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The Fine Structure of the Zoospores and Cysts of *Allomyces macrogynus*

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SUMMARY

Zoospores and cysts of *Allomyces macrogynus* have been studied by electron microscopy. A membrane-bound nuclear cap filled with ribosomes surrounded a single nucleus. Mitochondria and lipid bodies were found at the periphery of the nuclear cap. The flagellum was attached close to the nucleus by two tubular structures running anteriorly along both sides of the nuclear cap. During encystment the axoneme was retracted, the nuclear cap dissected with endoplasmic reticulum and the mitochondria increased in number and started to elongate. Concentric granules and/or mitochondria were found embedded in the nuclear cap.

INTRODUCTION

Examination of the posteriorly uniflagellate spores of the aquatic Phycomycetes by electron microscopy, primarily of thin sections, has revealed a structural organization from which generalizations can be made. The zoospores of *Monoblepharella* sp. (Fuller & Reichle, 1968), *Blastocladiella emersonii* (Reichle & Fuller, 1967), *Rhizidiomyces apophysatus* (Fuller & Reichle, 1965) and *Nowakowskiella profusa* (Chambers, Markus & Willoughby, 1967) all possess a ribosomal region (nuclear cap) closely associated with the single nucleus. The ribosomal region is membrane-bound in all except *Rhizidiomyces apophysatus*. They all possess a flagellum that in cross-section shows the typical 9+2 fibrillar configuration, a kinetosome and centrioles. However, the details of nuclear-cap organization and flagellar anchorage vary from organism to organism. All are surrounded by a cell membrane not enveloped by a cell wall. Mitochondria, endoplasmic reticulum, vacuoles, vesicles and lipid bodies are structures typically found in all of these spores.

This work was undertaken to determine the fine structure of *Allomyces macrogynus* zoospores and cysts as a preliminary to a study of sporogenesis and spore germination. Similarities and differences between these structures and closely related aquatic Phycomycetes are pointed out.

METHODS

The organism was grown in Petri dishes filled with Emerson (1958) YpSs agar for 5 days at room temperature in complete darkness. The plates were flooded with sterile tap water and allowed to stand for 45 min., after which the zoospores were in heavy concentration. The spores began to emerge from sporangia about 15–20 min. after being flooded. The time from flooding the plates to fixation was about 55 min. Spores prepared in this manner represented a heterogeneous population in which motile spores and those that had encysted were always present, although there were fewer encysted spores than motile spores.

The spores were collected by centrifugation and fixed in 1 % OsO_4 in 0·1 M-cacodylate buffer at pH 7·0 for 45 min. After fixation they were washed with cacodylate buffer, pelleted in 1 % water agar and dehydrated in a series of ethanol-water mixtures. The pellets were embedded in Epon 812 and sectioned with a Porter–Blum microtome employing glass knives. The sections were stained for 10 min. in 3·5 % aqueous uranyl acetate followed by lead citrate (Reynolds, 1963) and viewed with an RCA-EMU 3G electron microscope.

RESULTS

A near median section of a typical zoospore is shown in Pl. 1, fig. 1. Prominent among the features are the membrane-bound ribosomal region, herein referred to as the nuclear cap, a well-defined nucleus and a large nucleolus. A single large mitochondrion is usually found at the posterior end adjacent to the flagellum, with smaller mitochondria lining the periphery of the nuclear cap. Associated with most mitochondria is a small strand of endoplasmic reticulum. A few ribosomes are usually found lying between the strands of endoplasmic reticulum, Pl. 3, fig. 7. When the zoospore loses its motility it becomes spherical, the flagellum is retracted, the nuclear cap becomes dissected with endoplasmic reticulum and there is a proliferation of mitochondria; thus the motile spore develops into a cyst. A comparison of Pl. 1, fig. 1 and 2 shows these changes, which take place in a period of 30 min. in an aqueous environment.

Mitochondria, as shown in Pl. 1, fig. 1 and Pl. 3, fig. 5, are spherical in the motile spore with inclusions that are of the approximate size and staining character of ribosomes in the nuclear cap. The close association of the large mitochondrion with the nucleus is shown in Pl. 2, fig. 4 with the nucleus extending into the mitochondrion, although both are separated by their own membranes. During the encystment period the mitochondria become more numerous and prior to germination of the cyst they are found not only around the nuclear cap but also at the periphery of the spore (Pl. 1, fig. 2; Pl. 3, fig. 8). When the germ tube has just emerged few spherical and mostly elongated mitochondria are found (Hill, unpublished observations).

The endoplasmic reticulum as shown in Pl. 2, fig. 4 and Pl. 3, fig. 5 and 6 is closely associated with both the mitochondria and nuclear cap in the motile spore. In several instances the endoplasmic reticulum surrounding the nuclear cap extends around a mitochondrion.

