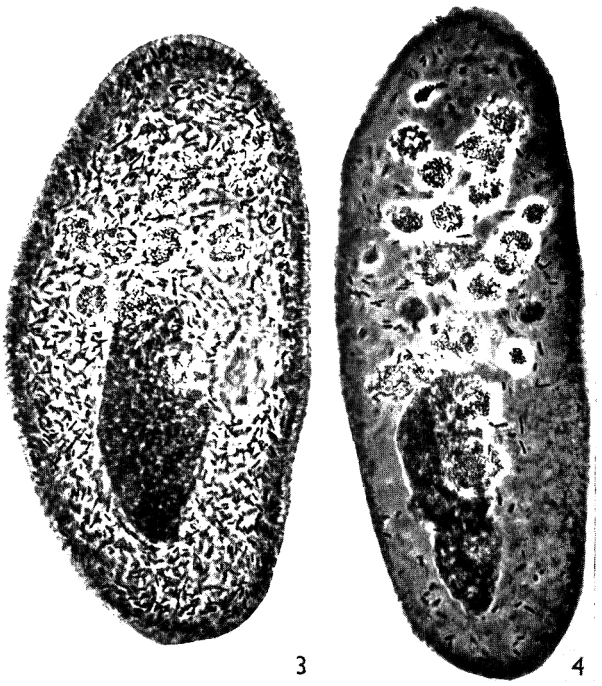
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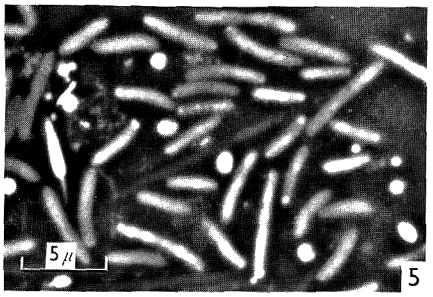
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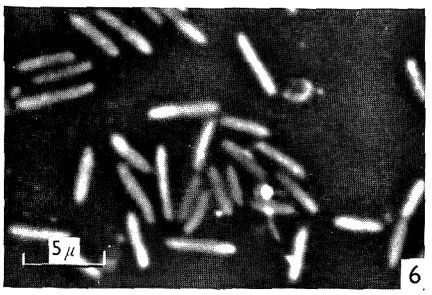
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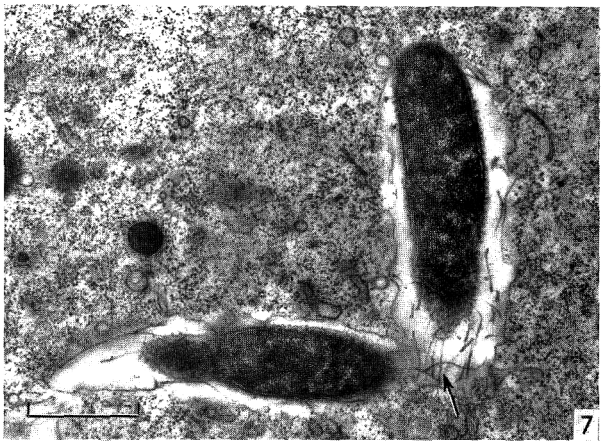
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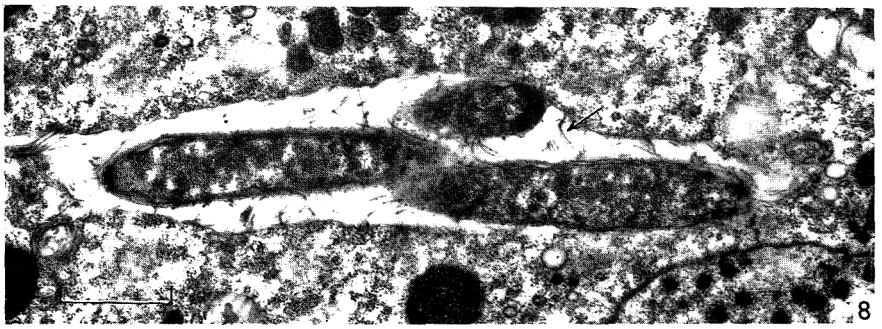
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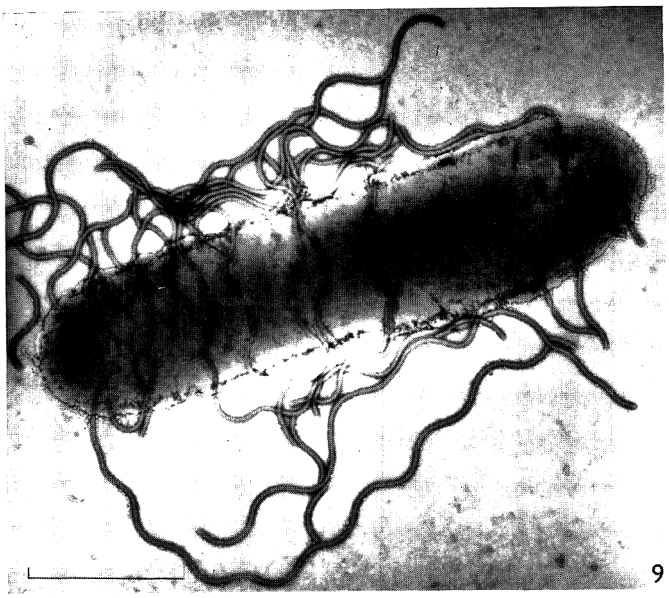
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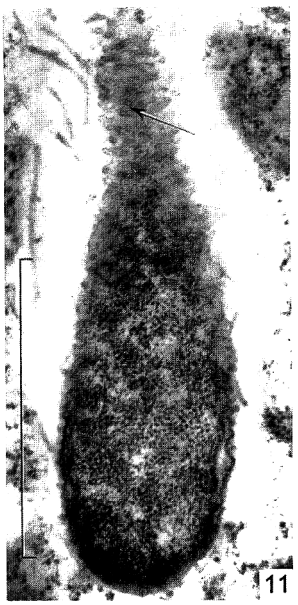
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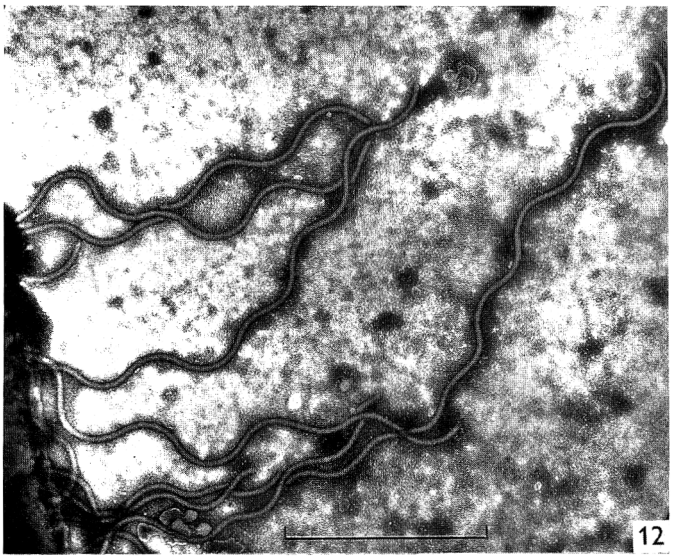

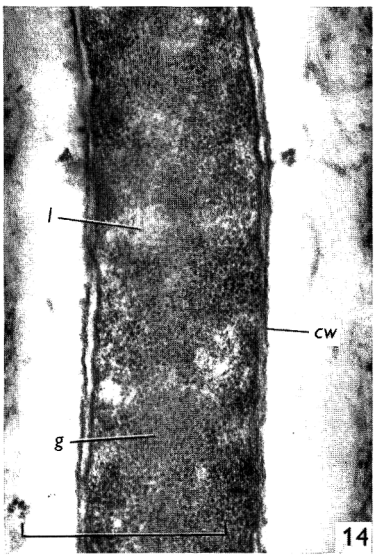
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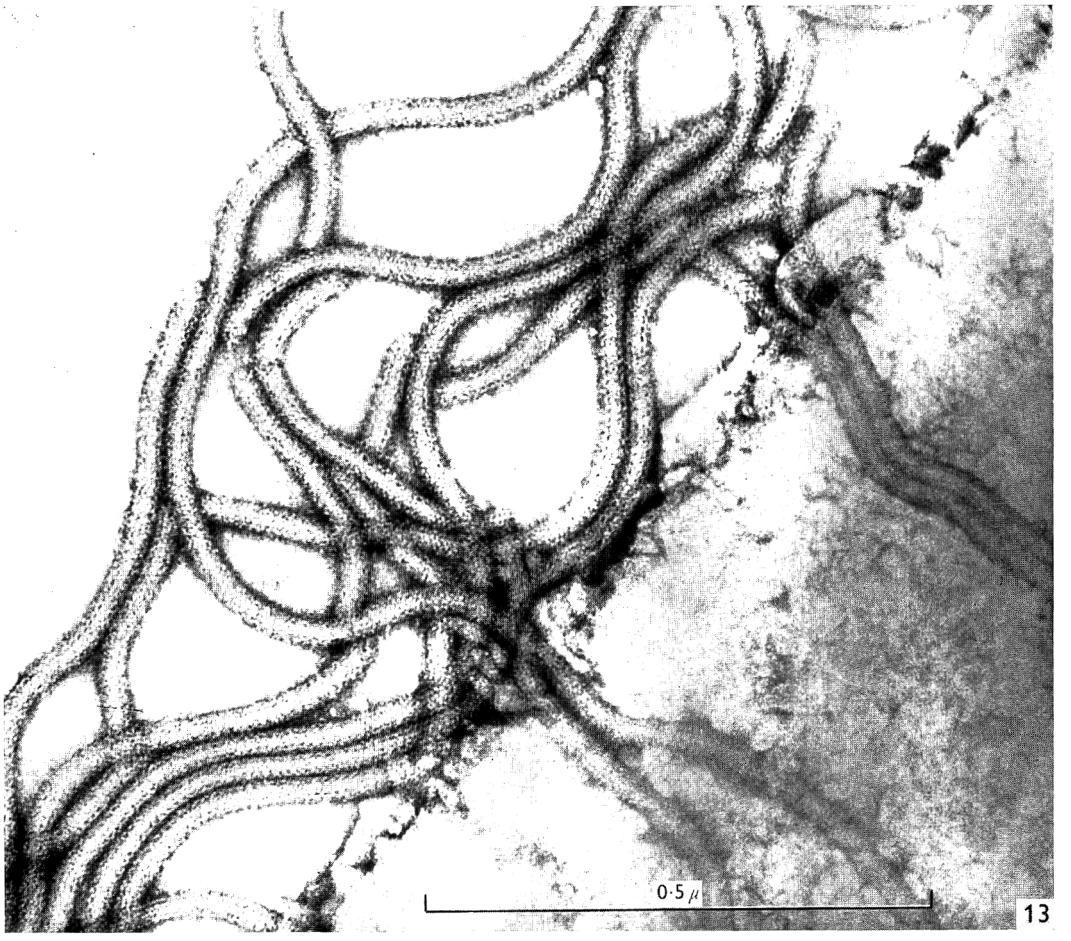
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Menaquinone Determination in the Taxonomy of Micrococcaceae

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SUMMARY

Staphylococci, micrococci and strains intermediate in guanine+cytosine (GC) content between these genera were all found to contain menaquinones. Two different types of menaquinone, described as 'normal' and 'hydrogenated' were revealed. Distinct and stable menaquinone patterns, formed by the percentages of individual isoprenologues, were found to characterize certain previously proposed species, groups or subgroups within the family. Studies on the distribution of menaquinones have also demonstrated heterogeneity within certain subgroups, notably Baird-Parker's Micrococcus subgroup 7, and the divisions within the yellow-pigmented micrococci revealed by Rosypal, Rosypalová & Hořejš have been substantiated. Normal menaquinones were found in strains extending over a wide range of reported GC ratios (31·0 to 61·4 %). These strains included staphylococci and non-pigmented micrococci, classified according to Baird-Parker, and also marine strains and others of uncertain taxonomic position. Hydrogenated menaquinones, however, were restricted to pigmented micrococci within the range 66·3 to 73·3 % GC. There is thus evidence of a correlation between pigmentation, high GC ratio and menaquinone type. Menaquinone assay may contribute to the classification of Micrococcaceae and thus complement existing techniques in chemotaxonomy.

INTRODUCTION

Menaquinones, vitamins of the K_2 group, are 2-methyl-1,4-naphthoquinones with an unsaturated polyisoprenoid side chain (MK); partially hydrogenated variants also occur in which at least one isoprene unit is saturated (MK(H)). Isoprenologues are designated by the number of isoprene units (n) in the side chain (Fig. 1). Menaquinones have been extracted from both Gram-positive and Gram-negative aerobic bacteria (review by Pennock, 1966) and also from Bacteroides and Veillonella species (Gibbons & Engle, 1964); one isoprenologue, MK-4, has been isolated from animal

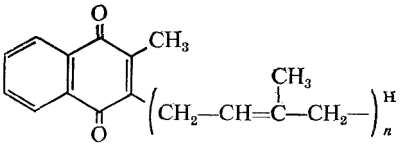

Fig. 1. Menaquinones, vitamins of the K_2 group, are 2-methyl-1,4-naphthoquinones with unsaturated polyisoprenoid side chains; n = number of isoprene units.

tissues (Martius & Esser, 1959) and also from a strain of *Staphylococcus aureus* (Cawthorne *et al.* 1957). Earlier investigations (Jeffries *et al.* 1967*a*) revealed the presence of menaquinones in all strains of staphylococci and micrococci examined. Several different menaquinone (MK) patterns, formed by different percentages of individual isoprenologues, were found and it was suggested that distinct MK patterns characterize *S. aureus* and certain accepted Micrococcus species. Subsequent studies of further strains of micrococci confirmed the taxonomic significance of their MK patterns (Jeffries *et al.* 1967*b*). The present paper describes our extended examination of aerobic Micrococcaeae and includes representatives of the genera *Staphylococcus* and *Micrococcus*, together with a number of mostly marine strains of intermediate and, at present, uncertain classification. Reported figures for guanine+cytosine content (GC ratio) in the DNA of the strains studied ranged from 31·0 to 74·0 %.

Baird-Parker (1965) divided the aerobic catalase-positive, Gram-positive cocci into two groups. Strains fermenting glucose were placed in group 1 (Staphylococcus) and those attacking glucose oxidatively, or not at all, in group 2 (Micrococcus). On physiological characters, Baird-Parker further divided the staphylococci into six subgroups, subgroup I representing *S. aureus*; the micrococci fell into eight subgroups, two of which corresponded to the accepted species *Micrococcus luteus* and *M. roseus*. More recently, Rosypal, Rosypalová & Hořejš (1966), in studies mainly on pigmented micrococci, proposed a classification into groups based on the GC content of their DNAs; the strains were further divided into subgroups on similarity of biochemical and physiological characters. They proposed that, pending further data on the GC content of species within the Micrococcaceae, strains of GC content within the range 30·7 to 36·4 % should be classified as Staphylococcus species and that only strains of GC content within the range 66·3 to 73·3 % should, at present, be accepted in the genus *Micrococcus*; attention was also drawn to a strain intermediate in GC content between these two genera. On DNA base composition Boháček, Kocur & Martinec (1967*a*) divided a number of strains previously classified as micrococci into three groups. The first group comprised strains with GC ratio of 63·5–73·0 % and included *M. luteus* and *M. roseus*, the second group consisted mainly of strains requiring reclassification, since they differed in GC content and also in other characters from the suggested ranges for the genera *Micrococcus* and *Staphylococcus*. Their third group consisted of motile strains, probably of marine origin, which were intermediate in GC content between the ranges for the genera *Staphylococcus* and *Micrococcus*, and it was suggested that these strains may be transferred to the genus *Planococcus*. Kocur & Martinec (1965) proposed that the genus *Sarcina* should include only the anaerobic species with fermentative metabolism and that the species *Sarcina lutea* should be considered identical with *M. luteus*. Canale-Parola, Mandel & Kupfer (1967) showed that the strict anaerobes, *Sarcina ventriculi* and *Sarcina maxima*, are distinct from aerobic micrococci in DNA base composition.

METHODS

Strains and cultivation. The strains studied included certain of those examined by Baird-Parker (1965), by Rosypal *et al.* (1966) and additional strains received from the Czechoslovak Collection of Micro-organisms (Brno), the Staphylococcus Reference Laboratory (Central Public Health Laboratory, London), and other sources (Table 1).

On receipt, all cultures were examined for purity and pigment production on nutrient agar at 26°, 30° and 37° after incubation for 1 week; agar-slope subcultures were then stored at room temperature or at 4°. With the exception of *Micrococcus morrhuae* (see below), Lemco agar (blood agar base, Oxoid Ltd.) was found to be suitable for the cultivation of all strains, including those of marine origin. Nutrient agar plates inoculated with peptone–water suspensions of growth from freshly prepared agar-slope cultures were incubated for 48 hr and the surface growth harvested for MK assay. Staphylococci were grown at 37° and micrococci at 30°; in strains growing well at 30° and 37° the MK patterns were found to be similar at both temperatures. With most strains between 1 and 3 g. wet weight of organism were extracted, requiring the inoculation of between 10 and 30 Petri dishes; with strains yielding only small amounts of MK quantities up to 9 g. wet wt organism were prepared. The extreme halophile, *M. morrhuae*, did not grow on the nutrient agar and a medium of high salt concentration (Payne, Sehgal & Gibbons, 1960) was used for maintenance of cultures and preparation of suspensions for extraction. Thirty plates inoculated from each strain of *M. morrhuae* were incubated at 37° for 3 weeks in sealed tins containing flasks of water to maintain high humidity.

Menaquinone assay

Extraction of lipids. Since menaquinones are light-sensitive all operations were done in subdued light. The bacterial growth scraped from the surface of the medium was dispersed in ethanol (15 ml.) to remove water. The dispersion was centrifuged (1250 *g*; 5 min.) and the supernatant liquor decanted; ethanol (15 ml.), containing 50 mg. pyrogallol as antioxidant, was added to the residue and the mixture then boiled under reflux for 30 min.; after cooling the supernatant fluid was removed. Water (25 ml.) was added to the combined ethanolic liquors, which were then extracted with light petroleum (b.p. 40 to 60°, 3 × 25 ml.). This extract (extract A) was then evaporated and dried by azeotropic distillation under reduced pressure with benzene + ethanol (1 + 1, v/v). To test the efficiency of ethanolic extraction the residue remaining from this process was further subjected to alkaline digestion. Since this procedure destroys some MK (Jeffries *et al.* 1967*a*) quantitative results must be treated with caution; however, we have no evidence that this results in changes in the isoprenologue pattern. The residue remaining from each 1 g. wet wt of extracted organisms was dispersed in 4 ml. ethanolic pyrogallol (pyrogallol 5%, w/v, in ethanol, prepared freshly each day); 2 ml. aqueous potassium hydroxide solution (KOH, 88 g.; water 54 ml.) was then added and the mixture boiled under reflux for 30 min. After cooling and the addition of water (10 ml.) the lipid was extracted with ether (3 × 25 ml.). The ether extracts were pooled, washed free from alkali with water and evaporated; the lipid extract (extract B) was then dried.

Thin layer chromatography. Extracts A and B were treated identically, but separately. Each extract was dissolved in a small volume of light petroleum and applied as a streak to a 20 × 10 cm. plate coated with a 300 μ layer of silica gel (Merck, A.G., Darmstadt) containing sodium fluorescein (50 p.p.m.). A spot of a pure MK solution was placed beside the streak to act as a marker and the chromatoplate was developed with benzene + light petroleum (1 + 1, v/v) to a distance of 8·5 cm. Menaquinones quench the green fluorescence when the plates are viewed under ultraviolet light, and thus appear purple against a green background; a marker of pure MK (R_F 0·4 in this

Table 1. *Classification and sources of strains*

Classification		Strains
STAPHYLOCOCCUS		
Staphylococcus aureus (Baird-Parker, 1965, group 1, subgroup I)		OXFORD 'H', WO133, B-P1650, WOOD 46, C1309, C1341, C2936, C2956, C2976, C2981, C3512, C4532, C4601, C5422, C5437, C5490, C6828, C6830, C8973, NCTC4163, NCTC8319, NCTC8325, NCTC8327, NCTC8329, NCTC8331, NCTC8354, NCTC8356, NCTC8357, NCTC8358, NCTC8363, NCTC8507, NCTC8509, NCTC8511, NCTC9315, NCTC9717, NCTC9754, NCTC9789, NCTC10033, NCTC10039, NCTC10455, NCTC10457
S. aureus (Brown, Sandvik, Scherer & Rose, 1967). Bovine strains, of proteinase groups G & H and 1 strain of undetermined group, not classifiable in Baird-Parker's system.		A197, A4, A5, A194
S. epidermidis		
Baird-Parker, 1965, group 1, subgroup	II	B-P12, B-P14, B-P317
	III	B-P34, B-P1654
	IV	B-P19, B-P35, NCTC4276, NCTC7291, M/C2
	V	M/C122, M/C193, M/C199, M/C223, M/BR11218
	VI	WO143, B-P21, B-P32, NCTC5955, NCTC7944
MICROCOCCUS (non-pigmented)		
Baird-Parker, 1965, group 2, subgroup	1	B-P1652, M/GP2, M/QWA8
	2	B-P5, B-16
	3	B-P3, B-P10, NCTC7292
	5	B-P45, B-P46, B-P47, B-P114, NCTC189
	6	B-P1653, NCTC8360, B-P8, B-P24
	7	B-P25, B-P26, B-P29, B-P89 (ATCC8456)
MICROCOCCUS (pigmented)		
Rosypal, Rosypalová & Hořejš, 1966, subgroup	1*a* (Baird-Parker, 1965, Micrococcus subgroup 7), yellow pigmented	CCM144, CCM210, CCM247, CCM248, CCM265, CCM266(i), CCM309, CCM331, CCM559, CCM851, CCM852, CCM853, CCM1335
	1*a* (Baird-Parker, 1965, Micrococcus subgroup 7), non-pigmented variant	CCM266(ii)
	1*b* (Baird-Parker, 1965, Micrococcus subgroup 8), pink-pigmented	CCM146, CCM633, CCM706, CCM385
	2*a* yellow-pigmented	CCM208, CCM547, CCM884, CCM1044
	3*a* grey-violet-pigmented, *M. cyaneus*	CCM856
	3*b* yellow-pigmented, *M. luteus*, neotype strain	CCM810 (ATCC398)

Table 1 *cont.*

Classification	Strains
Boháček, Kocur & Martinec, 1967*a* {yellow-pigmented *M. luteus* / pink-pigmented, *M. roseus*	CCM132, CCM169
CCM classification {yellow-pigmented *M. luteus* / non-pigmented variant *M. luteus*	CCM560, CCM679, CCM837
	CCM134, CCM840(i). CCM166
	CCM840 (ii)
Baird-Parker, 1965, Micrococcus subgroup 8, pink-pigmented, *M. corallinus*	B-P97 (ATCC966)
M. morrhuae, red-pigmented	CCM537, CCM859, CCM889, CCM2226
STRAINS OF UNCERTAIN CLASSIFICATION	
Rosypal *et al.* 1966, subgroup 4*a*, non-pigmented	CCM250
M. corallinus, non-pigmented	CCM678 (ATCC966)
M. eucinetus, brown-pigmented	CCM2387, CCM2389
Micrococcus spp. (marine), salmon-pink-pigmented	CCM1849, CCM2069, CCM2104

WO Walton Oaks collection of cultures.
B-P Strains received from Dr A. C. Baird-Parker, Bedford, England.
C Strains received from Dr M. T. Parker, Colindale, London, England.
NCTC National Collection of Type Cultures, London, England.
A Strains received from Dr R. W. Brown, Iowa, U.S.A.
M/ Strains received from Dr R. G. Mitchell, Oxford, England.
ATCC American Type Culture Collection, Rockville, Md., U.S.A.
CCM Czechoslovak Collection of Microorganisms, J. E. Purkyně University, Brno, Czechoslovakia.

system) also aided identification. The MK band was scraped from the plate and extracted from the silica gel with ether vapour.

Reversed phase paper chromatography. Zinc-ammine solution was prepared by dissolving zinc oxide (16 g.) and ammonium carbonate (25 g.) in a mixture of ammonium hydroxide (150 ml.) and water (600 ml.); 20 ml. of 0·1 % (w/v) aqueous fluorescein solution was then added. Sheets of Whatman no. 4 grade 'chromatography paper', 23 × 57 cm., were immersed in the zinc ammine solution, hung to dry at room temperature and then baked in a fan-draught oven at 95 to 100° for 1 hr. A portion of the sample was applied to a paper and partially overlapped with marker MKs. Since interfering substances were present the overlapping technique was necessary to ensure accurate identification of the MK isoprenologues. The remainder of the sample was applied to another paper, which was used for quantitative estimation of MKs. The phase of the papers was reversed by immersion in liquid paraffin solution (3 %, w/v, solution liquid paraffin B.P. in light petroleum, b.p. 60–80°). After evaporation of the light petroleum solvent the papers were developed in a filter-paper lined, vapour-saturated tank, by descending chromatography with 95 % (v/v) ethanol in water for 5 hr. at 27°. The papers were then dried with warm air and examined under ultraviolet light. Separation and migration of MKs in reversed-phase chromatography are related to isoprenoid side-chain length, thus MK-6 migrates farther than MK-7. After elution from the quantitative paper, the separate MK isoprenologues were measured.

Measurement and identification of isolated menaquinones. When examined by ultraviolet spectrometry menaquinones showed maxima at 239, 249, 260 and 270 nm. The absorption at 285 nm. was very small and the difference in absorption value (ΔE), observed at 285 and at 270 nm., was used as the basis of the assay. It was assumed that irrelevant absorption over this short range would have been constant. $\Delta E^{1\%}_{1\,cm}$ values determined experimentally in our laboratories on synthetic MKs were as follows: MK-9, 222; MK-8, 238; MK-7, 244; MK-6, 272. To complete the identification of MKs a complete u.v. scan from 230 to 350 nm. was made to ascertain that all four absorption maxima were present. Hydrogenated menaquinones, when present, ran more slowly than normal menaquinones on paper chromatography and thus occupied a position on the paper chromatogram between the normal isoprenologues.

RESULTS

The results of the menaquinone assays are shown in Tables 2*a* and *b*. Table 3 indicates the correlation between representative values for GC content in DNA and the type of menaquinone produced.

Menaquinones identified as MK-6, MK-7, MK-8 and MK-9 (referred to as 'normal' MKs, designated MK-n, with R_F values 0·318, 0·213, 0·143, 0·100, respectively) were found in staphylococci, micrococci and strains of intermediate uncertain classification. However, certain strains of micrococci with high GC values yielded menaquinone fractions which, when resolved in our paper chromatographic system, gave substances with R_F values 0·275, 0·182, 0·124 and 0·086. These substances gave a u.v. spectrum typical of menaquinones. Calculation of their R_F values suggested that they were partially hydrogenated MKs (referred to as 'hydrogenated' MKs and designated MK-n (H)). This conclusion was substantiated by their behaviour on thin layers of silver nitrate-impregnated silica gel.

Table 2*a*. *Distribution of normal menaquinones in staphylococci, micrococci and strains of uncertain classification*

Classification	No. of strains	Percentage of individual menaquinone isoprenologues* MK-9	MK-8	MK-7	MK-6
STAPHYLOCCOCUS					
Staphylococcus aureus (Baird-Parker, 1965, group 1, subgroup I)†	41	12±5	73±4	15±5	0
S. aureus (Brown, Sandvik, Scherer & Rose, 1967, bovine strains)	4	0	1±1	5±2	94±2
S. epidermidis					
Baird-Parker, 1965, group 1, subgroup II	3	0	53±12	47±12	0
Baird-Parker, 1965, group 1, subgroup III, B-P34	1	0	5	95	0
Baird-Parker, 1965, group 1, subgroup III, B-P1654	1	0	83	17	0
Baird-Parker, 1965, group 1, subgroup IV	4	0	17±6	83±6	0
Baird-Parker, 1965, group 1, subgroup IV, M/C2	1	0	0	100	0
Baird-Parker, 1965, group 1, subgroup V	3	0	7±6	93±6	0
Baird-Parker, 1965, group 1, subgroup V, M/C199	1	0	58	42	0
Baird-Parker, 1965, group 1, subgroup V, M/C223	1	0	Trace	Trace	0
Baird-Parker, 1965, group 1, subgroup VI	4	0	16±8	84±8	0
Baird-Parker, 1965, group 1, subgroup VI, NCTC7944	1	4	4	92	0
MICROCOCCUS (non-pigmented)					
Baird-Parker, 1965, group 1, subgroup 1, B-P1652	1	0	0	0	100
Baird-Parker, 1965, group 1, subgroup 1, M/GP2	1	0	Trace	100	Trace
Baird-Parker, 1965, group 1, subgroup 1, M/QWA8	1	0	0	Trace	0
Baird-Parker, 1965, group 1, subgroup 2	2	0	0	100	0
Baird-Parker, 1965, group 1, subgroup 3	3	0	5±5	95±5	0
Baird-Parker, 1965, group 1, subgroup 5	5	0	0	95±9	5±9
Baird-Parker, 1965, group 1, subgroup 6	3	0	15±13	85±13	0
Baird-Parker, 1965, group 1, subgroup 6, B-P24	1	0	0	0	100
Baird-Parker, 1965, group 1, subgroup 7	3	0	0	0	100
Baird-Parker, 1965, group 1, subgroup 7, B-P89 (ATCC8456)	1	0	0	100	0
Micrococcus morrhuae	2	0	Trace	0	0
CCM889	1	0	Trace	Trace	0
CCM2226	1	0	Trace	Trace	Trace
STRAINS OF UNCERTAIN CLASSIFICATION					
Rosypal, *et al.* 1966, subgroup 4*a*, CCM250	1	0	70	30	0
M. corallinus, CCM678 (ATCC966)	1	85	9	6	0
M. eucinetus, CCM2387	1	0	50	25	25
M. eucinetus, CCM2389	1	0	50	50	0
Micrococcus spp. (marine), CCM1849	1	0	0	Trace	0
Micrococcus spp. (marine), CCM2069	1	0	Trace	0	0
Micrococcus spp. (marine), CCM2104	1	0	Trace	Trace	0

* Mean values with standard deviations (individual results are obtainable from the authors).

† Ten additional strains of *S. aureus* examined after alkali digestion gave MK patterns similar to those shown in the table (Baird-Parker group 1, subgroup I).

Strain number abbreviations as in Table 1.

Table 2*b*. *Distribution of hydrogenated menaquinones in pigmented micrococci*

Classification	No. of strains	% of individual menaquinone isoprenologues* MK-9 (H)	MK-8 (H)	MK-7 (H)	MK-6 (H)	MK-5 (H)
MICROCOCCUS (pigmented)						
Rosypal, *et al.* 1966, subgroup 1*a* (Baird-Parker, 1965, Micrococcus subgroup 7)	13	0	84±6	16±5	0	0
Rosypal, *et al.* 1966, subgroup 1*a* (non-pigmented variant, CCM266(ii))	1	0	64	36	0	0
Rosypal, *et al.* 1966, subgroup 1*b* (Baird-Parker, 1965, Micrococcus subgroup 8)	4	21±2	74±4	5±3	0	0
Rosypal, *et al.* 1966, subgroup 2*a*	4	1±1	15±10	82±8	2±2	0
Rosypal, *et al.* 1966, subgroup 3*a* CCM856 (*M. cyaneus*)	1	94	6	0	0	0
Rosypal, *et al.* 1966, subgroup 3*b* CCM810 (ATCC 398) (*M. luteus* neotype strain)	1	80	20	0	0	0
Boháček, *et al.* 1967*a* CCM132 (*M. luteus*)	1	0	12	83	5	0
Boháček, *et al.* 1967*a* CCM169 (*M. luteus*)†	1	0	21	0	0	0
Boháček, *et al.* 1967*a* (*M. roseus*)	3	14±4	79±6	7±1	0	0
CCM classification CCM134 (*M. luteus*)	1	11	77	2	1	9
CCM classification CCM840 (*M. luteus*)	1	4	79	17	0	0
CCM classification CCM840(ii) non-pigmented variant (*M. luteus*)	1	7	81	3	0	9
CCM classification CCM166 (*M. luteus*)‡	1	0	26	11	0	10
Baird-Parker, 1965, Micrococcus subgroup 8, B-P97 (ATCC966)	1	95	5	0	0	0

* Mean values with standard deviations (individual results are obtainable from the authors).

† The following normal menaquinone isprenologues were also present: MK-9, 13 %; MK-8, 28 %; MK-7, 21 %; MK-6, 17 %.

‡ The following normal menaquinone isoprenologues were also present: MK-9, 12 %; MK-8, 30 %; MK-7, 11 %.

Strain number abbreviations as in Table 1.

Table 3. *Correlation between guanine+cytosine content (% GC) in DNA of various cocci and type of menaquinone produced*

Name or designation of strain	Strain no.	Guanine+cytosine content in DNA: % GC	Reference	Menaquinones
Staphylococcus aureus (Baird-Parker, 1965, group 1, subgroup I)*	NCTC4163	31·0	1	
Micrococcus candicans (Baird-Parker, 1965, group 2, subgroup 7)*	ATCC8456, B-P89	36·4	2	
Staphylococcus epidermidis (Baird-Parker, 1965, group 1, subgroup VI)*	NCTC7944	36·5	1	
Micrococcus sp. (marine)	CCM1849	39·6	3	
M. eucinetus	CCM2387	40·0	3	
Micrococcus sp. (marine)	CCM2069	42·2	3	
Micrococcus sp. (marine)	CCM2104	42·2	3	
M. eucinetus	CCM2389	50·3	4	Normal (MK-n)
Rosypal, *et al.* 1966	CCM250	54·2	5	
M. morrhuae	CCM859	57·1	3	
M. morrhuae	CCM537	57·8	3	
M. corallinus (non-pigmented)	CCM678, ATCC966	59·6	3	
M. morrhuae	CCM889	61·0	3	
M. morrhuae	CCM2226	61·4	3	
Rosypal *et al.* 1966: Micrococcus subgroup 3b†	CCM810	66·3	5	
Rosypal *et al.* 1966: Micrococcus subgroup 2a†	CCM1044	68·0	5	Hydrogenated (MK-n(H))
Rosypal *et al.* 1966: Micrococcus subgroup 1b†	CCM633	70·8	5	
Rosypal *et al.* 1966: Micrococcus subgroup 1a†	CCM309	73·3	5	

* GC content in DNA within suggested range for *Staphylococcus* spp. (Rosypal *et al.* 1966)

† GC content in DNA within suggested range for *Micrococcus* spp. (Rosypal *et al.* 1966).

References: 1, Silvestri, L. G. & Hill, L. R. (1965); 2, Boháček, J., Kocur, M. & Martinec, T. (1967*b*); 3, M. Kocur, personal communication; 4, Boháček, *et al.* (1967*a*); 5, Rosypal, *et al.* (1966).

Strain number abbreviations as in Table 1.

Although, for a given strain, the yield of MK was found to vary somewhat between cultures grown on different occasions, the isoprenologue ratios (MK patterns) were found to be constant. The reasons for this variation in MK yield are not known and our limited experiments yielded negative results. However, it has been shown, with a vitamin-K dependent strain of *Fusiformis melaninogenicus*, that MKs diffuse into agar from growing bacteria and this may represent a variable loss, since we only extracted the bacterial mass and not the culture fluid. The amounts of MK extracted ranged from a trace (less than 1 μg.) to more than 400 μg./g. wet wt organism. In general, the highest yields were from the *Staphylococcus aureus* strains and the lowest were from the strains originally isolated from marine sources, including the extreme halophile, *Micrococcus morrhuae* (Table 2*a*). The MK patterns attributed to strains producing very small amounts of MK should be regarded as approximate, since the errors involved in such calculations may be considerable; but the identification of the type of MK produced by these strains was, in all cases, unequivocal.

Menaquinone patterns

As shown in Table 2*a*, MK-7 and MK-8 were common to both staphylococci and certain micrococci; with the exception of one strain of uncertain classification (CCM 678, ATCC966, discussed below), MK-9 was found only in staphylococci. Menaquinone-6, although present as the major isoprenologue in strains of a physiologically distinct group of coagulase-producing staphylococci, was not found in any other strains within this genus. Menaquinone-6 was, however, present in certain non-pigmented micrococci, and in some strains it was the only isoprenologue detected. Hydrogenated MKs were restricted to yellow, pink and violet-pigmented strains (*Micrococcus luteus*, *M. roseus*, *M. cyaneus*) and the non-pigmented variant colonies derived from two *M. luteus* strains (Table 2*b*). As discussed below, normal MKs were present in strains ranging widely in GC content, whereas hydrogenated MKs were extracted only from strains within the highest GC range.

The MK patterns of all strains have remained stable after repeated subculture and also after freeze-drying.

DISCUSSION

Staphylococcus

Staphylococcus aureus. With the exception of a single culture, described below, all the coagulase-producing strains comprising the accepted species, *S. aureus*, classifiable in group 1, subgroup I of Baird-Parker (1965) showed a single and characteristic MK pattern, with MK-8 as the major isoprenologue. MK-9 and MK-7 were present as minor isoprenologues in all strains, but their relative proportions differed between strains. The strains studied varied widely in sensitivity to antibiotics and in bacteriophage type; they included the twenty-one propagating strains of the International Phage Typing series.

Four cultures of *Staphylococcus aureus* NCTC7511 were received from different laboratories; this strain has been widely distributed as propagating strain 53 of the International Phage Typing series. Three of the cultures gave the MK pattern characteristic of *S. aureus* Baird-Parker group 1 subgroup I as shown in Table 2*a*. One culture, however, which had been employed in genetical studies by Korman (1963), revealed an anomalous MK pattern (MK-8, 17%; MK-7, 48%; MK-6, 11%;

MK-5, 15%; MK-4, 9%) including two isoprenologues, MK-4 and MK-5, not previously encountered in a bacterium. This culture was indistinguishable, in bacteriophage studies, from the other three cultures of NCTC8511. Detailed studies on the menaquinones of these cultures of NCTC8511, and on mutants allegedly derived from one of them, were described by Jeffries, Harris & Price (1967*c*). Identical GC ratios (32·4%) were found in the culture of NCTC8511 giving an anomalous MK pattern and in one of the cultures of this strain from another source (L. R. Hill, personal communication).

Four bovine strains, isolated by Brown, Sandvik, Scherer & Rose (1967), were coagulase and phosphatase positive but did not produce acetoin from glucose or ferment maltose or mannitol. These strains, with features common to both *Staphylococcus aureus* (Baird-Parker group I subgroup I) and *S. epidermidis* (Baird-Parker group I subgroup III), were therefore not classifiable in Baird-Parker's system; they were, moreover, clearly separable on MK pattern from the strains of *S. aureus* studied by Baird-Parker (1965), all of which fell into his subgroup I.

Staphylococcus epidermidis. Of the twenty strains classified in Baird-Parker's subgroups II to VI, all except one of the subgroup IV strains, which contained MK-7 only, produced both MK-7 and MK-8. Two strains (NCTC7291, 7944), one in subgroup IV and one in subgroup VI, also produced small amounts of MK-9; MK-6 was not detected in any of the *S. epidermidis* strains. The strains could be broadly divided into two groups, according to the relative proportions of MK-7 and MK-8. The ratio of MK-7 : MK-8 : varied from about 0·5 to 10, or even higher; the highest ratios tending to predominate within subgroups IV, V and VI. A strain of the violet-pigmented *Micrococcus violagabriellae* (strain B-P317 in Table 1), now reclassified as a subgroup II Staphylococcus (Baird-Parker, 1965), showed an MK pattern similar to that of the two other strains of subgroup II staphylococci examined.

Micrococcus

Non-pigmented strains. The nineteen strains examined belonged to Baird-Parker's Micrococcus group 2, subgroups 1, 2, 3, 5, 6 and 7; they produced normal MKs only. MK-7 was the major isoprenologue in most strains and in ten of them either MK-7 or MK-6 was the sole isoprenologue detected. Menaquinone-8 was found, in relatively small amounts, in a few strains. Certain MK patterns were common to strains in different Baird-Parker subgroups. Strain NCTC8360, formerly classified as *Staphylococcus aureus* and alleged to clot sheep plasma, but not human or rabbit plasma (K. J. Steel, cited by Baird-Parker, 1965) was found by Baird-Parker to belong to Micrococcus subgroup 6. The MK pattern of this strain supports its separation from *S. aureus.*

Pigmented strains. In contrast to the non-pigmented strains, considered above, the yellow and pink-pigmented strains classified in Baird-Parker's subgroups 7 and 8 respectively, together with other pigmented strains classified by Rosypal *et al.* (1966), produced hydrogenated MKs (Table 2*b*). All except one of the pigmented strains B-P97, ATCC966) were from the Czechoslovak Collection of Micro-organisms and included representatives of the groups and subgroups proposed by Rosypal *et al.* and a few of the strains studied by Boháček *et al.* (1967*a*).