Although the nuclear cap is bound by a double membrane that is continuous with the nuclear membrane, as shown by the arrow in Pl. 2, fig. 4, Pl. 4, fig. 9, and Pl. 5, fig. 15, it was seldom found to be homogeneous. In most cases mitochondria and/or other granules, as seen in Pl. 1, fig. 1 and Pl. 4, fig. 11, appear to be embedded in it. Some of the endoplasmic reticulum that dissects the nuclear cap during encystment (Pl. 1, fig. 2; and Pl. 2, fig. 3) appears to originate in the cap itself. As the spore progresses toward encystment dissection of the cap progresses so that ribosomal areas are cut off into smaller units each surrounded by a double membrane (Pl. 3, fig. 8). Plate 4, fig. 9 shows a large area of ribosomes cut off anterior to the nuclear cap. When the spore (now cyst) germinates the nuclear cap is no longer intact or recognizable as such and the ribosomes are dispersed throughout the germ tube and spore body (Hill, unpublished observations).

The single whiplash flagellum, posteriorly located and closely associated with the

nuclear membrane as found in the motile spore, is shown in Pl. 1, fig. 1, Pl. 4, fig. 9, 10 and Pl. 5, fig. 13. The outer membrane is continuous with the cell membrane, which could account for the numerous spores observed with flagellar bulbs (Pl. 5, fig. 15). The axoneme, as shown in Pl. 4, fig. 9 and 10, appears to fuse with the outermost unit of the nuclear membrane. The kinetosome is not bound at the cell membrane by a terminal plate. A cross-section of an extended flagellum showing the 9+2 configuration is shown in Pl. 5, fig. 16. A centriole, indicated by the arrow in Pl. 4, fig. 10, is attached to the kinetosome. The retracted axoneme observed around the inside periphery of the encysted spore can be seen in longitudinal section in Pl. 5, fig. 14. A cross-section of a retracted axoneme is shown in Pl. 1, fig. 2, Pl. 3, fig. 8 and Pl. 5, fig. 17.

A unique inclusion found in most sections was a granule of concentric rings either embedded in or lying close to the nuclear cap. It was usually enclosed by a double membrane and usually surrounded by an electron-transparent space, as shown in Pl. 4, fig. 11 and 12.

Lipid bodies were present in both motile and encysted spores (Pl. 1, fig. 1 and 2), being more abundant in the latter than the former. They were usually found scattered around the periphery of the nuclear cap. Small vacuoles were always observed in the electron-transparent area found between the nuclear cap and cell membrane (Pl. 1, fig. 1).

DISCUSSION

The zoospores of *Allomyces macrogynus* are strikingly similar in gross morphology to the zoospores of *Blastocladiella emersonii* (Reichle & Fuller, 1967; Lessie & Lovett, 1968) because of the position of the nucleus and nuclear cap at the posterior end and ribosomal composition of the nuclear cap. However, they differ from *B. emersonii* zoospores in that they have several mitochondria, compared to one in *B. emersonii*, no structures corresponding to the gamma bodies in *B. emersonii* and lipid bodies scattered around the periphery of the nuclear cap as compared to the lipid sac of *B. emersonii*.

The nuclear cap is clearly a distinguishing feature of the Blastocladiella zoospore. If fine structure analysis is to be used not only for comparative purposes but as an aid to the establishment of relationships among aquatic Phycomycetes, then the nuclear cap would appear to be a logical structure for this purpose. No criteria have yet been established to indicate what constitutes a 'characteristic nuclear cap' of any order of the aquatic Phycomycetes. This is due, in part, to insufficient evidence based on electron-micrograph analysis and to the lack of the number of species in which the motile phase has been examined.

No evidence was obtained in this study to indicate, as Fuller & Reichle (1968) suggested, that the membrane surrounding the nuclear cap is derived from the nuclear membrane even though as shown in Pl. 2, fig. 4 and Pl. 5, fig. 15 they are connected. Ribosomes in *Allomyces macrogynus* zoospores are not confined to the nuclear cap as they are in the fully developed zoospore of *Blastocladiella emersonii* (Fuller, 1966; Fuller & Reichle, 1968) since they are also found surrounded by a double membrane as pockets and lying individually, unattached between strands of endoplasmic reticulum (Pl. 3, fig. 5, 6, 7 and Pl. 4, fig. 9). Dissection and subsequent disintegration of the nuclear cap is apparently a fundamental process that occurs during the develop-