Two species (*Micrococcus luteus, M. roseus*) are recognized in the genus *Micrococcus* (Evans, 1965) but, in the tests described by Baird-Parker (1966), pigment is the only character which reliably separates these species from each other, their separation from

micrococci in the other subgroups being mainly on negative evidence. Differences in cell-wall composition and in the protein contents of the cocci are regarded as criteria justifying the separation of the species, although stable pink-pigmented mutants are alleged to have been produced from a yellow-pigmented strain classifiable in Baird-Parker subgroup 7 (G. W. Gould, cited by Baird-Parker, 1965).

Baird-Parker (1965) classified the yellow-pigmented *Micrococcus luteus* strains, together with non-pigmented strains, in Micrococcus subgroup 7, but our MK studies show that this subgroup is not homogeneous: of the strains examined by us all the yellow-pigmented ones contained hydrogenated MKs, and the non-pigmented strains normal MKs. We suggest a division of these strains into two subgroups, based on type of menaquinone produced. This separation is further supported by the higher sensitivity of the yellow-pigmented strains to lysozyme (unpublished observations). Non-pigmented variants (CCM266 (ii); CCM840 (ii)) isolated in our laboratory from two yellow strains have, like the parent strains, contained hydrogenated MKs.

Rosypal *et al.* (1966) divided the yellow-pigmented Micrococcus strains into three groups on differences in GC content within the range 66·3 to 73·3 %; they found that strain ATCC398 proposed as the type strain of *M. luteus* by Breed (1952), was in a different group (3) from the other strains classified as *M. luteus* which they studied and placed in group 1. These authors considered that, since the name of the type species should not be changed, the application of the specific name *luteus* to strains within their subgroup 1 *a*, corresponding to Micrococcus subgroup 7 of Baird-Parker, should be questioned. Although the significance of minor differences in GC content, such as those found by Rosypal *et al.*, remains to be substantiated, a correlation has been found between the proposed subgroups of Rosypal *et al.* and MK patterns. Although the yellow-pigmented subgroup 2a strains of Rosypal *et al.* were alleged to correspond to Micrococcus subgroups 5 and 6 of Baird-Parker, we were unable to confirm this finding; we found that, in the tests recommended by Baird-Parker, these strains more closely resembled those in subgroup 7.

Correlation between MK patterns and the classification of micrococci proposed by Rosypal et al. (1966)

Subgroup 1 a. Thirteen strains were tested; all were yellow and some produced violet pigment also. They gave a distinct MK pattern, with MK-8(H) as the main isoprenologue and MK-7(H) as minor; small amounts of MK-6(H) were found in some strains and one strain also produced a trace of MK-9(H). Strain CCM266 yielded unstable variants lacking pigment (226 (ii)); in MK pattern these were identical with the pigmented parent strain (266(i)).

Subgroup 1 b. The four strains examined produced pink pigment and revealed a single MK pattern, with MK-8(H) as major and MK-9(H) and MK-7(H) as minor isoprenologues. The presence of MK-9(H) and the lower relative percentage of MK-7(H) differentiates this subgroup from the strains in subgroup 1a; MK-8(H) as the main isoprenologue is common to both subgroups, and perhaps supports their suggested close relationship.

Subgroup 2 a. Four strains (two previously classified as *Micrococcus varians* and two as *M. conglomeratus*) showed a single MK pattern, in which MK-7(H) predominated, MK-8(H) was present in all strains and two of them also contained small amounts of MK-6(H) or MK-9(H).

Subgroups 3a and 3b. The single strains comprising these two subgroups gave a similar and distinct MK pattern in which MK-9(H) was the main isoprenologue and MK-8(H) was the minor. *Micrococcus cyaneus* (CCM856) represents subgroup 3a and produces grey/violet rough colonies. It was regarded by Kocur & Martinec (1963) as an atypical strain of *M. luteus*; Rosypalová *et al.* (1966), however, regarded this species as distinct. The type strain of *M. luteus* (ATCC398) represents subgroup 3b.

Subgroup 4a. The single strain in this subgroup (CCM250), differing in GC ratio (56·4%) from staphylococci and micrococci, contained normal MKs only; the pattern (MK-8, 70%; MK-7, 30%) resembles that of certain coagulase-negative staphylococci.

The yellow-pigmented Micrococcus strains studied by Rosypal *et al.* (1966) fell into three ranges in GC content; each has now been shown to have a distinct MK pattern. Furthermore, the subgroup 1a strains have been shown to be sensitive to lysozyme by a plate incorporation method, whereas the subgroup 2a strains were relatively resistant (unpublished observations).

The menaquinone patterns of other yellow and pink-pigmented Micrococcus strains studied. Three pink-pigmented strains classified as *M. roseus* by Boháček *et al.* (1967*a*) gave an MK pattern identical with the strains of this species classified by Rosypal *et al.* (1966) in their subgroup 1*b*, thus all seven *M. roseus* strains examined were homogeneous in MK pattern.

Two allegedly non-pigmented strains of *Micrococcus luteus*, unlike the thirteen strains of this species classified in subgroup 1a of Rosypal *et al.* (1966), produced an MK pattern similar to that of the *M. roseus* strains. On nutrient agar at 30° one of these strains (CCM840; GC 71%) produced numerous colonies lacking pigment, together with a single yellow colony; on subculture these colonies remained stable with respect to pigmentation. Although the MK patterns of the two colony types were similar, the non-pigmented variant produced an additional isoprenologue (tentatively identified as MK-5(H)), which was not encountered in any of the other strains in our series. The presence of MKs of the hydrogenated type suggests that the non-pigmented variant colonies (CCM840, (ii)) unlike the non-pigmented strains in Baird-Parker subgroup 7 which produced normal MKs, arose as variants from an originally yellow-pigmented strain. The other strain (CCM134), received as nonpigmented, produced a pale yellow pigment at 30° which intensified at room temperature; the MK pattern of this strain was also like that of the *M. roseus* strains. A pink-pigmented strain (B-P97, ATCC966) of *M. corallinus* (Baird-Parker Micrococcus subgroup 8) showed an MK pattern similar to that of the type strain of *M. luteus*, classified in subgroup 3 of Rosypal *et al.* Menaquinone patterns may thus substantiate the suggested close relationship between the yellow and pink-pigmented strains at present accorded specific status.

Two yellow-pigmented *Micrococcus luteus* strains (CCM169, GC 73·3%; CCM166, GC 73·4%) each contained normal and hydrogenated menaquinones; we have not encountered a mixture of normal and hydrogenated meanquinones in any of the other strains examined. However, it should be stressed that hydrogenated menaquinones have been found to be characteristic of pigmented micrococci and have not been detected in any other strains.

Strains of uncertain classification

Strains originally described as Micrococcus species, and subsequently found to be of intermediate position in GC content (48 to 51 %) between the suggsted ranges for the genera *Staphylococcus* (30·7 to 36·5 %) and *Micrococcus* (66·3 to 73·3 %) were described by Boháček *et al.* (1967*a*). These authors suggested that certain motile strains, including the light-brown pigmented *M. eucinetus* should, in view of their % GC, motility and marine habitat, be transferred to the genus *Planococcus*. The two strains of *M. eucinetus* examined (GC 40 and 50·3 %) each produced less than 20 μg. MK of the normal type/g. wet wt organism. Three other strains of salmon-pink pigmented marine micrococci (CCM1849, 2069 and 2104) were received from Dr M. Kocur, together with estimates of their GC content (39·6 to 42·2 %); these strains also yielded normal MKs, but in insufficient amounts for quantitative estimation of individual isoprenologues. Normal MKs (mainly MK-8) were found in very small quantities, necessitating the extraction of between 7 and 9 g. wet wt organism, in four strains of the red-pigmented *M. morrhuae*, grown for 3 weeks on a special medium of high salt content formulated for halophilic cocci; a fifth strain (CCM2224) grew relatively poorly, yielding only 2·4 g. wet wt organism, from which we were unable to extract sufficient MK for characterization. The GC content of the strains of *M. morrhuae* ranged from 57·1 to 61·4 % (M. Kocur, personal communication).

A strain submitted as a non-pigmented variant of *Micrococcus corallinus* (CCM678, ATCC966) was classified by us on negative characters and placed in Baird-Parker's Micrococcus subgroup 7. This strain, with a reported GC ratio of 59·6 % (M. Kocur, personal communication), has a value somewhat lower than those within the range for pigmented Micrococcus strains. This strain produced small quantities of menaquinones of the normal type but, as shown in Table 2*a*, the MK pattern, with MK-9 as major isoprenologue, was not encountered in any other strain that we examined. The GC ratio of the pink-pigmented strain, ATCC966, B-P97, has not been determined, but the MK pattern, with hydrogenated isoprenologues (Table 2b) suggests that the alleged non-pigmented variant strain is not related to it.

The strain with 56·4 % GC in DNA, representing subgroup 4a of Rosypal *et al.* (1966) discussed above, like the other strains intermediate in position between micrococci and staphylococci, also yielded normal MKs.

Strains NCTC7292 and NCTC189 were received as *Staphylococcus saprophyticus* and *S. lactis*, respectively. Both strains were submitted to Dr A. C. Baird-Parker, who classified them as micrococci (NCTC7292 in Micrococcus subgroup 3; NCTC89 in Micrococcus subgroup 5). In the standard medium for the determination of anaerobic utilisation of glucose with a paraffin oil seal, proposed by the International Subcommittee on Staphylococci and Micrococci (1965), we found that NCTC7292 produced acid only at the top of the tube, and NCTC189 did not produce acid. Mortensen & Kocur (1967) confirmed that, although neither strain visibly fermented glucose in the proposed standard medium containing the indicator bromocresol purple, both strains formed acid weakly and slowly, as revealed by potentiometric pH determination of anaerobic cultures, in a fluid medium containing glucose. These two strains, together with others of different GC ratios, were thus found by Mortensen & Kocur to be intermediate between strains rapidly fermenting glucose, all of which were of low GC ratio (staphylococci), and strains forming little if any acid from glucose by oxidation

and all of high GC ratio (micrococci). The GC ratios of strains 7292 and 189 (Table 1) suggest their classification with the genus *Staphylococcus*, but this is not supported by their relative resistance to novobiocin, determined according to Mitchell & Baird-Parker (1967). The presence of MK-6 in both strains suggests that they may be closer to strains at present classified as non-pigmented micrococci.

Micrococcus candicans, ATCC8456, B-P89, was classified on physiological characters in Baird-Parkers' Micrococcus subgroup 7; however, the GC ratio of this strain, reported as 36·4% by Boháček, Kocur & Martinec (1967*b*), suggests that it should be classified in the genus *Staphylococcus*. As shown in Table 2*a*, all four non-pigmented subgroup 7 Micrococcus strains produced normal menaquinones; but strain ATCC8456 showed a different pattern from the other three strains, on which GC values have not been established.

It would appear, from the literature, that GC ratios have been determined on a disproportionately large number of strains of pigmented micrococci and on relatively few staphylococci and non-pigmented micrococci. A few non-pigmented strains, at present classified as micrococci on biochemical and physiological characters, have GC ratios which suggest that, in fact, these strains should not be classified in the genus *Micrococcus* (A. C. Baird-Parker, personal communication).

Association between menaquinone type and GC ratio

In addition to the values shown in Table 3, GC ratios, which fell within the range 66 to 74%, were provided for all the pigmented micrococci received from the Czechoslovak Collection of Micro-organisms. Thus 45 of the 129 strains of Micrococcaceae on which we have determined menaquinones were of known GC content.

According to the type of menaqinone produced, aerobic Micrococcaceae may be divided into two groups. The first, broader, group would include strains with GC ratios lower than 61·4%. The second group would comprise all strains producing hydrogenated menaquinones and would include those at present classified as *Micrococcus luteus* and *M. roseus* (% GC 66 to 74). Within these groups, subgroups may be defined on MK patterns. It will be interesting to see whether further data on GC ratios supports the differences suggested by the menaquinone distribution.

We are grateful to Dr M. Kocur, Curator, Czechoslovak Collection of Micro-organisms, who supplied the strains of marine cocci and most of the pigmented micrococci, together with GC values where they were not available from the literature. To Dr A. C. Baird-Parker we are indebted for providing strains representative of his proposed classification and also for many helpful discussions during the course of our work. For supplying other strains, together with details of isolation or classification, we are pleased to record our thanks to Mrs E. Asheshov, Dr M. T. Parker, Dr S. P. Lapage and Dr R. G. Mitchell. Dr O. Isler (Hoffman la Roche, Basle, Switzerland) kindly provided samples of synthetic menaquinones. For constructive criticism during the course of this work and in its presentation we thank Mr S. A. Price and Dr J. Green.

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An Electron Microscope Study of Spore Structure and Development in *Alternaria brassicicola*

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SUMMARY

Each cell of the mature spore of *Alternaria brassicicola* has a two-layered wall, the layer distant from the protoplast being melanized. The mature septa are five-layered, having two layers of secondary wall on either side of the septal partition which is itself three-layered. Each septum has one simple pore. New spores are produced by an outgrowth, through a pore, of the inner wall layer of the mother cell. Young spores have many small mitochondria and much vesicular endoplasmic reticulum; as they mature, lipid bodies and an unknown polyglucoside are produced. Mature spores have glycogen but very little if any lipid. The suggestion by other workers that vesicular endoplasmic reticulum, multivesicular bodies and lomasomes are involved in wall formation is supported.

INTRODUCTION

Alternaria brassicicola (Schw.) Wiltshire (= *A. oleracea* Milbraith) produces acropetal chains of dictyospores with melanized walls (Pl. 1, fig. 1). In the classification of Hughes (1953) the spores are porospores (his group VI), that is, spores that are produced by the extrusion of the protoplast through a pore in the conidiophore wall. Terminal cells of the spores produce new spores in a similar fashion, and side branches to the chain may form by the growth of functional conidiophores from the cells of the spores (Pl. 1. fig. 1). The taxonomy, nutrition and light-microscope morphology have been summarized and extended by Joly (1964). He describes how the new spores are budded out from mother cells at the tips of the conidiophores or spores. He states that the protoplasm of the mother cell retracts and leaves the mother cell empty, thereby isolating the newly formed spore. If this is so, it is difficult to see how the nutrients for the development of the new spores are moved along the chain to the tip where the spores are developing. The electron microscope studies described in this paper clarify this.

The cytology of Alternaria species has been studied by Stall (1958) and Hartmann (1966), both of whom found that the cells of the vegetative mycelium and the spores are multinucleate. Hartmann (1966) also reported that all the nuclei in a spore chain are derived from one or a very small number of nuclei which migrate into the first spore budded from the conidiophore.

The electron microscopy of mature conidia of higher fungi is usually difficult because of the impermeability of the wall to fixatives and embedding materials. The ultrastructure of the conidia of several species has, however, been described (Fusarium by Acha, Aguirre, Uruburu & Villanueva (1966), Marchant (1966*a*, *b*); Botrytis by Buckley,

Sjaholm & Sommer (1966); Cochliobolus by Matsui, Nozu, Kikumoto & Matsuura, (1962); Neurospora by Turian (1966) and his co-workers). Reviews of general fungal and spore ultrastructure have been published, such as those by Hawker (1965) and Bracker (1967). Melanized dictyospores and the formation of acropetal chains, the subjects of this study, have not been investigated at the ultrastructural level, possibly because of the difficulty of fixing and embedding such thick-walled spores, and the difficulty of preventing fragmentation of the chain.

METHODS

Alternaria brassicicola was selected because of its abundant sporulation and genetical stability in culture; the large size of the spores enabled them to be orientated for sectioning. The culture used was kindly supplied by Dr Q. Ruscoe of Exeter University.

The cultures were grown on potato glucose agar (Oxoid CM 139) at 25° for 4 to 10 days, depending on the degree of maturity that was required for the majority of the spores. A technique was developed to maintain chain continuity during fixation and embedding and to orientate the spores for sectioning: the results of this technique are assessed later. Pyramidal epon blocks were prepared with Beem (LKB Products Ltd.) capsules as moulds. The blocks were very thinly coated with Haupt's adhesive (Jensen, 1962). The small square tops of the pyramids were then touched very lightly onto a colony of the fungus; some chains of spores (Pl. 1, fig. 1), and many individual ones, adhered to the surface. The block was quickly chilled on an ice-cold metal sheet to gel the adhesive and was then put into cold fixative. The fixative denatures the protein in the adhesive, preventing a later return to sol at room temperature.

Fixation was extremely difficult. Numerous attempts to use mixtures of glutaraldehyde, osmium tetroxide and acrolein produced poor results, but the following methods were satisfactory.

(1) Permanganate (modified from Luft (1956)). Unbuffered 2% (w/v) potassium permanganate (with one drop of 1% (v/v) Brij (British Drug Houses Ltd.) bio-inert wetting agent/200 ml.) was used at 4° for 2 hr.

(2) Paraformaldehyde + glutaraldehyde + acrolein (modified from an unpublished method of Dr E. M. Rodriguez, Bristol University). Two g. paraformaldehyde were placed in 25 ml. of water at 90° and 0·1 N-sodium hydroxide added dropwise until the solution cleared; 25 ml. of this was mixed with 5 ml. of 25% (w/v) glutaraldehyde and 0·6 ml. acrolein. This solution was diluted 1:1 with 0·2 M-phosphate buffer (pH 7·4). The material was fixed for 6 hr, washed in 0·1 M-buffer, and then fixed in 1% (w/v) osmic acid in 0·1 M-buffer for 16 hr, all at 4°.

After fixation the material, still attached to the epon block, was stained in a saturated aqueous solution of uranyl acetate for 2 hr at 4°, dehydrated at 4° in an ethanol + water series followed by propylene oxide, placed in two changes of 50 + 50 propylene oxide + epon (with accelerator) at room temperature for periods of 24 hr each, transferred to fresh epon for 24 hr with three changes, and finally placed in Beem capsules with fresh epon and polymerized for 3 days at 35°, 1 day at 45°, and 2 days at 60°. Pyramids were cut for sectioning to include selected spores or spore chains whose approximate maturity had been determined under the light microscope. Photomicrographs were made of the pyramids before sectioning, and these were used to identify particular spores under the electron microscope by their relative positions in the section. This

greatly assisted in the determination of the relative spore age in electron micrographs. Sections were cut on an LKB ultratome, mounted on Formvar-coated carbon-stabilized copper grids, stained with alkaline lead citrate (Reynolds, 1963) and viewed with an AEI 6B electron microscope.

Some spores were prepared for the Stereoscan electron microscope by attaching them to the metal specimen-holders with Haupt's adhesive and coating them with gold + palladium after drying.

RESULTS

The sequence of events in the spore production and maturation of *Alternaria brassicicola* described below is compiled from many individual observations; to place them in the correct sequence was difficult. The method of preparation and embedding, involving the attachment of the spores to epon blocks, achieved two objects. First, the somewhat delicate chains were held together, though shrinkage during dehydration frequently separated the spores slightly. Secondly, the arrangement of the spores in a flat plane enabled them to be studied with the light microscope before sectioning, and made longitudinal sections possible. All the structures described have been seen after at least two different fixation treatments, unless otherwise stated. This, together with information from light microscopy and the relative spore age, makes it virtually certain that the described sequence is the correct one. In the following description the development of the spore is traced from its emergence until maturity. The process is considered in three parts: wall development; the formation of new spores; changes in the cytoplasmic membranes and organelles.

Wall and septum formation and maturation

Three main parts of the wall are distinguished: the primary wall, which is the original wall formed when the spore was budded out; the septal partition, which is three-layered and forms the main part of the septum; the secondary wall, which surrounds individual cells of the spore and may line either the primary wall or both the primary wall and the septal partition (Fig. 1).

The wall of the young spore is quite thin and has a granular electron-dense substance concentrated in the outer region (Pl. 1, fig. 2). It remains in this condition throughout the very rapid increase in size of the spore which takes place during the first hour or so of growth. As the rate of enlargement slows, the wall develops two ill-defined layers (Pl. 1, fig. 3), the outer one having a granular texture and darker colour probably because of the deposition of melanin and the inner one being electron-transparent. The outer layer (the primary spore wall) is derived from the original wall of the young spore (Pl. 1, fig. 2; Pl. 3, fig. 17; Fig. 1A), while the inner layer (the secondary wall) surrounds individual cells of the spore as they are delimited by septum formation (Pl. 1, fig. 3; Figs. 1B, C). The septal partition abuts onto the primary wall when the septum is laid down before the secondary wall formation begins (Pl. 1, fig. 3 at A; Fig. 1B), but when the septal partition is laid down after the secondary wall it will abut onto the latter (Pl. 1, fig. 4; Fig. 1C). Longitudinal septal partitions abut onto previous septa (Pl. 1, fig. 3 at B; Fig. 1D), giving the muriform condition. In a large muriform spore there may be a succession of walls formed over some days as the spore matures. The only method of determining the order in which the septa have been formed is to study the place where the septal partition abuts in relation to

the other wall layers. The secondary walls are thinner over the newer septal partitions than over the older ones (Pl. 1, fig. 3), and are thickest of all where they are laid directly on the primary wall.

The details of the septal partition formation are difficult to observe; because the process takes only a matter of minutes (light microscope observations) it is fixed in a very small number of spores. The septum is first apparent as an invagination of the

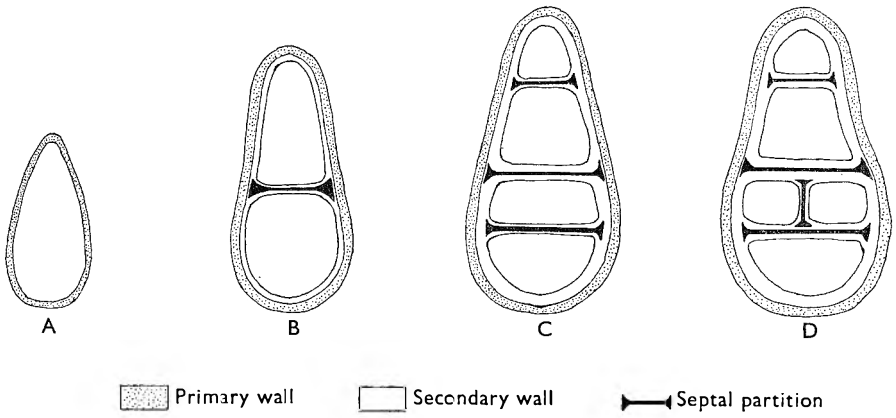

Fig. 1. *Alternaria brassicicola*. Diagram of wall layers in relation to septum formation (not to scale). A. The young spore having only the primary wall. B. The first septal partition is laid down abutting onto the primary wall, and the secondary wall is laid down in each cell. C. Further septal partitions are formed; the secondary wall has continued to grow in area and thickness and covers the septal partition. D. A mature spore in which a longitudinal septum has formed.

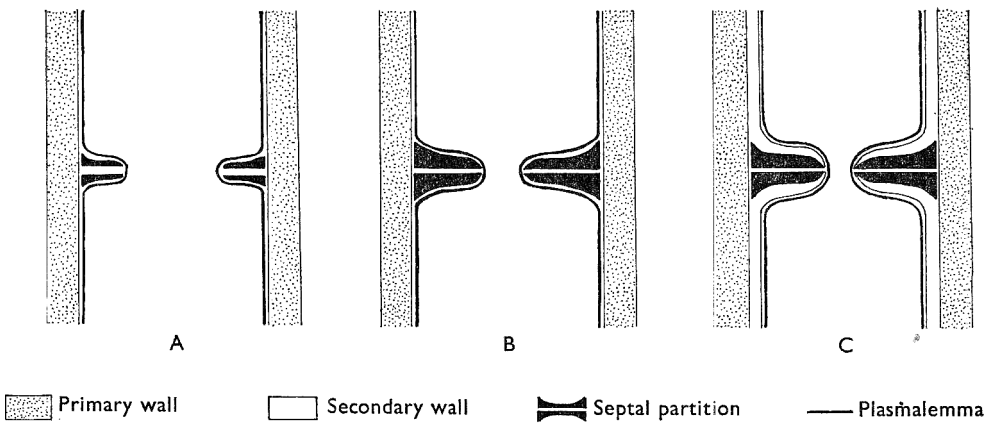

Fig. 2. *Alternaria brassicicola*. Diagram of septum formation (not to scale; in median section). A. The plasmalemma invagination lays down the two layers of the septal partition, each in contact with the plasmalemma producing it and separated from each other by the electron-transparent layer. B. The invagination reaches the centre and presumably either leaves the pore open or fuses to give a complete septum in which the pore is subsequently formed. C. Secondary wall is laid over the septal partition so that each of the cells now formed, though in cytoplasmic continuity, has its own double wall of half the septal partition and a secondary wall. The walls of the two cells are separated by the electron-transparent layer.

plasmalemma which develops on both sides of the spore in section and is presumably annular (Pl. 1, fig. 5, 6; Fig. 2A). Within the invagination the septal partition is formed in two layers, each maintaining close contact with the membrane and separated from the other layer on the opposite side of the invagination by an electron-transparent layer (Pl. 1, fig. 6; Fig. 2A). The invagination grows towards the centre of the cell. The origin of septal pores, which are invariably present, has not been observed, but presumably either the closure of the invagination is incomplete or the pore is formed by perforation of the completed septal partition. In the young septal wall (Pl. 2, fig. 7; Fig. 2B) the electron-transparent dividing layer between the two layers of the septal partition is clearly seen. The secondary wall is laid down on this septal partition; what determines the change from one wall type to another is not clear. Each of the new cells is thus surrounded by its own double wall. The fully formed septum has five layers, these being the septal partition in which the two layers are separated by the electron-transparent layer and a layer of secondary wall which covers the partition on each side (Pl. 1, fig. 3; Fig. 2C).

The pore in the completed septum is simple (Pl. 1, fig. 3, which is not quite median; Pl. 2, fig. 8). The septal partition and the secondary wall, when present, narrow abruptly and the plasmalemma passes around the lip where it is in direct contact with the layers of the septal partition. The pores are nearly always situated in the middle of the septum, and occur in longitudinal as well as transverse walls. In developing and mature spores the pores are invariably open; no pore plugs have been seen. Spherical membrane-bounded structures often occur near the pores (Pl. 1, fig. 3). These are usually electron-dense and are probably Woronin bodies (Bracker, 1967); sometimes, however, they appear electron-transparent, probably being poorly fixed and having lost their usual dense contents. Various organelles, including nuclei, have been seen passing through the pores.

Melanin deposition occurs from the time of spore formation, but is restricted, except in the very last stages of maturation, to the primary wall and septal partition of the spore, which become progressively more electron-dense (Pl. 2, fig. 9) and finally completely opaque (compare Pl. 1, fig. 2 to 4; Pl. 2, fig. 9). Very little, if any, melanin is laid down in any secondary wall even when it occurs between a septal and a primary wall (Pl. 2, fig. 10). The electron-transparent layer which separates the two parts of the septal partition may become obscured by the melanin at maturity (Pl. 2, fig. 9).

The rough and cracked appearance of the mature melanized wall (Pl. 2, fig. 9) is probably an artifact introduced during preparation and sectioning; it first becomes apparent when the spores are placed in propylene oxide. Optical and Stereoscan electron microscope photographs (Pl. 2, fig. 11 to 13) show smooth walls.

The spore walls are perforated by basal and apical pores which presumably maintain cytoplasmic continuity along the chain and allow transport of nutrients to the developing tip. The basal pore (Pl. 2, figs. 11, 13; Pl. 3, fig. 14, 15) is the connexion between the spore and its mother cell. The pore is usually a more or less cylindrical tube passing through the wall (Pl. 3, fig. 14), but there may be a constriction at the boundary of the primary and secondary walls (Pl. 3, fig. 15). The apical pore is originally formed as a simple perforation of the primary wall through which the secondary wall blows out as a new bud (Pl. 3, fig. 17). However, within an hour or two the wall around the pore starts to develop a ring of thickening, hereafter called the annulus, which lies within or just inside the secondary wall (Pl. 3, fig. 16).

This increases in size and becomes electron-dense, possibly due to the deposition of melanin, until it is clearly visible even with the light microscope (Pl. 2, fig. 12). The function of this structure is unknown, but it is probably connected with mechanical support of the rest of the chain above the spore in question, the whole weight of which is borne by the very small area of contact around the pore. In the spores used in this study, which were mature but not dormant, the basal and apical pores were always open and cytoplasm could sometimes be seen 'leaking out' when the chain was broken during preparation (note, for example, the space left in Pl. 3, fig. 15). How, or whether, the pores are occluded in the dormant spore of a broken chain is not known; investigations are proceeding.

The basic wall structure is similar to that described for Botrytis (Buckley *et al.* 1966), though in *Alternaria brassicicola* the wall is much thicker and the structure and development much complicated by the presence of septa and the formation of a chain of spores. The multiseptate spore of Fusarium might be expected to be similar in some respects, but Marchant (1966*a*, *b*) described the septa as completely continuous with the outside wall and similar to it in structure. This is not true of Alternaria, the wall of the latter being melanized, without the three layers described for Fusarium, and the spore having a much more complicated septum structure. *A. brassicicola* also has different septal partitions from Ascodesmis (Moore, 1963) which are not obviously composed of two dark layers separated by an electron-transparent one, though there is a suggestion of such a structure in one of the photographs (fig. 11C of Moore, 1963). Bracker & Butler (1963) described a similar septal partition (in the hyphae of Rhizoctonia) to that reported here, but no secondary wall was laid down on the partition and the septal pore was of the dolipore type.

The formation of spores

Spores are formed by the growth of a bud through a fairly well defined pore, which is apparently produced enzymically, for there is no sign of mechanical rupture (Pl. 3, fig. 17, 18). The mother spore has usually formed one or more septa and has a secondary wall before it produces a spore. The pore is produced in the primary wall and the secondary wall grows out through it to form the primary wall of the new spore (Pl. 3, fig. 17). As this matures and melanin is deposited, the distinction between the new spore primary and the mother spore primary walls becomes less clear (Pl. 3, fig. 18), but the discontinuity is indicated on the left of Pl. 3, fig. 18, and is also shown by the fact that spores break away from the chain cleanly, without a tear in the melanized wall (Pl. 3, fig. 15). The discontinuity of melanized walls was pointed out by Simmons (1967) who used the optical microscope, though he did not observe the secondary wall and reported complete discontinuity between the walls of the mother cell and the daughter spore. The young spore rapidly expands, laying down the secondary wall and the septa as described above.

Spores are also produced from conidiophores; either from the original one, attached to the mycelium, which gave rise to the first spore in the chain, or, alternatively, after the enlargement of the spore has stopped, a growing point may appear in one of the lateral cells. A short conidiophore is rapidly produced (in about 10 min., from optical microscope observations) and is usually walled off from the rest of the spore, apparently by a normal septum (Pl. 1, fig. 1; Pl. 3, fig. 19). The apical cell may also produce a conidiophore rather than a spore direct. What determines this, and why a lateral cell

develops a new growing point, is unknown. The formation of the conidiophore has not been seen on the electron microscope, but optical microscope observations suggest that growth takes place over a wide region of the spore wall rather than through a pore as in spore production. On some of the electron micrographs of mature conidiophores there is a suggestion of a collar of primary wall around the base (Pl. 3, fig. 19), indicating that the secondary wall may have mechanically broken and grown through the primary wall to form the conidiophore.

The conidiophore, which has both primary and secondary walls (Pl. 3, fig. 19, 20), produces a new spore at its apex in the same way as described above for spores, and an annulus forms around the pore.

The cytoplasm of the conidiophore is sometimes very sparse and apparently degenerate (Pl. 3, fig. 20). This probably accounts for the reports from light-microscope work (Joly, 1964) that the tips of the conidiophores and spores were empty.

Cytoplasmic maturation

Cytoplasmic maturation is a complex process because of the large numbers of organelles and membrane systems involved and will for convenience be divided into stages related to the wall maturation. It is, however, a continuous process. The very young spore (Pl. 1, fig. 2) has many small, irregularly shaped mitochondria, and much endoplasmic reticulum which is often vesicular. The plasmalemma is convoluted and associated with small vesicles (Pl. 4, fig. 28); these are thought to be connected with the wall formation and will be discussed later when considering the maturing spore, in which they are more prominent. One or two nuclei are always present and their double membranes show well-defined pores. The presence of many mitochondria and extensive endoplasmic reticulum is compatible with a rapidly metabolizing and growing cell.

The cytoplasm of the maturing spore (Pl. 1, fig. 3; Pl. 4, fig. 21) also has plentiful endoplasmic reticulum and mitochondria which are possibly somewhat larger than in the young spore. The main difference is the presence of lipid bodies and some vacuoles. An unknown substance is also present: it has an amorphous structure and appears as electron-transparent aggregates usually located around the edges of the protoplast (Pl. 1, fig. 3; Pl. 4, fig. 22). Preliminary chromatography of its hydrolysis products indicates that it is a polymer of glucose. It gives none of the usual cytochemical reactions of starch or glycogen and its exact composition is unknown, though some of its staining properties, particularly with toluidine blue (Pearse, 1953), suggest that it may be a dextran. The structure shown on the photographs suggests that the substance has probably been removed during fixation and/or dehydration.

The lipid bodies have the usual structure (Pl. 1, fig. 3, Pl. 4, fig. 21), and have also been shown to be present by standard Sudan and osmium-staining methods for optical microscopy. There are also structures that are thought to contain phospholipid (Pl. 4, fig. 23, 24); they are very similar to those described by Buckley *et al.* (1966). Smith & Marchant (1968) reported similar bodies in yeast and concluded that they were spherosomes whose phospholipid was used to form myelin bodies in vacuoles.

The vacuoles are for the most part empty (Pl. 1, fig. 3). Some of them occasionally have multiple membranes around them (Pl. 4, fig. 25) or membrane complexes within them (Pl. 4, fig. 26). The membranes are too widely spaced for true myelin bodies; Smith & Marchant (1968) pointed out that these loose aggregates of membranes,

which have been reported in various fungi, should not be confused with true myelin bodies. It is noticeable that in *Alternaria brassicicola* they are seen only in material fixed with formaldehyde + glutaraldehyde + acrolein; this suggests that they may be an artifact of this particular method of fixation.

There are also complex membrane structures associated with the convoluted plasmalemma of maturing spores, which together with the multivesicular bodies and endoplasmic vesicles in the cytoplasm, are thought to be concerned with extensive secondary wall formation which is occurring at this time. This role in wall formation was proposed by Marchant, Peat & Banbury (1967); they postulated that vesicles and multivesicular bodies were produced by the endoplasmic reticulum and then fused with the plasmalemma, discharging their contents, possibly enzymes or wall material, onto the developing wall. Multivesicular bodies can be seen in the cytoplasm and also outside the plasmalemma (Pl. 4, fig. 22, 27). Extensive endoplasmic reticulum in the region of the walls appears to be budding off small vesicles (Pl. 1, fig. 2; Pl. 4, fig. 21, 28) into the cytoplasm which fuse with the plasmalemma (Pl. 1, fig. 2; Pl. 2, fig. 8; Pl. 4, fig. 21, 28). There are more complex elaborations of membranes in the region of the plasmalemma; some of these appear to be the fusion of membrane-containing vacuoles (Pl. 4, fig. 29) and presumably the vacuolar contents pass into the wall. These have not been seen in permanganate-fixed material. Finally, there are complex lomasomes (Pl. 4, fig. 30) very similar to those shown by Moore & McAlear (1961, Fig. 8) in their original paper on these structures. Marchant *et al.* (1967) illustrated rather more organized bodies and referred to them as plasmalemma elaborations. The latter authors pointed out that all these vesicular systems, interpreted by them and by Moore & McAlear (1961) as fusing with the plasmalemma and contributing to wall formation, might in fact be moving in the reverse direction and be pinocytotic vacuoles in the sorts of hyphae from which they were first described. However, Marchant *et al.* (1967) found them in aerial sporangiophores of Phycomyces, and they are here shown to be present in aerial chains of spores of *Alternaria brassicicola*, where pinocytosis could not occur. This supports the argument that they are in fact moving outwards and depositing material in the walls.

The cytoplasm of mature spores is very different from that of either the young or the maturing spore. There are few mitochondria and generally very little endoplasmic reticulum (Pl. 2, fig. 9). The plasmalemma is relatively smooth and the vesicular systems largely absent. This indicates a great decrease of the metabolic rate and a cessation of wall synthesis. There are a few small vacuoles, but these are not as prominent as they are in the maturing spore. Lipid is absent or rare and no phospholipid bodies have been seen in mature spores. The polyglucoside is present in small amounts. The only additional substance found in mature spores is glycogen, which is frequently abundant (Pl. 2, fig. 9; Pl. 4, fig. 31). It appears as electron-dense composite particles (α particles) randomly distributed in the cytoplasm.

These changes in lipid and polysaccharide content of the spore have been checked by light microscope observations with standard cytochemical techniques for the localization of the substances. The following methods were used: Sudan dyes and osmium tetroxide for lipids, periodic acid Schiff's reagent for insoluble carbohydrates, Bauer-Feulgen and Best's carmine for glycogen, toluidine blue for the dextran-like substance (Glick, 1949; Jensen, 1962; Pearse, 1953).

Permanganate fixation does not preserve ribosomes, and the other fixatives used

did not generally give good enough results of all stages in the maturation for the changes in ribosome distribution to be found. Optical microscope stains for RNA showed the greatest density in young and maturing spores, with practically none in the mature ones.

DISCUSSION

The study of chains of spores of *Alternaria brassicicola* has allowed detailed observations on maturation. Changes in the cytoplasmic structures follow a readily interpretable pattern, linked with the rate of metabolism, that would be expected in the various ages of spore; changes in mitochondria and endoplasmic reticulum are particularly obvious. The significance of the shift in the energy reserves from lipid and a polyglucoside in maturing spores to glycogen and a polyglucoside in mature spores is unknown. The sequence of the wall structure changes is complex. Investigations on germinating spores and hyphae are being continued to complete the full cycle of wall production in *A. brassicicola.* The wall structure of the spores is unlike any previously described and the distinction between euseptate and distoseptate spores as proposed by Luttrell (1963) on the basis of light microscopy is difficult to make. In euseptate spores the cells remain together as a single unit; in distoseptate spores each individual cell is surrounded by its own wall and separates from the other cells of the spore when the enclosing spore envelope is broken. *Alternaria brassicicola* would seem, from optical microscopy, to be euseptate, but the electron microscopy shows that each cell is surrounded by its own wall. Furthermore, in a spontaneous albino mutant of this strain the spores seem to be distoseptate, for the individual cells may fall apart when the outer envelope ruptures (unpublished results). The multiseptate spore of Fusarium (Marchant, 1966*a*) is euseptate, for the septa are continuous with the outer wall and the spores show no tendency to disintegrate.

The mode of spore production by *Alternaria brassicicola* poses problems about the exact differences between the primary and secondary walls. The secondary wall of the mother cell gives rise to and is continuous with the primary one of the new spore, which in turn has a secondary wall lining it. Primary walls seem incapable of much further growth in thickness after they start to become melanized. Only the secondary or young primary walls, next to the cytoplasm, can grow in area or thickness, and this is probably linked with the deposition of new wall material by the plasmalemma and associated organelles. It would appear that the difference between the primary wall and septal partition and the secondary wall may be not so much in structure as in their position relative to the plasmalemma. It is not known how, or why, the deposition of melanin occurs only in the walls farthest away from the protoplast. Transport of materials up the chain to the developing spores at the tip must occur, and a well-developed system of pores is present which maintains cytoplasmic continuity along the chain. Even cells that are apparently empty when viewed with the optical microscope do in fact contain some cytoplasm and therefore maintain this continuity. This transport up the chain raises problems of compartmentalization within the chain, for the spores at the base are maturing while material is passing through them to the developing ones at the tip. How neighbouring spores maintain a different physiological state while in close cytoplasmic connexion is not understood. It is possible that chain length is determined by the maturation of spores at the base, growth stopping either when all the nutrients are removed from the translocation stream before it reaches the

tip or when transport through the mature spores at the base is for some reason impossible. Both these possibilities would explain why spores at the tip of a mature chain are often smaller than those at the base; they have been cut off from the nutrient supply and have matured before reaching their full size. Similarly spores produced on a poor culture medium are smaller than those from a rich medium (Joly, 1964). However, this does not imply that maturation is caused by lack of nutrients, for spores will mature at the base of a chain while new ones are still being produced at the tip.

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EXPLANATION OF PLATES

Key to symbols used on plates: PW = primary wall; SW = secondary wall; SP = septal partition; P = plasmalemma; ER = endoplasmic reticulum; N = nucleus; M = mitochondrion; V = vacuole; L = lipid body; G = glycogen; PG = polyglucoside; MC = mother cell. Scale lines represent distances in microns (μ) as indicated.

PLATE 1

Fig. 1. Light microscope picture of a spore chain of *Alternaria brassicicola*. Note the branching chains, conidiophores on the spores and the very young spores at the chain tips. The scale line should read 20μ not 10μ as shown. × 600.

Fig. 2. Young spore. There are numerous mitochondria and plentiful endoplasmic reticulum, but no vacuoles, lipid or storage products. $KMnO_4$. × 7750.

Fig. 3. Part of a medium-aged spore. The wall is two-layered and the septa have pores. Note the positioning of the ends of each septal partition in relation to the other wall layers. At A the septal partition abuts onto the primary wall and at B it abuts onto another septum. The cytoplasm has vacuoles, lipid bodies and the unknown polyglucoside. Formaldehyde + glutaraldehyde + acrolein, postfixed in osmium tetroxide. × 7500.

Fig. 4. The septal partition abuts onto the secondary wall, having been formed after it. $KMnO_4$. × 30,000.

Fig. 5. The start of septum formation, with the plasmalemma invaginating. $KMnO_4$. × 11,000.

Fig. 6. Detail of fig. 5. Note how the plasmalemma on each side of the invagination lays down its own layer of the septal partition, leaving an electron-transparent layer between the two dark layers of the partition. $KMnO_4$. × 25,000.

PLATE 2

Fig. 7. The young septal partition formed between the primary wall and another septum: the partition is composed of three layers, two granular ones separated by an electron transparent-one. The section is not quite median, hence no pore is visible. $KMnO_4$. × 17,500.

Fig. 8. Median section of a septal partition with a pore, before the secondary wall is laid down. The septal partition is divided by the electron-transparent layer and the plasmalemma is continuous over the lip. Note the large amounts of endoplasmic reticulum and vesicles formed from it. $KMnO_4$. × 27,500.

Fig. 9. A cell of a mature spore. The primary wall and septal partition are heavily impregnated with melanin. There are few vacuoles and mitochondria, little polyglucoside and no lipid in the cytoplasm, but glycogen is present. $KMnO_4$. × 15,400.

Fig. 10. Part of a mature spore showing an unmelanized secondary wall between the primary wall and the septal partition. $KMnO_4$. × 24,375.

Fig. 11. Light-microscope photograph of a spore with a two-layered wall and a basal pore where it was joined to the mother cell. × 2000.

Fig. 12. As fig. 11, but showing the apical pore with the annulus. × 2100.

Fig. 13. Stereoscan electron microscope photograph showing the smooth wall and basal pore. × about 2350.

PLATE 3

Fig. 14. Basal pore in the nearly mature spore: the secondary wall lines the pore. $KMnO_4$. × 35,000.

Fig. 15. As fig. 14, but the pore has a constriction where it passes through the junction between the primary and secondary walls. $KMnO_4$. × 26,250.

Fig. 16. Apical pore of a spore with a well-developed annulus. $KMnO_4$. × 13,500.

Fig. 17. A very young spore just being produced. The bud is surrounded by a wall which is continuous with the secondary wall of the mother cell. $KMnO_4$. × 29,000.

Fig. 18. A part of a more mature spore attached to its mother cell. There is continuity between the secondary wall of the mother cell and the new primary wall of the spore. $KMnO_4$. × 24,000.

Fig. 19. A conidiophore produced by one of the lateral cells of a spore, forming a branching point in the chain. Formaldehyde + glutaraldehyde + acrolein; postfixed in osmium tetroxide. × 8000.

Fig 20. An apical conidiophore, on a spore, whose contents are degenerating. The annulus has not been cut in median section. Formaldehyde + glutaraldehyde + acrolein; postfixed in osmium tetroxide. × 15,000.

PLATE 4

Fig. 21. Cell of a medium-aged spore. The cytoplasm contains mitochondria and many lipid bodies, endoplasmic reticulum is plentiful and in many places seems to be budding off small vesicles. $KMnO_4$. × 17,500.

Fig. 22. Detail of the polyglucoside appearing as amorphous electron-transparent aggregates. $KMnO_4$. × 50,000.

Fig. 23, 24. Bodies thought to contain phospholipid. 23, $KMnO_4$. × 25,000; 24, Formaldehyde + glutaraldehyde + acrolein; postfixed in osmium tetroxide. × 48,000.

Figs. 25, 26. Vacuoles surrounded by or containing multiple membrane complexes. 25, × 50,000; 26, × 58,000. Formaldehyde + glutaraldehyde + acrolein; postfixed in osmium tetroxide.

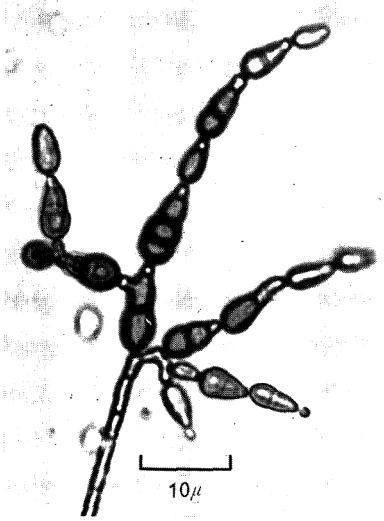
Fig. 27. A lomasome in a medium-aged spore. Formaldehyde + glutaraldehyde + acrolein; postfixed in osmium tetroxide. × 72,500.

Fig. 28. Detail of fig. 2. The endoplasmic reticulum has cysternae which are cut off to form vesicles which subsequently fuse with the plasmalemma. $KMnO_4$. × 52,500.

Fig. 29. A membrane complex associated with the plasmalemma. Formaldehyde + glutaraldehyde + acrolein; postfixed in osmium tetroxide. × 37,500.

Fig. 30. A complex lomasome in a medium-aged spore. Formaldehyde + glutaraldehyde + acrolein; postfixed in osmium tetroxide. × 30,000.

Fig. 31. Detail of fig. 9 showing abundant glycogen. $KMnO_4$. × 30,000.



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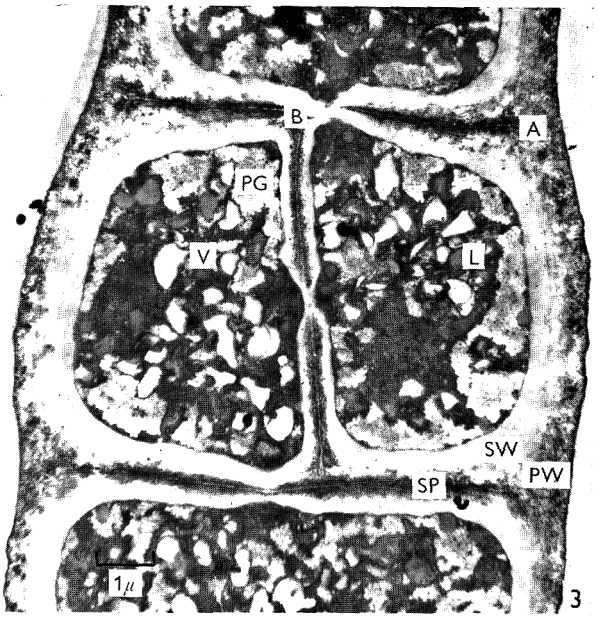

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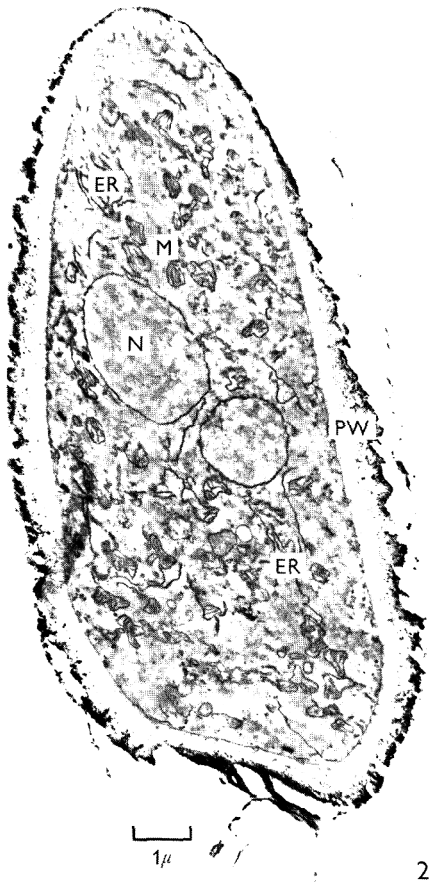

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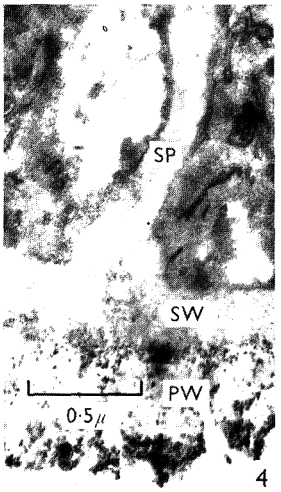

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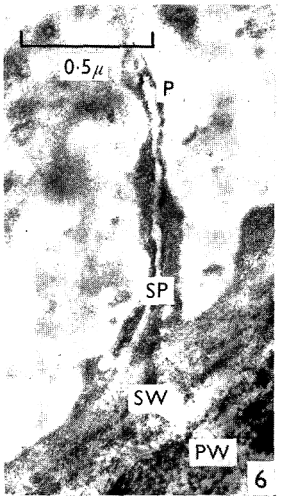

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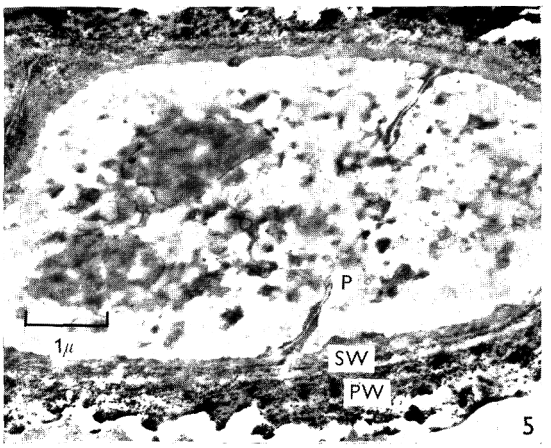

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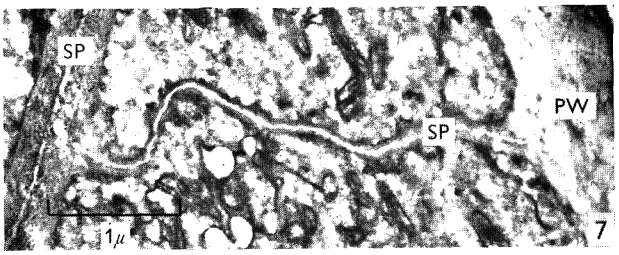

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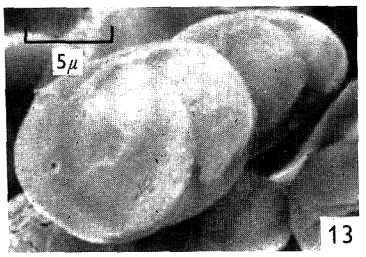

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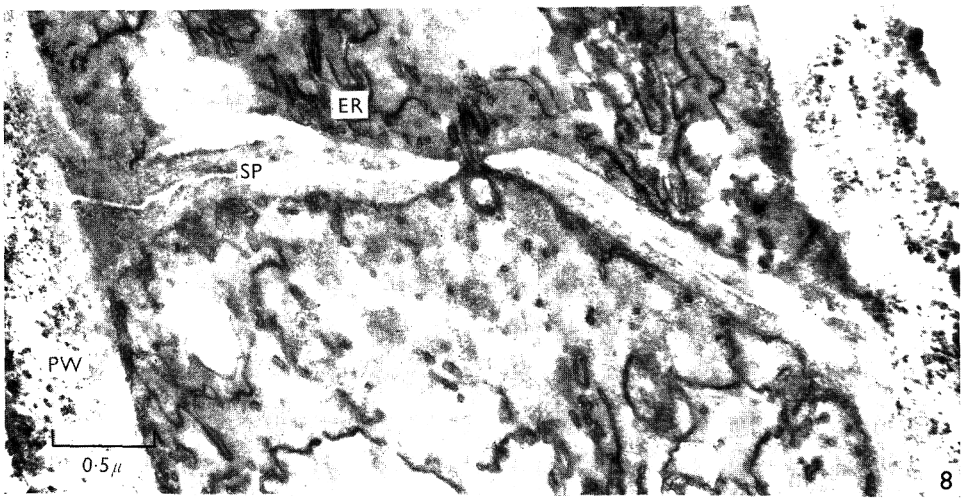

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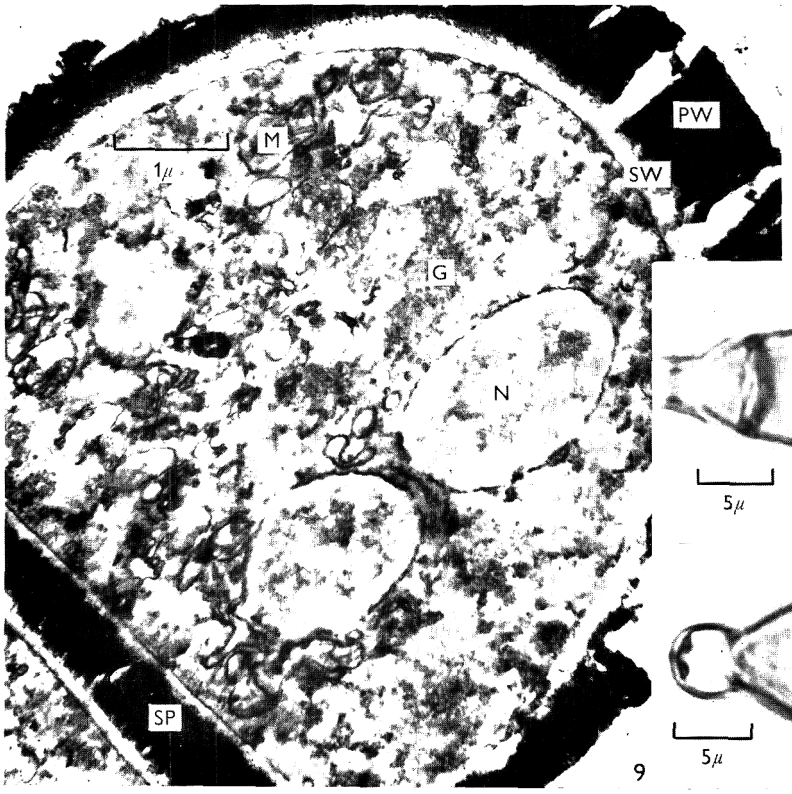

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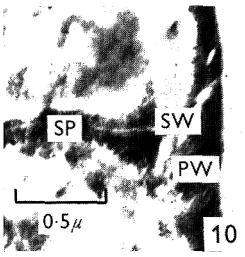

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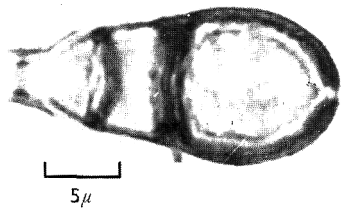

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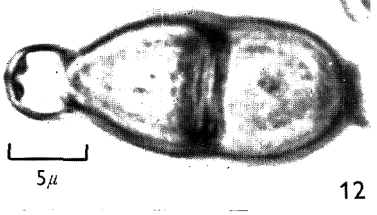

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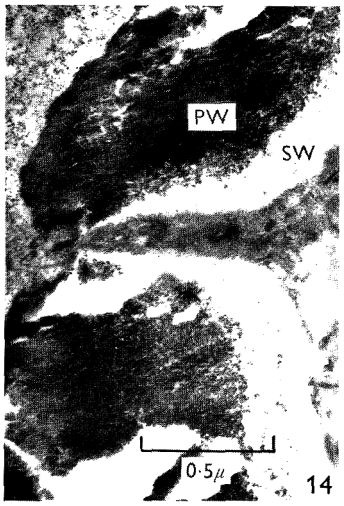

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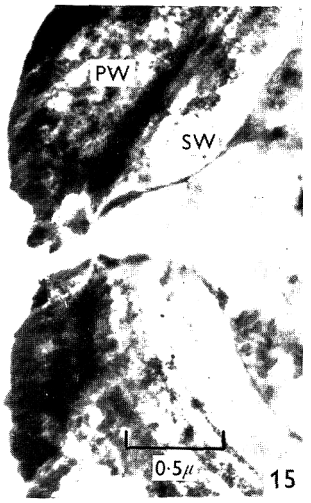

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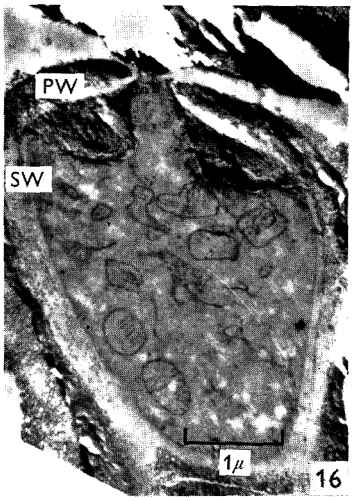

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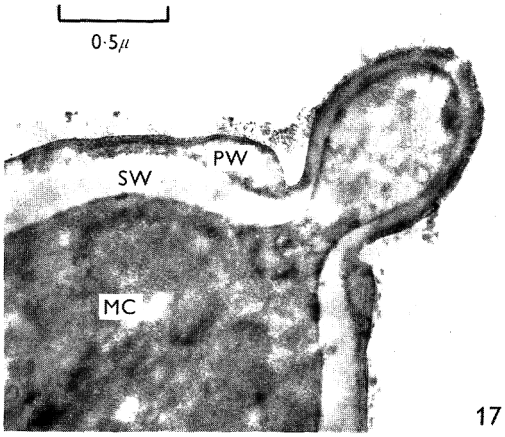

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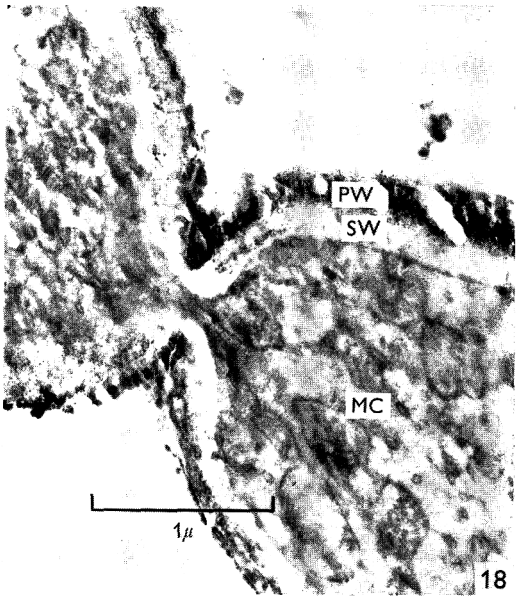

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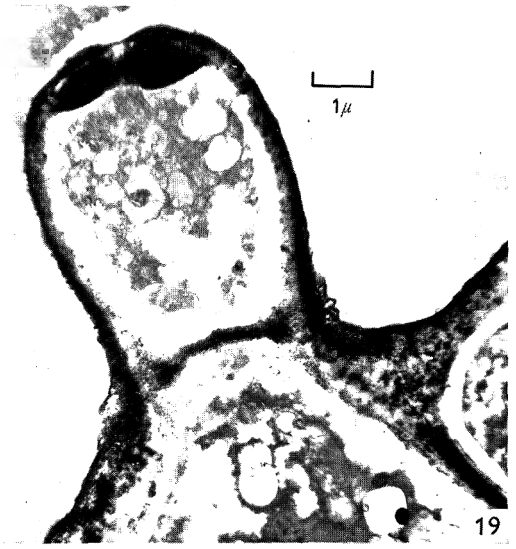

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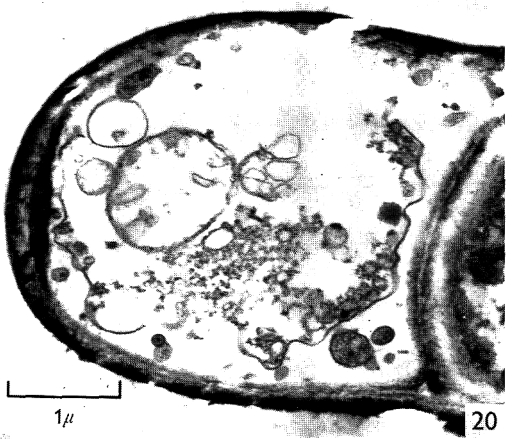

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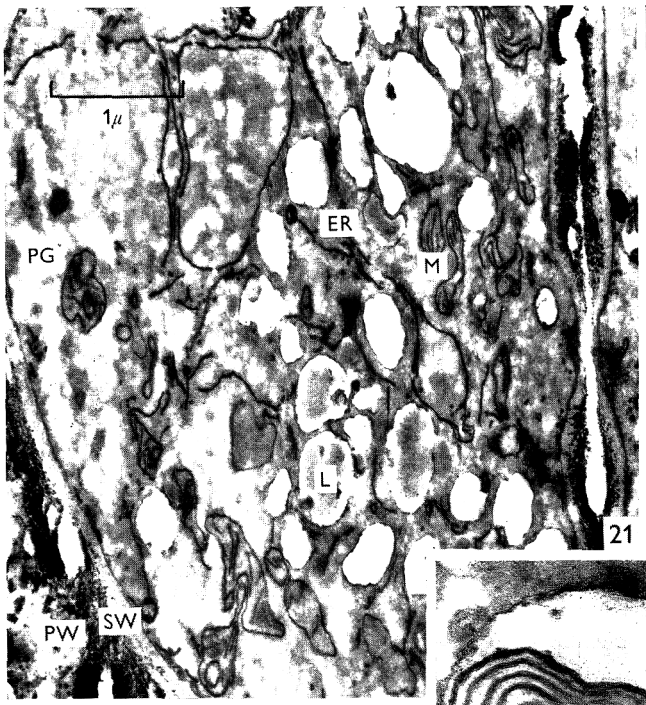
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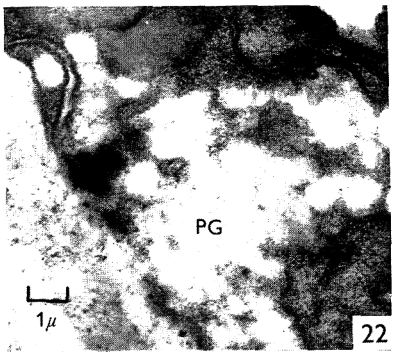
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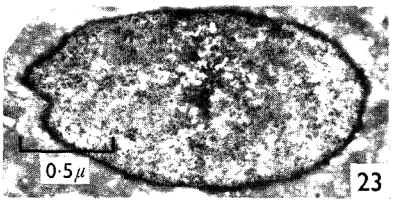
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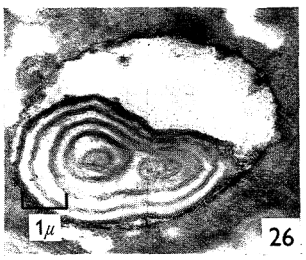
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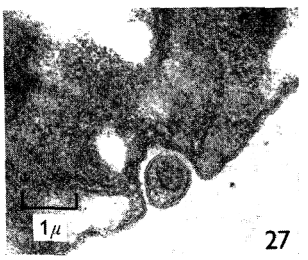
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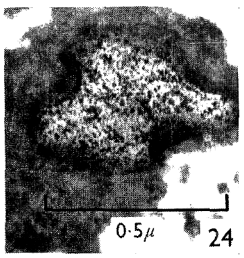
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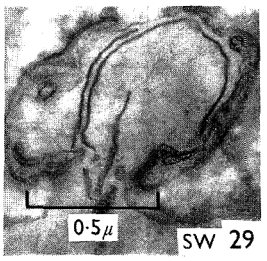
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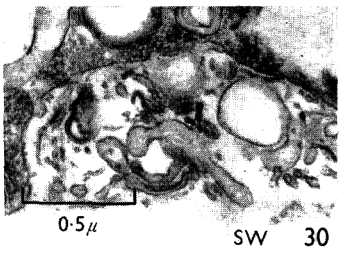
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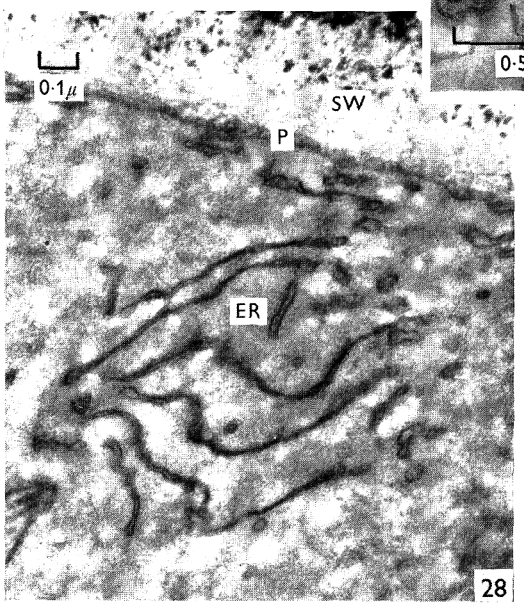
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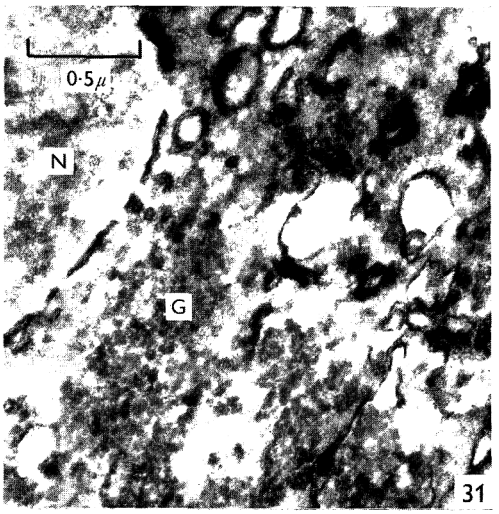
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Printed in Great Britain

The Wall Composition of Micrococci

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SUMMARY

The distribution of teichoic acids and extractable polysaccharides in the walls of the various micrococci examined did not show a direct correlation with the conventional systematic classification of these organisms. Three main groups were distinguished: in one group (A) ribitol teichoic acids containing glucosamine were found; in a second group (B) glycerol teichoic acids with a variety of sugar substituents were found; in a third group (C) wall teichoic acids were absent but polysaccharides were extracted from the walls. The third group (C) represents the species *Micrococcus conglomeratus*, and the lack of order in the structure of the extractable polysaccharides supports previous criticism of the validity of this species.

INTRODUCTION

The cell walls of staphylococci contain distinct teichoic acids (Davison & Baddiley, 1963). In the species *Staphylococcus aureus* the polymer is of the ribitol type with α- and β-*N*-acetylglucosamine residues (Baddiley, Buchanan, RajBhandary & Sanderson, 1962), whereas in the species *S. epidermidis* it is of the glycerol type with glucose residues (Davison & Baddiley, 1964). Immunochemical specificity of these teichoic acids is related to differences in glycosidic configuration (Davison, Baddiley, Hofstad, Losnegard & Oeding, 1964; Nathenson, Ishimoto, Anderson & Strominger, 1966; Oeding, Myklestad & Davison, 1967). In strains of *S. lactis* no similar homogeneity in type of teichoic acid is found, and one strain (13) contains a polymer of glycerol phosphate and *N*-acetylglucosamine 1-phosphate, that has been extensively studied (Archibald, Baddiley & Button, 1965; Button, Archibald & Baddiley, 1966; Blumsom, Douglas & Baddiley, 1966; Archibald, Baddiley & Button, 1968; Baddiley, Blumsom & Douglas, 1968). The majority of strains of *S. lactis* are micrococci (Baird-Parker, 1965). The present paper investigates further their cell-wall composition.

METHODS

Organisms and preparation of walls. Cultures were obtained from the National Collection of Industrial Bacteria, the National Collection of Type Cultures, the Czechoslovak Collection of Micro-organisms and from Drs A. C. Baird-Parker, R. G. Mitchell and M. S. Pohja. All the bacteria studied were Gram-positive catalase-positive cocci. Oxidation of glucose, mannitol and lactose was examined by the recommended method (*Subcommittee*, 1965). All strains of *Micrococcus conglomeratus*

oxidized glucose but not mannitol or lactose, with the exception of NCIB 2677, which did not oxidize glucose.

Walls were prepared by mechanical disintegration of cocci with Ballotini beads in a shaker head or a Braun homogenizer. Teichoic acids or polysaccharides were extracted with cold dilute trichloroacetic acid as described previously (Davison & Baddiley, 1963; Davison, 1968). Teichoic acids and polysaccharides were identified by paper chromatography of hydrolysates (Armstrong, *et al.* 1958; Baddiley *et al.* 1962; Davison & Baddiley, 1963).

Analyses. For the polysaccharides from Micrococcus species 740, 836 and 2677, glucose was determined after hydrolysis in 2 N-HCl for 1 hr at 100° by the Glucostat reagent (Worthington Chemical Corporation, New Jersey, U.S.A.), total hexose by the phenol + sulphuric acid method of Dubois *et al.* (1956), and total hexosamine by the method of Rosen (1957). For the teichoic acid from Micrococcus species 8, which contained alanine, galactosamine and glucosamine were determined in the Technicon autoanalyser, glucose by the method of Dubois *et al.* (1956), and phosphorus by the method of Chen, Toribara & Warner (1956).

RESULTS

Group A. Walls of these strains contained ribitol teichoic acid with *N*-acetylglucosamine residues; traces of glucose were detected in the preparations. The organisms were: Micrococcus species 234 and 238 (Pohja, 1960); 34 and 51877 (Alder, Brown & Mitchell, 1966); NCIB 8609; NCTC 189. Ribitol and its glucosaminide were detected after hydrolysis with alkali and then enzymic dephosphorylation of the teichoic acid from NCTC 189. This degradation procedure was not done on the other strains in this group.

Group B. Walls of these strains contained complex glycerol teichoic acids; some of the preparations contained both hexose and hexosamine residues. The organisms were: Micrococcus species 8 and 24 (Baird-Parker, 1965); 13 (Davison, 1968); NCIB 8174. The teichoic acid preparation from Micrococcus species strain 8 had an optical rotation + 93·5° (*c.* 0·4 water), and contained glucose, glucosamine and galactosamine in a molecular ratio total sugar to phosphate of 0·9:1. It was stable to hydrolysis by alkali, indicative of a fully glycosylated α-linked polymer. Teichoic acid from strain 24 gave glycerol diphosphates, glucosamine and traces of ribitol derivatives on acid hydrolysis; a saccharinic acid was formed in alkali. This polymer is presumably similar to the polymer of glycerol phosphate and glucosamine 1-phosphate in walls of Micrococcus species 13 (Archibald *et al.* 1968), but contains ribitol units. Teichoic acid from the strain NCIB 8174 contained glucose, glucosamine, rhamnose and galactose. This polymer preparation is complex, as both teichoic acid and a polysaccharide were seen on paper electrophoresis (0·05 M-veronal + hydrochloric acid buffer (pH 8·5), voltage gradient 8·5 V/cm.).

Group C. Walls of these strains contained non-phosphorylated polysaccharides; no teichoic acids were detected. The organisms were: *Micrococcus conglomeratus* CCM, 825, 836, 740; NCIB 2677. Polysaccharide material from strain CCM 825 contained glucose, galactose and traces of glucosamine; that from CCM 836 contained galactose and glucosamine in a molecular ratio of 3:1. Strain CCM 740 and NCIB 2677 both contained polysaccharides of glucose, galactose and galactosamine. These differed structurally, with respective molecular proportions of sugars of 1:2:1 and 1:3:2.

DISCUSSION

In this study micrococci have been divided into three groups: in group A the walls contained ribitol teichoic acid with *N*-acetylglycosaminyl substituents; in group B they contained glycerol teichoic acids with a variety of carbohydrate substituents; in group C no teichoic acids were found in the walls but these contained polysaccharides with varying carbohydrate components. The physiological characteristics of the micrococci show little correspondence with the distribution of polymers in the walls. Thus, ribitol teichoic acids are wall components in some (group A) glucose-oxidizing acetoin-negative micrococci, whereas others (groups B, C) contain either glycerol teichoic acids or polysaccharides.

The nature of the wall polymers in some individual cases is noteworthy. Thus, Micrococcus species NCTC 189 (group A) contains a ribitol techoic acid in which several of the ribitol phosphate residues lack the usual *N*-acetylglucosaminyl substituent; this differs markedly from strains of *Staphylococcus aureus* where such a substituent is probably present on each ribitol phosphate (Baddiley *et al.* 1962; Davison *et al.* 1964). The teichoic acid preparation from strain 8 (group B) contains glycerol phosphate residues, each of which must possess a glycosyl substituent since the polymer is stable towards alkali. The substituents are glucosamine, galactosamine (presumably both as *N*-acetyl derivatives) and glucose; it is not yet known whether this strain contains a mixture of teichoic acids, or whether the three different glycosyl substituents occur on the same polymer chain. Strain 24 (group B) contains wall teichoic acid showing chemical similarities to the extensively studied strain 13, but the preparation gives, on acid and alkali hydrolysis, degradation products of ribitol in addition to glycerol. The strains of *M. conglomeratus* (group C) contain in their walls a heterogeneous collection of polysaccharides; strain CCM 825 possesses a polysaccharide of glucose and galactose, whereas that from strain CCM 836 contains galactose and glucosamine. A glucose-oxidizing strain CCM 740 and a strain NCIB 2677 that does not oxidize glucose both contain structurally different polysaccharides of glucose, galactose and galactosamine.

The validity of the species *Micrococcus conglomeratus* is questionable (Baird-Parker, 1965; Evans, 1965). It is significant that walls of highly pigmented cocci received as this species (group C) differ from the rest of the micrococci studied, in that they have polysaccharides but no wall teichoic acids. These cocci are weak glucose oxidizers (Mortensen & Kocur, 1967) and may be representative of a group distinct from the predominantly non-pigmented more halotolerant cocci with teichoic acid.

Many glucose-oxidizing acetoin-negative micrococci are physiologically indistinguishable (Baird-Parker, 1965), and differences we have observed in the teichoic acid and polysaccharide composition of their cell walls may be of value in their delineation and in the establishment of the validity of reputed species.

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Modification of the Antigenic Surface of *Rhizobium trifolii* by a Deficiency of Calcium

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SUMMARY

Quantitative absorption of antisera was used to study the effect of calcium on the antigenic surface of *Rhizobium trifolii*. Antisera to Ca-deficient and Ca-adequate rhizobia (whole or broken) revealed two parts in the absorption curve: *range 1* in which there was ready removal of most of the agglutinating antibody with a very small absorbing dose; *range 2* in which the remaining agglutinating antibody resisted absorption. When Ca-adequate bacteria were used for absorption, range 1 consisted of about 87% of the total titre. The corresponding figure with Ca-deficient bacteria was 95%. These values have been attributed to three types of antibody; avid 1 (readily absorbed by either kind of bacterium), avid 2 (readily absorbed by Ca-deficient bacteria), non-avid (difficult to absorb with either bacterium). The fact that avid antibody 2 was absorbed readily by Ca-deficient bacteria but with difficulty by Ca-adequate bacteria may be due to a quantitative deficiency of a particular antigen on the surface of the Ca-adequate bacterium, or to a structural condition which gives the antigen lower affinity for its homologous antibody. Absorption characteristic of Ca-deficient rhizobia was obtained with the Ca-adequate bacteria treated with EDTA under conditions known to remove 90% of Ca from the cell. Broken bacteria to some extent simulated the absorption curve found with Ca-deficient bacteria. It is suggested that Ca located in the surface lipopolysaccharide layer of the wall of rhizobium grown with a sufficiency of this element obscures or modifies an antigenic group. Glucuronic acid found in the somatic antigen fraction of this bacterium is suggested as a possible site of Ca action.

INTRODUCTION

Earlier work showed that when *Rhizobium trifolii* was grown in a defined medium deficient in calcium, its shape was altered to an enlarged sphere (Vincent & Colburn, 1961) which, although not osmotically fragile, showed a greatly increased susceptibility to lysozyme without the potentiating action of EDTA (Vincent & Humphrey, 1963), and increased permeability or fragility (Humphrey & Vincent, 1965). The small amount of Ca contaminating the medium was concentrated in the walls of the Ca-deficient bacteria and amounted to only half that of normal walls (Humphrey & Vincent, 1962). It was concluded that Ca played a role in securing wall components in a firm steric arrangement, although the more loosely knit structure consequent on a shortage of this element retained a degree of stability and function. It seemed likely that differences in the surface of Ca-adequate and Ca-deficient rhizobia would be reflected in their detailed antigenic composition. However, simple agglutination and gross cross-absorption tests failed to reveal any differences of this kind; and dif-

ferences in respect of diffusing antigens by the gel immune diffusion technique were attributable to internal antigens leaking from the Ca-deficient cells (Humphrey & Vincent, 1965). The present work is an attempt at a more critical analysis of the surface antigens of both kinds of rhizobia by relating the amount of antibody removed to the quantity of cells used for absorption. Quantitative absorption could be expected to reveal disorganization of surface antigens that would be hard to detect by other methods. This approach has the additional advantage of being applicable to the untreated bacterial surface.

METHODS

Organism and cultural conditions. Rhizobium trifolii (strain SU297/31) was grown in a defined liquid medium (Vincent, 1962) having mM total divalent cation made up either of 0·5 mM-Ca^{2+} and 0·5 mM-Mg^{2+} (Ca-adequate) or mM-Mg^{2+} (Ca-deficient).

Breakage of bacteria. When broken organisms were used as antigens in the preparation of antisera, whole Ca-adequate culture was shaken for 20 min. at 4° with ballotini beads in a Mickle disintegrator. Washed organisms for absorption were similarly broken in some experiments.

Preparation of antisera. Antisera to Ca-adequate, Ca-deficient and Mickle-disintegrated Ca-adequate whole organisms were produced in rabbits by the method previously described (Humphrey & Vincent, 1965). For brevity, these antisera will be referred to as 'Ca-adequate', 'Ca-deficient' and 'broken'. Agglutination titres of the antisera so obtained were greater than 3200.

Absorption of antisera. Antisera were diluted to a suitable agglutination titre (400–600 in earlier, 1000 or greater in later experiments) and absorbed, at a further dilution of 1/25 in saline (0·85%, w/v, NaCl), by a range of increasing amounts (50 to 2500 μg./ml.) of Ca-adequate and Ca-deficient bacteria. In the case of whole bacteria these had been centrifuged out of a standardized suspension of known dry weight. Disintegrated cells were not centrifuged but were added in appropriate volume to a more concentrated serum. Absorption took place under controlled conditions: shaken at 37° on a wrist shaker for 1 hr followed by standing at 4° for 18 hr. The sedimented material was then removed by centrifugation.

Determination of titre. The residual titre of the absorbed serum was determined at closely spaced dilution intervals. With sera absorbed at 1/25, the least dilution possible, using concentrated testing antigen, was 1/27. For dilutions 1/50 upwards, the testing antigen was a suspension in saline containing 280 μg. unheated, washed, Ca-adequate bacteria per ml. The suspension so prepared showed no flagellar agglutination. Agglutination was recorded after 24 hr (5 hr at 55°, 19 hr as the water bath cooled to room temperature). The end-point was taken as the highest dilution at which there was some deposit and definite reduction in the opacity of the suspension. An initial, pre-absorption, titre of 1000 was used in later experiments, rather than 400 to 600 as was first used. The higher starting level allowed definite end-points to be determined in range 2 of the residual titre: absorptive dose curve.

Ca-deficient bacteria used as testing antigen at the same concentration as Ca-adequate bacteria showed a condition of antigen excess in that the suspension showed no definite reduction in turbidity though showing a deposit of agglutinated bacteria. When the concentration of Ca-deficient test suspension was halved, the resulting absorption curve resembled that obtained with Ca-adequate testing bacteria.

Expression of results. Residual titres have been expressed as a proportion of the titre of the unabsorbed antiserum. This has proved a satisfactory way of making use of results with antisera of starting titre between 400 and 1200.