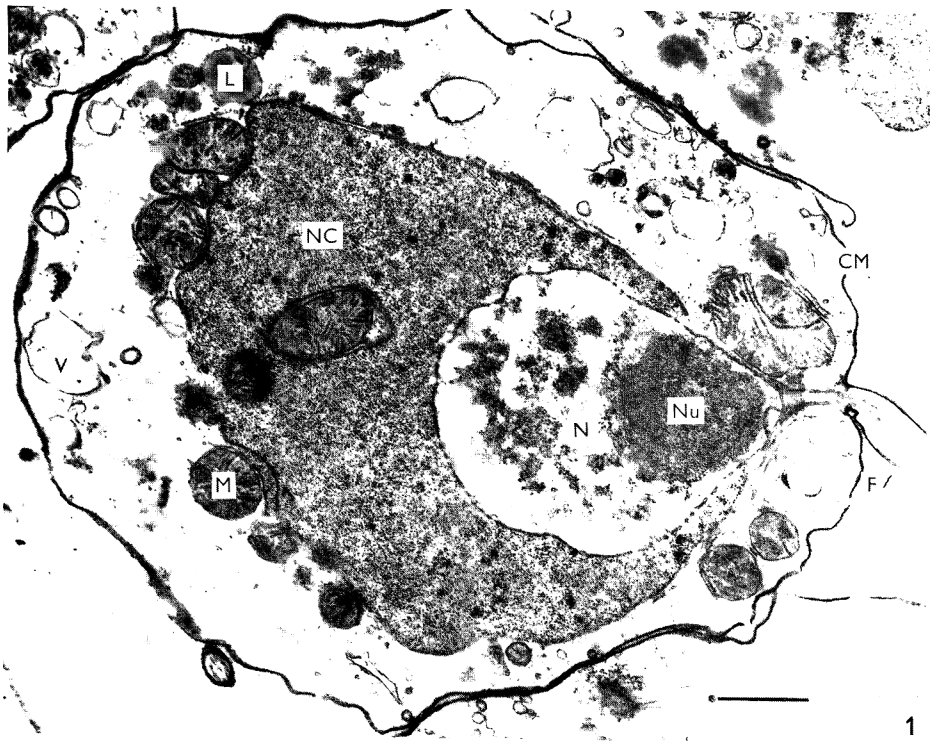
ment of flagellated fungal spores since it is known to occur in zygote formation in *A. macrogynus* (Blondell & Turian, 1960), encysting zoospores of *B. emersonii* (Reichle & Fuller, 1967), post-encysted zoospores of *Rhizidiomyces apophysatus* (Fuller & Reichle, 1965) and *A. macrogynus* zoospores (Pl. 1, fig. 2; Pl. 2, fig. 3).

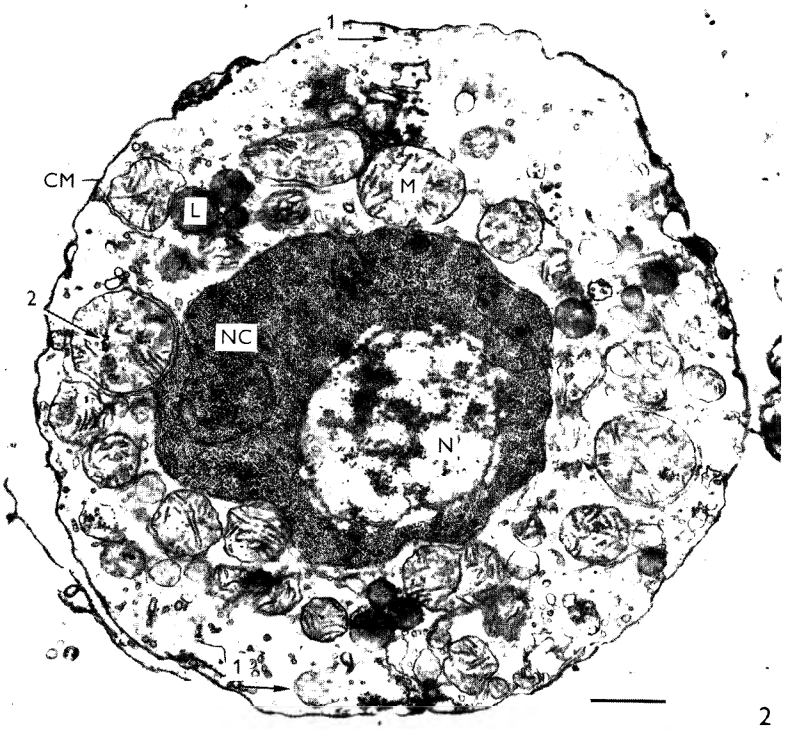
The centriole, indicated by the arrow in Pl. 4, fig. 10, does not show the striations characteristic of *Blastocladiella emersonii* (Reichle & Fuller, 1967) or the gametes of *Allomyces arbusculus* (Renaud & Swift, 1964) and *A. macrogynus* (Blondell & Turian, 1960). The kinetosome does not have a well-defined terminal plate nor are microtubules present. These observations are probably indicative of developmental events that take place prior to flagellar withdrawal and subsequent encystment. Although Fuller (1966) mentioned the presence of microtubules in *Rhizidiomyces apophysatus*, *B. emersonii* and *Monoblepharella* sp. they were not observed in *A. macrogynus* zoospores and they were neither apparent nor mentioned by Renaud & Swift (1964) for *A. arbusculus* gametes nor by Blondell & Turian (1960) during gametogenesis of *A. macrogynus*. However, as seen in Pl. 1, fig. 1 and Pl. 4, fig. 9, the kinetosome is connected to two tubular structures running on either side of the nuclear cap. These structures may function as microtubules in giving internal stability to the cell during the motile phase.