Treatment of antisera with 2-mercaptoethanol (ME) and heat. Treatment was by 0·1 M-ME in saline buffered with 0·05 M-phosphate buffer pH 7·2. Antisera were diluted 1/5 with this solution, incubated at 26° for 24 hr in sealed tubes, and diluted with saline to 1/25 prior to absorption. Heat treatment consisted of heating antisera for 30 min. at 75°, either at a dilution of 1/25 in unbuffered saline, or at a dilution of 1/5 in buffered saline, pH 7·2.

RESULTS

Form of absorption curve

Residual titres obtained in a large number of experiments, after varying degrees of absorption, are shown for Ca-adequate antiserum (Fig. 1). The same form of curves was obtained with both Ca-deficient antisera and the broken antiserum. Most of the antibody was removed by the smallest practical dose of absorbing bacteria

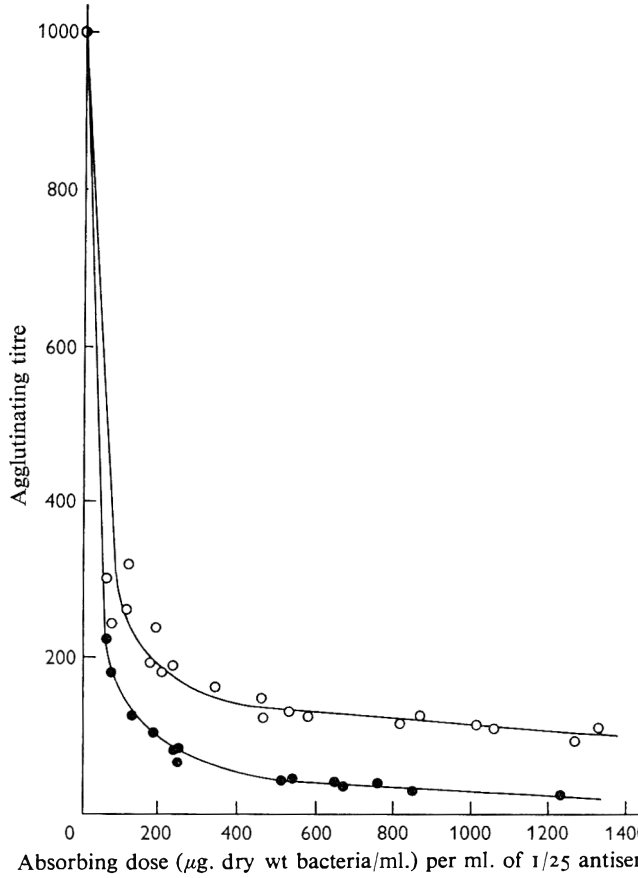

Fig. 1. Absorption of rhizobial antiserum by Ca-adequate (○——○) and Ca-deficient (●——●) bacteria. Points represent average values of all experiments with the antiserum developed against Ca-adequate rhizobia (see Table 1). The same form of curves was obtained with Ca-deficient and broken antisera.

(50 μg./ml.) but the remainder was absorbed much less readily so that a significant amount remained after treatment with more than 500 μg./ml.

Experiments in which the starting titre was varied between 400 and 1600 showed that 50 μg./ml. absorbing bacteria provided considerable excess of the antigen(s) required for the removal of most of the antibody (range 1 absorption, as defined below). In these experiments more than 80% of the antibody was removed whether the initial titre was 400, 800 or 1200. The proportion removed when the starting titre was 1600 was less but represented an additional absolute amount removed by the same weight of cells.

These results have been interpreted in terms of at least two kinds of antibody: (i) the larger part with high affinity for the absorbing bacteria (range 1 absorption); (ii) a smaller part having very low affinity (range 2 absorption). From the curve shown in Fig. 1 it is apparent that the residual titre after absorption with 500 μg./ml. bacterial suspension can be used to estimate the proportion of low affinity antibody.

Table 1. *Absorption of Ca-adequate, Ca-deficient and broken antisera by Ca-adequate bacteria (whole, broken and EDTA-treated) and Ca-deficient bacteria*

	Proportion (%) of antibody unabsorbed by 500 μg./ml. bacterial suspension*			
		Ca-deficient antiserum†		
Absorbing suspension	Ca-adequate antiserum	(1)	(2)	Broken antiserum
Ca-adequate	13·1 ± 0·7 (8)	19·8 ± 1·5 (10)	8·9 ± 0·5 (20)	14·6 ± 1·1 (5)
Ca-deficient	4·7 ± 0·6 (7)	< 5 (4)	3·3 ± 0·3 (12)	6 (2)
Ca-adequate, broken	—	9·3 ± 0·7 (3)	7 ± 1 (2)	8·5 ± 0·7 (2)
Ca-adequate, EDTA-treated	—	8·0 ± 1·1 (3)	3·9 ± 0·3 (10)	7·5 ± 2·2 (2)
Ca-adequate, EDTA-treated + divalent cation	—	9·1 ± 0·2 (11)	4·8 ± 0·6 (10)	—
	Partition of antibody (%)			
		Ca-deficient antiserum†		
Type of antibody	Ca-adequate antiserum	(1)	(2)	Broken antiserum
Avid 1	87	80	91	85
Avid 2 (avid with Ca-deficient bacteria)	8	> 15	6	9
Non-avid	5	< 5	3	6

* Standard errors based on deviations from the mean based on the number of determinations shown within parentheses. The two values without a standard error do not justify this treatment.

† Separate values for two antisera.

Absorption of Ca-adequate, Ca-deficient and broken rhizobia

Table 1 summarizes the results for a large number of experiments with Ca-adequate, Ca-deficient and broken antisera. Absorbing with Ca-deficient bacteria (consistently with all antisera) reduced the titre to a half or a third of the range 2 level remaining when Ca-adequate cells were used. This means that some of the antibody which was difficult to absorb with Ca-adequate bacteria was absorbed avidly by the Ca-deficient bacteria.

Because the supply of Ca-adequate bacteria was already in excess of that required for the removal of readily absorbed range 1 antibody, the capacity of Ca-deficient cells to absorb readily a further significant fraction must be attributed to a different antigen having greater affinity for this part of the antibody complex.

A sufficiently large dose (2500 μg./ml.) of Ca-adequate bacteria removed difficultly absorbable antibody to below the limit of detection (residual titre, < 27). The slopes of the range 2 curves were similar with Ca-adequate and Ca-deficient bacteria but, because they started at a lower level, the latter reached the limit of detection at 500 to 1000 μg./ml.

Mechanical breakage of Ca-adequate bacteria increased their range 1 absorption to a degree intermediate between intact Ca-adequate and Ca-deficient bacteria.

Effect of EDTA on the absorbing capacity of Ca-adequate rhizobia

EDTA under conditions which remove 90% Ca from the wall (Humphrey & Vincent, 1962) modified the Ca-adequate bacteria in the direction of the absorbing properties of Ca-deficient bacteria (Table 1). It was not possible to obtain a significant restoration of Ca-adequate absorption characteristics by subsequent washing of the EDTA-treated bacteria (either in water, or HCl at pH 3·5) followed by exposure to Ca^{2+}, Sr^{2+} or Mg^{2+}. Rhizobia grown with a deficiency of Ca were not further affected by EDTA, nor converted to the Ca-adequate condition by exposure to Ca^{2+} after growth had occurred.

Possible role of internal antigens

The possibility that the internal antigens revealed by gel diffusion using Ca-deficient and broken rhizobia (Humphrey & Vincent, 1965) might be responsible for the agglutinin absorption pattern of Ca-deficient and broken bacteria observed in the present work was investigated. A strain of *Rhizobium trifolii* SU 157 which does not cross-agglutinate, reveals, after breakage, internal precipitinogens, identical with the homologous, when diffused in agar against antisera to *R. trifolii*, SU 297/31. Treatment with such broken SU 157 failed to reduce the agglutination titre of antiserum developed against SU 297/31. Further it did not, when used with Ca-adequate SU 297/31, increase percentage absorption in range 1. That is, the extra range 1 absorption of antibody with Ca-deficient bacteria cannot be attributed to internal antigens.

The effect of heat and mercaptoethanol (ME) on antisera

It seemed possible that the antibodies responsible for various ranges of absorbability might prove to be macroglobulin or γ-globulin, in which case macroglobulin would be distinguishable by its sensitivity to heat and ME (Pike, 1967). In the present system, however, it was not possible to show complete destruction of antibody concerned with either range by heat or ME. Antisera heated in unbuffered saline at a dilution of 1/25 showed a proportionate decrease of a half to a third of their titre, reflected evenly throughout the curve. On the other hand, the application of heat or ME to antisera diluted 1/5 in saline buffered at pH 7·2 showed partial reduction of titre specifically in range 1 (Table 2). However, range 1 was not destroyed completely and the portion destroyed (ME, 40%; heat, 20%) could not be related to the difficultly absorbable fractions.

Table 2. *Partial destruction of antibody by heat and ME**

	Titre			
	Unheated	Heated	Untreated	ME
Unabsorbed	1000	800	1000	600
Absorbed (500 μg./ml.) Ca-adequate bacteria	80	90	80	113
Absorbed (500 μg./ml.) Ca-deficient bacteria	40	34	40	40

* Both treatments were carried out a serum dilution of 1/5 in saline buffered at pH 7·2.

DISCUSSION

Quantitative absorption was used many years ago in an attempt to study the nature of the agglutination reaction (e.g. Eisenberg & Volk, 1902; Craw, 1905). Later, Wilson & Miles (1932) used it to provide a quantitative basis to the analysis of cross-reacting species of the genus *Brucella* which had two common antigens in varying proportion on their surface. Their data, like ours with Rhizobium, showed a marked degree of absorption with a small dose of absorbing antigen but a residual titre that required much heavier doses for absorption beyond the limit of detection. These characteristics they related to the relative amount of A and M antigens on the surface of the absorbing suspensions and the amounts of the respective agglutinins in the antiserum. However, not enough absorbing doses were used to enable the quantitative relationships to be fully expressed. The sharp inflection in the curve of the dose/response relationship between residual antibody and absorbing antigen found by Wilson & Miles (1932) and our own more detailed data contrast with the relatively smooth curves obtained by Heidelberger & Kabat (1937). In the latter case only one antigen/antibody system was thought to be operating.

We interpret the marked difference we have found in all the antisera between ready absorption of the larger part of the agglutinins (range 1) and the more difficult absorption of the residual agglutinin (range 2) as evidence that each contain at least two kinds of agglutinating antibody, distinguished by different avidity for the absorbing bacteria. Further the Ca-deficient and, to a less degree, Mickle-disintegrated bacteria have antigens in a form that permits them to combine readily with an antibody fraction non-avid for the Ca-adequate surface. On this basis antibody in the four antisera given in Table 1 can be partitioned on an average percentage basis, thus: avid antibody 1 (removed by Ca-adequate or Ca-deficient bacteria), 87%; avid antibody 2, non-avid with Ca-adequate, avid with Ca-deficient bacteria, 8%; non-avid, 5%. The consistent marked capacity of the Ca-deficient bacteria to absorb avid antibody 2 would seem to indicate a marked difference in the antigens concerned with this combination, consequent on the shortage of Ca in the cell wall. The difference in the Ca-adequate and Ca-deficient surfaces points either to a direct role of Ca in the antigenic surface layer of the rhizobium or to considerable but less direct modification of the detailed architecture of the surface due to absence of a specific bonding cation. That is, the difficult absorption of this fraction of antibody by Ca-adequate bacteria may be due to (1) a modification of a surface antigen in the presence of Ca-bonding, whereby only one part of an effective grouping is fully exposed, resulting

in lowered affinity for a corresponding antibody; or (ii) fewer but whole particular antigenic sites on the surface of the bacterium when the surface is both intact and firmly bonded. EDTA conversion of the surface to simulate the absorbing characteristics of the Ca-deficient bacteria suggests a fairly direct role for Ca but failure to restore *in vitro* the Ca-adequate condition to the Ca-deprived or EDTA-treated bacteria argues for a more complex structural relationship.

The fact that Mickle-breakage, as well as Ca removal or absence (due to nutritive deficiency), makes the absorbing surface more avid for a significant proportion of difficultly absorbable antibody suggests that Ca obscures rather than combines directly with an antigenically active group. It is also apparent that the surface of the Ca-adequate rhizobia is modified to provide this antigenic form when inoculated into the rabbit.

The lipopolysaccharide somatic antigen extracted by the phenol method (O'Neill & Todd, 1961) from the strain of *Rhizobium trifolii* used in the present study (Humphrey & Vincent, unpublished data) while showing sugars similar to the 'core' and 'side-chain' sugars of lipopolysaccharides of the Enterobacteriaceae (Lüderitz, Staub & Westphal, 1966) was unusual in that it included a significant amount of glucuronic acid and was sufficiently acid to be precipitated by the cetyltrimethyl ammonium ion. This would provide a ready site for Ca-combination, which might either directly prevent glucuronic acid acting as an antigenic determinant group or, by forming a cross link, block a potential antigenic site and render it subsurface.

These results add to a growing body of evidence which points to the outer lipopolysaccharide/lipoprotein layers as a probable site of fixed divalent cations. It is an advantage of the present investigation over those that depend solely on EDTA treatment for the removal of divalent cation, that by studying deprivation by both methods one can observe parallelism in behaviour, and distinguish, in the case of Rhizobium, an effect for Ca not substituted by Mg. The fact that it has not been possible to show the same specific requirement for Ca in the growth of Escherichia and Aerobacter (A. Chan, P. Y. Yao & J. M. Vincent, unpublished data) parallels the experience of others with Escherichia and Pseudomonas for which the requirement is a more general one for divalent cation, including Ca^{2+}, Mg^{2+}, Zn^{2+} (Asbell & Eagon, 1966). Compared to the wall of Pseudomonas (Eagon, Simmons & Carson, 1965), that of *Rhizobium trifolii* contains twice as much Ca, but only one-fifth the Mg (Humphrey & Vincent, 1962).

The several effects of EDTA on walls of Gram-negative bacteria: potentiation of lysozyme action (Repaske, 1956; Salton, 1958; Noller & Hartsell, 1961); increased permeability to toxic reagents (Gray & Wilkinson, 1965; Leive, 1965); and leakage of intracellular components (Gray & Wilkinson, 1965) could be due to disturbance of the lipopolysaccharide/lipoprotein outer layer. In fact, Leive found that up to 50% of the wall lipopolysaccharide of *Escherichia coli* could be liberated by EDTA, an effect not attributable to any alkaline detergent acton. Relating these effects more directly to shortage of divalent cation we were able to show parallel effects such as immediate lysozyme sensitivity (Vincent & Humphrey, 1963) and leakage of intracellular antigens (Humphrey & Vincent, 1965) in rhizobia that had been deprived of Ca during growth.

Recent work on the respective effects of lysozyme and EDTA on the rigidity of the wall of Pseudomonas (Carson & Eagon, 1966; Eagon & Carson, 1965) and on the conversion of rods of Escherichia to spheres by phospholipase C (Weinbaum *et al.*

1967) points to the distruption of divalent cation cross-links involving phospholipid. It would seem from these studies with other bacteria that one need not invoke deficiency of Ca in the mucopeptide layer itself to account for the changed shape of Ca-deficient rhizobia. The present experiments make it seem probable that some at least of the Ca is situated either at the antigenic surface or, if below the surface, so as to affect the configuration of the lipopolysaccharide. Failure to detect any difference in electrophoretic mobility between Ca-adequate and Ca-deficient rhizobia (Humphrey, Marshall & Vincent, 1968) suggests either that the general configuration of the lipopolysaccharide is affected without exposing additional groups or that any such additional charged groups, freed because of Ca-deficiency, are too deeply situated to contribute to the charge on the surface.

The method of quantitative absorption described in this paper is applicable to other types of investigation of the bacterial surface. For example, a cross-reacting strain could be expected to absorb more slowly in range 1, displacing the curve to the right, if it had an antigen (or antigens) related to the principal antigen(s) of the homologous bacterium but not identical and with lower affinity for the corresponding antibody. On the other hand, a sharp but incomplete range 1 absorption would indicate that the bacteria shared some, but not all, high affinity antigens. Modification of antigenically active groups, either chemically of as a result of lysogenization can also be studied by this method. Investigations along these lines are in progress in this laboratory.

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The Structure of Cell Walls of Phycomycetes

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SUMMARY

Dilute acid treatment of isolated hyphal walls from *Phytophthora heveae* and *Pythium butleri* separates two fractions: an acid-soluble carbohydrate protein containing glucosamine (about three-fifths of the total glucan weight) and an acid-insoluble pure glucan. Acid and enzymic hydrolyses of the soluble fraction indicate two major glycosidic linkages, β (1 → 3) and β (1 → 6), and a little of the β (1 → 4). Digestion of the residual material by cellulase confirmed the presence of a cellulose-like glucan in these walls. A small amount of laminaribiose and gentibiose confirms the presence of β (1 → 3) and β (1 → 6) linkages. Comparable analyses indicate that the walls of both fungi possess similar glucan structures, the differences being quantitative. Proteins in the Phycomycetes walls seem to act as binding points between carbohydrate chains.

INTRODUCTION

Novaes-Ledieu, Jiménez-Martínez & Villanueva (1967) reported that cell walls of two oomycetes, *Phytophthora heveae* and *Pythium butleri*, contained 81–90 % of carbohydrates, glucose being the most abundant sugar. Acid yielded a glucan quickly solubilized in N-HCl at 100° and another resistant to hydrolysis. This last glucan resembled a crystalline cellulose, and accounted for 35 and 25 % of the hyphal walls of *P. heveae* and *P. butleri*, respectively. The current investigation reports more information about the structure of cell wall glucans and the possible role of proteins in the wall.

METHODS

Organisms, media and preparation of cell wall material. Hyphal walls of *Phytophthora heveae* and *Pythium butleri* were prepared as described by Novaes-Ledieu *et al.* (1967). Mycelium was disrupted in a Ribi fractionator (Sorvall). Cell walls were freed of cytoplasmic material by washing and differential centrifugation. Lipids were extracted from the cell walls by the method of Ballesta (1966) and extracted materials were lyophilized and stored *in vacuo* at room temperature

Partial acid digestion of cell walls. About 400 mg. *Phytophthora heveae* walls were hydrolysed in 10 ml. of N-HCl in a boiling water-bath 14 times for 5 min. and finally 5 times for 10 min. *Pythium butleri* walls were treated with the acid 8 times for 10 min. and 5 times for 15 min. After each operation the digested walls were centrifuged and the residues washed twice with N-HCl. The combined washings and HCl extracts were neutralized with NaOH and stored at 4°.

Cellulolytic hydrolysis of the acid-insoluble fraction. About 50 mg. residue in 0·03 M citrate buffer, pH 5·8, were homogenized in a MSE ultrasonic disintegrator for

30 min. and incubated on a reciprocating shaker for 24 hr at 40° with 100 units of crude Streptomyces QM 814 cellulase (Reese & Mandels, 1963). The digestion repeated for another 24 hr with fresh cellulase. This enzyme preparation contained a little β (1 → 3) glucanase but only negligible glucosidase. Phosphoric acid-swollen, Whatman cellulose, prepared following the method of Walseth as described by Bartnicki-Garcia & Lippman (1967), was used as a control in the enzymic experiments. During incubation the suspensions were protected from bacterial contamination with a layer of toluene. After centrifugation the supernatant liquids were boiled for 5 min. and analysed.

Proteolytic hydrolysis of acid-soluble fraction. Non-dialysable components (3 mg. dry weight) were dissolved in 1 ml. formic-acetic acid buffer, pH 2, and an amount of crystalline pepsin added to obtain a ratio pepsin:protein of 1:40. The mixture was incubated at 37° for 24 hr under toluene and then dialysed against distilled water at 5° for a day using cellophane tubing. Dialysable products were analysed by chromatography and electrophoresis.

Analytical procedures. Total carbohydrate was determined by the anthrone procedure (Chung & Nickerson, 1954) and reducing sugar by the method of Somogyi (1952), using glucose as standard. Quantitative determination of protein was made by the procedure of Lowry, Rosebrough, Farr, & Randall (1951), with crystalline bovine serum albumin as standard, and by the ninhydrine method (Moore & Stein, 1954). Hexosamine was estimated by the method of Rondle & Morgan (1955) after hydrolysis of the cell wall with 4 N-HCl for 17 hr at 105°.

Paper chromatography. Digestion products were desalted on a mixed resin bed (Dowex-1 × 4; Dowex-50 × 2) and chromatographed on Whatman no. 1 paper for biose and triose sugars in *n*-butanol + acetone + water (4 + 5 + 1 by vol.) for 48 hr; ethyl acetate + pyridine + acetic acid + water (5 + 5 + 1 + 3 by vol.) for 36 hr; *n*-butanol + pyridine + water (6 + 4 − 3 by vol.) for 40 hr and detected with aniline hydrogen phthalate in *n*-butanol. Semi-quantitative determination of monosaccharides was made on chromatograms as described by Gottschalk & Ada (1956). For amino acids, glycopeptides and glycoproteins, butanol + formic acid + water (75 + 15 + 10 by vol.) was used for 24 hr. Amino compounds were detected with ninhydrin (0·5 % w/v, in acetone).

Gentibiose was prepared by paper chromatography of partial acid hydrolysates of postulan, a β (1 → 6) glucan, extracted from the lichen *Umbilicaria pustulata* (Lindberg & McPherson, 1954). Disaccharide was purified with a modification of the procedure described by Reese, Parrish & Mandels (1962). Laminaribiose and laminaritriose were isolated in a similar way from partial acid hydrolysates of laminarin. The nature of a trisaccharide on a chromatogram was confirmed by elution, followed by second acid hydrolysis and rechromatography of further products. Glucose was assayed with glucose oxidase reagent (Glucostat 'special', Worthington Biochemical Corp., Freehold, New Jersey, U.S.A.).

Zone electrophoresis was carried out on paper (Whatman no. 1) or, for components with a high molecular weight subjected to high voltages, on glass-fibre paper (Whatman GF 81) in pyridine-acetate (pH 6·5), acetic acid–formic acid (pH 2·25) or 0·5 M carbonate (pH 11·5; 750 V; 3 hr) for amino acids. A borate buffer at pH 8·7 was used to separate gentiobiose and laminaritriose (2500 V; 2 hr). The buffers at pH 2·25 or 6·5 and a 0·5 M sodium tetraborate buffer at pH 9·2 separated glycoproteins (1500 to

2500 V; 2 hr). Proteins were revealed with amido black (Smithies, 1955) and polysaccharides on glass-fibre paper with α-naphtol H_2SO_4.

Electron microscopy. Intact cell walls and acid-insoluble fractions of *Phytophthora heveae* and *Pythium butleri* were shadowed with an alloy of gold and palladium with a Zeiss EM 9 electron microscope.

Sedimentation diagrams. Sedimentation values were obtained at 59,780 rev./min. in a Beckman Spinco Model E analytical ultracentrifuge in a 12 mm. aluminium cell at 20°.

Enzymic attack of walls of living oomycetes. Mycelia were suspended in 0·5 ml. of 0·05 M-citrate buffer, pH 6, containing 0·25 to 0·6 M-$MgSO_4$. Enzyme preparations (usually 125 units) were added and the mixtures incubated at 27° without shaking. The process was followed by microscopic observations. The β-glucanases preparations tested were: crude cellulase of Basidiomycete QM 806 grown with cellulose possessing a large amount of β (1 → 3) exoglucanase; cellulase of Streptomyces QM 814 containing a small amount of β (1 → 3) glucanase; a β (1 → 6) glucanase of *Penicillium brefeldianum* QM 1872 (Reese & Mandels, 1959) also high in β (1 → 3) glucanase and β glucosidase but with very little if any β (1 → 4) glucanase.

RESULTS

Partial acid hydrolysis of hyphal walls. The cell walls of *Phytophthora heveae* and *Pythium butleri* are very similar in soluble carbohydrate and in percentage of cell wall solubilized by the acid treatment (Table 1). Most, if not all, of the protein was in the soluble fractions and represented 6 to 11 % of the wall weight. The cell-wall glucosamine was also recovered in the acid-soluble portion.

Table 1. *Analysis of hyphal walls*

Pythium butleri and *Phytophthora heveae* walls were digested with N-HCl as described in the text. Solubilized and residual fractions were analysed.

Organism	Initial weight (mg.)	Soluble carbohydrates*		Soluble protein†		Insoluble residue‡	
		mg.	%	mg.	%	mg.	%
P. heveae	388	200	52	25	6	160	41
P. butleri	361	160	44	40	11	151	42

* Anthrone positive carbohydrate calculated using glucose as standard.
† Protein calculated as serum albumin according to the Lowry procedure.
‡ Dry weight.

Practically all the protein components of the soluble fractions remained in the cellophane bag during dialysis of 100 ml. against 3 l. distilled water for 36 hr at 4°. About 22 and 35 % of the soluble carbohydrate of *P. butleri* and *P. heveae*, respectively, passed through the membrane. These results indicate that the N-HCl treatment hydrolysed a significant portion of the wall-carbohydrates, whereas it had much less effect on the protein. The fact that most protein was recovered in the soluble fraction suggests an association of glucan and protein in the wall of these two oomycetes.

Dialysable soluble fraction. Qualitative analysis of this fraction showed only carbo-

hydrate. Glucose was the dominant single sugar though trace amounts of galactose and mannose were identified (more from *Phytophthora heveae*). Laminaribiose was the principal disaccharide: about 60 % of total disaccharides for *Pythium butleri* and 70 % for *P. heveae*. The *P. butleri* fraction contained considerably more gentibiose (30 %) than the *P. heveae* fraction. Cellobiose was 6–8 % in *P. butleri* and 12–15 % in the *P. heveae* fraction. A small amount of laminaritriose was found in both.

Non-dialysable soluble fraction. This fraction contained 85 % carbohydrate for *Phytophthora heveae* and 66 % for *Pythium butleri*. Protein was 14 % and 33 % respectively. Glucosamine was found exclusively in this fraction. Acid hydrolysates of the same fraction showed not only a high glucose content but also a significant and unexpected amount of mannose (8 to 10 % of total monosaccharides). A small quantity of galactose (1–2 % of monosaccharides) was also found for *P. heveae*. Acid hydrolysates of the protein revealed all of the common acids of walls in the usual proportions. In order to calculate approximately the proportion of β (1 → 3)

Table 2. *Relative amount of di- and tri-saccharides in the various fractions of Oomycetes walls*

The oligosaccharides were estimated on paper chromatograms (Gottschalk & Ada, 1956) after either acid or enzymic hydrolysis (see text).

			P. butleri	*P. heveae*
First acid soluble fraction	Non-dialysable	Laminaribiose	+ + +	+ + +
		Gentiobiose	+ + + +	+ + + +
		Laminaritriose	+ +	+ +
		Cellobiose	+ +	+ +
	Dialysable	Laminaribiose	+ + + + +	+ + + + +
		Gentibiose	+ + +	+ +
		Laminaritriose	+ +	+ +
		Cellobiose	+ +	+ + +
Second acid soluble fraction		Laminaribiose	+	+
		Gentiobiose	+ + + + +	+ + + + +
		Laminaritriose	−	−
		Cellobiose	+	+
Supernatant from enzymic hydrolysis of residue		Laminaribiose	+ +	+
		Gentiobiose	−	−
		Laminaritriose	+	+
		Cellobiose	+ + + + + +	+ + + + + +
Residual material		Laminaribiose	+ +	+
		Gentiobiose	+ + + +	+ + + +
		Laminaritriose	+ +	+ +
		Cellobiose	+ + +	+ + +

Carbohydrates in percentage: over 80 % of total di-trisaccharides (+ + + + + +); 60 to 80 % (+ + + + +); 35 to 60 % (+ + + +); 10–35 % (+ + +); 2–10 % (+ +); less than 2 % (+).

and β (1 → 6) glucan linkages, we chromatographed a large number of hydrolysates obtained under varying conditions of partial hydrolysis. Pure β (1 → 3) and β (1 → 6) glucans were submitted to similar hydrolyses, and the degradation products also chromatographed. An approximate ratio of gentiobiose to laminaribiose of 2 was found for walls of both organisms. In all the chromatograms, a small amount of laminaritriose was encountered while traces of cellobiose were detected, chiefly in *P. heveae* fraction. According to chromatographic and electrophoretic assays, acid hydrolysis of phosphoric acid-swollen cellulose did not release disaccharides in

appreciable quantity, so the exact contents of β (1 $\rightarrow$ 4) linkages in glucan complexes were not calculated exactly. Cellulolytic hydrolysis of fraction components confirmed the presence of β (1 $\rightarrow$ 4) linkages and indicated their approximate value. Semi-quantitative estimation of the moni, di- and trisaccharides of wall fractions is shown in Table 2. Minor amounts of other oligosaccharides, not resolved, appeared in paper chromatograms. It is possible that sophorose exists in *P. butleri* walls.

The degree of polymerization of components of two non-dialysable soluble fractions was indicated by values of reducing power (Somogyi, 1952) in relation to the total anthrone carbohydrate content. The percentages obtained (3·2 % for *P. heveae* and 8·5 % for *P. butleri*) suggested a low degree of polymerization of carbohydrate chains. On the other hand, the sedimentation constants of 2·02 S for *P. heveae* and 1·68 S for *P. butleri* suggested median molecular weights of about 15,000, which agrees with the behaviour on dialysis. The combined data indicate the presence of non-carbohydrate portion in the the non-dialysable complexes.

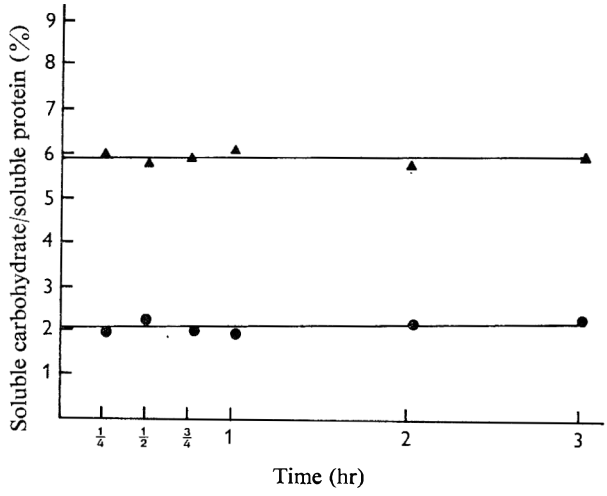

Fig. 1. Ratio of soluble carbohydrate to soluble protein extracted with diluted acid from hyphal walls, at various times of hydrolysis: ●, *P. butleri*; ▲, *P. heveae*.

The ratio of carbohydrate to protein in solubilized components at various times of acid digestion was constant (Fig. 1), which permits one to think that protein and carbohydrate were always extracted in constant proportion. Hence a macromolecular glucan-protein complex may exist in the wall.

Glycoproteins were demonstrated in both *Phytophthora heveae* and *Pythium butleri* fractions by chromatography in butanol-formic acid and, after elution of the spots without spraying, by electrophoresis at various pH values; specific reagents for both proteins and polysaccharides were sprayed after cutting the paper into two equivalent strips.

The *Phytophthora heveae* fraction contained a predominant amido black and α-naphthol positive spot of R_F: 0·10. The eluted material was separated at 2500 V, pH 2·25, into two compounds which ran 10 and 15 cm. in 2 hr on Whatman no. 1 paper when

placed up to 7 cm. from the anode. For *Pythium butleri* two main amido black and α-naphthol positive spots were detected with respective R_F values of 0·08 and 0·12. The material with the lower R_F gave two glycoprotein spots running 8 and 28 cm. on similar electrograms, and the material from the second compound gave a single spot running 35 cm. In both the cases additional spots were detected, which corresponded to glycoproteins in lower concentrations. Complete hydrolysates of material removed from each spot always contained glucose and amino acids.

Control acid hydrolyses of laminarin, laminaribiose, cellulose and cellobiose indicate that gentiobiose does not appear to any significant extent on our chromatograms as a reversion product.

Proteolysis of non-dialysable acid fractions. Both *Phytophthora heveae* and *Pythium butleri* yielded similar amino acids and peptides from suspected glucan-protein complexes or protein components, according to the method of Moore & Stein (1954). Dialysable sugars assayed with anthrone were also similar. Peptic digestion released 20 to 25 % of the total protein. In contrast, only 1 to 2 % of carbohydrate passes through the dialysis bag after this treatment.

β (1 → 3) glucanases digestion of isolated walls and soluble non-dialysable fractions. Two β-glucanase preparations were used to obtain more information about glucan structure of walls: a practically pure β (1 → 3) endoglucanase from *Rhizopus arrhizus* and a β (1 → 3) exoglucanase from Basidiomycete QM 806, here grown with starch. Both the preparations were relatively free of β (1 → 4) glucanase. Cell walls and soluble fractions of two oomycetes were treated with exo and endo β (1 → 3) glucanases in 0·05 M citrate buffer, pH 4·8 at 40°, for 4 hr and the products of enzymic hydrolysis studied. Enzymic treatment of each cell wall and its respective soluble non-dialysable fraction with β (1 → 3) glucanase from *R. arrhizus* did not show substantial digestion of these components, although the glucan linked in β (1 → 3) represents about one-third of the walls. On the contrary, the β (1 → 3) glucanase from Basidiomycete QM 806 was able to digest isolated walls and soluble acid fractions, and liberate a large amount of glucose.

Purification of the undigested acid-residue. Dry walls of *Phytophthora heveae* contained 36 % and those of *Pythium butleri* 23 % cellulosic glucan (Novaes-Ledieu *et al.* 1967). The ratios of insoluble residue to dry wall, under the conditions specified above, were somewhat higher, particularly for *P. butleri* (Table 1). The differences might derive from a contaminant glucan that was not digested by acid in the present experiments, so the digested acid residue was subjected to a second acid hydrolysis under more drastic conditions: in 2 N-HCl at 105° for 2½ hr. Only carbohydrates were present in the totally dialysable supernatant. Anthrone analyses indicated that the glucan digested was 10 % dry-wall weight for *P. heveae* and 23 % for *P. butleri.* Glucose was the only monosaccharide, and gentiobiose the most abundant disaccharide, from *P. heveae*; from *P. butleri* a small amount of galactose was also detected. Both hydrolysates contained laminaribiose and cellobiose.

Enzymic attack of cellulosic residue. The cellulase of Streptomyces dissolved about 70 % of the glucan in the case of *Phytophthora heveae* and 62 % in *Pythium butleri* to give mainly cellobiose, but also traces of glucose, laminaritriose and laminaribiose, this last sugar being more plentiful in *P. butleri.* Further enzyme treatment of the residue did not liberate any significant amount of carbohydrate. The material remaining after cellulase digestion was hydrolysed for 4 hr, in 2 N-HCl at 105° and chromato-

graphed. Both had a high content of glucose and gentiobiose as well as lesser amounts of cellobiose. *P. heveae* yielded traces of laminaribiose and laminaritriose; in *P. butleri* these amounted to 10 to 15 % of solubilized glucan. A component suspected to be sorbose was also encountered in *P. butleri*. In spite of all the treatments a very small amount of the wall still remained insoluble. This material seems to be a complex carbohydrate.

Electron microscopic observation. Intact cell walls of both fungi showed the mass of crossed fibres embedded in an amorphous matrix typical of hyphal walls of fungi. The acid-insoluble residue conserved the fibrillar structure of the original material.

β-glucanase digestion of living mycelium walls. Nicolas (1965) treated mycelium of two oomycetes, *Pythium ultimum* and *Phytophthora heveae*, with a crude cellulase from *Trichoderma lignorum*, and observed lysis of hyphal walls with release of vacuoles and vacuolated protoplasts. Our studies with Streptomyces and basidiomycete cellulases showed considerable digestion of the walls of the mycelium with both preparations, but only the basidiomycete cellulase liberated protoplasts. Failure to obtain protoplasts with other cellulase preparations may be explained by the presence of proteolytic activity in the enzyme preparation. A *Penicillium brefeldianum* enzymic preparation, which does not contain cellulase activity, did not dissolve the walls of the living mycelium of the oomycetes studied while showing a large release of reducing sugars in experiments with both isolated cell walls.

DISCUSSION

The acid-soluble material studied here was a glucan, or glucans, predominantly linked β (1 → 3) and β (1 → 6) with an approximate ratio of 1/1, but containing also 5–10 % of β (1 → 4) linkages. Since parallel hydrolyses of an acid-swollen cellulose showed only minor degradation, we assumed that the β (1 → 4) linkages came from non-cellulosic glucan. The acid-insoluble fraction revealed mainly β (1 → 4) glucosidic linkages and also a low content of β (1 → 3) and β (1 → 6) glucosidic linkages. This acid resistant glucan was not a pure cellulose as was supposed from preliminary experiments. It could be a glucose polymer of mixed linkage, though dominantly β (1 → 4), or it could be a mixture of cellulose with another glucan which is very resistant because of a high degree of branching.

The low effectiveness of the β (1 → 3) endoglucanase from *Rhizopus arrhizus* on the non-dialysable soluble fraction of two oomycetes walls, containing a considerable percentage of β (1 → 3) glucan linkages, may be due to the fact that this polymer is highly branched in such a way as to be inaccessible to the enzyme. Besides the digestion of isolated walls and soluble non-dialysable fractions releasing a large amount of glucose when used, a β (1 → 3) exoglucanase from Basidiomycete QM 806 may suggest that β (1 → 3) glucan exists in an acid soluble complex as branches, easily attackable by the exoenzyme. On the other hand, the fact that a β (1 → 3), β (1 → 6) glucanase preparation from *Penicillium brefeldianum* degraded isolated walls, with liberation of sugars but not living cells, indicates the presence of resistant glucan, very insoluble in acid, in the outer layer of the cell wall. Our results differ a little from those obtained by Bartinicki-Garcia & Lippman (1967) with *Phytophthora cinnamomi*. These authors confirmed, in effect, the presence of cellulosic-glucan in cell walls and reported the existence of glucan(s) soluble in acid, but highly insoluble in alkali, with an undetermined proportion of β (1 → 3) and β (1 → 6) linkages.

Most of the protein of walls was found in the acid-soluble fractions. A chemical extraction of walls with N-KOH showed a very low release of carbohydrates (less than 5 %), whereas the major portion of protein was solubilized. Hence protein is probably not indispensable to cell wall integrity. Possibly protein units are absent from linear carbohydrate chains but serve to bind them together. This hypothesis is in agreement with the fact that pepsin liberated no nitrogenous material from cell walls, whereas it released a large quantity of peptide and amino acids from acid-soluble material. Mild acid treatment presumably solubilized a glucan and the consequent rupture of carbohydrate linkages made the protein units more accessible to proteolytic digestion. As was published previously (Novaes-Ledieu *et al.* 1967), the protein of two oomycetes walls contains a significant proportion of hydroxy amino acids and, in the case of *Pythium butleri*, a high content of aspartic acid. These results could be interpreted as possible binding points between carbohydrate and protein. On the other hand, glucosamine, which has always been found in carbohydrate-protein fractions, could also be a binding point between protein and carbohydrate chains.

The larger protein percentage in *Pythium butleri* could explain the more drastic treatment necessary to solubilize a glucan-protein complex from the wall of this phycomycete and the different behaviour of the digested material on dialysis.

It may be concluded that the major component in cell walls of the two oomycetes is a glucan-protein soluble in dilute acid. The main chain of carbohydrate may contain various β-glycosidic linkages, side chains linked β (1 $\rightarrow$ 3), and protein chains serving to bind together different glucan molecules. This glucan might form the inner layer of the hyphal walls. In addition both phycomycetes contain an acid-resistant glucan with dominant β (1 $\rightarrow$ 4) linkages and a lower content of other kinds of linkages.

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Elimination by Ethidium Bromide of Antibiotic Resistance in Enterobacteria and Staphylococci

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SUMMARY

Ethidium bromide, a trypanocidal drug affecting nucleic acid synthesis, was found to be a powerful agent in eliminating some antibiotic resistance in bacteria. In staphylococci, penicillinase production was eliminated in mercury-resistant organisms, but not in mercury-sensitive ones. Among enterobacteria, two resistance factors showing the same resistance pattern were differently eliminated, and correlation between elimination and transfer of resistance factors was not always observed. F′-lac$^+$ factor was also eliminated by ethidium bromide in *Escherichia coli* K12. Elimination of antibiotic resistance was observed generally at high frequency, and could be better reproduced than with acridine dyes.

INTRODUCTION

Many genetic determinants of antibiotic resistance in Enterobacteriaceae and Staphylococcus have been shown to belong to extrachromosomal elements; genetic studies on this subject have been reviewed by Mitsuhashi (1965, 1967), Watanabe (1967) and Novick (1967). These drug-resistance determinants are transmissible to sensitive bacteria independently of chromosomal genes and like the sex factor they can be eliminated by acridine dyes, but generally at low frequencies. Recently, Ikeda, Iijima & Tajima (1967) reported that the sex factor in a male strain of *Escherichia coli* K12 was eliminated by sarkomycin, which was however less active than acridine orange. Elimination of antibiotic resistance at high frequency is of interest to assert extrachromosomal location of genetic determinants, and drugs with better eliminating or 'curing' effect are needed. Previous investigations have shown that ethidium bromide binds to DNA and RNA, and inhibits DNA-polymerase and RNA-polymerase (Waring, 1966). Like the acridines, ethidium bromide was intercalated between base-pairs of DNA. The complex between ethidium bromide and nucleic acids has been described by Lepecq & Paoletti (1967), and biochemical effects of this drug on bacteria were reported by Tomchick & Mandel (1964). The present paper summarizes the effects of ethidium bromide on antibiotic resistance in some multiresistant enterobacteria and staphylococci.

METHODS

Bacteria and culture media. Resistant enterobacteria (obtained from International Centre for Salmonella, Pasteur Institute, and Pasteur Hospital laboratory) were as described by Chabbert & Baudens (1966) and Baudens & Chabbert (1967). *Escherichia coli* K12 54117 F$^-$lac$^+$ and *E. coli* K12 C600 F$^-$lac$^-$ were provided by Dr E. L. Wollman, and *E. coli* K12 × 200 PS lac$^-$ carrying F′-lac$^+$ factor by Dr F. Jacob (Pasteur Institute,

Paris). The conjugation conditions used were as described by Watanabe & Fukasawa (1961). Nine strains were used for the present work: *Samonella oranienburg* LA39.R4 (resistant to Sm Km Cm Tc Su); *S. paratyphi B* LA43.R8 (Sm Cm Su); *S. panama* LA46.R11 (Am Km Tc); *S. paratyphi B* LA47.R13 (Am Sm Cm Tc Su); *E. coli* (111:B4) LA49.R15 (Am Sm Cm Tc Su); *Shigella flexneri* LA56.R22 (Am Sm Cm Tc Su); *S. stanleyville* LA 68.R32 (Am Sm Km Cm Tc Su); *S. oranienburg* LA69. R33 (Am Sm); *S. derby* LA71.R35 (Am Sm Su); *E. coli* K12 54117.R22; C600.R22; 54117.R15; C600.R15 were obtained by transfer of factors R22 and R15 from *Sh. flexneri* LA56.R22 and *E. coli* LA49.R15 to sensitive strains of *E. coli* K12.

Eight strains of *Staphylococcus aureus* (coagulase-positive) isolated from clinical material were used. Drug-resistance patterns were: LA72: P Hg Sm Tc Em; LA75: P Sm Km Tc Em; LA78: P Hg Tc Em; LA80: P; LA82: P Hg Sm Tc Em; LA86: P Hg Em; LA87: P Sm Tc Em; LA89: P Hg Sm Km Tc Em. Penicillin resistance determinant was transduced from LA75 to *S. aureus* 209P (ATCC: 65.38P) by phage 53 (NCTC 8406), as previously described (Chabbert, Baudens & Gerbaud, 1964). Code key: Am, ampicillin; Sm, streptomycin; Km, Kanamycin; Cm, chloramphenicol; Tc, tetracycline; Su, sulphonamide; P, benzylpenicillin; Em, erythromycin; Hg, $HgCl_2$.

Tryptic soy broth (0370 Difco) and tryptic soy agar (0369 Difco) were routinely used, and the effects of different pH values were separately studied.

Chemicals. Ethidium bromide (3,8-diamino-5-ethyl-6-phenyl-phenanthridinium) was a gift from Boots Pure Drug Co., Ltd., Nottingham, Great Britain. Benzylpenicillin, dihydrostreptomycin sulphate and sulphamethoxypyridazine were obtained from 'Specia' Rhône-Poulenc, Paris; ampicillin from Delagrange Laboratories, Paris; chloramphenicol and erythromycin from Roussel Laboratories, Paris; tetracycline hydrochloride from Pfizer-Clin Laboratories, Paris.

Determination of ethidium bromide activity on bacterial growth. Minimal inhibitory concentrations of ethidium bromide were determined by the spot-technique on serial dilutions of drug in nutrient agar, and expressed in molarity. Growth curves in nutrient broth were read at 620 mμ where light adsorption by ethidium bromide was minimal (Tomchick & Mandel, 1964).

Determination of antibiotic resistance. Bacteria (10^4) were inoculated by the spot-technique, using a modification of Steers's apparatus (1959), on nutrient agar supplemented with the following concentrations of antibiotics: benzylpenicillin, 0·1 i.u./ml.; ampicillin, 20 μg./ml.; streptomycin, 32 μg./ml.; chloramphenicol, 20 μg./ml.; tetracycline, 16 μg./ml.; erythromycin, 10 μg./ml. kanamycin, 12·5 μg./ml.: sulphamethoxypyridazine, 100 μg./ml.

Resistance to mercuric ion was determined with the sensitivity discs described by Novick (1967).

Elimination of antibiotic resistance. A small inoculum (10^4 bacteria/ml.) was grown overnight at 37° in nutrient broth containing a subinhibitory concentration of ethidum bromide, giving incomplete inhibition (from 6 to 10×10^{-6}M for staphylococci, and from 60 to 2500×10^{-6} M for enterobacteria). The culture was plated on agar, and isolated colonies tested for antibiotic resistance. In other cases, 10^5 bacteria were plated on nutrient agar containing serial dilutions of ethidium bromide and incubated overnight. The plates were flooded with 2 ml. broth. Bacterial suspension were homogenized, and bacteria were re-isolated on agar and tested for drug resistance.

Effect of ethidium bromide on induced synthesis of penicillinase in staphylococci. Exponentially growing staphylococci (strain LA78) were induced for penicillinase synthesis with $1{\cdot}5 \times 10^{-6}$ M-methicillin (Novick & Richmond, 1965) and ethidium bromide was added 30 sec. later to give final concentrations 0, 5, 10, 20, 50 and 100×10^{-6} M. Synthesis of penicillinase was stopped at 30 min. by adding chloramphenicol (50 μg./ml.) to all samples. Bacteria were centrifuged down (20,000 *g* for 5 min. at 4°), washed and suspended in phosphate buffer (0·1 M, pH 5·8). The extinctions of bacterial suspensions were adjusted at 620 mμ, and various dilutions were assayed iodometrically for penicillinase activity (Perret, 1954).

RESULTS

Elimination of antibiotic resistance in enterobacteria. Typical results are shown in Table 1, and others will be reported separately. All drug-resistance determinants in *Salmonella oranienburg* LA39.R4, *Shigella flexneri* LA56.R22, *Escherichi coli* K12 54117.R22 and *E. coli* K12 C600.R22 were eliminated at a high frequency. Spontaneous loss of complete drug resistance was low, and no loss of isolated resistance characters was observed. No significant differences in the elimination of resistance factor R22 were observed in the originally resistant *Sh. flexneri* LA56.R22, and *E. coli* K12 54117.R22 and C600.R22. In *S. derby* LA71.R35 (resistant to ampicillin, streptomycin, sulphonamide) ampicillin resistance was suppressed in 100% of the organisms by ethidium bromide treatment, but streptomycin and sulphonamide resistances were not modified. The determinant for ampicillin resistance was easily transferred to *E. coli* K12 54117, but determinants for streptomycin and sulphonamide resistance were not transferred.

Kanamycin resistance was significantly eliminated in *Salmonella panama* LA46.R11, change in tetracycline resistance was not significant, and loss of ampicillin resistance was not observed. It has been reported that kanamycin resistance was significantly eliminated by acriflavine in this strain (Baudens & Chabbert, 1967). Nevertheless, all resistance characters were transferred jointly or separately to sensitive bacteria.

With *Salmonella stanleyville* LA68.R32, *S. paratyphi B* LA47.R13, and *S. oranienburg* LA69.R33, spontaneous loss of single resistance characters was observed, but the frequency was not significantly increased by ethidium bromide treatment. In *S. paratyphi B* LA43.R8 and *Escherichia coli* LA49.R15, no elimination of antibiotic resistance was observed.

Elimination of F′-lac$^+$ factor by ethidium bromide. Escherichia coli K12 ×200 PS lac$^-$ carrying F′-lac$^+$ factor was treated by ethidium bromide, and organisms were isolated on Drigalski medium. Ethidium bromide, like acridine dyes (Hirota & Iijima, 1957), eliminated F′-lac$^+$ factor: 20% of tested colonies were unable to ferment lactose. No spontaneous loss of this factor was observed in controls (104 colonies).

Elimination of antibiotic resistance in staphylococci. Frequencies of loss of resistance in nine strains of staphylococci are shown in Table 2. Penicillin resistance was eliminated at high frequency (8 to 100%) in five multiresistant strains. These five strains were resistant to mercuric chloride, and mercury resistance was co-eliminated in all penicillin-sensitive colonies. Induced synthesis of penicillinase in *Staphylococcus aureus* LA78 was partially inhibited at 50×10^{-6} M and completely inhibited at 100×10^{-6} M-ethidium bromide. With four strains, no elimination of penicillin resistance was ob-

Table 1. *Elimination by ethidium bromide of antibiotic resistance from enterobacteria*

		Frequency						
		Plus ethidium bromide				Control		
Organisms	Lost characters	Conc. (10^{-6} M)	No. examined colonies	No. sensitive colonies	%	No. examined colonies	No. sensitive colonies	%
S. oranienburg LA39.R4 (Sm Km Cm Tc Su)	Sm Km Cm Tc Su	625	154	75	49	309	0	0
Sh. flexneri LA56.R22 (Am Sm Cm Tc Su)	Am Sm Cm Tc Su	60	156	120	77	104	3	2·9
E. coli K12 54117.R22 (Am Sm Cm Tc Su)	Am Sm Cm Tc Su	1300	104	34	32	134	1	0·7
E. coli K12 C600.R22 (Am Sm Cm Tc Su)	Am Sm Cm Tc Su	75	104	73	71	104	4	3·9
S. derby LA71.R35 (Am Sm Su)	Am	2500	104	104	100	96	2	2·0
S. panama LA46.R11 (Am Km Tc)	Km Tc	625	390	0	0	554	5	0·9
	Km		390	61	15·6	554	44	7·9*
	Tc		390	5	1·2	554	5	0·9

* Corrected $\chi^2 = 10{\cdot}0$ significant ($P = 0{\cdot}01$).

Table 2. *Elimination by ethidium bromide of antibiotic resistance from mercury-resistant and mercury sensitive staphylococci*

Strains and original drug resistance	No. examined colonies	Lost characters		Control*		Strains and original drug resistance	No. examined colonies	Lost characters	Control*
		P	Hg	P	Hg				
LA72 (P Hg Sm Tc Em)	130	97 (75 %)	97 (75 %)	0	0	LA75 (P Sm Km Tc Em)	104	0	0
LA78 (P Hg Tc Em)	104	104 (100 %)	104 (100 %)	0	0	LA80 (P)	134	0	0
LA82 (P Hg Sm Tc Em)	252	82 (32 %)	82 (32 %)	0	0	209P (ATCC 65.38) (P)	104	0	0
LA86 (P Hg Em)	138	75 (54 %)	75 (54 %)	0	0	LA87 (P Sm Tc Em)	104	0	0
LA89 (P Hg Sm Km Tc Em)	52	4 (8 %)	4 (8 %)	0	0				
		Mercury resistant						Mercury sensitive	

* 104 colonies were tested for all controls.

served. These strains were multiresistant or resistant to benzylpenicillin only, but all mercury sensitive.

Relations between inhibitory activity and resistance elimination by ethidium bromide. As observed with acridine and other basic dyes, staphylococci were more sensitive to ethidium bromide than were the enterobacteria examined. All strains of *Staphylococcus aureus* were inhibited at low concentrations (8 to 12×10^{-6} M), but very important differences in minimal inhibitory concentrations were observed with the enterobacteria: 75×10^{-6} M for *Shigella flexneri* LA56.R22, and 3125×10^{-6} M for *Salmonella derby* LA46.R33. Subinhibitory concentrations with maximal eliminating effects are given in Table 1. The relation between ethidium bromide concentration, inhibition of growth and elimination of resistance in *S. aureus* LA78 and *Sh. flexneri* LA56.R22 are shown in Fig. 1.

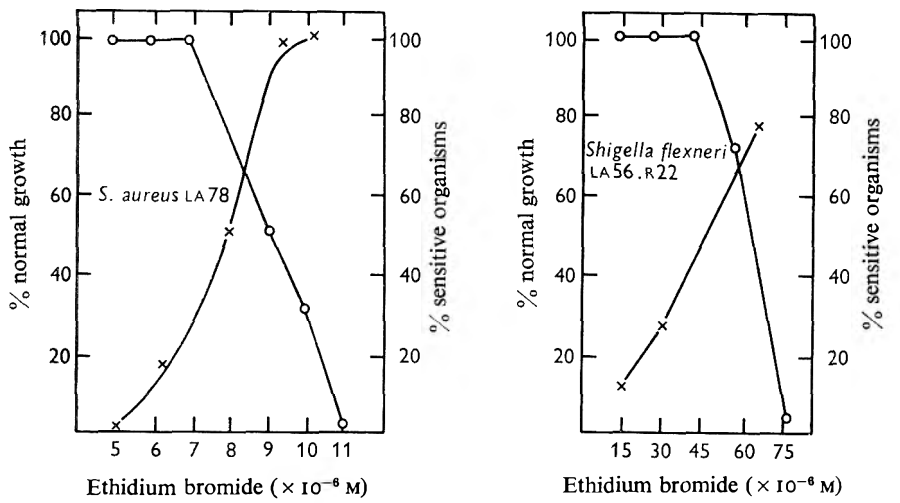

Fig. 1. Relations between ethidium bromide concentration, inhibition of growth and percentage of antibiotic sensitive colonies in *Staphylococcus aureus* LA78 and *Shigella flexneri* LA56.R22. O – – – O, Percentage of normal growth in overnight culture (turbidimetric reading at 620 mμ); × – – – ×, percentage of sensitive organisms.

Effects of light, manganese concentration and pH value on growth and elimination of resistance. The inhibitory activity of ethidium bromide on growth was significantly increased when organisms were incubated in serial concentrations of ethidium bromide in the presence of light (40 cm. from a white lamp of 100 W); the growth of *Staphylococcus aureus* LA78 was inhibited at 7×10^{-6} M (11×10^{-6} M in absence of light). At subinhibitory concentration of ethidium bromide in the presence of light, 50 to 100% of the bacteria lost antibiotic resistance. Although it has been shown that 0·33 mM-manganese sulphate antagonized ethidium bromide induced growth inhibition (Tomchick & Mandel, 1964), no effect of manganese was observed on the elimination of antibiotic resistance in *Shigella flexneri* LA56.R22. Effects of different pH values on growth and elimination of resistance are shown in Table 3.

Absence of ethidium bromide selective effect. The possibility of selection by ethidium bromide of spontaneously sensitive bacteria was studied in *Shigella flexneri* LA56. Growth of an originally resistant strain carrying factor R22 and of a 'cured' sensitive

strain was inhibited at exactly the same concentration of ethidium bromide (75×10^{-6} M). There was no significant difference in growth rates of these strains with 60×10^{-6} M ethidium bromide, which eliminates factor R22 (Fig. 1). Identical results were obtained by Watanabe & Fukasawa (1961) with acridine dyes and according to them, a selective effect of ethidium bromide can be excluded.

Table 3. *Effects of variations of pH value on elimination by ethidium bromide of resistance to penicillin and mercury from Staphylococcus aureus* LA78

Nutrient broth was adjusted to various pH values with N-HCl or N-NaOH; ethidium bromide was added at final concentration of 8 and 5×10^{-6} M; 104 colonies were tested in each case.

	Ethidium bromide, 8×10^{-6} M		Ethidium bromide, 5×10^{-6} M	
pH value	Growth*	% of sensitive colonies	Growth*	% of sensitive colonies
6·8	+	4	+	0
7·0	±	20	+	0
7·2	±	50	+	0
7·4	±	35	+	0
7·6	±	20	+	0
7·8	0	—	±	4

* Normal growth, + ; partial growth, ± ; no growth, 0.

DISCUSSION

It is reported here that ethidium bromide eliminates some antibiotic resistance determinants and the F′-lac$^+$ factor. Elimination of R factors by acridine dyes in Enterobacteriaceae has been reported by Mitsuhashi, Harada & Kameda (1961), and Watanabe & Fukasawa (1961); the frequencies of elimination were generally low, particularly in *Escherichia coli*. In our experiments, factors R4 and R22 were eliminated by ethidium bromide at high frequencies, from originally resistant strains and from two strains of *E. coli* K12 to which factor R22 had been transferred. The bacteria became sensitive to all the drugs tested, and in contrast to the results of Mitsuhashi *et al.* (1961), no difference was observed in elimination of factor R22 in *Shigella flexneri* and *E. coli* K12. With *Salmonella derby* LA71, only one isolated resistance character was eliminated at a very high frequency (100 %). In this strain, only this suppressible character was transferred to sensitive bacteria. Conversely, no elimination of resistance characters was observed with *E. coli* LA49.R15 and *S. paratyphi B* LA43.R8 carrying R factors transferable to sensitive *E. coli* K12. Factor R15 was successively transferred to *E. coli* K12 C600 F$^-$-lac$^-$ and to *E. coli* K12 54117 F$^-$-lac$^+$; 90 to 100 % of sensitive recipient cells became resistant in each case. Although factor R15, transferred at very high frequency was apparently extra-chromosomal and de-repressed (Meynell & Datta, 1967), ethidium bromide did not eliminate factor R15 from originally resistant *E. coli* LA49.R15, or from *E. coli* K12 54117 and C600 carrying R15. The influence of recipient strains can be excluded in this system, because factor R22 (showing the same resistance pattern as R15) was eliminated at high frequency from originally resistant *Sh. flexneri* and *E. coli* K12 54117.R22 and C600.R22.

It has been previously shown that with some strains of *Staphylococcus aureus*, penicillin resistance and erythromycin resistance were eliminated by acridine dyes, separately or jointly, at generally low frequencies: 0·1 to 3·5 % (Hashimoto, Kono & Mitsuhashi, 1964), 0·2 to 0·5 % (Harmon & Baldwin, 1964) and 0·6 to 3·9 % (Baudens, Gerbaud & Chabbert, 1965) for penicillin resistance; 2 to 8 % (Mitsuhashi, Morimura, Kono & Oshima, 1963) for erythromycin and other macrolides resistance; 0·1 to 3·5 % (Mitsuhashi, Hashimoto, Kono & Morimura, 1965) for joint elimination of penicillin and erythromycin resistances. Novick (1963) reported that no elimination of antibiotic resistance by acriflavine was observed with his strains.

Penicillin resistance was eliminated by ethidium bromide in five multiresistant strains of *Staphylococcus aureus*, at higher frequencies (8 to 100 %); 'curing' concentrations of ethidium bromide (5 to 10×10^{-6} M) were lower than acriflavine concentrations reported by different workers (50 to 100×10^{-6} M). Elimination of penicillin resistance was observed at a concentration which did not inhibit penicillinase synthesis. This finding agrees with previous results showing that protein formation was relatively unaffected by ethidium bromide (Tomchick & Mandel, 1964). Also, in our experience, penicillin sensitivity remained stable in absence of ethidium bromide.

The five strains of *Staphylococcus aureus* in which penicillin resistance was eliminated were mercury resistant, and mercury resistance was always co-eliminated with penicillin resistance. It has been reported that these two markers were co-transduced and co-eliminated spontaneously or by acriflavine (Richmond & John, 1964; Asheshov, 1966). In four strains of *S. aureus*, penicillin resistance was not eliminated. These strains were resistant to penicillin or to several antibiotics, but were sensitive to mercury salts. Chromosomal location for penicillin resistance determinants, not eliminated by acriflavine or high temperature, has been suggested (Asheshov, 1966).

Although it has been reported by Miller & Harmon (1967) that genetical determinants controlling penicillin and mercury resistance in one strain of *Staphylococcus aureus* were co-transduced with chromosomal determinant responsible for methionine synthesis, it seems that in our strains, mercury resistance is generally associated with 'curable' and probably extra-chromosomal penicillin resistance determinants.

In strains of *Staphylococcus aureus* where penicillin and mercury resistances were eliminated at high frequencies (8 to 100 %), no elimination of erythromycin resistance was observed. There is no close linkage of penicillin and erythromycin resistance determinants in these strains, which seem to offer a different system from those previously described by Novick (1967).

The partially selective effect of acriflavine and ethidium bromide on extra-chromosomal DNA synthesis remains unclear. It was reported (Lepecq & Paoletti, 1967) that there was no difference in ethidium bromide fixation on DNA of different origin, but that the linear or circular form of DNA affects drug fixation (Hudson & Vinograd, 1967). Although there are some differences in drug penetration in different strains of Enterobacteriaceae, resistant organisms grown on high concentrations of ethidium bromide contained large amounts of drug, and it can be suggested that differences in DNA polymerase and RNA polymerase sensitivities are responsible for differences in ethidium bromide sensitivity of bacterial strains.

The present work has shown that ethidium bromide is a powerful drug in eliminating

some resistance factors at high frequency with excellent reproducibility, and would appear to be a useful tool for further study of the lack of elimination of other resistance factors.

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Phototropism and Light-growth Responses of the Tall Conidiophores of *Aspergillus giganteus*

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SUMMARY

Aspergillus giganteus conidiophores are positively phototropic to unilateral stimulation with white light. The response is restricted to the apical 240 μ of the conidiophore. The curvatures start about 22 min. after the onset of stimulation and proceed at a rate of about 2·5° min.$^{-1}$. During the response the rate of extension of the proximal wall (with respect to the direction of the incident illumination) decreases by about 5 % as compared to its previous rate of growth, whilst that of the distal wall increases by about 5 %; thus there is no net change in the rate of wall growth during phototropism. The sign of the phototropic response is reversed when conidiophores are unilaterally stimulated with ultraviolet radiation (280 mμ) or with white light whilst submerged in liquid paraffin. Conidiophores cultured on media containing l-lyxoflavin or diphenylamine show normal phototropic responses.

A tenfold increase or decrease in light intensity is followed after approximately 12 min. by a slight change (about 11 %) in the rate of conidiophore growth. However, changes in light intensity between 139 and 268 lux do not affect the growth rate. There is an acceleration in growth rate when conidiophores previously held in red light for some hours are re-illuminated with white light.

INTRODUCTION

Although most fungal hyphae are insensitive to unilateral stimulation with white light there are some, particularly those which bear reproductive structures, which are phototropic. These hyphae are usually positively phototropic but negative responses have also been reported (Welty & Christensen, 1965). The selective advantage of reproductive structures being positively phototropic is presumably because spore dispersal is directed towards what is usually open space.

A second light-induced response which has been studied in fungi is the so-called light-growth response. A light-growth response may be defined as a change in growth rate in response to a change in light intensity. When growth is accelerated on increasing the light intensity, the light-growth response is said to be positive, whilst when there is a decrease in growth rate the response is said to be negative.

The hyphae most frequently chosen for studying phototropism and light-growth responses in fungi are the sporangiophores of *Phycomyces blakesleeanus*. The advantage of using this species is that its sporangiophores are large, robust and relatively xerophytic. As a result of intensive investigation the growth kinetics and pattern of wall extension of *P. blakesleeanus* sporangiophores during phototropic and light-

growth responses have been precisely defined (Shropshire, 1963). However, this study has not led to full understanding of the nature of the perceptive and response mechanisms involved. Much more needs to be known about normal features of hyphal growth before the nature of the changes induced by external stimuli can be fully understood.

There are some advantages in using *Phycomyces blakesleeanus* sporangiophores to study phototropic and light-growth responses but there is also a need to investigate these phenomena in other species. To a certain extent this has already been done (Banbury, 1959; Carlile, 1965). The advantage of using *Aspergillus giganteus* is that phototropism can be studied in the absence of geosensitivity (Trinci & Banbury, 1967) and that conidiophore elongation, unlike the growth of *P. blakesleeanus* sporangiophores, is a light dependent process; *P. blakesleeanus* sporangiophores grow at the same rate in continuous darkness or light, but frequent light stimuli are essential if the elongation of *A. giganteus* conidiophores is to be maintained (Trinci & Banbury, in press). In previous studies on *Aspergillus giganteus* we have described the kinetics of conidiophore growth, the influence of light on conidiophore production and carotenogenesis and the ultrastructural details of sporulation (Trinci & Banbury, 1967; Trinci, Peat & Banbury, 1968; Trinci & Banbury, 1969).

METHODS

Strain w-267 of *Aspergillus giganteus* from Professor A. Weston's collection at Harvard University was used. The fungus was cultured on Oxoid malt extract agar except when the action of l-lyxoflavin or diphenylamine were being tested, in which case the the following medium was used (g./l.): D-glucose, 50·0; KH_2PO_4, 1·0; NH_4NO_3, 1·0; $MgSO_4.7H_2O$, 1·0; $ZnSO_4.7H_2O$, 0·002; $FeSO_4.7H_2O$, 0·002. When diphenylamine was added to this medium the glucose concentration was decreased from 5 to 1 %. The media were sterilized by autoclaving for 15 min. at 121°.

Microscopic studies of conidiophore growth were made on cultures growing in observation cells (Pl. 1, fig. 1) under the conditions described by Trinci & Banbury, 1967, or in the apparatus shown in Fig. 1. The red observation light was on for the duration of each experiment; tall conidiophores were not produced when it was the sole source of illumination and no phototropic response was induced when it was used to illuminate conidiophores unilaterally.

Conidiophore growth was followed by time-lapse photography, with measurements made on tracings taken of the projected film (final magnification × 335), or by direct measurement with a micrometer eyepiece (one division of the micrometer equalled 0·83 μ). The concave and convex flanks of curved conidiophores were measured by 'walking' dividers along their lengths and adding up the secant distances. The length of the apical phototropically sensitive segment of the conidiophore was estimated by superimposing the outlines of responding conidiophores traced from successive photographs.

The intensity of the white light illuminating cultures was measured in lux by using an SEI exposure photometer or a Weston Master IV exposure meter (both supplied by Ilford Ltd., London). One lux of white cycle = 1·6 erg./cm.^{2} (Westphal, 1947).

Cultures were initially incubated for 2 to 3 days at 22° ± 1° in an illuminated incubator and then transferred to the horizontal microscope for observation. All experiments

were made in a dark room at 23·0 ± 0·5° on conidiophores which were 4 to 8 mm. tall, i.e. they were in the linear phase of growth (Trinci & Banbury, 1967).

The effect on growth rate of altering the light intensity between 20 and 200 lux was studied by using conidiophores which had been grown for 20 to 26 hr at one or other light intensity. In these experiments the effect on growth rate of either one step up or step down in light intensity was followed. When the light intensity illuminating the

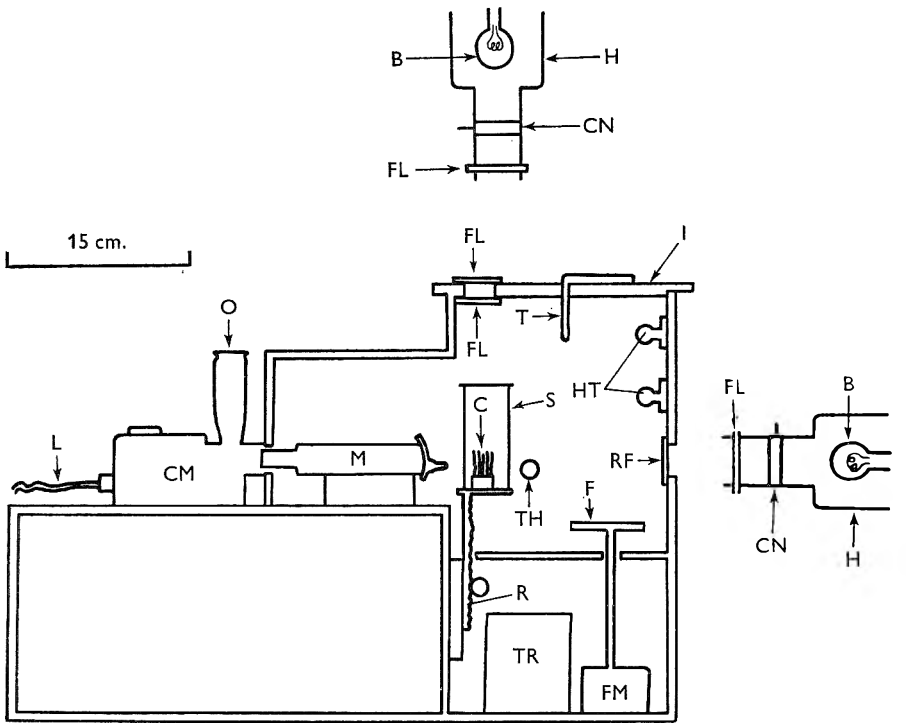

Fig. 1. Apparatus used to follow conidiophore growth and phototropism.

B	6 V, 48 W tungsten filament bulb	L	Lead to intervalometer
C	Conidiophores	M	Microscope barrel, objective and eyepiece
CM	Camera	O	Observation eyepiece
CN	Condenser	R	Rack for vertical movement of specimen
F	Fan	RF	Red filter emitting no light below 620 mμ
FL	Infra red absorbing glass filter	S	Specimen chamber
FM	Fan motor	T	Thermometer
H	High intensity lamp connected to rheostat	TH	Thermostat barrel
HT	Incubator heat source (blacked out cycle bulbs)	TR	Transformer supplying cycle bulbs
I	Wooden incubator		

cultures was varied between 139 and 268 lux, each conidiophore was subjected to four successive changes in light intensity at hourly intervals.

When the phototropic response was studied, the conidiophores were first illuminated with white light from above and then this light was switched off and the cultures were illuminated from one side. Statistical analysis of the experimental results was based on formulae from Moroney (1956).

RESULTS

Influence of changes in light intensity on the rate of conidiophore elongation

The effect on the rate of conidiophore growth of a step up or a step down in light intensity between 20 and 200 lux was determined. Statistical analysis of the results (Table 1) indicated that about 12 min. after the increase or decrease in light intensity there was a significant change in the rate of conidiophore growth. In each case the new rate of growth was persistent during the 1 to 2 hr. period of observation.

Table 1. *Statistical comparison between the initial rate of conidiophore growth and the subsequent rates after altering the light intensity*

Measurements of conidiophore growth were taken at 2 min. intervals. Statistical comparisons were based on data obtained during successive 4 min. intervals.

Time since the light intensity was changed (in min.)	Light intensity decreased from 200 to 20 lux. Mean growth rate at 200 lux = 8·7 μ/min.		Light intensity increased from 20 to 200 lux. Mean growth rate at 20 lux = 6·7 μ/min.	
	Mean rate of growth (μ/min.)	Probability† (Student's t)*	Mean rate of growth (μ/min.)	Probability† (Student's *t*)*
4	8·7	—	6·3	< 0·2
8	8·5	< 0·4	6·7	—
12	8·3	< 0·2	6·8	< 0·7
16	8·0	< 0·05	7·3	< 0·1
20	7·6	< 0·01	7·7	< 0·01
24	7·6	< 0·01	7·4	< 0·01
28	7·8	< 0·02	7·5	< 0·01
32	8·0	< 0·05	7·1	< 0·05
36	8·1	< 0·10	7·0	< 0·2
40	7·8	< 0·02	7·1	< 0·05
44	7·8	< 0·02	7·5	< 0·01
48	7·8	< 0·02	7·7	< 0·01
52	8·0	< 0·10	8·0	< 0·01
56	8·0	< 0·10	8·0	< 0·01
60	7·9	< 0·05	7·7	< 0·01

† Probability of the difference between the mean growth rates before and after change in light intensity resulting from chance variation.

* Student's *t* 0·05 = difference probably significant, 0·01 = significant difference, 0·001 = highly significant difference.

The mean rate of growth of the eight conidiophores cultured at 200 lux was 8·7 μ/min. (standard deviation ± 1·32 μ) whilst their mean rate of growth from a period 12 min. after the decrease in light intensity was 7·9 μ/min.; thus there was on darkening an 11 % decrease in growth rate. The mean rate of growth of the five conidiophores cultured at 20 lux was 6·7 μ/min. (standard deviation ± 0·56 μ), whilst the mean rate of growth from a period 12 min. after increasing the light intensity was 7·5 μ/min.; thus in this case there was about a 12 % increase over the previous growth rate.

We previously reported that the degree of apical oscillation of the conidiophore about its vertical axis of growth was greater at 20 lux than at 200 (Trinci & Banbury, 1967). This variation is oscillation with light intensity (Pl. 1, fig. 2, 3) may have resulted in an underestimation of the rate of growth at the lower intensity used (in

these experiments measurements of growth were made with the micrometer eyepiece). In view of this possibility it was decided to determine the effect on growth rate of changing the light intensity between 139 and 268 lux. In this case the degree of conidiophore oscillation was slight even at the lower intensity and it was thus less likely that a variation in the degree of oscillation would cause a significant error in the measurement of growth rate. The effect on conidiophore growth of a step up or a step down in light intensity between 139 and 268 lux is shown in Fig. 2.

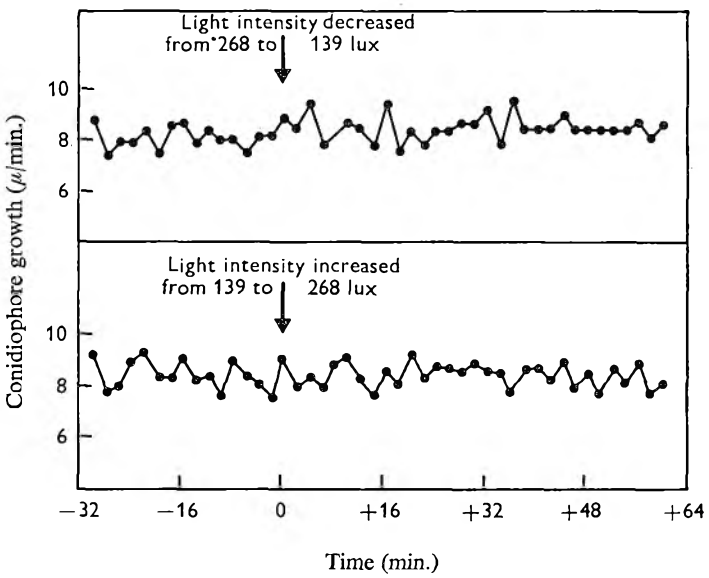

Fig. 2. Influence of an increase or decrease in light intensity between 139 and 268 lux on the rate of conidiophore growth. Each graph represents the mean of five changes in light intensity. Light intensity changes at arrow.

The results were analysed statistically in the same way as in the previous experiment. There was no significant change in the rate of conidiophore growth when the light intensity was either increased or decreased. For the step down in light intensity the calculated probabilities (probability of the difference between the mean growth rates before and at 4 min. intervals after the change in light intensity resulting from chance variation) ranged from 0·2 to 0·8 whilst for the step up in light intensity they ranged from 0·3 to 0·9. Thus in these experiments the conidiophores did not show a positive growth response on increasing the light intensity or a negative growth response on darkening. The light-growth response of the sporangiophores of *Phycomyces blakesleeanus* (taken from Delbrück & Reichardt, 1956) and *Thamnidium elegans* (taken from Lythgoe, 1961) and the conidiophores of *Aspergillus giganteus* are presented in Fig. 3 so that they may be compared. To our knowledge these are the only three species in which the light-growth response of positively phototropic hyphae has been investigated.

Light-growth response and phototropism of conidiophores grown for a period in red light

In a previous paper (Trinci & Banbury, 1967) we described the effect of white light on conidiophores whose rate of growth had decreased following a 24 hr period in red light. After a lag of about 30 min. the rate of conidiophore growth increased slowly from about 4 μ/min. to about 7·5 μ/min. In the present experiments conidiophores

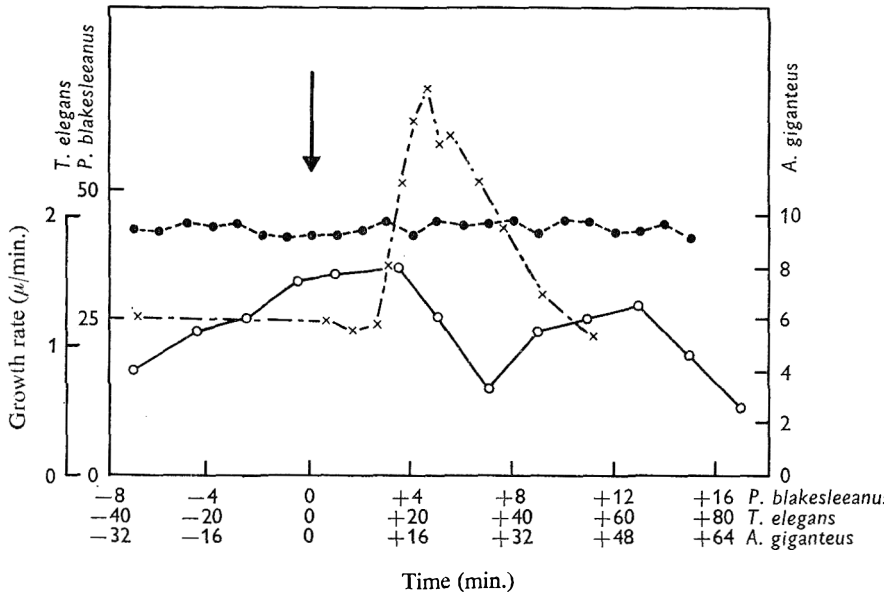

Fig. 3. Comparison between the light-growth responses of *Phycomyces blakesleeanus* (from Delbrück & Reichardt, 1956), *Thamnidium elegans* (from Lythgoe, 1961) and *Aspergillus giganteus*. *P. blakesleeanus*, ×——×, transient increase in light intensity at arrow. *T. elegans*, ○——○, change from darkness to light at arrow. *A. giganteus*, ●- - - - -●, step up in light intensity from 139 to 258 lux at arrow.

which had been grown for 15 hr in red light were unilaterally illuminated with white light (200 lux); 35 min. later the conidiophore started to respond with a positive phototropic curvature. The curvature, which proceeded at a rate of about 3·5°/min. was completed some 20 min. later. The change in rate of growth of the conidiophore wall during phototropism is presented in Table 2 and Fig. 4. During the phototropic response there was a 41 % increase in the mean rate of wall extension as compared to the original growth rate and there was a difference of about 35 % between the growth rates of the distal and proximal walls. After completion of the phototropic response there was a decrease in the extension rate.

This type of response occurred in the three conidiophores which were unilaterally stimulated in this manner, although there was not always as great an increase in growth rate during phototropism. In the case of the conidiophore drawn in Fig. 4, the apical 200–225 μ of the conidiophore responded to the unilateral stimulus; this is almost exactly the length calculated previously for the extension zone (Trinci & Banbury, 1967) and would seem to indicate that the decrease in growth rate which occurred in red light or darkness was not accompanied by a shortening of the extension zone.

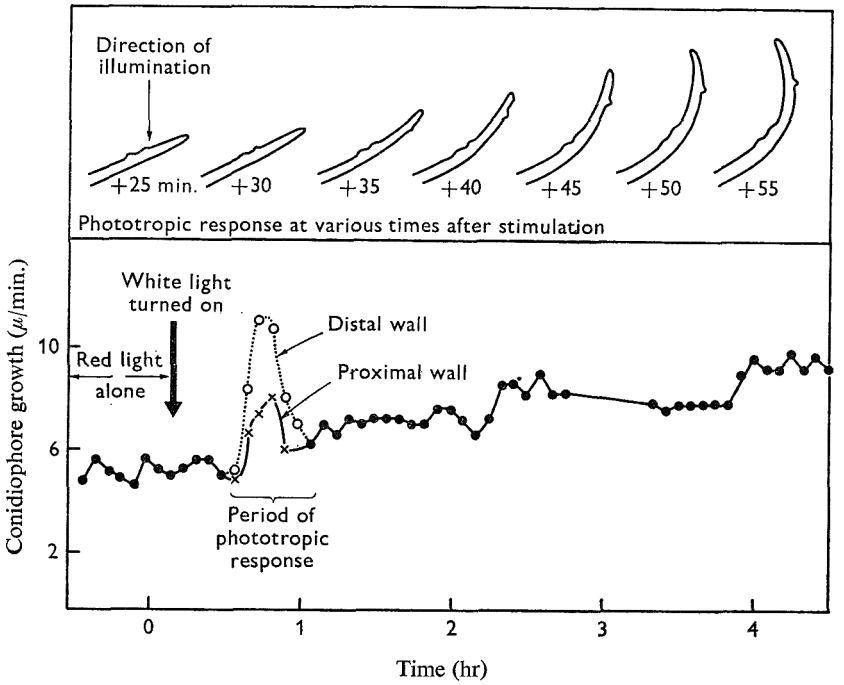

Fig. 4. Influence of white light on the rate of growth and phototropism of a unilaterally illuminated conidiophore previously held in red light for 15 hr. Illuminated with white light at arrow.