Two explanations of the disappearance of the flagella at the end of the motile phase are that they are cast off or resorbed (Kole, 1965). A third possible explanation, presented by evidence shown in Pl. 1, fig. 2, Pl. 3, fig. 8 and Pl. 5, fig. 14 and 15, is that the cell membrane surrounding the axoneme is probably resorbed, while the axoneme is retracted at the termination of the motile phase. That the flagellar sheath found in *Allomyces macrogynus* zoospores is not withdrawn is evident by comparing Pl. 5, fig. 13 and 14 and fig. 16 and 17. Several spores were seen with bulbs similar to that shown in Pl. 5, fig. 15, while in no instance was the cell membrane surrounding the axoneme observed attached to the cell membrane, except at the junction of the cell body. Although it is evident that the axoneme is withdrawn, no mechanism nor structure as to how this is accomplished is known.

The close association between the large mitochondrion, the nucleus (Pl. 2, fig. 4) and the flagellum (Pl. 4, fig. 9 and 10) suggests an intimate relationship between control and energy supply and utilization during the motile phase. This association is even more pronounced in *Blastocladiella emersonii* zoospores (Reichle & Fuller, 1967), where the single mitochondrion is perforated through which passes the kinetosome. During the encystment period the mitochondria become more numerous (compare Pl. 1, fig. 1 and 2), and are dispersed throughout the spore. In early germlings most of the mitochondria are elongate rather than spherical. These changes are apparently common during the course of development in fungi because they are known to occur in conidia of *Botrytis cinerea* (Hawker & Hendy, 1963), ascospores of *Neurospora tetrasperma* (Lowry & Sussman, 1968) and *B. emersonii* zoospores (Reichle & Fuller, 1967). Furthermore, the mitochondria are found during the encystment period dispersed and lying close to the cell membrane, suggesting that they function in cell wall synthesis during the period of mycelial growth.

The presence of the concentric granule, Pl. 4, fig. 11 and 12, apparent in various stages of the life-cycle (Skucas, 1968) except the gametes (Blondell & Turian, 1960), is of interest because it seems to be found only in the genus *Allomyces* among the aquatic Phycomycetes that have been studied with the electron microscope. Its