Table 2. *Changes in the rate of wall growth during the phototropic response of a conidiophore previously held in red light for 15 hr*

	Mean growth rate (μ/min.)	Growth as a % of rate prior to the response (5·4 μ/min.)
1. Growth prior to response	5·4	100
2. Growth during the response period		
(*a*) Distal wall	8·8	163
(*b*) Proximal wall	6·5	120
(*c*) Mean of proximal and distal walls (axis)	7·6	142
3. Growth during the 15 min. following completion of the response	6·9	128

The above experiments confirmed the fact that there is an acceleration in growth when conidiophores held in red light for some hours are re-illuminated with white light. However, it is clear that this response is essentially different from the positive light-growth response of *Phycomyces blakesleeanus* sporangiophores. In *P. blakesleeanus* an increase in light intensity (Fig. 3) or a change from darkness to light has only a transitory stimulatory effect on the rate of sporangiophore elongation but in *Aspergillus giganteus* continued growth of the conidiophores is dependent upon frequent white light stimuli (Trinci & Banbury, 1969).

Phototropic response to white light in air

Conidiophores which had previously been illuminated from above were photographed at 2 min. intervals before and after unilateral stimulation with white light. The response of five conidiophores treated in this manner was analysed. The mean lag after stimulation before the onset of the response was 22 min. The mean length of the apical segment of the conidiophore which responded to the unilateral stimulus was 241 μ (standard deviation $\pm$ 41 μ) and the phototropic curvature proceeded at a mean rate of 2·5°/min. (standard deviation $\pm$ 0·5°). The region of the conidiophore which responds phototropically is restricted to the extension zone. We previously reported that experiments in which starch grains were dusted on to the condiophore indicated that the extension zone varied between 170 and 240 μ (Trinci & Banbury, 1967). There is thus quite good agreement between the two methods used to determine the length of the extension zone.

The mean rate of growth of the conidiophores before unilateral stimulation and the rates of growth of the proximal and distal walls (with respect to the direction of incident illumination) during the response are presented in Table 3. The rate of extension of the distal wall accelerated by 5·3 % during the phototropic curvature, whilst that of the proximal wall decreased by 4·5 %. The mean between the rates of extension of the distal and proximal walls was almost exactly the same as the growth rate of the conidiophore before stimulation. Thus during the phototropic response there was a redistribution of growth within the extension zone but apparently no net increase or decrease in the rate of wall extension.

Table 3. *Change in the rate of wall growth during phototropism*

	Mean of five conidiophores	
	Mean growth rate (μ/min.)	Growth as % of original rate (10·04 μ/min.)
1. Growth prior to unilateral stimulation	10·04 ($\pm$ 0·82)*	100
2. Growth during the phototropic response		
(*a*) Distal wall	10·57 ($\pm$ 0·59)*	105·3
(*b*) Proximal wall	9·59 ($\pm$ 0·82)*	95·5
(*c*) Mean of proximal and distal walls (axis)	10·08	100·4

* Standard deviation.

The response of conidiophores to unilateral illumination is to some degree dependent upon their previous history. The rate of phototropic curvature was faster when the conidiophores had been held in red light before stimulation than when they had previously been illuminated with white light.

Phototropic response to ultraviolet light and under liquid paraffin

A phototropic response is induced by the establishment of a light gradient across the hypha; when the radiation intensity is greater on the distal side of the hypha (with respect to the direction of incident illumination) the tropic response is positive; when it is greater on the proximal side, the tropic response is negative. The side which re-

ceives the greater illumination is determined by which of the optical properties of the hypha predominates, the 'lens effect' (the hypha behaving as a cylindrical lens focusing light on the rear wall) or attenuation of light within the hypha. These two properties of the sporangiophore work in opposition; the 'lens effect' tends to promote positive tropic curvatures whilst severe attenuation of the light across the cytoplasm results in negative curvatures.

Two experiments were undertaken to determine whether the optical system operative in *Aspergillus giganteus* conidiophores is the same as that found in *Phycomyces blakesleeanus* sporangiophores (Shropshire, 1963). To determine if the 'lens effect' is operative the conidiophores were submerged in liquid paraffin and unilaterally illuminated with white light. All the conidiophores which continued to grow after this treatment responded to the stimulation with sharp negative phototropic curvatures (Pl. 1, fig. 4). The formation of droplets along the wall continued during the period in which the conidiophores grew in liquid paraffin, demonstrating that they arose by exudation of material from within the conidiophore rather than by condensation.

The effect of light attenuation across the conidiophore was demonstrated by unilaterally stimulating conidiophores with ultraviolet radiation of wavelength 280 mμ; a spectrophotometer was used as the source of monochromatic ultraviolet radiation. The conidiophores responded to the unilateral stimulus by sharp negative phototropic curvatures (Pl. 1, fig. 5). Curry & Gruen (1957) showed that the tropic response of *Phycomyces blakesleeanus* sporangiophores was negative for any wavelength shorter than 302 mμ. In this case the negative curvature is due to strong absorption of the short wavelength radiation on the side of the sporangiophore nearest to the light source, probably by gallic acid (Dennison, 1959).

Action of l-lyxoflavin and diphenylamine

Riboflavin and carotene have both been considered as the photoreceptive pigment in fungal phototropism (Carlile, 1965). Page (1956) found that l-lyxoflavin, a riboflavin analogue, inhibited the photoinduction of trophocyst formation in Pilobolus and interpreted the results as indicating that riboflavin functions as the photoreceptor in this process. The effect of l-lyxoflavin on the phototropic response of *Aspergillus giganteus* conidiophores was therefore examined and also that of diphenylamine which strongly inhibits the synthesis of carotene in fungal mycelium when added to the culture medium. *A. giganteus* was grown on media to which one or other of these two inhibitors was added and the effect on the reaction time before initiation of a phototropic response studied. The results are presented in Table 4. The fact that conidiophores took longer to respond on the medium containing diphenylamine was probably a reflection of the slower growth of the fungus rather than to any selective inhibition of the photoreceptive mechanism (40 μg. diphenylamine/ml. of medium decreased the radial growth rate of colonies by about 40 %). Gressel & Hartmann (1968) reported that incorporation of l-lyxoflavin and diphenylamine in the culture medium inhibited the growth of *Trichoderma viride* to a greater extent than the photoinduction of sporulation.

Table 4. *Effect on the duration of the reaction time of the phototropic response of the addition of l-lyxoflavin and diphenylamine to the medium*

Treatment	Period between onset of unilateral stimulation and onset of the phototropic response (min.)
1. Control*	33
2. L-Lyxoflavin (100 μg./ml.) added to medium	32
3. Diphenylamine (40 μg./ml.) added to medium	42

* The longer reaction time of the conidiophores in these experiments as compared with those described previously is probably due to the fact that a different medium was employed.

DISCUSSION

There appear to be two distinct responses in *Aspergillus giganteus* to light. First, light is essential for the maintenance of conidiophore elongation and causes an acceleration in the growth of conidiophores previously held in red light or darkness for some hours, and secondly, asymmetrical illumination causes a redistribution of the growth potential and results in phototropism. This conclusion seems to be supported by experiments in which it was shown that by varying the frequency of illumination the influence of light in inducing conidiophore growth could be divorced from its orientating effect; illumination of cultures for 12 min./12 hr or 10 sec./10 min. (same total light dose) resulted in the induction of the same amount of conidiophore growth (as measured on a dry-weight basis) but only under the latter light régime were the conidiophores orientated with respect to the direction of the incident illumination (Trinci & Banbury, 1969).

The fact that negative phototropic curvatures result both from unilateral stimulation of conidiophores submerged in liquid paraffin in white light, and with ultraviolet radiation in air, demonstrates that the optical properties of *Aspergillus giganteus* conidiophores are essentially the same as those of *Phycomyces blakesleeanus* sporangiophores. Unilateral illumination with white light in air results in the establishment of a light gradient across the conidiophore which in turn causes a redistribution of the growth potential within the hypha (as distinct from any net stimulation of growth) and hence a phototropic curvature. Under our experimental conditions the rate of growth of the distal wall is increased by about 5 % during phototropism whilst that of the proximal wall is depressed by about the same amount.

The light-growth responses of the three positively phototropic species which have been examined to date do not show a common pattern (Fig. 3). As far as *Aspergillus giganteus* is concerned, it seems that at least for small changes in light intensity there is no significant change in growth rate. The slight change in growth rate recorded for a tenfold increase in light intensity may not represent a true acceleration in growth for the reasons already stated. Thus it does not seem possible to explain phototropism in *A. giganteus* on the basis of unequal light-growth responses on the 'near' and 'far' sides of the curving conidiophore. Even in *Phycomyces blakesleeanus* it has proved

possible to induce a phototropic curvature without any light-growth response (Castle, 1961*a*). In the case of *Thamnidium elegans*, Lythgoe (1961) found that a positive phototropic response is coupled with a negative light-growth response (Fig. 3). He suggested that this apparent contradiction could be explained by the fact that the onset of the phototropic response coincides in time with the recovery phase of the negative light-growth response. However, Lythgoe did not conduct the critical experiment which might have substantiated this hypothesis, viz. comparing the recovery rates in light and in darkness after an initial period in light during which the growth rate was reduced.

At this stage it is only possible to speculate on the nature of the perceptive mechanism involved in phototropism. Work with algal systems may be of relevance to fungal phototropism. It has, for example, been shown that if part of a Vaucheria filament is illuminated, chloroplasts accumulate at this point. The light inducing this movement is absorbed by the cortical cytoplasm rather than by the chloroplasts themselves (Haupt, 1965). A postulate that a comparable type of system operates in the fungi would explain some of the puzzling features of the light responses which this group show. Various workers have suggested that hyphal growth involves the transport of vesicles containing cell-wall precursors to the apical region of the wall extension (Marchant, Peat & Banbury, 1967; McClure, Park & Robinson, 1968; Brenner & Carroll, 1968; Manocha & Colvin, 1968). We would postulate that the rate of growth of a hypha is determined by the rate of supply of these vesicles to its apical extension zone. It is possible that under conditions of symmetrical illumination the vesicles are randomly distributed within the conidiophore and are delivered at a more or less equal rate to the whole of the inner surface of the extending hyphal tip. However, when a light gradient is established across a hypha the peripherally located photoreceptor may induce an increase in the rate of supply of vesicles to the wall in the more brightly illuminated half of the cell in a manner similar to chloroplast migration in Vaucheria. Curvatures would thus result from more vesicles being delivered to one part of the extending conidiophore tip than to the other; thus the rate of growth of one side of the conidiophore tip would increase whilst that of the other would decrease by a corresponding amount. This is, of course, exactly what one observes experimentally. We would suppose that the rate of supply of vesicles to the region of the extension zone is governed by factors other than light intensity. This type of model would help to explain how it is possible to have a phototropic response in the absence of any net increase in growth or any demonstrable positive light-growth response. In some respects this hypothesis is similar to that put forward by Castle (1961*a*, *b*) to explain 'phototropic inversion' and the fact that phototropic bending can be initiated in *Phycomyces blakesleeanus* without the changes in growth speed that characterize a light-growth response.

It is hoped that future studies using the electron micoscope will help to substantiate this hypothesis.

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EXPLANATION OF PLATE

Abbreviations used: a, aluminium cap containing water and a cylinder of blotting paper; c, culture cell; e, exudation droplet formed under liquid paraffin; o, observation cell; cn, conidiophores.

Fig. 1. Culture chamber used to make microscopic observations of conidiophore growth.

Fig. 2. *Aspergillus giganteus* conidiophores grown at a light intensity of 20 lux.

Fig. 3. *A. giganteus* conidiophores grown at a light intensity of 200 lux.

Fig. 4. *A. giganteus* conidiophores unilaterally illuminated with white light whilst submerged in liquid paraffin. The arrow indicates the direction of illumination.

Fig. 5. *A. giganteus* conidiophores unilaterally illuminated with ultraviolet radiation (280 mμ). The arrow indicates the direction of illumination.

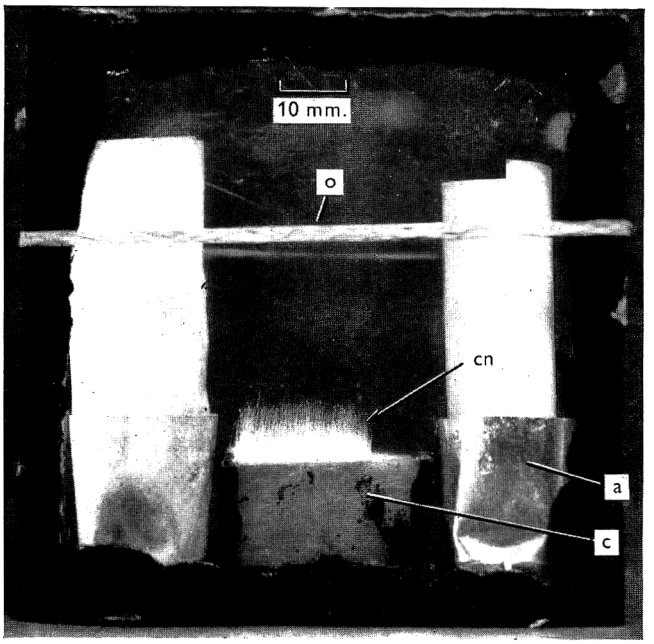


Fig. 1

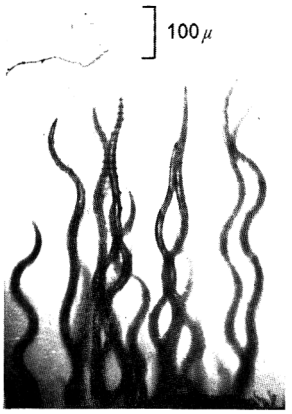
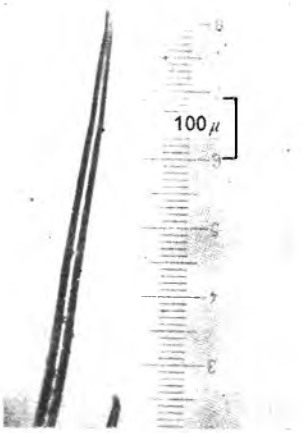


Fig. 2



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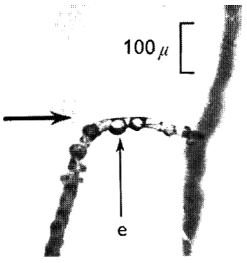

Fig. 4

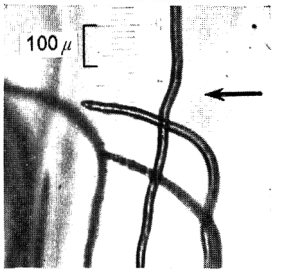

Fig. 5

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Loss of Sensitivity to EDTA by *Pseudomonas aeruginosa* Grown under Conditions of Mg-Limitation

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(*Accepted for publication* 8 *August* 1968)

SUMMARY

Pseudomonas aeruginosa was grown in batch culture in simple salts medium under conditions of Mg-limitation and varying degrees of Mg-excess. Sensitivity to EDTA was measured in terms of lysis and decrease in colony count. The greater the degree of Mg-limitation the greater was the resistance to loss of viability and lysis. Loss of viability of sensitive bacteria occurred more rapidly than lysis. This suggests that bacterial death preceded cell lysis.

INTRODUCTION

EDTA has been shown to render Gram-negative bacteria sensitive to the action of lysozyme and it has been suggested that EDTA increases the permeability of the outer layers of the cell wall, allowing lysozyme to penetrate to the underlying mucopeptide layer (Costerton *et al.* 1967). Treatment of Gram-negative organisms with EDTA has been shown to result in lysis, loss of viability and release of substances absorbing at 260 mμ (Gray & Wilkinson, 1965*a*; Neu, 1966) and to increased sensitivity to a variety of antibacterial agents (Brown & Richards, 1965; Leive, 1965). The degree of sensitivity of Gram-negative organisms has been shown by Wilkinson (1967) to vary between species. Gray & Wilkinson (1965*b*) found that this sensitivity was related to the ability of EDTA to solubilize components of isolated cell walls of the particular organism and they concluded that the lipopolysaccharide portion was probably involved. The presence of divalent cations in the cell walls of *Pseudomonas aeruginosa* has been demonstrated (Eagon, Simmons & Carson, 1965), and Asbell & Eagon (1966) suggested that EDTA acted by chelating cations involved in cross-linkages with lipopolysaccharide components of the cell wall. Costerton *et al.* (1967) suggested that Mg might be one of the cations involved. The object of the present work was to determine the sensitivity to EDTA of *P. aeruginosa* organisms grown with various Mg concentrations. To avoid changes in sensitivity due to washing procedure (Brown, 1968), unwashed cultures were used.

METHODS

Organism. Pseudomonas aeruginosa NCTC 6750 was used throughout this study.

Chemicals. All chemicals used were of AnalarR grade.

Cleaning procedures. All glass-ware was treated with sulphuric+chromic acid mixture, washed with tap water and finally in glass-distilled water.

Culture methods. The culture was maintained in a liquid medium without added Mg

which consisted of: 0·001 M-D(+)glucose, 0·01 M-$(NH_4)_2HPO_4$, 0·01 M-$(NH_4)_2SO_4$, 0·0005 M-NaCl, 0·0005 M-KCl dissolved in glass-distilled water (pH 7·3). Bacteria used in the following experiments were grown in media identical to the above, but containing graded concentrations of $MgSO_4$. Cultures (500 ml.) were grown in 2 l. flasks in a Mickle shaker bath (The Mickle Laboratory Engineering Co. Gomshall, Surrey) at 37·5°. The growth rate was initially the same in all cultures but became increasingly decreased because of Mg limitation. Cultures with the lower concentrations of Mg showed the greater degree of Mg limitation of growth. Cell division eventually ceased owing to depletion of glucose at an extinction (E) of about 0·180 measured at 470 mμ by using a Unicam SP 600 spectrophotometer. This corresponded to a colony count of about $3·5 \times 10^8$ bacteria/ml. Unwashed cultures in this condition were used for all studies with EDTA and were taken for treatment when the E_{470} value had remained constant for 1 hr. The growth medium was at pH 7·3 at inoculation and decreased to 7·2 by the time cell division ceased. This pH value was unaltered by the EDTA treatments used.

Treatment of bacteria with EDTA. Samples (93 ml.) of unwashed culture at 37·5° were added to 7 ml. volumes of EDTA solutions of various concentrations in 250 ml. flasks in a water bath at 37·5°. The EDTA treated cultures (pH 7·2) were maintained at 37·5° throughout the experimental period.

Measurement of lysis. Samples (3 ml.) of EDTA-treated cultures were taken and the extinction of the sample measured at 470 mμ (E_{470}).

Colony count estimations. Samples (1 ml.) of EDTA-treated bacteria were removed and after inactivation of the EDTA by dilution in nutrient broth (Oxoid) to which $CaCl_2$ was added, 0·5 ml. volumes were spread on surface-dried nutrient agar plates. Colonies were counted after incubation for 36 hr at 37·5°.

RESULTS

Preliminary experiments indicated a definite Mg requirement for growth by *Pseudomonas aeruginosa*. This accords with the findings of other workers (Webb, 1949) and of Tempest, Hunter & Sykes (1965) who showed Mg to be an essential component of the ribosomes of *Aerobacter aerogenes*. Figure 1 illustrates representative results of growing pseudomonads in the basal medium with the addition of graded amounts of Mg. The lag period was inversely proportional to the Mg concentration originally in the medium. Cell division did not cease abruptly as Mg became limiting, but progressively slowed. This finding was in agreement with that of Tempest *et al.* (1965). Eventually cell division ceased in all cultures owing to depletion of glucose, but at the lower Mg concentrations the final extinction value was lower than that attained by cultures containing higher concentrations of Mg. Presumably during the period of slower growth (depending on Mg concentration) glucose was depleted by cell respiration. Mg concentrations were selected such that, although at the lowest concentrations Mg limitation of growth had occurred, the final E_{470} value was close to the maximum possible for the glucose concentration (0·001 M in every case).

Effect of Mg-limitation on lysis of Pseudomonas aeruginosa by EDTA

Suspensions of bacteria were taken for EDTA treatment when the E_{470} value had remained constant for 1 hr. Lysis of bacteria grown in the basal medium with the

addition of Mg 1 μg./ml. resulted after treatment with various EDTA concentrations (Fig. 2). This procedure was repeated with bacteria grown in a range of Mg concentrations and the decrease in E_{470} value after 180 min. taken as a measure of sensitivity.

Figure 3 illustrates the effectiveness of several EDTA concentrations in causing lysis, measured for bacteria grown in 0·05, 0·2, 0·5, 1·0, 2·0 and 4·0 μg. Mg/ml. Two distinct

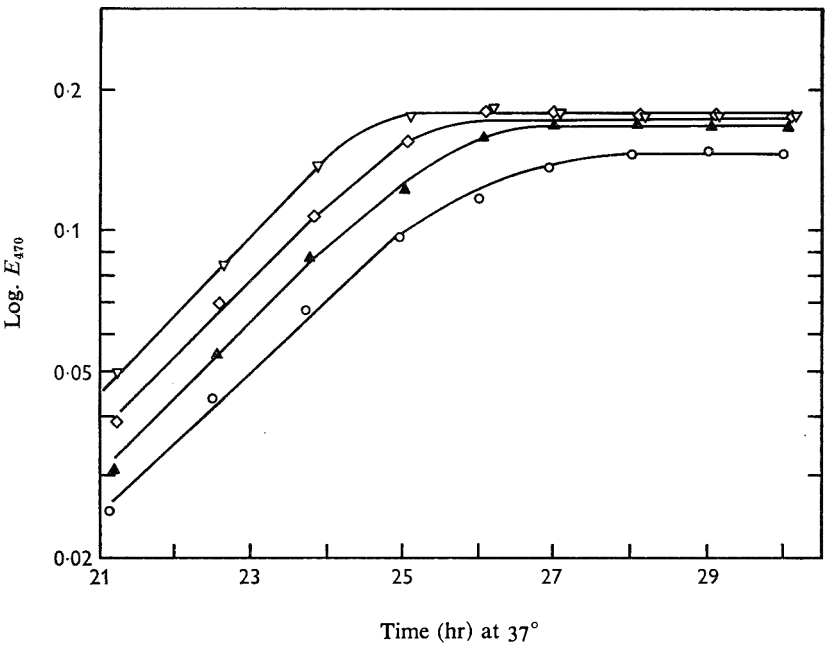

Fig. 1. Effect of magnesium content of medium on growth of *Pseudomonas aerugionosa*. Mg concentrations (μg./ml.) were: ○, 0·05; ▲, 0·1; ◇, 0·45; ▽, 1.

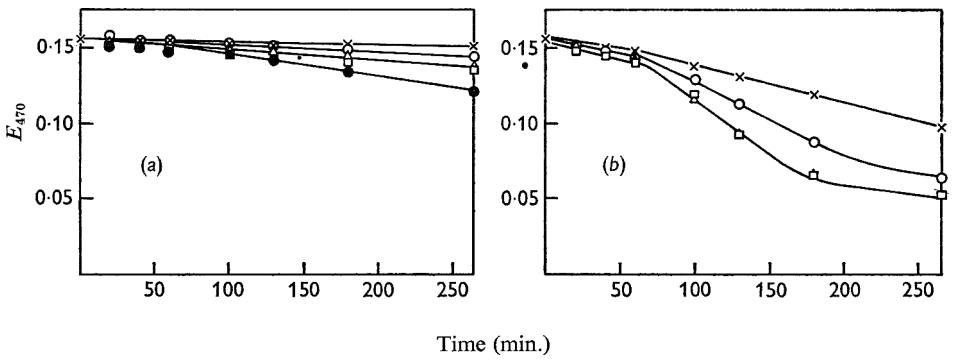

Fig. 2. Effect of EDTA concentration on lysis of *Pseudomonas aeruginosa* grown in medium with 1 μg.Mg/ml. Bacteria not Mg-limited. EDTA concentrations (μg./ml.) were—(*a*): ×, zero; ○, 10; △, 20; □, 25; ●, 37·5; and (*b*): ×, 50; ○, 100; △, 375; □, 1000.

effects were observable. First, as the EDTA concentration was increased the rate of lysis increased to a maximum for bacteria grown in any one Mg concentration. The EDTA concentration required to produce a maximum effect was greater, the higher

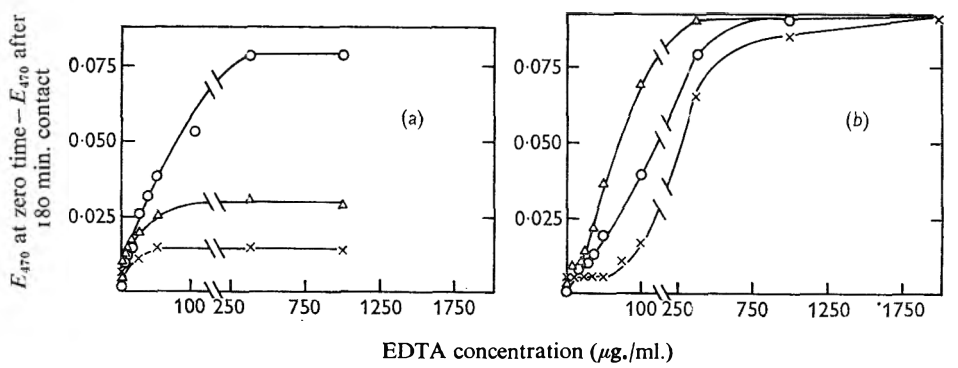

Fig. 3. Effect of EDTA concentration on rate of lysis of *Pseudomonas aeruginosa* grown in media with different Mg concentrations. Mg concentrations (μg./ml.) were—(*a*): ○, 0·5; △, 0·2; ×, 0·05 and (*b*): ×, 4; ○, 2; △, 1.

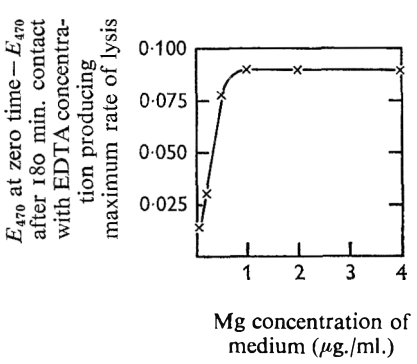

Fig. 4

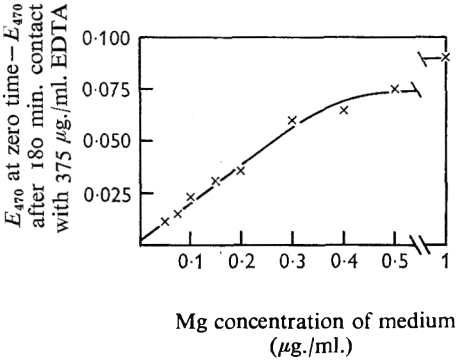

Fig. 5

Fig. 4. Maximum rate of lysis by EDTA of *Pseudomonas aeruginosa* grown in media with different Mg concentrations. Maximum rate of lysis at any one Mg concentration can be deduced from Fig. 3.

Fig. 5. Relationship between maximum rate of lysis of *Pseudomonas aeruginosa* by EDTA and Mg concentration in the medium. 375 μg. EDTA/ml. produced maximum rate of lysis over this Mg range.

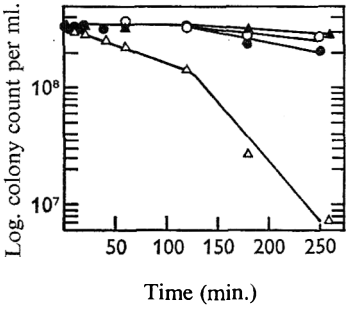

Fig. 6. Effect of EDTA 375 μg./ml. on the colony count of *Pseudomonas aeruginosa* grown in a basal medium with different Mg concentrations. ○, 0·05 μg. Mg./ml. untreated; ●, 0·05 Mg. μg./ml. treated with 375 μg./ml. EDTA; ▲, 1 μg. Mg/ml. untreated; △, 1 μg. Mg/ml. treated with 375 μg. EDTA/ml.

the Mg concentration in the growth medium. This agrees with earlier work which showed that cations in excess of growth requirements reduced the activity of EDTA against unwashed bacteria (Brown & Richards, 1965). Secondly, over the range of Mg concentrations 0·05 to 0·5 μg./ml. a large change in maximum rate of lysis occurred, while further increases in the Mg concentrations produced only a small increase in the maximum rate of lysis (Fig. 4). To investigate this phenomenon further, bacteria grown in Mg concentrations over the range 0·05 to 0·5 μg./ml. were treated with 375 μg. EDTA/ml., a concentration shown to produce maximum rate of lysis over this range of Mg concentrations. The results (Fig. 5) indicated a linear relationship between the maximum rate of lysis and the concentration of Mg in the growth medium below about 0·3 μg./ml.

Effect of EDTA on viable counts of Mg-limited cultures

Colony counts were made at intervals upon bacteria grown in (*a*) 1 μg. Mg/ml., (*b*) 0·05 μg. Mg/ml., which had been treated with 375 μg. EDTA/ml. The results (Fig. 6) indicated once again that bacteria grown in the low Mg concentration were much more resistant to EDTA than bacteria produced in the high concentration. After 250 min. the colony count of the Mg-limited bacteria had decreased about 25 %, whereas the bacteria not Mg-limited had a decrease in colony count of about 40-fold. Comparison of these data with details of lysis resulting from EDTA treatment indicated that loss of viability proceeded more rapidly than lysis. This suggested that bacterial death preceded cell lysis.

DISCUSSION

It has been suggested that EDTA acts by removal of divalent cations, probably Ca or Mg, from the cell wall of *Pseudomonas aeruginosa* where these ions occupy positions of structural importance (Asbell & Eagon, 1966). The present results support this concept. Calcium was not added to the medium used in this study and any traces present would have been impurities of the Analar chemicals. In fact, the basal medium contained 0·06 μg. Ca/ml. impurity as determined by flame photometry (Unicam SP 900). The fact that the bacteria grown in the lowest Mg concentration were almost completely resistant to the action of EDTA may have been due to a lack of such cations in the cell-wall architecture. The linear relationship between lysis and Mg concentration in the growth medium probably reflected a relationship between Mg in the medium and the wall structure of the eventual Mg-limited cells. It may also be that, as in the case of *Aerobacter aerogenes*, the organic components of the cell wall differed when the bacteria were deprived of Mg (Ellwood & Tempest, 1967). The above findings indicated that the use of EDTA in classification of pseudomonads (Wilkinson, 1967) must be approached with caution since more or less Mg in the growth medium could greatly affect the result. The effect of Mg in the growth medium may also be important where spheroplast or protoplast production involves EDTA treatment.

We thank the Medical Research Council for a grant which supported part of this work.

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Morphological Changes of *Vibrio cholerae* Organisms in Glucose Saline

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SUMMARY

Morphological changes in *Vibrio cholerae* harvested from 18 hr growth on nutrient agar surface and incubated in glucose saline at 37° have been studied by electron microscopy using metal shadowing, uranyl staining and thin sectioning techniques. Within the 6 hr incubation period, a vacuolar region has been found to separate the cell wall from the protoplasmic body presumably at one polar end. Subsequently such separation has been found all round the periphery of the protoplasmic body, which assumes a round form of average dimension $0{\cdot}4 \pm 0{\cdot}05\ \mu$. Within the 24 hr incubation period, majority of the cells (60 to 70 %) are rounded and of these a significant fraction (15 to 20 %) contained bodies limited by one single membrane. It is suggested that these bodies represent the protoplasts of *V. cholerae*.

INTRODUCTION

Gallut (1954) reported that a toxic substance is produced when the *Vibrio cholerae* organisms are incubated in glucose saline. He also mentioned that the toxin is produced by the solubilization of the cell wall and that the resultant bodies are not agglutinated by the homologous anti-O-immune sera (Gallut, 1965). No systematic study has yet been made by electron microscopy on the morphological changes these vibrios undergo during incubation in glucose saline. In our earlier studies (Chatterjee & Das, 1966, 1967) it was reported that during the early period of incubation (within 1·5 hr), *V. cholerae* organisms excrete small particles which are bounded by the cell-wall material. The present paper deals with the morphological changes of the vibrios at subsequent periods of incubation in glucose saline as studied by the thin sectioning, metal shadowing and uranyl staining techniques.

METHODS

Bacterial strains and condition of growth. Two strains of *Vibrio cholerae*, INABA C84 (obtained through the courtesy of Dr K. N. Neogy, Department of Bacteriology of this Institution) and OGAWA 154 (obtained through the courtesy of Dr S. Mukherjee, Indian Institute of Experimental Medicine, Calcutta) were used throughout this investigation. Organisms were grown either in nutrient broth or in 1 % (w/v) peptone water (pH 8·0) for about 18 hr at 37°. 0·1 ml. of this culture was inoculated and spread over the surface of a nutrient agar plate and incubated at 37° for 16 to 18 hr, so as to obtain a uniform surface growth.

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Incubation in glucose saline. Vibrios from the 18 hr growth on the nutrient agar surface were suspended in 0·85 % saline and washed twice by centrifugation. The washed vibrios were then suspended in suitable volumes of glucose saline (NaCl 0·85 g., glucose 0·5 g., distilled water 100 ml.) (Bernard & Gallut, 1943; Gallut, 1954) distributed in Erlenmeyer flasks (100 ml. capacity) in volumes of 20 ml., the initial optical density of the suspension thus being adjusted in the range 0·8 to 1·0. The suspension was then incubated at 37° for a maximum period of 24 hr.

Fixation of incubated cells. Vibrio cholerae organisms were harvested from the glucose saline at different periods of incubation by centrifugation and fixed immediately by resuspending in Ringer solution containing either 1 % (w/v) osmium tetroxide or 4 % (w/v) formaldehyde. Fixation was carried out at 0 to 4° for 4 to 24 hr. In some cases fixation was carried out by dissolving the osmium tetroxide or formaldehyde in the glucose saline solution to the desired concentration and keeping the whole at 0 to 4°.

Metal shadowing. For metal shadowing, a small drop of the suspension after fixation was deposited on carbon collodion-coated copper wire mesh and the excess liquid was withdrawn a few seconds later. The preparation was then washed thoroughly by rinsing in distilled water, dried and shadowed obliquely with chromium.

Uranyl staining. Vibrio cholerae organisms deposited on the carbon collodion substrate after the desired period of incubation in glucose saline solution and subsequent fixation, were stained positively by floating the grids, film side down, on the surface of a 1 % (w/v) uranyl acetate solution in distilled water for about 1 hr. The grids were then washed thoroughly in distilled water and dried.

Thin sectioning. The cells after incubation in glucose saline solution for the desired period were harvested by centrifugation and the pellet was fixed in 1 % (w/v) solution of osmium tetroxide in Kellenberger buffer (pH 6·1) for 16 to 20 hr at room temperature (Kellenberger, Ryter & Sechand, 1958). The fixed cells were washed in 0·5 % solution of uranyl acetate in the said buffer for about 2 hr. The washed cells were placed between layers of agar, dehydrated in graded ethanol and embedded in a methacrylate mixture (methyl+butyl, 2+3) or in Epon (Luft, 1961). Methacrylate mixture was prepolymerized (Borysko & Sapranauskas, 1954) to reduce the polymerization damage and the corresponding sections after being stained were overlaid in most cases with a thin film of carbon (Watson, 1957) to reduce the sublimation artifact. Thin sections were cut with glass knives on a Porter Blum ultramicrotome. Sections were stained with uranyl acetate and/or lead citrate or potassium permanganate (Lawn, 1960). Vibrios harvested from 24 hr growth on nutrient agar were fixed, embedded and sectioned under identical conditions as control.

Electron microscope. All electron micrographs were taken by a Hitachi HS-6 electron microscope at instrumental magnification ranging from ×6000 to 15,000. Measurements were made from the enlarged prints by a micrometer eyepiece (accuracy 0·1 mm).

RESULTS

During the incubation of *Vibrio cholerae* organisms in glucose saline at 37° interesting changes in cellular morphology were noted. Within the first 1 to 1·5 hr incubation, the vibrios were found to release in the environmental medium cell-wall materials in the form of approximately round particles. The dimension of these particles varied between 400 and 1100 Å in most cases as described in detail in our earlier publications

(Chatterjee & Das, 1966, 1967). At this period all the vibrios maintained their rod-like structure as in the native stage (Pl. 1, fig. 1). Also thin sectioning of the vibrios harvested at this stage of incubation in glucose saline revealed no significant difference in the ultrastructure of their protoplasmic constituents as compared to what was found in the vibrios grown on agar surface (Pl. 2, fig. 5).

At subsequent stages of incubation the normal relative disposition of the cell wall and plasma membrane was changed gradually with time in the majority of the vibrios. Organisms harvested from 2 to 6 hr incubation in glucose saline revealed that the cell wall was separated from the protoplasmic mass by a wide vacuolar region (Pl. 2, fig. 6) at one end only. A similar picture was also found in metal-shadowed preparations (Pl. 1, fig. 2) where due to the limitation of the technique the presence of the vacuolar region separating the extended cell wall and the protoplasm was not evident. Plate 1, fig. 2, illustrates that the separation has taken place at one polar end. The cell wall, presumably, got weakened at this stage and the protoplasmic mass began to assume a spherical form out of the native rod-shaped structure (Pl. 1, fig. 3). It appeared from Pl. 1, fig. 3, that more than one rounded protoplasmic bodies would form per single bacterium.

In the 6 to 24 hr incubation period, majority of the vibrios assumed rounded forms having a much denser round protoplasmic core covered by a larger envelope of weakened or degraded cell wall and presenting the appearance of a poached egg (Pl. 1, fig. 4*a*). Thin section at this stage revealed that the separation between the cell wall and the protoplast was complete practically all over the latter's periphery (Pl. 2, fig. 8). The structure of the cell wall became considerably degraded and breaks appeared in the cell-wall structure (Pl. 3, fig. 9). At this stage, round protoplasmic bodies were found in the process of getting rid of the cell-wall envelope (Pl. 2, fig. 7). Envelopes discarded in this process apparently did not contain any protoplasmic body (Pl. 3, fig. 9, 11). In Pl. 1, fig. 4*b*, many of the round protoplasmic bodies apparently did not retain any remnant of the envelope. Some of them presented a doublet structure and one appeared to be in the process of being pinched off a rod-shaped body (arrow). At higher magnification, thin section revealed the presence of one limiting membrane (thickness 70 to 90 Å) around many of these protoplasmic bodies (Pl. 3, fig. 10). These bodies contained a nuclear vacuole surrounded by a denser cytoplasmic area and have a diameter between 0·3 and 0·6 μ with an average of $0{\cdot}4 \pm 0{\cdot}05\ \mu$.

By counting the electron micrographs of the thin sections as well as the shadow-cast preparations of the cells after the 24 hr incubation period, a rough estimate of the percentage of the different types of bodies formed was made. It was found that over 60 to 70 % of the *Vibrio cholerae* organisms were converted into round forms and of these a significant fraction (15 to 20 %) contained bodies limited by one single membrane. The rest of the bodies still retained the cell-wall layer separated from the protoplast by a wide vacuolar area.

DISCUSSION

The different stages of the formation of spherical bodies from *Vibrio cholerae* organisms incubated in glucose saline, as obtained in the present investigation, are of interest. Evidences obtained suggest that each rod-shaped protoplasmic mass of the *V. cholerae* organism transforms into more than one spherical bodies whose diameter is on the average not greater than the width of the intact cells. Individual

spherical bodies are separated from the parent protoplasmic mass apparently by a pinching off process. These observations are not in accord with the mode of formation of spheroplasts from *Escherichia coli* and *Proteus vulgaris* grown in presence of penicillin (McQuillen, 1960; Lederberg, 1956) or of *E. coli* deprived of diaminopimellic acid (McQuillen, 1958). However, the formation of a large vacuolar region where the outer wall and the inner plasma membrane of the spherical bodies have separated, as found in the present investigation, has been commonly encountered in spheroplasts of Gram-negative bacteria induced by the growth in the presence of penicillin or glycine or by the deprivation of diaminopimellic acid (Thorsson & Weibull, 1958).

Action of different physico-chemical agents on a varietyof bacteria and the consequent changes induced in them have been investigated by many authors and reviewed recently by Stolp & Starr (1965). Antibiotics such as penicillin, enzymes such as lysozyme and amino acids such as glycine have been observed to lyse bacteria unless otherwise protected by an osmotically stable system. Under the action of these agents many bacteria in an osmotically protected system have been observed to transform into spherical forms (Brenner *et al.* 1958; Hines, Freeman & Pearson, 1964; McQuillen, 1960; Mitchell & Moyle, 1956; Salton, 1961; Weibull, 1953) which are usually termed protoplasts or spheroplasts in accordance with the complete absence or not of any cell-wall material covering such spherical bodies. Jeynes (1961) reported that the *Vibrio cholerae* organisms grown in the presence of glycine transform into true protoplasts by the complete loss of outer wall component. According to Gallut & Giuntine (1963) if the *V. cholerae* organisms are incubated in glucose saline at 37° true protoplasts are formed by the solubilization of the cell wall. However, thin sections of the resulting bodies were not examined in any of the above investigations on *V. cholerae*. The problem of deciding about the presence or absence of the cell wall in Gram-negative bacteria in the osmotically sensitive form was discussed by Brenner *et al.* (1958) and the need for the adoption of thin sectioning technique in such cases was emphasized.

The action of glucose saline on the Gram-positive or Gram-negative bacteria has not been investigated so far. Considerable evidence has, however, been obtained in the present investigation to conclude that *Vibrio cholerae* organisms are mostly converted into spherical forms by incubation in glucose saline. Uranyl stained as well as shadowed preparations of unsectioned cells have indicated that the spherical forms shed the considerably weakened and degraded cell wall. Thin sections have revealed the presence of wide vacuolar region between the spherical protoplasmic mass and the cell wall and also breaks or discontinuities in the cell-wall structure. These observations suggest the possibility of the complete separation of the protoplasmic mass from the degraded cell-wall layer. At higher magnification a membrane of thickness between 70 and 90 Å has been found to limit many of these round protoplasmic bodies. No other membrane beneath this has been evident in the electron micrographs. It is unlikely that this membrane represents the cell wall since wide separation between the protoplast and the cell wall has been noticed even at a shorter period of incubation of the vibrios in glucose saline. Hofschneider (1960) observed the presence of a single membrane limiting a number of *E. coli* treated with EDTA and lysozyme and suggested that it was the cytoplasmic membrane. Birdsell & Cota-Robles (1967) confirmed these findings. Costerton *et al.* (1967) also recorded the presence of a single membrane

limiting a marine pseudomonad after treatment with EDTA and lysozyme. It is thus suggested that the single membrane found, in this investigation, to limit many of the round bodies represents the plasma membrane and that the corresponding bodies are the protoplasts of *V. cholerae*.

This work forms part of a thesis submitted by J. Das and accepted for the D.Phil. degree by the University of Calcutta.

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EXPLANATION OF PLATES

PLATE 1

Fig. 1. *Vibrio cholerae* harvested from 18 hr growth on nutrient agar surface. Metal shadowed. × 21,000.

Fig. 2. *V. cholerae* organism harvested from 2 to 6 hr incubation in glucose-saline. Metal shadowed. CW, cell wall. × 48,000.

Fig. 3. Vibrios harvested from 2 to 6 hr incubation period. Arrow indicates the formation of round protoplasmic body. Metal shadowed. × 13,000.

Fig. 4*a*. A round protoplasmic body covered by a larger envelope of the cell wall (CW). Metal shadowed. × 30,000.

Fig. 4*b*. Round protoplasmic bodies obtained in the 6 to 24 hr incubation period. DS, doublet structure. Arrow indicates the formation of a round body apparently by a pinching off process. Metal shadowed. × 18,000.

PLATE 2

Fig. 5. Ultrathin section of *V. cholerae* harvested from 18 hr growth on nutrient agar surface. × 45,000.

Fig. 6. Ultrathin section illustrating the presence of vacuolar region (VAC) separating the cell wall (CW) from the protoplasmic body. × 55,000.

Fig. 7. Uranyl stained preparation of *V. cholerae* harvested from 6 to 24 hr incubation in glucose saline. The protoplasmic body has become round with the cell wall (CW) still attached to it. Arrow indicates one such body which is apparently free of the cell wall material. × 16,800.

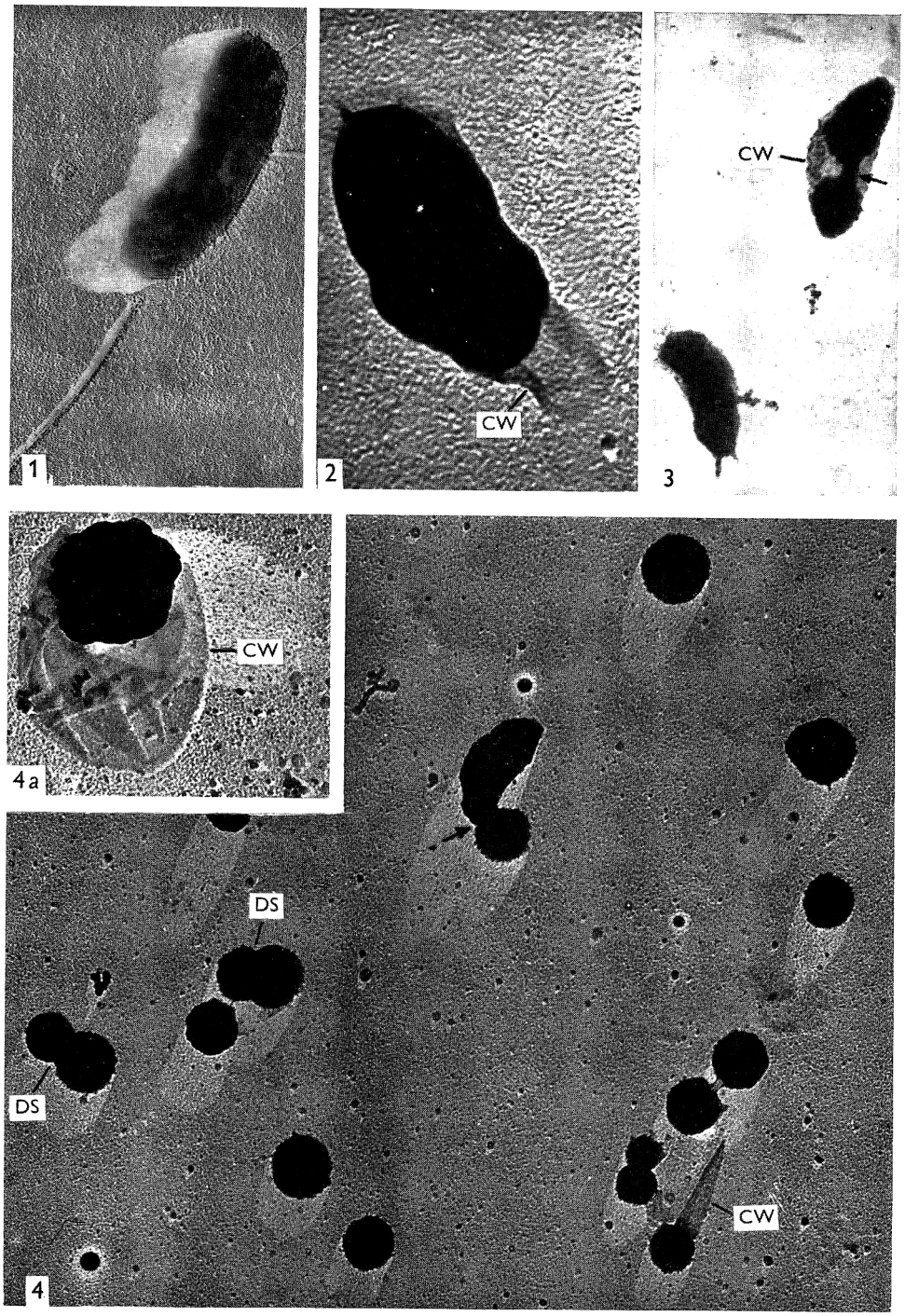
Fig. 8. Ultrathin section of a vibrio harvested from 6 to 24 hr incubation period shows the separation of the cell wall (CW) from the protoplasmic body all round the latter's periphery. × 80,000.

PLATE 3

Fig. 9. Ultrathin section of vibrios harvested from 6 to 24 hr incubation period shows the presence of a break (arrow) in the cell wall (CW) structure. The protoplasmic bodies B and C are apparently free of the cell wall. An apparently discarded cell wall material (CW) can be seen in the same field. × 66,000.

Fig. 10. One single membrane (M) is seen to limit the round protoplasmic bodies obtained in the 6 to 24 hr incubation period. × 100,000.

Fig. 11. Apparently discarded cell-wall material (CW) in thin section. × 60,000.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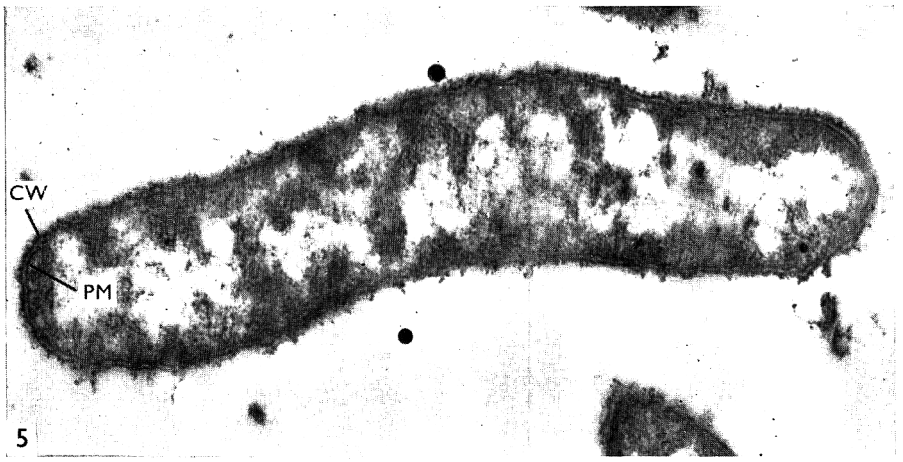
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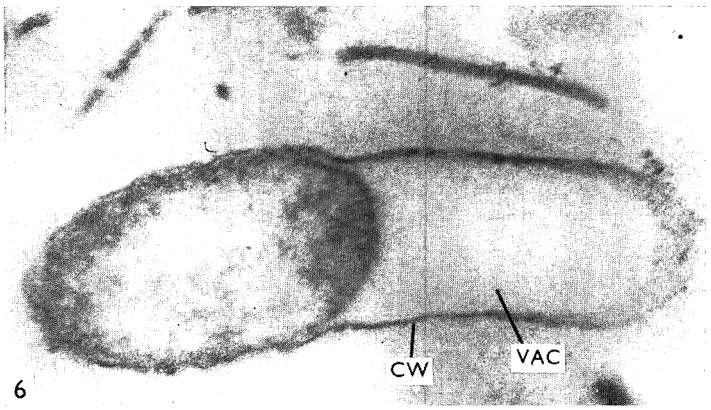
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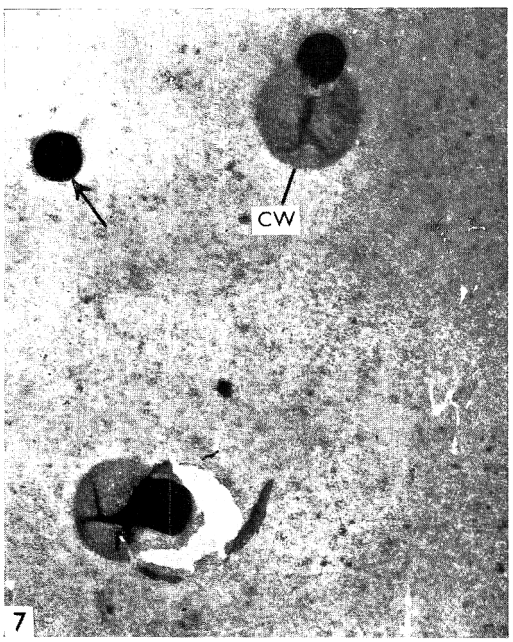
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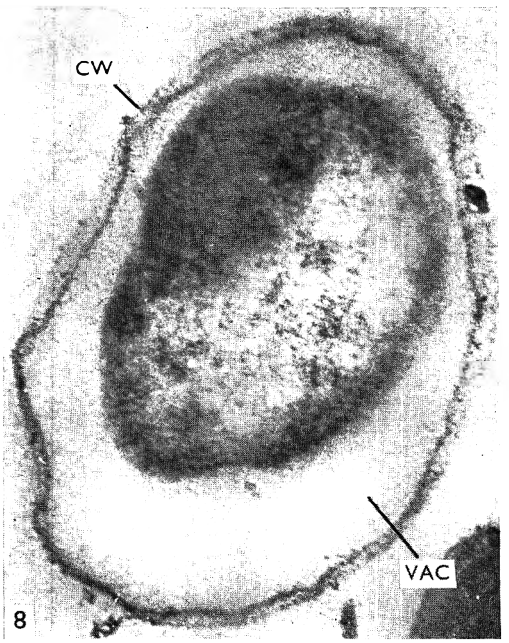
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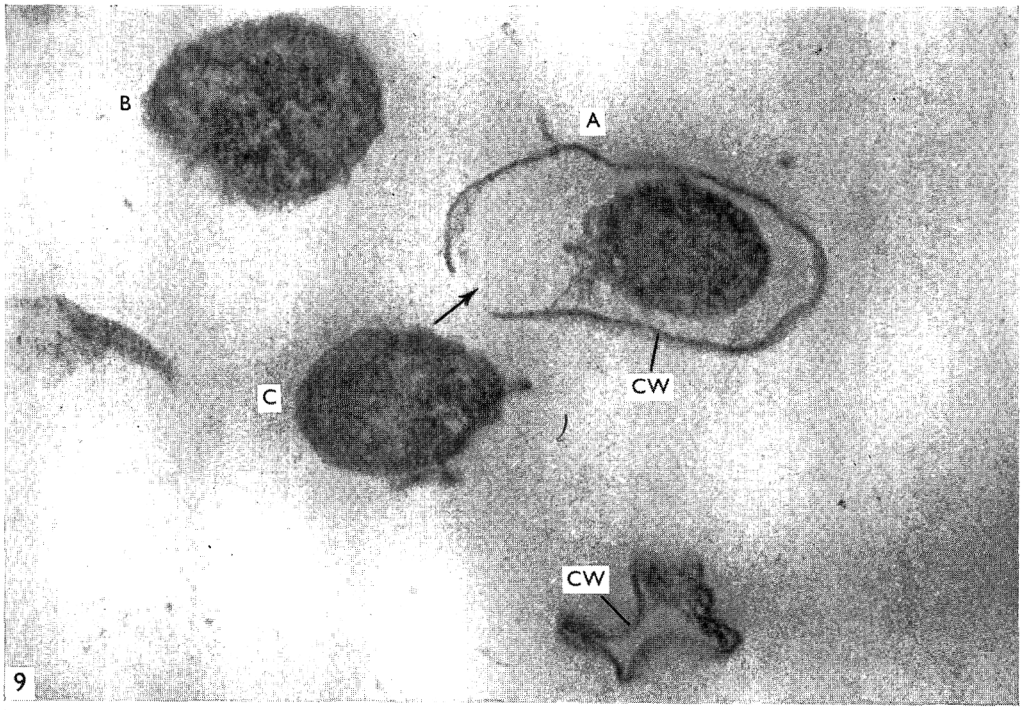
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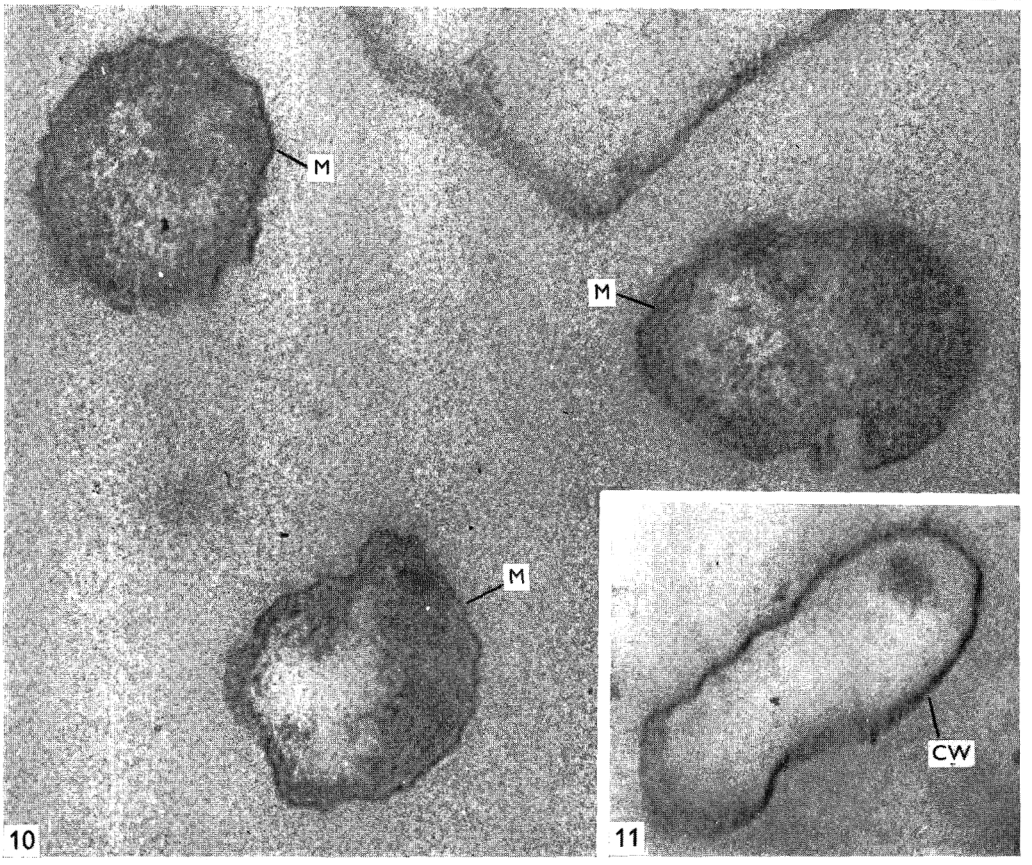
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The Incorporation and Metabolism of Glucose by *Anabaena variabilis*

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SUMMARY

The incorporation and metabolism of glucose by the blue-green alga *Anabaena variabilis* is described. Experiments with [^{14}C]glucose indicated that this compound contributed up to 46% of the total dry wt of organism. Respiratory studies with [1-^{14}C] and [6-^{14}C] showed that the pentose phosphate pathway was the major route of glucose dissimilation. No evidence of alteration in enzyme concentration after growth in the presence of glucose was found in the ten examples of glycolytic and pentose phosphate pathway enzymes examined. Enzymes from both pathways were affected by the end-products. These results are discussed in relation to autotrophic metabolism in blue-green algae.

INTRODUCTION

In an earlier paper (Pearce & Carr, 1967*a*) we described the metabolism of acetate by two species of blue-green algae which until recently were considered to be strict photoautotrophs. Such metabolism, and that of other carbon compounds, by blue-green algae hitherto considered to be autotrophs, is now well established (Hoare & Moore, 1965; Hoare, Hoare & Moore, 1967; Carr & Pearce, 1966; Pearce & Carr, 1967*a*; Smith, London & Stanier, 1967). A noticeable feature of *Anabaena variabilis* is that even though glucose is incorporated and metabolized its addition to the growth medium produces no increase in growth rate and only slight increase in respiratory rate (Kratz & Myers, 1955*a*). In contrast, the growth of some species of blue-green algae is stimulated by glucose or other sugars, and indeed they may be maintained heterotrophically in the dark (Kiyohara, Fujita, Hattori & Watanabe, 1960; Fay, 1965). Studies on carbohydrate metabolism in blue-green algae have usually centred upon the synthesis of polysaccharide (Fredrick, 1954; Kindel & Gibbs, 1963) and on the operation of fructose diphosphate aldolase (Richter, 1959; Wildon & ap Rees, 1965; Willard, Schulman & Gibbs, 1965). An examination of [^{14}C]sugar metabolism in autotrophically grown *Tolypothrix tenuis* (a species capable of heterotrophic growth) led Cheung & Gibbs (1966) to conclude that the oxidative pentose phosphate cycle was the main pathway of carbohydrate dissimilation. Several of the enzymes necessary for this pathway were detected in extracts of *Anacystis nidulans* by Richter (1959). The work described in the present paper establishes the incorporation and metabolism of glucose by *A. variabilis* and examines some of the enzymes concerned in glucose breakdown in cell-free extracts prepared from organisms grown in the presence and absence of glucose.

METHODS

Growth of Anabaena variabilis. The blue-green algae used were maintained and cultured as previously described (Carr & Hallaway, 1965; Pearce & Carr, 1967*a*); *Anabaena variabilis* was used for most of the work; certain enzyme activities of *Anacystis nidulans* were also measured. The basic autotrophic growth medium was medium C (Kratz & Myers, 1955*b*) supplemented with 0·05% (w/v) sodium bicarbonate and gassed with air+carbon dioxide (95+5, v/v). Glucose was added to this medium, after separate sterilization, where indicated in the text.

Incorporation of [U-^{14}C]glucose. The growth medium (20 ml.) was supplemented with [^{14}C]glucose and incubated at 34° in boiling tubes illuminated with 30 W. warm-white strip lights at 9 in. distance. Vessels were gassed with air or air+carbon dioxide (95+5, v/v) as indicated. The extent of isotopic incorporation was measured in a harvested and thoroughly washed (6 times) suspension by counting, after drying, at infinite thinness in a Nuclear-Chicago Gas-Flow Automatic Planchette Counter of efficiency 34% and a background of 16 counts/min. Each sample was counted in duplicate to an accuracy of at least 2%.

Oxidation of [^{14}C]glucose. Bloom & Stetten (1953) devised a procedure for estimating the relative activities of the two main pathways of glucose breakdown by measuring the ratio of carbon dioxide production from different carbon atoms within the glucose molecule. Although the relative participation of the pentose phosphate pathway and glycolytic pathway cannot be absolutely determined (see Hollmann, 1964) the comparison of rate of [^{14}C]carbon dioxide evolution from the 1-C and 6-C positions of glucose indicate the extent of each pathway. The ratio of [^{14}C]carbon dioxide from [6-^{14}C]glucose compared with [^{14}C]carbon dioxide from [1-^{14}C]glucose (C_6:C_1 ratio) will be unity when glycolysis is the only operative pathway. C_6:C_1 ratios of less than unity indicate the contribution of the alternative pentose phosphate pathway.

Double side arm Warburg flasks contained a suspension of *Anabaena variabilis* in medium C in the main compartment, hyamine hydroxide (0·3 ml) in the centre-well (Snyder & Godfrey, 1961), with sodium dodecylsulphate (10%, w/v) in one side arm and [^{14}C]glucose in the other. At various times after the addition of glucose to the main compartment the respiration was terminated by adding sodium dodecylsulphate. After standing for 45 min. the carbon dioxide was totally absorbed by the hyamine hydroxide and this was transferred, by a syringe, to 5 ml. scintillation fluid. The centre-well was washed with toluene (0·2 ml.) and the washing added to the scintillation fluid. Samples were placed in glass vials together with 5 ml. scintillation fluid (in g./l. toluene:2,5-diphenyloxazole, 5; dimethyl-1,4-bis-2-(4-methyl-5-phenyl-oxazoyl-benzene, 0·3) and counted in a Packard Tri-Carb Liquid Scintillation Spectrophotometer Series 314E to an accuracy of at least 2%. The efficiency for counting [^{14}C] was 60% (±1%); the background counting rate was 20 counts/min.

Estimation of glucose. The colorimetric procedure of Roe (1955) was used; absorption at 620 mμ was measured in a Hilger and Watts Spectrochem and glucose concentration obtained from a standard curve.

Preparation of cell-free extract and protein estimation. These procedures were as described by Pearce & Carr (1967*a*).

Enzyme assays

In the assays described the amount of commercially prepared enzymes added are expressed as units of activity; one unit metabolized 1 μmole substrate/min. Except where stated assays were done at room temperature (20°) and the spectrophotometer used was a Unicam SP700.

Hexokinase [EC2.7.1.1]. The method of assay (Slein, Cori & Cori, 1950) determined the rate of glucose-6-phosphate formation by measuring the rate of NADP reduction in the presence of glucose-6-phosphate dehydrogenase. The reaction mixture contained (μmole): potassium phosphate buffer (pH 7·0), 200; $MgCl_2$, 10; ATP, 5; NADP, 0·5; cell-free extract containing 2 to 7 mg. protein; final volume 2·7 ml. The rate of NADP reduction at 340 mμ was determined after the addition of 10 μmoles glucose to the experimental cuvette. In this assay no coupling glucose-6-phosphate dehydrogenase was added as the extracts of *Anabaena variabilis* and *Anacystis nidulans* contained a relatively high glucose-6-phosphate dehydrogenase activity (see Table 1). During the first few minutes of the reaction the rate of glucose-6-phosphate formation was limiting and the constant rate attained was taken as the measure of hexokinase activity.

Glucose-6-phosphate dehydrogenase [EC1.1.1.43]. The reaction mixture was the same as above except that 5 μmole glucose-6-phosphate were added to start the reaction (Kornberg & Horecker, 1955).

6-Phosphogluconate dehydrogenase [EC1.1.1.49]. The same reaction mixture was used as above but in this case 6-phosphogluconate (5 μmole) was added to initiate the assay (Horecker & Smyrniotis, 1955).

Phosphoriboisomerase [EC5.3.1.6]. The reaction assay system was essentially that of Axelrod & Jang (1954) and contained (μmole): potassium phosphate buffer (pH 7·0) 50; ribose-5-phosphate, 3; cell-free extract (3 to 5 mg. protein); total volume 1·0 ml. After incubation at 34° the reaction was stopped by adding concentrated H_2SO_4. Ribulose was detected colorimetrically by the carbazole procedure of Dische & Borenfreund (1951).

Phosphohexoisomerase [EC5.3.1.9]. The formation of glucose-6-phosphate from fructose-6-phosphate was assayed by coupling the isomerase with glucose-6-phosphate dehydrogenase (commercial enzyme added). The reaction mixture contained (μmole): potassium phosphate buffer (pH 7·0), 200; NADP, 0·5; 20 μl. glucose-6-phosphate dehydrogenase (equiv. 3 units of activity); cell-free extract (3 to 6 mg. protein) in 2·7 ml. total volume. Fructose-6-phosphate (5 μmole) was added to the experimental cuvette to start the reaction.

Phosphofructokinase [EC2.7.1.11]. The rate of production of fructose-1,6-diphosphate from fructose-6-phosphate and ATP was measured by coupling the kinase reaction with aldolase, triose phosphate isomerase and α-glycerophosphate dehydrogenase (Slater, 1953). The reaction mixture contained (μmole): potassium phosphate buffer (pH 7·0), 200; ATP, 9; $MgCl_2$, 10; cysteine, 20; with 20 μl. of a mixture of triose phosphate isomerase and α-glycerophosphate dehydrogenase (containing 14 units of each activity) + 50 μl. aldolase (containing 5 units of activity) + cell-free extract containing 2 to 5 mg. protein; total volume 2·7 ml. $NADH_2$ (0·3 μmole) was added to the experimental cuvette and after the measurement of the very slight $NADH_2$ dehydrogenase activity, 10 μmole fructose-6-phosphate were added and the

decline in absorption at 340 mμ was recorded. When the algal extract was omitted from the assay system there was no decline in absorption on the addition of fructose-6-phosphate, indicating that the samples of commercial enzymes employed did not contain any phosphofructokinase activity.

Fructose diphosphate aldolase [EC4·1·2·13]. The formation of triose phosphate from fructose diphosphate was measured by coupling this reaction with triose phosphate isomerase and α-glycerophosphate dehydrogenase (Racker, 1947). Identical cuvettes contained 200 μmole potassium phosphate buffer (pH 7·0)+20 μl. of a mixture of triose phosphate isomerase and glycerophosphate dehydrogenase (containing 14 units of each activity)+cell-free extract (3 to 7 mg. protein); 2·7 ml. final volume. $NADH_2$ (0·3 μmole) was added to the experimental cuvettes and after the initial measurement of $NADH_2$ dehydrogenase activity in the extract, 5 μmole fructose-1,6-diphosphate were added and the decrease in absorption at 340 mμ was determined. Control assays were made in which algal extract was omitted. No decrease in absorption at 340 mμ was found on adding fructose diphosphate in the absence of algal extract, indicating that the commercial enzymes did not contain contaminating aldolase activity.

Triose phosphate dehydrogenase ([EC1.2.1.12] NAD dependent; [EC1.2.1.13] NADP dependent). The rate of reduction of NAD or NADP resulting from the oxidation of glyceraldehyde-3-phosphate was followed at 340 mμ. The assay method was essentially that of Gibbs (1955). The reaction mixture contained (μmole): potassium phosphate buffer (pH 7·5), 200; sodium arsenate, 10; cysteine, 12; NAD (or NADP), 0·5: cell-free extract containing 3 to 7 mg. protein; 2·7 ml. total volume. The reaction was initiated by adding 3 μmole glyceraldehyde 3-phosphate to the experimental cuvette.

Pyruvate kinase [EC2.7.1.40]. The production of pyruvate from phosphoenolpyruvate was determined by measuring the rate of oxidation of $NADH_2$ in the presence of lactic dehydrogenase (Bücher & Pfleiderer, 1955). Identical cuvettes were made up containing (μmole): tris (pH 7·6), 100; $MgCl_2$, 10; KCL, 50; ADP, 2·5; 20 μl. lactic dehydrogenase (containing 30 units of activity); cell-free extract, containing 3 to 8 mg. protein; 2·7 ml. total volume. $NADH_2$ (0·3 μmole) was added to the experimental cuvette and after the initial measurement of $NADH_2$ dehydrogenase activity in the extract, 9 μmole phosphoenolpyruvate were added and the decrease in absorption at 340 mμ determined. Controls showed that the sample of phosphoenolpyruvate used did not contain any contaminating pyruvate and also that the commercial lactic dehydrogenase did not have any pyruvate kinase activity.

Chemicals and commercially prepared enzymes. Unless otherwise stated, all chemicals were analytical grade or of the purest commercial grade available and were purchased from British Drug Houses Ltd., Poole, Dorset. NAD, $NADH_2$, NADP, ATP, AMP and glucose-6-phosphate dehydrogenase were from C. F. Boehringer (Mannheim, Germany). Phosphoenolpyruvate, triose phosphate isomerase, α-glycerophosphate dehydrogenase, aldolase and lactic dehydrogenase were from Sigma London Chemical Co. (12, Lettice Street, London, S.W.6). Sodium dodecylsulphate was obtained from K and K Laboratories (177-10, 93rd Avenue, Jamaica 33, New York, U.S.A.) and hyamine hydroxide from the Packard Instrument Co. (10–12 St John's Road, Wembley, Middlesex). All radioisotope-labelled material was purchased from the Radiochemical Centre, Amersham, Buckinghamshire.

RESULTS

Growth and respiration

In agreement with the results of Kratz & Myers (1955*a*) the growth rate of *Anabaena variabilis* was not increased or retarded when glucose (10 to 50 mM) or sucrose (30 mM) was present in the autotrophic growth medium. When the available carbon dioxide was decreased by changing the gas phase from air+carbon dioxide (95+5, v/v) to air (about 0·05 %, v/v, CO_2) the growth rate was decreased, this decrease being unaffected by adding glucose (to 30 mM) to the medium. No growth of *A. variabilis* occurred in the complete absence of carbon dioxide and the presence of glucose. When *A. variabilis* was suspended in growth medium (see Methods) the respiratory rate was several times higher than that obtained with suspensions in 0·1 M-potassium phosphate (pH 7·0; Fig. 1*a*); in neither case did glucose stimulate the respiration rate.

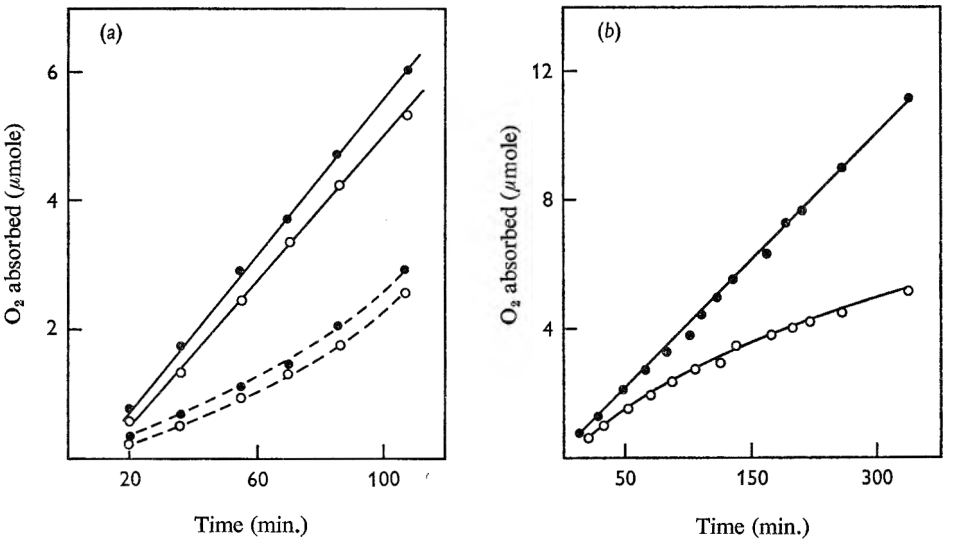

Fig. 1. (*a*) Oxygen uptake by *Anabaena variabilis*. Washed organisms (equiv. 9·5 mg. dry wt) were incubated in growth medium+glucose (33 mM) (-●—●-); growth medium alone (○—○); phosphate buffer (pH 7·0) 0·01 M+glucose (33 mM) (●-●); phosphate buffer (pH 7·0) 0·01 M alone (○-○). (*b*) Oxygen uptake by starved *Anabaena variabilis*. Oxygen uptake in the presence of glucose (33 mM) (●—●); endogenously (○—○), by a starved suspension of *A. variabilis* (equiv. 7·4 mg. dry wt).

Starvation of organisms of carbon dioxide for 4 hr before harvesting decreased the endogenous rate of oxygen uptake; the addition of glucose or acetate largely restored this to that of the non-starved control (Fig. 1*b*). The lack of any permeability barrier to glucose metabolism in this organism was indicated by measurements of glucose utilization by a washed suspension of *A. variabilis*. Glucose was removed from the medium at a rate equal to 1·7 mμmole/min./mg. dry wt organism. (Fig. 2)

Experiments with [^{14}C]glucose

The utilization of glucose by *Anabaena variabilis* was confirmed by measurement of incorporation of [U-^{14}C]glucose into cell material during growth. When this organ-

ism was grown under the standard procedures in the presence of 2 μc [U-^{14}C]glucose (20 mM), 33% of the total dry wt after 5 days of growth was derived from the [^{14}C]-labelled glucose (Table 1). This proportion increased to 46% when air replaced air + carbon dioxide (95 + 5, v/v) as gas phase, and the addition of acetate (20 mM) to the medium decreased the extent of glucose incorporation to 20% of the dry wt. The figures in Table 1 were calculated after measurement of isotope incorporation and

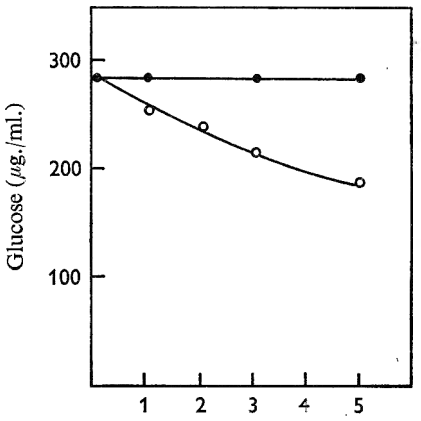

Fig. 2

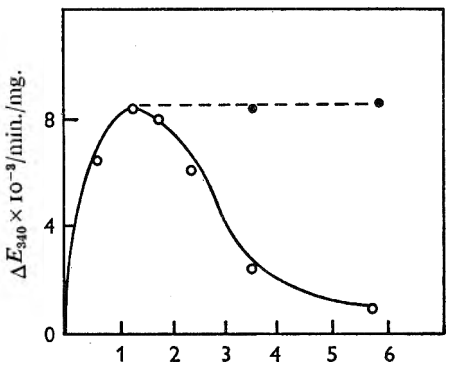

Fig. 3

Fig. 2. Decrease in glucose concentration during incubation with *Anabaena variabilis*. *A. variabilis* (equiv. 1·2 mg. dry wt/ml.) was suspended in growth medium (100 ml.) containing glucose (300 μg./ml.) and illuminated by four 60 W. lamps at 34°. Organisms were kept in suspension by a magnetic stirrer. Samples were taken at intervals, the algae removed by centrifugation, and the glucose content determined (see Methods). O—O, *A. variabilis*; ●—●, control of boiled organisms.

Fig. 3. Effect of ATP on phosphofructokinase activity in *Anabaena variabilis*. The assay was as in Methods except that ATP concentration was varied as shown in the absence (O—O) or presence (●–●) of AMP (0·3 mM).

Table 1. *Incorporation of* [*U-*^{14}*C*]*glucose into growing cultures of Anabaena variabilis*

Growth medium (20 ml.) was supplemented as indicated with glucose (2 μc) or acetate and organisms grown as in Methods. Results 1 and 2 are mean values; the range and number of experiments are given in parentheses.

Expt	Addition (20 mM)	Gas phase	% dry wt derived from glucose
1	Glucose (U-^{14}C)	Air + CO_2 (95 + 5, v/v)	33 (29 to 34; 4 expts)
2	Glucose (U-^{14}C)	Air	46 (43 to 48; 4 expts)
3	Glucose (U-^{14}C) + acetate	Air	20

amount of cell material formed (as dry wt); they do not take into consideration any contribution to respiration by the [U-^{14}C]glucose during the growth period. As might be expected, the inclusion of glucose or acetate into the growth medium decreased the degree of incorporation of [^{14}C]carbon dioxide into the cell material of *A. variabilis* by an amount about equivalent to the [U-^{14}C]glucose incorporation (Table 2).

The relative contribution of carbon atoms 1 and 6 of glucose to respiratory carbon

dioxide has been used as an indication of the relative participation of glycolysis or the pentose phosphate pathway in glucose dissimilation (see Methods). A washed suspension of *Anabaena variabilis* was incubated with either [1-^{14}C]- or [6-^{14}C]glucose and the rate of [^{14}C]carbon dioxide production measured (Table 3). The C_6:C_1 ratio obtained was low and indicated an extensive operation of the pentose phosphate pathway. This ratio was not altered by incubation in light or dark, nor by growth of the organism in the presence of glucose. Furthermore, *A. variabilis* grown with glucose did not exhibit higher rates of carbon dioxide production from the 1-C or 6-C positions.

Table 2. *Incorporation of* [^{14}C] CO_2 *by Anabaena variabilis in the presence of glucose or acetate*

A. variabilis was grown in 20 ml. medium under conditions of illumination and temperature as described in the Methods section. The cultures were gassed by recycling a mixture of air + carbon dioxide. The carbon dioxide was released into the closed re-cycling system by the acidification of sodium [^{14}C]carbonate. The [^{14}C]carbon dioxide continually recycled through the cultures contained 100 μc, and the extent of incorporation was measured after incubation for 3 days.

Growth medium	Specific radioactivity (counts/min./mg. dry wt)	% inhibition of [^{14}C]CO_2 assimilation
CO_2 alone	108,000	0
CO_2 + acetate (20 mM)	88,600	18
CO_2 + glucose (20 mM)	52,000	51

Table 3. *Relative release of* [^{14}C]*carbon dioxide from 1-C and 6-C positions of glucose by Anabaena variabilis*

Double side arm Warburg flasks contained *A. variabilis* (equiv. 8 mg. dry wt) in 1·7 ml. growth medium; hyamine hydroxide (0·3 ml.) in the centre-well, 10% sodium dodecylsulphate (1 ml.) in one side-arm and glucose ([1-^{14}C] or [6-^{14}C], 100 μmole containing 2 μc) in the other. The [^{14}C]carbon dioxide evolved was dissolved by the hyamine hydroxide, and after termination of respiration by the addition of sodium dodecylsulphate the radioactivity in the hyamine hydroxide determined.