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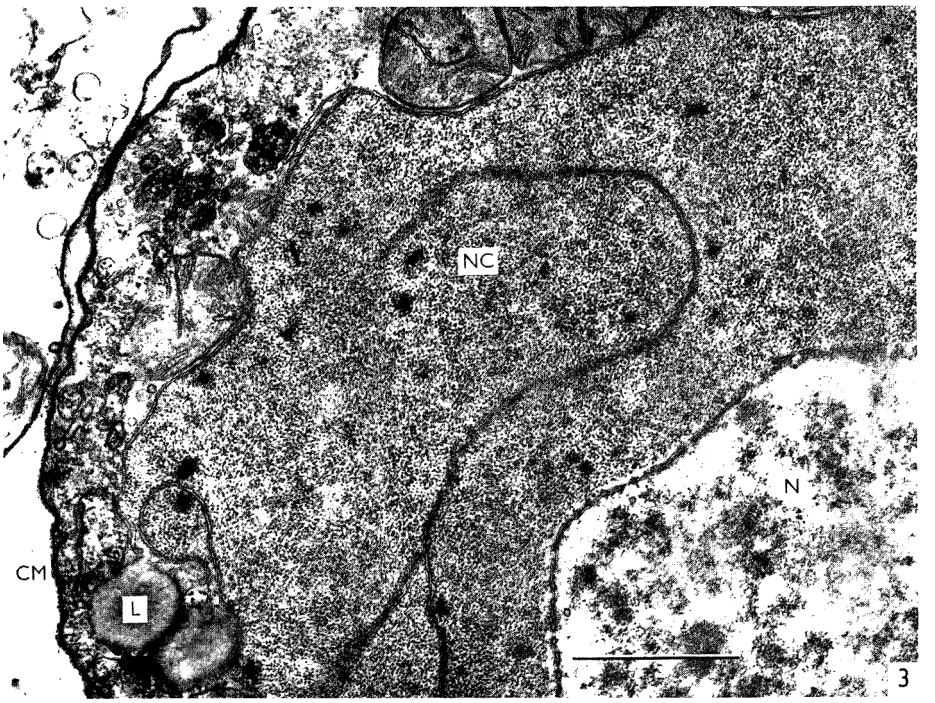
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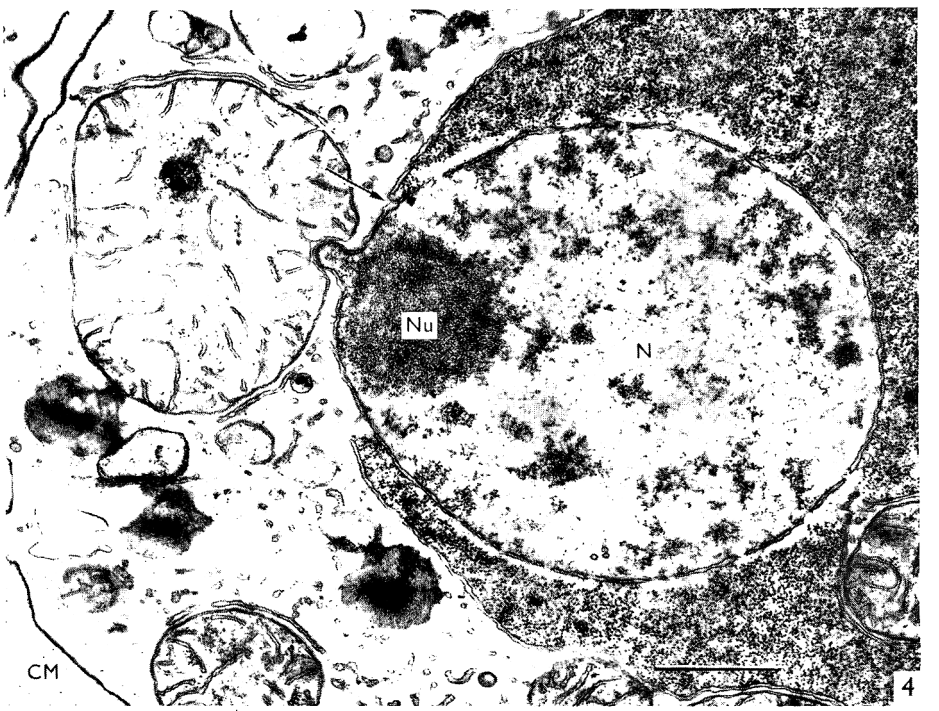
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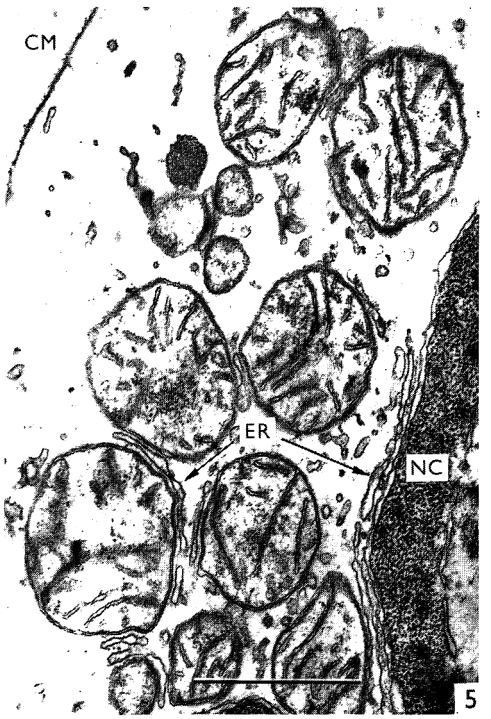
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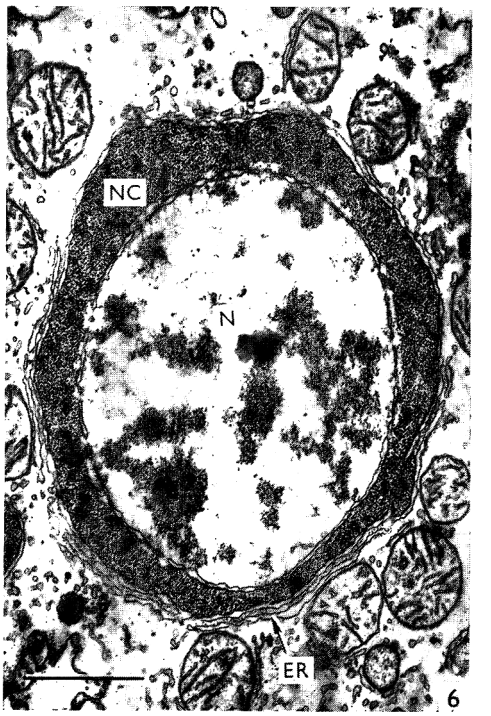
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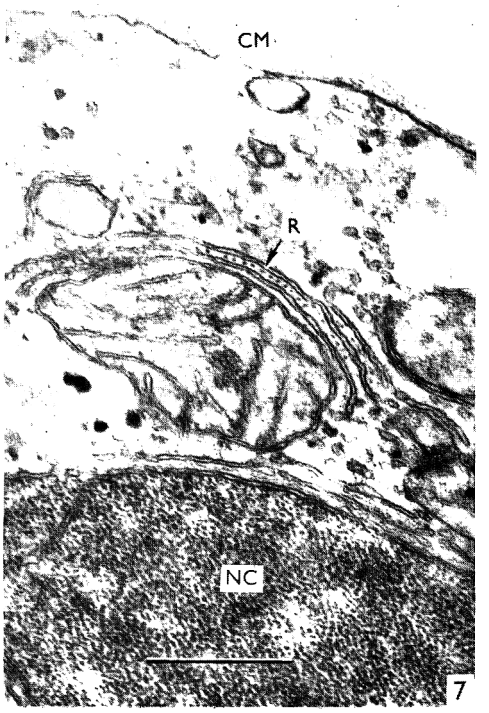
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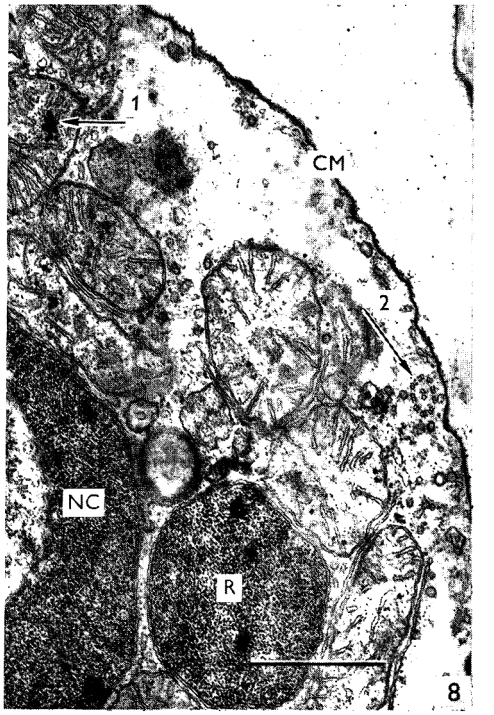
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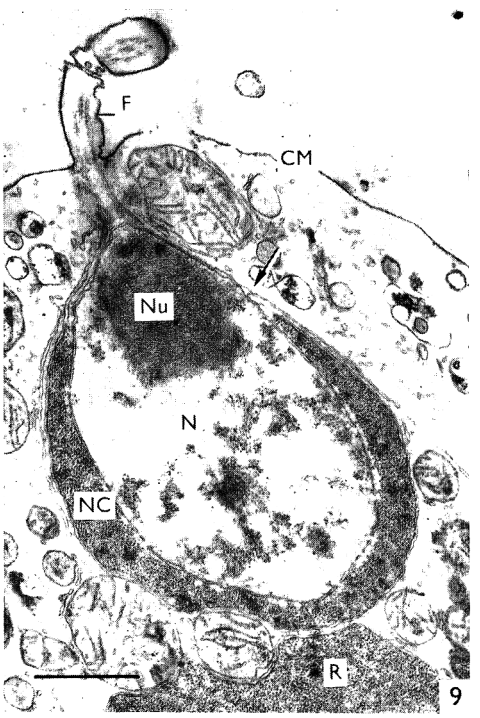
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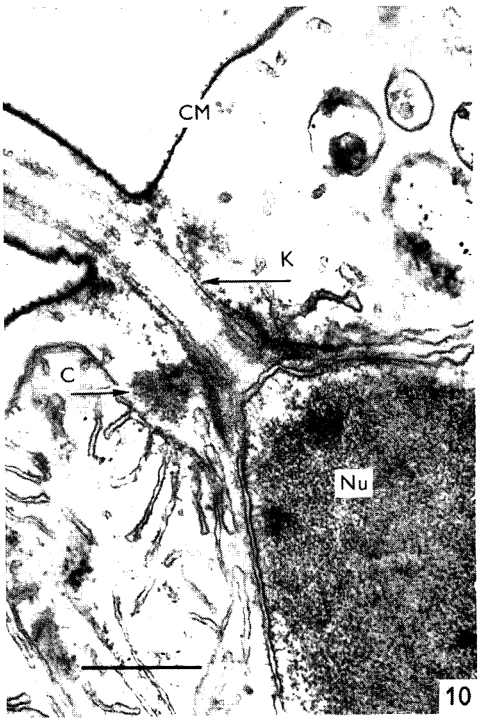
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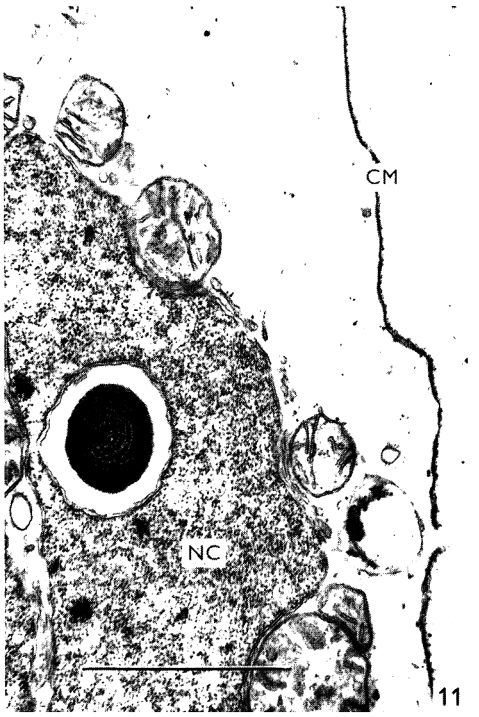
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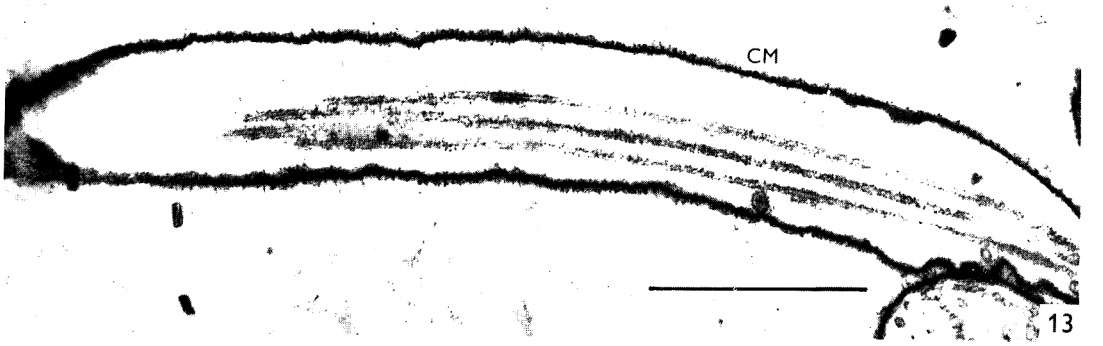