Carbon source for growth	Incubation	[^{14}C]carbon dioxide* derived from 1-C position	[^{14}C]carbon dioxide* derived from 6-C position	C_6:C_1 ratio
5% CO_2	Dark	2830	390	0·14
	Light	2690	430	0·16
5% CO_2 + glucose (20 mM)	Dark	2800	390	0·14
	Light	3100	410	0·13

* Expressed as counts/hr/mg. dry wt organism.

Enzymic activities

Many of the enzymes involved in glucose metabolism may readily be detected in extracts of *Anabaena variabilis* prepared either by extrusion through a French pressure cell or by ultrasonic disintegration. The specific activities of such enzymes are often low when compared with enzymes from heterotrophic bacteria, as is the growth rate of the blue-green alga under study. The most striking feature of the results presented in Table 4 is the similarity in activities found in extracts from organisms grown under different conditions. None of the enzymes examined was significantly altered by growth in the presence of glucose. Glucose-6-phosphate dehydrogenase and 6-phosphoglu-

conate dehydrogenase were specific for NADP. Triose phosphate dehydrogenase was active with either NAD or NADP. Recent work in this laboratory has indicated that these two enzymic activities are carried on the same protein and share the same active site (Hood & Carr, 1967). Triose phosphate formation by fructose diphosphate aldolase was measured by the reduction of dihydroxyacetone phosphate by $NADH_2$ to α-glycerophosphate with a commercial α-glycerophosphate dehydrogenase (see Methods).

Hexokinase, glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase were also measured in extracts of *Anacystis nidulans*, after growth in the presence or absence of glucose. Inclusion of glucose in the growth medium resulted in enzyme activities very slightly higher (not more than 10 %) than those found in cultures grown with carbon dioxide alone.

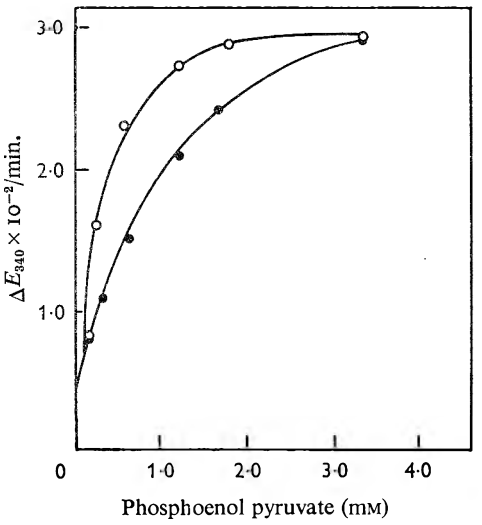

Fig. 4

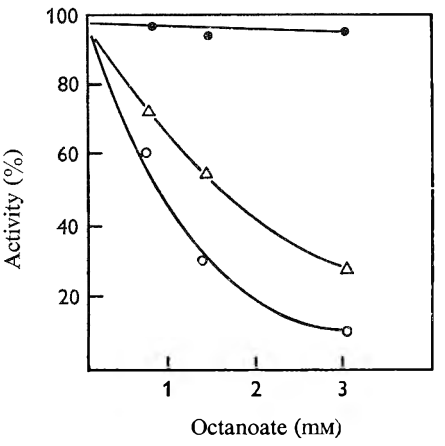

Fig. 5

Fig. 4. *Anabaena variabilis*. Activation of pyruvate kinase by fructose-1,6-diphosphate. Assay procedure as described in Methods, with various concentrations of substrate as shown, in the presence (○—○) or absence (●—●) of fructose-1,6-diphosphate (1 mM).

Fig. 5. Effect of octanoate of the activities of three glycolytic enzymes in extracts of *Anabeana variabilis*. Enzyme assays as in Methods except that octanoate was added as indicated. Results are expressed as the percentage activity of control without octanoate. ●—●, Pyruvate kinase; △—△, phosphofructokinase; ○—○, and glucose-6-phosphate dehydrogenase.

In contrast to the lack of adaptive response that enzymes of glucose metabolism of *Anabaena variabilis* displayed, inhibition and activation of enzyme activity by end-products and associated molecules was readily demonstrated. The effect of increasing concentrations of ATP upon phosphofructokinase activity is illustrated in Fig. 3. Activity was maximal with 1·2 mM-ATP; above this concentration ATP became increasingly inhibitory. This inhibition was annulled by AMP (0·3 mM) and to a lesser extent by ADP (3 mM); inorganic phosphate did not annul the inhibition caused by excess ATP. Citrate (1 mM) inhibited phosphofructokinase activity completely and phosphoenolpyruvate (0·5 mM) did so to 44 % of the standard assay control. Pyruvate kinase was activated by fructose-1,6-phosphate (Fig. 4), there being no evidence of a

sigmoidal character to the reaction curve. This enzyme was similarly activated by fructose-6-phosphate and glucose-6-phosphate. Citrate, which may be considered to be an end-product of glycolysis, inhibited pyruvate kinase (Table 5). Free fatty acids inhibit the activity of glycolytic enzymes in rat liver preparations (Weber, Convery, Lea & Stamm, 1966). The inhibition by different concentrations of octanoate of glucose-6-phosphate dehydrogenase, phosphofructokinase but not of pyruvate kinase, activity in extracts of *A. variabilis* is shown in Fig. 5.

Table 4. *Glycolytic and pentose phosphate pathway enzymes in extracts of Anabaena variabilis grown autotrophically and in the presence of glucose*

Except where indicated activity is expressed as mμmole/min./mg. protein; values are mean of several determinations.

	Carbon source	
Enzymes	CO_2	CO_2 + glucose (20 mM)
Hexokinase	1·2	1·2
Phosphohexoisomerase	5·4	5·3
Phosphofructokinase	8·1	7·8
Fructose diphosphate aldolase	3·6	3·6
Triose phosphate dehydrogenase (NAD)	8·3	7·5
Triose phosphate dehydrogenase (NADP)	4·2	3·7
Pyruvate kinase	13	11
Glucose-6-phosphate dehydrogenase	8·7	7·8
6-Phosphogluconate dehydrogenase	12	12
Phosphoriboisomerase*	0·35	0·32

* Activity expressed as E_{540}/min./mg.

Table 5. *Effect of metabolites on pyruvate kinase activity in extracts of Anabaena variabilis*

The pyruvate kinase assay was as in Methods except that 3 μmole phosphoenolpyruvate was used.

Addition	Concentration (mM)	Activity (%)
None	—	100
Citrate	0·1	81
	1·0	0
Fructose-6-phosphate	0·3	116
	3·0	144
	10·0	145
Glucose-6-phosphate	1·0	112
	10·0	155

DISCUSSION

The concept that autotrophic micro-organisms utilize only carbon dioxide for the biosynthesis of cellular material has undergone some revision in recent years. It is clear from the data presented here, and from those mentioned in the Introduction, that some species of blue-green algae (namely *Anabaena variabilis*, *Anacystis nidulans*) assimilate and metabolize exogenous organic compounds, even though carbon dioxide still serves as a major source of carbon. Similarly, species of chemosynthetic bacteria

which are 'obligate' autotrophs, can assimilate various reduced carbon compounds (see Kelly, 1967; Smith *et al.* 1967). The studies on incorporation of organic materials into autotrophs have led to interest in intermediary metabolism of these organisms. An incomplete tricarboxylic acid cycle has been found in blue-green algae and in chemosynthetic bacteria (Kelly, 1967; Pearce & Carr, 1967*b*; Smith *et al.* 1967). The latter workers suggested that the lack of NADH oxidase and of α-oxoglutarate dehydrogenase can account for obligate autotrophism.

The present results with *Anabaena variabilis* show no abnormalities of metabolic pathway in glucose metabolism. Although enzymes necessary for the glycolytic and pentose phosphate pathways are both present, the respiratory experiments with [1-^{14}C]- and [6-^{14}C]glucose indicate that the latter is the dominant pathway. The labelling pattern of polysaccharides, respiratory data and the use of inhibitors led Cheung & Gibbs (1966) to conclude that this was the main route of glucose dissimilation in *Tolypothrix tenuis*. Earlier work did not detect aldolase in blue-green algal extracts (Richter, 1959; Fewson, Al-Hafidh & Gibbs, 1962) but later experiments measured and examined this enzyme in extracts of the same species (Willard *et al.* 1965; Van Baalen, 1965; Willard & Gibbs, 1967). In *Anabaena cylindrica* experiments with isotope-labelled compounds indicated that the pentose phosphate pathway was the major but not the only operative pathway (Wildon & ap Rees, 1965).

The most striking feature of the enzymic activities measured in *Anabaena variabilis* in the present work is the failure of glucose in the growth medium to alter the activities of enzymes concerned in its metabolism (Table 4) or the over-all rate of glucose dissimilation (Table 3). Incorporation and respiratory studies showed that exogenous glucose was metabolized and could contribute up to 46% of the total cell material. The inclusion of glucose in the growth medium has been shown to increase, by up to three-fold, hexokinase activity in *Escherichia coli* (Scott & Chu, 1959) and *Clostridium perfringens* (Ivanov, 1959) and glucose-6-phosphate dehydrogenase in *Pseudomonas aeruginosa* (Hamilton & Dawes, 1960) and *E. coli* (Pakoskey, Lesher & Scott, 1965).

The control in *Anabaena variabilis* of enzymic activity by end-product inhibition and allosteric activation was readily demonstrated. The inhibition of phosphofructokinase by 5 mM-ATP and the annullment of inhibition when 0·3 mM-AMP was present is similar to that found in yeast (Ramaiach, Hathaway & Atkinson, 1964) and in *Escherichia coli* (Lowry & Passonneau, 1964). The activation of pyruvate kinase by fructose-1,6-phosphate found in *A. variabilis* has also been observed in rat liver preparations which show distinct sigmoidal character and is clearly an allosteric effect (Taylor & Bailey, 1967). The inhibition by octanoate of several liver enzymes involved in glucose metabolism, including glucose-6-phosphate dehydrogenase and phosphofructokinase, has been described (Weber *et al.* 1966). Octanoate inhibited these two enzymes in *A. variabilis* extracts (Fig. 5) but had no effect on pyruvate kinase which, in contrast, was markedly inhibited in the liver preparation of Weber *et al.* (1966).

The failure to control enzymic activity in *Anabaena variabilis* by repression or de-repression has been noted previously with respect to acetate metabolism, and its possible role in autotrophy has been discussed (Pearce & Carr, 1967*a*). The identical activities of glucose metabolizing enzymes, after growth in the presence and absence of glucose, is consistent with the view that *A. variabilis* does not adjust its metabolism as efficiently as many other microbial species and exerts control at the level of pre-formed enzymes rather than at enzyme synthesis. This may account, fully or in part,

for the inability of *A. variabilis* and other autotrophic blue-green algae to increase their growth rate in the presence of exogenous substrates. However, at least one example of enzyme repression, that of nitrite reductase by ammonium ion, exists in blue-green algae (Carr, 1967) and others may await detection.

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Effect of Oxygen on Growth of *Azotobacter chroococcum* in Batch and Continuous Cultures

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SUMMARY

Initiation of growth of nitrogen-fixing Azotobacter species was prevented by efficient aeration but proceeded normally with gentle aeration; addition of CO_2 to the air did not relieve inhibition. The ratio of oxygen solution rate to concentration of organisms determined whether growth would be inhibited or not. Populations growing in media containing fixed nitrogen (NH_4^+) showed no unusual sensitivity to oxygen though inhibition could be induced at a P_{O_2} value of 0·6 atm. Nitrogen-limited continuous cultures fixed about twice as much N_2/g. carbon source utilized at 0·03 atm. O_2 than at the atmospheric value (0·2 atm.); even at relatively high cell concentrations growth was inhibited at 0·6 atm. O_2. Carbon- and phosphate-limited continuous cultures showed even more sensitivity to oxygen when fixing nitrogen but none when growing with NH_4^+; excessive oxygen was lethal to phosphate-limited populations. These observations suggest that two mechanisms exist in the cell to protect the oxygen-sensitive components of nitrogenase from oxygen: augmented respiration to scavenge excess oxygen and a conformational state of nitrogenase that prevents damage by O_2.

INTRODUCTION

High oxygen tensions inhibit or delay the growth of many aerobic or facultatively anaerobic bacteria (Moore & Williams, 1911) including Azotobacter species (Meyerhof & Burk, 1928). However, the mechanisms of such oxygen toxicity remain, to a large extent, obscure. Barron (1955) proposed that harmful effects of oxygen on biochemical material were due mainly to non-specific oxidation of enzyme sulphydryl groups. Chance, Jamieson & Coles (1965) found that hyperbaric oxygen inhibited energy-linked pyridine nucleotide reduction in mitochondria at 11 to 17 atm. and suggested that oxidation of sulphydryl groups may be important. Certain nutrients may protect in the case of Achromobacter P6, since inhibition at high oxygen tensions depended on the nature of the carbon and energy source and could be reversed by amino acid supplements (Gottlieb, 1966).

Oxygen toxicity in Azotobacter at partial pressures of oxygen above a P_{O_2} of 0·6 atm. seems fairly well established (Meyerhof & Burk, 1928; Tschapek & Giambiagi, 1955; Schmidt-Lorenz & Rippel-Baldes, 1957), although Fife (1943) reported that respiratory activity of Azotobacter increased up to a P_{O_2} of 0·8 atm. Meyerhof & Burk orginally regarded oxygen toxicity as an inhibition of nitrogen fixation, but the phenomenon was later considered to be a general inhibition of growth (Burk, 1930). Parker (1954) argued that, if metabolic hydrogen is involved in the reduction of nitro-

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gen to ammonia, then oxygen should compete for this hydrogen and hence depress fixation. Parker & Scutt (1960), assuming that spot analyses for N were measures of nitrogen assimilation rates, obtained kinetic data from growth experiments which showed oxygen to be a competitive inhibitor of nitrogen fixation. Parker (1954) found that nitrogen fixation was more efficient at low oxygen tensions: three times as much nitrogen was fixed per unit glucose consumed at a P_{O_2} of 0·04 atm. than a P_{O_2} of 0·2 atm. Tschapek & Giambiagi (1955) proposed that the influence of P_{O_2} on nitrogen fixation was intimately linked with respiration but offered no explanation of the inhibition observed. Attempts to shift the inhibitory P_{O_2} to above 0·6 atm., by using different substrates, were unsuccessful and served only to indicate that the mechanism was complex. Schmidt-Lorenz & Rippel-Baldes (1957) found that increased P_{O_2} only affected nitrogen fixation and not assimilation of bound nitrogen; they came to the conclusion that the unfavourable influence of increasing P_{O_2} on the efficiency of nitrogen fixation was due more to an effect on the E_h of the environment than a direct effect on some enzyme system. Khmel, Gabinskaya & Ierusalimsky (1965) observed that yields of *Azotobacter vinelandii* fermentations were highest at low aeration rates and that maximum nitrogen fixation occurred at an aeration rate of 18·7 mmole O_2/l./hr. Dilworth (1962) found that increasing the P_{O_2} above 0·6 atm. with *A. vinelandii* caused pyruvate accumulation and attributed the O_2 inhibition observed to inactivation of the pyruvic oxidase system.

All the results so far have been obtained from studies with batch cultures. Under such conditions the environment is continually changing and reproducibility in the behaviour of populations is sometimes difficult to obtain (Malek, 1966). In this paper we have re-investigated the effect of P_{O_2} on growth and nitrogen fixation in *Azotobacter chroococcum* by using both batch cultures and continuous cultures.

METHODS

Organisms and media. Stock cultures of *Azotobacter chroococcum* NCIB 8003, *Azotobacter vinelandii* NCIB 8660 and *Azotobacter macrocytogenes* NCIB 8700, were maintained on slopes of medium 'mannitol B' (Burk's medium as prescribed by Newton, Wilson & Burris (1953) with mannitol in place of sucrose) and stored at room temperature. For continuous cultures a modified Burk's medium ('mannitol B_6') was used which contained (g./l.): mannitol, 10; K_2HPO_4, 0·64; KH_2PO_4, 0·16; NaCl, 0·2; $MgSO_4.7H_2O$, 0·2; $CaCl_2$, 0.1; distilled water plus these trace elements (mg./l.): $FeSO_4.7H_2O$, 2·5; H_3BO_3, 2·9; $CoSO_4.7H_2O$, 1·2; $CuSO_4.5H_2O$, 0·1; $MnCl_2.4H_2O$, 0·09; $Na_2MoO_4.2H_2O$, 2·5; $ZnSO_4.7H_2O$, 1·2; nitrilotriacetic acid, 100. NH_4Cl was 200 mg./l. when added; final pH, 7·4 ± 0·2. Concentrations of mannitol, phosphate and other constituents were altered occasionally as mentioned below.

Twenty-litre batches of medium were autoclaved at 121° for 45 min., after which time a precipitate was visible. This disappeared after several days at room temperature; the medium was not used until all the precipitate had dissolved.

Viabilities. Slide culture (Postgate, Crumpton & Hunter, 1961) on mannitol B_6 medium solidified with agar was used. Annuli were of stainless steel, 1·2 mm deep; incubation was for 18 to 24 hr at 25° and viabilities were reproducible to ± 3 %.

Apparatus. Continuous culture apparatus with working volumes of approximately 200 ml. as described by Baker (1968) was used and maintained at 30°. For batch-

culture experiments similar vessels without side arms were used. Aeration was effected by magnetic stirring which gave oxygen solution rates of up to 180 mmole O_2/l./hr (see Results). Oxygen solution rates were determined by the sulphite oxidation method (Cooper, Fernstrom & Miller, 1944).

The oxygen concentration in the culture was continuously measured by using the membrane-covered Clark electrode (Protech Ltd., Rickmansworth). The partial pressures of gases in the gas stream were regulated by gas flowmeters (Rotameter Mfg Co., Croydon), corrected for differences in gas density, the rates of flow being taken as proportional to the partial pressures of gas in the final mixture. At P_{O_2} values below atmospheric, the P_{N_2} was kept constant at 0·8 atm. and argon was introduced into the gas stream to give the required P_{O_2}. Above atmospheric P_{O_2}, no argon was used and P_{N_2} was equal to $(1-P_{O_2})$. The buffering action of the medium was sufficient to maintain a constant pH value of 6·8 with nitrogen-fixing populations, so automatic pH control was necessary only with ammonia-grown bacteria.

Criteria of nutrient limitation. Chemostat cultures were considered limited by a given nutrient when: (1) reduction in concentration of the nutrient by 50% decreased bacterial concentrations proportionately; (2) a doubling in concentration of all other nutrients in the medium had no effect on bacterial concentration. In the case of N_2 limitation, the first criterion was not readily applicable, but a doubling of all medium constituents produced no increase in bacterial concentration. Electrode readings indicated that oxygen was present in excess and so, by elimination, N_2 gas was considered to be growth-limiting.

Analytical procedures. To determine organism concentration, duplicate 10 ml. samples of culture were filtered through weighed membrane filters (Oxoid Ltd.), the filters washed with an equal volume of distilled water, dried in an oven and weighed. Frequent determinations were made by nephelometric measurements, but these served only as a general indication of cell mass and were not always proportional to it. In continuous cultures a variation in the opacity $\pm 2\%$ or less over four doubling times was taken as an indication of steady state conditions. Mannitol was estimated by periodate oxidation (Neish, 1950). Cultures were sampled into a few drops of concentrated H_2SO_4 (to stop metabolism), centrifuged, and 0·2 to 1 ml. of supernatant fluid analysed. Nitrogen was determined by the micro-Kjeldahl technique, followed by colorimetric analysis of ammonia with Nessler's reagent (Meynell & Meynell, 1965). Respiratory activity was measured by conventional Q_{O_2} estimations in Warburg manometers at 30°. The samples were transferred as rapidly as possible from the culture vessel to the Warburg flask and underwent no centrifuging or washing procedure. For cytochrome spectra, samples were centrifuged and the deposit suspended in medium B_6 to give a final concentration equivalent to 10 mg./ml. dry wt bacteria. Two ml. of the concentrated sample were transferred to a diffuse reflectance cell and extinction measured over the range 400–600 mμ in a Unicam S.P.700 spectrophotometer. Sodium dithionite was added to obtain reduced spectra.

RESULTS

Effect of aeration on growth in batch cultures

Batch cultures of *Azotobacter chroococcum* did not grow after inoculation when subjected to vigorous aeration, but grew when the aeration was initially only slight (Dalton & Postgate, 1967). Inhibition by high aeration of various aerobic bacteria has

been generally attributed to lack of CO_2, which is necessary for the initiation of growth of many aerobes (Walker, 1932; Gladstone, Fildes & Richardson, 1935). Such an explanation did not apply to the nitrogen-fixing organisms *A. chroococcum*, *A. vinelandii* and *A. macrocytogenes*, which could be inhibited by efficient aeration even under an atmosphere containing 0·02 atm. CO_2. *Azotobacter chroococcum* was chosen for further investigation since its behaviour was also being studied in continuous cultures, which were a source of reproducible inocula.

Table 1. *Aeration and growth of Azotobacter chroococcum*

Batch cultures were inoculated with *A. chroococcum* and stirred at different rates corresponding to the oxygen solution rates quoted for 0·2 atm. O_2. Growth was assessed as visible turbidity; for further details see text.

	N_2-grown			NH_4^+-grown		
O_2 solution rate (mmole O_2/l./hr)	42	42(+CO_2)	3·2	42	42(+CO_2)	3·2
P_{O_2} (atm.)						
0·08	+	+	+	+	+	+
0·2	−	−	+	+	+	+
0·4	−	−	+	+	+	+
0·6	−	−	+	−	−	+

Three batch cultures were set up in vessels resembling those used for continuous culture and containing the regular medium with 10 g. mannitol/l. Two of these were stirred to give the relatively high aeration rate of 42 mmole O_2/l./hr, one of these had 0·02 atm. CO_2 in the atmosphere passed over the culture, the third was stirred to give the relatively low aeration rate of 3 mmole O_2/l./hr. The cultures were inoculated to equiv. 0·3 μg. dry wt *Azotobacter chroococcum*/ml. from a nitrogen-limited continuous culture and incubated for up to 4 days at 30°. The results are illustrated in Table 1; inhibition by high aeration rates was complete with nitrogen-fixing populations even at atmospheric P_{O_2} values; only at very high P_{O_2} values were NH_4^+-utilizing strains inhibited. Inhibition depended on inoculum size: at equiv. 0·75 μg. dry wt organism/ml. growth was not completely inhibited but merely delayed for about 20 hr; at 1·5 μg./ml. high aeration induced a lag of about 10 hr as compared with the normal lag of 4 to 5 hr. These observations showed that inhibition of growth, apparently due to oxygen toxicity and not to CO_2 deprivation, could occur among Azotobacter species in batch cultures. The problem was studied further in continuous flow culture systems in which various parameters influencing growth were more readily controlled.

Effect of aeration on growth and nitrogen fixation in continuous culture

A steady-state population of *Azotobacter chroococcum* growing at a dilution rate of 0·2 hr^{-1} and P_{O_2} 0·2 atm. was established (P_{N_2} 0·8 atm.) in continuous culture. The culture was sampled on three separate days and analysed for nitrogen content, mannitol consumption, dry weight of organism and respiratory activity. The P_{O_2} was then altered and the analyses repeated when the culture was considered to be in a new steady state. Respiratory activity increased with P_{O_2} from Q_{O_2} 250 μl.mg./hr at 0·025 atm. O_2 to a plateau of Q_{O_2} 950 between 0·2 and 0·5 atm. O_2.

Figures for the steady state yields of organism and of nitrogen fixed under various partial pressures of oxygen are plotted in Fig. 1. The yield of organisms showed a

plateau about the range of normal air and decreased at very low or high P_{O_2} values. In contrast, the nitrogen fixed per g. mannitol increased at low P_{O_2} values to uncommonly high values. The efficiency of nitrogen fixation at the ordinary atmospheric P_{O_2} of 0·2 (37 mg. N/g. mannitol) is higher than most values recorded in the literature; we attribute this fact to the use of continuous culture, because the criteria given in Methods, together with other evidence, indicated that the growth-limiting substrate in these conditions was nitrogen gas, and cultures in such conditions would select for bacterial variants of high nitrogen-fixing activity.

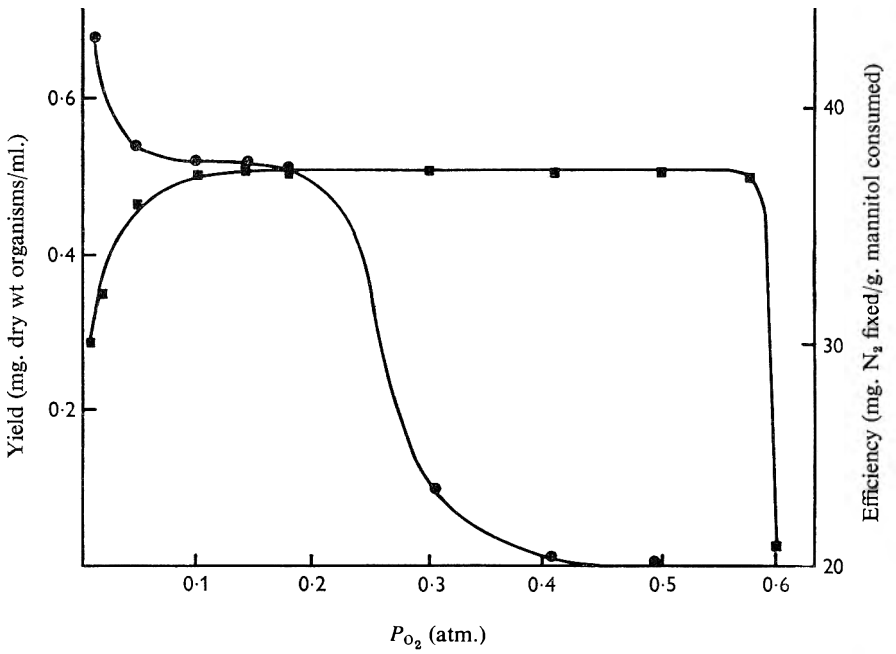

Fig. 1. Concentrations of bacteria and efficiencies of nitrogen fixation in continuous cultures of *Azotobacter chroococcum* under various partial pressures of oxygen at $D = 0{\cdot}2\ hr^{-1}$. ●, efficiency; ■, organism concentration.

These cultures were not particularly sensitive to high oxygen tensions until P_{O_2} values in excess of 0·6 atm. were reached; this figure is comparable to figures given by earlier workers and discussed in the introduction. Steady-state populations were then established at $P_{O_2} = 0{\cdot}2$ atm. and the mannitol concentration decreased until this substrate became formally the growth-limiting substrate and determined the steady-state yield of organisms. Such populations proved difficult to establish and unstable when established because they showed hypersensitivity to oxygen even at normal atmospheric P_{O_2}: at a dilution rate of $0{\cdot}14\ hr^{-1}$ a mannitol-limited nitrogen-fixing population (grown with mannitol 0·15 g./l. medium) showed 30% inhibition of growth (yield declined from 0·35 mg./ml. to 0·24 mg./ml.) in 2 hr when the oxygen supply to the organism was increased, either by increasing the stirring rate from 680 to 1100 rev./min. or the P_{O_2} from 0·2 to 0·5 atm. (the stirring rates quoted approximate to O_2 transfer rates of 16·0 mmole O_2/l./hr and 70 mmole O_2/l./hr respectively). The population recovered when the oxygen supply was brought back to normal.

Carbon-limited populations growing with fixed nitrogen (1·5 g./l. NH_4Cl under $A + O_2$ mixtures) did not show such sensitivity to aeration.

Tests of the carbon-limited nitrogen-fixing population by using oxygen electrodes showed that an ambient oxygen concentration in the actual culture of 10 μM (equivalent to 0·01 atm. O_2 at saturation) was the highest tolerated. When the dissolved oxygen concentration exceeded this value, growth was inhibited; when it fell much below this value, oxygen tended to become the growth-limiting substrate. Nitrogen-limited populations required an ambient O_2 of about 20 μM for maximum growth and were more tolerant of increased O_2 supply than C-limited populations. However,

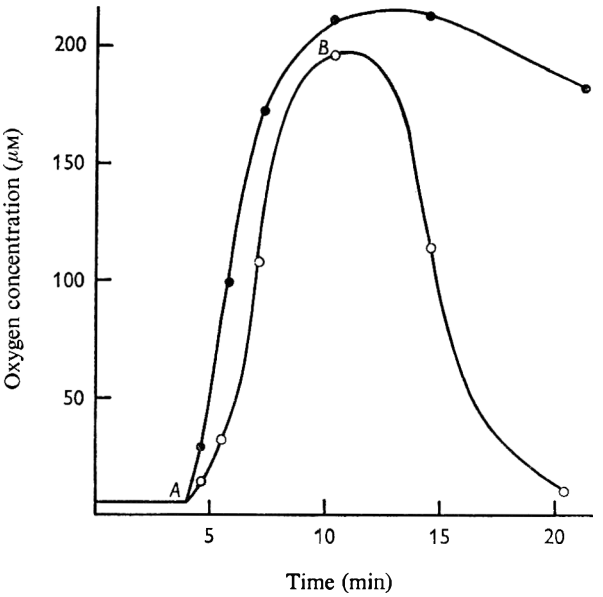

Fig. 2. Effect of increasing P_{O_2} on dissolved O_2 in a steady-state culture of *Azotobacter chroococcum* at $D = 0{\cdot}15$ hr^{-1}. ●—●, P_{O_2} increased from 0·24 to 0·39 atm. at *A*. ○—○, P_{O_2} increased from 0·24 to 0·39 atm. at *A*, returned to 0·24 atm. at *B*.

inhibition of growth did occur when the dissolved O_2 concentration was in excess of 30 μM. Oxygen inhibition was reflected in dramatic changes in the dissolved oxygen concentration when aeration conditions were altered. Figure 2 illustrates the effect of increasing the P_{O_2} over a nitrogen-limited culture in a steady state at $D = 0{\cdot}15$ hr^{-1}.

The hypersensitivity of carbon-limited populations to oxygen inhibition suggested that nitrogen-limited populations, if grown slowly so as to allow maximum use of available carbon, might show comparable hypersensitivity. Such a population, grown slowly at 0·05 hr^{-1} but with mannitol (10 g./l.), which would normally be sufficient, showed inhibition by high aeration (190 mmole O_2/l./hr) under the normal P_{O_2} of 0·2 atm.; it did not show such inhibition at faster growth rates. The question whether the population was shifted from a formal N_2 limitation to a formal mannitol limitation by an increase in the aeration rate will be considered in the Discussion.

Phosphate-limited populations were also hypersensitive to oxygen. A phosphate-limited, nitrogen-fixing population (1·78 mg./P/l.), growing at a dilution rate of 0·115 hr^{-1} with an oxygen solution rate of 15 mmole O_2/l./hr (P_{O_2} = 0·2 atm.), gave

a yield of 0·95 mg. dry wt organism/ml. Increasing the oxygen solution rate to 70 mmole O_2/l./hr, by increasing the stirring rate, decreased the yield to 0·35 mg./ml. over a 14 hr period. Similarly, an increase in P_{O_2} from 0·08 to 0·20 atm. at the higher stirring rate (1050 rev./min.) caused a decrease in yield from 0·7 mg./ml. to 0·25 mg/ml. over a 24 hr period at a dilution rate of 0·14 hr^{-1}. As with mannitol-limited populations, the effect was reversible; however, the culture could be sterilized completely when high aeration was continued for 3 days at a dilution rate of 0·05 hr^{-1}. Populations using NH_4^+ instead of fixing atmospheric N_2 again showed no such oxygen sensitivity.

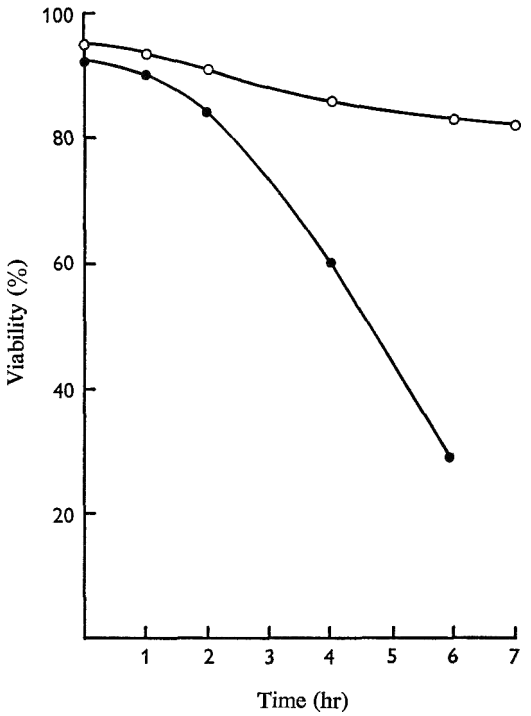

Fig. 3. Survival of over-aerated populations of *Azotobacter chroococcum* from continuous cultures. Steady-state populations of *A. chroococcum* were established at $D = 0{\cdot}2\ hr^{-1}$ in mannitol-limited (1·5 g. mannitol/l.) and phosphate-limited (0·05 g. K_2HPO_4/l.) conditions and gave 0·4 and 0·45 mg. dry wt organisms/ml. respectively. At zero time, flow of medium was interrupted and aeration was increased from 25 to 60 mmole O_2/l./hr. Viabilities were then assessed by slide culture (see text). ○, Carbon-limited population; ●, phosphate-limited population.

Lethality of oxygen

The lethality of oxygen to sensitive populations was studied by obtaining steady-state carbon-limited and phosphate-limited populations of similar population densities to avoid population effects on survival (Postgate & Hunter, 1963). Then, simultaneously, the flow of medium was stopped and the aeration rate increased just over twofold; the viability was then monitored by slide culture during several hours. This procedure gave a measure of the lethality of oxygen to the populations, uncomplicated by 'cryptic' growth which occurred when the cultures were left running. Figure 3 shows that oxygen was lethal to 70% of a phosphate-limited population over 6 hr;

it was much less lethal to the carbon-limited population, though the decline in viability recorded is significant. Nitrogen-limited populations showed no decline in viability in comparable conditions.

DISCUSSION

Increased efficiency of nitrogen fixation at low P_{O_2} values observed with batch cultures of Azotobacter by Parker (1954) was attributed to competition between oxygen and nitrogen for electrons by Parker & Scutt (1960), nitrogen fixation being regarded as a form of respiration. Our findings confirm the observations of Parker and his colleague and extend them to organisms in continuous culture; the complete inhibition of growth by high levels of oxygenation which we have observed presumably reflects an extreme state of oxygen competition, since it is specific to nitrogen-fixing populations. Inhibition was most marked with carbon-limited and phosphate-limited populations when these were fixing nitrogen and in these circumstances oxygen could be lethal to the organisms. We present an interpretation of the effects of oxygen on the physiology of Azotobacter on the basis of these findings and present knowledge of the behaviour of cell-free nitrogenase preparations.

Nitrogen-fixing systems have been extracted from *Azotobacter vinelandii* (Bulen, Burns & LeComte, 1965) and *A. chroococcum* (Kelly, 1966) in particulate forms which, unlike the soluble extracts of *Clostridium pasteurianum* (Carnahan, Mortenson, Mower & Castle, 1960), are reasonably stable in air. They can be further resolved into two soluble components, both of which are necessary for nitrogen fixation but which have become extremely sensitive to oxygen and readily damaged unless handled in anaerobic conditions (Bulen & LeComte, 1966; Kelly, Klucas & Burris, 1967). Nitrogen-fixing cell-free extracts of blue-green algae also appear to be oxygen-sensitive (Fay & Cox, 1967). These observations provide evidence that, even in aerobic bacteria, the components of nitrogenase are intrinsically sensitive to oxygen. The living organism, since it grows in air, must possess a mechanism for protecting the nitrogen-fixing site from oxygen. The oxygen-tolerance of the particulate preparations suggests that some steric arrangement of the components makes for oxygen tolerance and, in principle, this 'conformational protection', as we shall term it, could operate in two ways. Either the oxygen-sensitive sites could be inaccessible to oxygen, or they could be stabilized by conformational features of the nitrogenase complex so that, though accessible to oxygen, they would be undamaged by it.

Our findings lead us to assume that a second protective mechanism also operates in the living organism: that respiration is used to scavenge oxygen from the neighbourhood of the nitrogen-fixing site. Such a mechanism was proposed by Phillips & Johnson (1961) as a result of their observations that *Azotobacter vinelandii*, given excess oxygen, consumed sugar at rates greater than those necessary to supply the energy requirement. They suggested that respiration functioned as an 'oxygen-wasting system' which maintained a low E_h value within the cell, presumed to be necessary for nitrogen fixation. Evidence pointing to respiration as a protective process is provided by numerous other publications, including Meyerhof & Burk (1928), Burk (1930), Parker (1954), Schmidt-Lorenz & Rippel-Baldes (1957), Parker & Scutt (1960), Dilworth & Parker (1961) and Khmel *et al.* (1965), even though their findings were not always interpreted as implying a protection process. Our reasons for regarding respiration as protective to the nitrogenase complex are the following. (1) Respiration

in nitrogen-limited nitrogen-fixing cultures is adjusted to balance oxygen supply and leaves nitrogen fixation largely unaffected. In conditions of high aeration or high P_{O_2} nitrogen fixation became remarkably inefficient in terms of carbon substrate consumed; in near anaerobic conditions it became highly efficient, though the growth yield of the population was then not impressive. (2) In carbon-limited nitrogen-fixing cultures, which could not adjust their respiration to match oxygen tension, extreme oxygen sensitivity was observed. (3) The inhibitory P_{O_2} in the gas phase varied according to the population density but the maximum tolerated molarity of oxygen in the culture medium remained much the same, indicating that a balance between solution and consumption of oxygen was being actively maintained. (4) Hypersensitivity to oxygen was not shown by populations that were not fixing nitrogen.

Our direct measurements of Q_{O_2} values showed a trend in the direction of high Q_{O_2} corresponding to high P_{O_2}, as we should expect on this hypothesis, but we do not attach great significance to the actual Q_{O_2} values because of the rapid alteration in Q_{O_2} that follows handling of bacterial populations. Nevertheless, it seems logical to accept that the high Q_{O_2} values for which the Azotobacteriaceae are famous (4000 to 5000 μl. O_2/mg. dry wt/hr; Williams & Wilson, 1954) represent a mechanism for excluding oxygen from parts of the cell.

The existence of some kind of conformational protection, supplemented in the actively growing and fixing organism by respiratory protection, accounts for the behaviour of nitrogen-limited and putatively carbon-limited populations. It raises the question whether the so-called carbon-limited populations obtained here were truly carbon-limited. The yield certainly depended on the carbon concentration in the inflowing medium, but it was likely that the rates of carbon and nitrogen assimilation were finely balanced such that a small excess of carbon was available for the respiratory protection mechanism. The concept of limitation by single nutrients in chemostats thus becomes rather involved in a circumstance in which oxygen concentration is critical and access of nitrogen to nitrogenase is limited by some intracellular mechanism.

Ammonia-grown organisms showed no abnormal sensitivity to oxygen; the failure of such inocula to grow at P_{O_2} values of 0·6 is probably analogous to the sensitivity to hyperbaric oxygen shown by other bacteria. Many microbes respond to plentiful availability of oxygen by bringing in non-cytochrome respiratory pathways (Lenhoff, Nicholas & Kaplan, 1956; Rosenberger & Kogut, 1958). Reflectance spectra (Giovanelli, 1957), as adapted to Azotobacter by Moss & Tchan (1958), were determined on bacteria grown with high and low aeration. No impressive change in the heights of the cytochrome peaks between 400 and 650 mμ appeared, suggesting that a non-cytochrome pathway was not used by *Azotobacter chroococcum*. Moreover, the sensitivity of phosphate-limited populations to oxygen suggests that the respiratory protective mechanism operates through the cytochrome pathway, since otherwise there is no reason for such cells to be sensitive. The ATP:ADP ratio is known to exert a controlling effect on mitochondrial respiration (Klingenberg & Schollmeyer, 1960), and there are indications that phosphate compounds and nucleotides exert some respiratory control in bacteria (Ishikawa & Lehninger, 1962; Revsin & Brodie, 1967); ADP will stimulate and ATP inhibit the respiration of *A. chroococcum* (M. G. Yates, personal communication). If, in the phosphate-limited population, respiration is suddenly augmented to compensate for an increase in oxygen concentration, then ADP would be converted to ATP. If the control mechanism were analogous to that in

mitochondria, the ATP:ADP ratio would thus be shifted in favour of decreased respiration and, because the respiratory protective mechanism had been thwarted, the nitrogenase would no longer function properly and growth would cease. In these circumstances permanent damage to the nitrogenase might ensue and O_2 might become lethal; the exceptional lethality of oxygen to phosphate-limited populations might well be a reflexion of this condition.

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