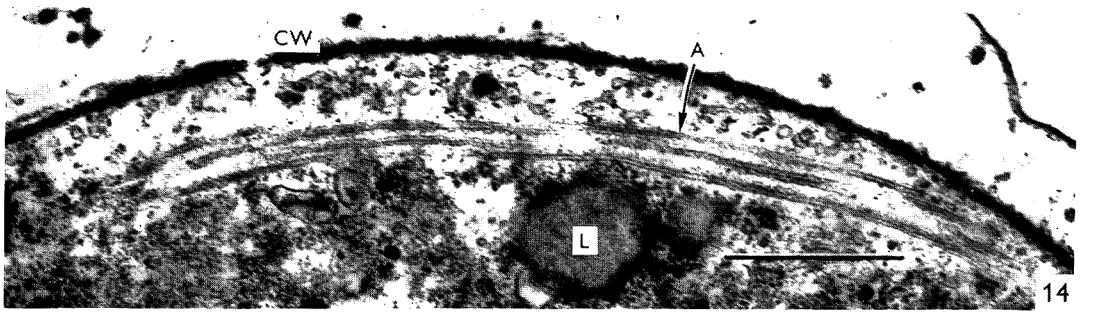

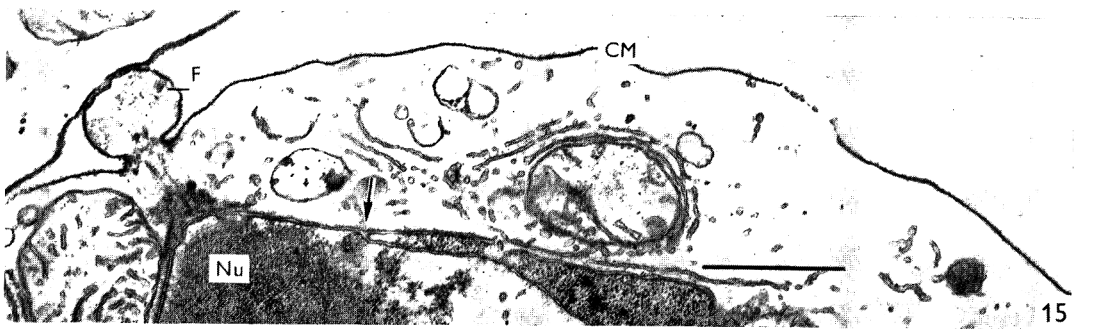

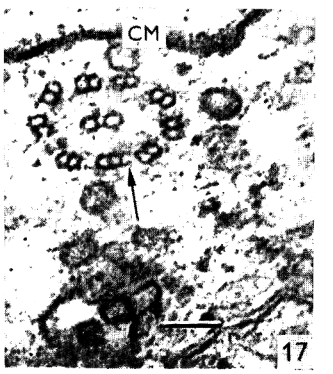

composition, function and formation are not known, although it may be a site of accumulation of metabolic products. Finally, the appearance of granules found only in the mitochondria of encysted spores (Pl. 1, fig. 2) are very similar to the polysaccharide granules reported by Lessie & Lovett (1968) during the formation of *Blastocladiella emersonii* zoospores.

The formation of the membrane that surrounds the nucleus and nuclear cap, the position and number of centrioles and the nature of the change, if any, that takes place during the time just after the zoospore is released, and its appearance in the sporangium just prior to its release, are points which are currently being studied.

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EXPLANATION OF PLATES

Abbreviations used. A, axoneme; C, centriole; CM, cell membrane; CW, cell wall; ER, endoplasmic reticulum; F, flagellum; K, kinetosome; L, lipid body; M, mitochondrion; N, nucleus; NC, nuclear cap; Nu, nucleolus; R, ribosomes; V, vacuole.

The bar represents 1 μ unless otherwise indicated. All magnifications are approximate.

PLATE 1

Fig. 1. A median longitudinal section through a motile spore.

Fig. 2. A near median section through an encysted spore. Arrow 1 indicates a cross-section of the retracted axoneme. Arrow 2 indicates polysaccharide bodies in mitochondrion.

PLATE 2

Fig. 3. Enlarged portion of an encysted spore. Note endoplasmic reticulum dissecting nuclear cap.

Fig. 4. Enlargement of a portion through the posterior area of a motile space. Arrow indicates continuity of nuclear cap and nuclear membrane.

PLATE 3

Fig. 5. A portion of an area of mitochondria from a motile spore. Note spherical shape.

Fig. 6. A cross-section through the nucleus near the anterior end of a motile spore. Arrow indicates abundant endoplasmic reticulum surrounding the nuclear cap.

Fig. 7. A portion of a motile spore showing an enlarged view of ribosomes (arrow) lying between two strands of endoplasmic reticulum. Bar indicates 0·5 μ.

Fig. 8. An enlarged section of an encysted spore. Arrow 1 indicates polysaccharide granules in a mitochondrion. Arrow 2 indicates a cross-section of a retracted flagellum.

PLATE 4

Fig. 9. A section through the posterior third of a motile spore showing the relationship between the flagellum and nucleus. Arrow indicates junction of nuclear and nuclear cap membrane.

Fig. 10. An enlargement of a median longitudinal section showing the region of flagellar attachment. Bar indicates 0·5 μ.

Fig. 11. A concentric body embedded in the nuclear cap.

Fig. 12. An enlargement of the concentric body.

PLATE 5

Fig. 13. A longitudinal section of the flagellum.

Fig. 14. A section through an encysted spore showing a longitudinal view of a retracted axoneme (arrow).

Fig. 15. A section through a spore just prior to encystment showing the flagellar bulb. Arrow indicates junction of nuclear and nuclear cap membrane.

Fig. 16. Cross-section of flagellum with the cell membrane surrounding the axoneme. Bar indicates 0·5 μ.

Fig. 17. Enlarged view of a retracted flagellum in an encysted spore. Note loss of cell membrane. Arrow indicates arm on one of the doublets. Bar indicates 0·1 μ.

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Deoxyribonucleic Acid Base Compositions of Mycoplasma Strains of Avian Origin

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SUMMARY

The guanine + cytosine (GC) base compositions of deoxyribonucleic acids (DNA) of avian mycoplasmas, as well as strains from other sources, were determined from buoyant density in CsCl. The values from our work ranged from 24·0 to 35·7 moles %. There were small clusters at about 35 % and 32 % but the rest of the values ranged continuously from 24·0 % to 30·5 %. From reports in the literature, the values for the Mycoplasma group ranged from 22·8 to 41·0.

INTRODUCTION

Knowledge of the mean base composition of deoxyribonucleic acid (DNA) of organisms is an important criterion in classifying species. These techniques, however, have not been applied to the avian mycoplasmas. Nucleic acid homology techniques have been used to determine the lack of relatedness between mycoplasmas and particular bacteria (McGee, Rogul, Falkow & Wittler, 1965; Rogul, McGee, Wittler & Falkow, 1965) and to show relationships among the mycoplasmas of human origin (Reich, Somerson, Hybner, Chanock & Weissman, 1966; Reich, Somerson, Rose & Weissman, 1966). DNA studies were reported at the Second Conference on Biology of the Mycoplasma (N.Y. Academy of Sciences) comparing mycoplasma strains with each other and with L-forms and bacteria (McGee, Rogul & Wittler, 1967; Neimark, 1967; Somerson, Reich, Chanock & Weissman, 1967).

This study was undertaken to determine the guanine + cytosine (GC) contents of the DNA of mycoplasmas and to contribute data that may lead to more precise classification of these organisms.

METHODS

The sources of some strains have been reported (Kelton, Gentry & Ludwig, 1960; Kelton & Van Roekel, 1963). The three bovine strains were obtained from Drs Edward and Leach, the Wellcome Research Laboratories, Beckenham, Kent, England; the porcine strain, *Mycoplasma granularum*, from Dr W. Switzer, Iowa State University; *M. gallisepticum* strains 801, 1010 and 1150 from Dr H. Yoder, Iowa State

University; *M. meleagridis* strain 529 from Dr R. Yamamoto, University of California, Davis. The strains representing avian serotypes I, J, K, L, and M were obtained from Dr M. Frey, Iowa State University, and *M. synoviae* strain 1853 from Dr N. Olsen, West Virginia University. Strain 4330 is designated *M. hominis*, type 1 (Morton &

Table 1. *DNA base compositions of Mycoplasma strains of avian origin*

Species	Strain	Serotype	CsCl buoyant density (g./cm.^{3})	GC content (mole %)
M. gallisepticum	801	A	1·695	35·7
M. gallisepticum	PG-31	A	1·695	35·7
M. gallisepticum	1010	A	1·695	35·7
M. gallisepticum	S-6	A	1·6945	35·2
M. gallisepticum	1150	A	1·694	34·7
M. synoviae	1853	S	1·6935	34·2
Mycoplasma sp.	694	L	1·6915	32·1
Mycoplasma sp.	TU	C	1·6899	30·5
Mycoplasma sp.	C	C	1·6885*	29·1
M. iners	O	G	1·689	29·6
M. iners	PG-30	G	1·6885	29·1
Mycoplasma sp.	PSU-4	D	1·688*	28·6
Mycoplasma sp.	NY	D	1·6875	28·1
M. meleagridis	529	H	1·688	28·6
M. meleagridis	N	H	1·6875	28·1
M. gallinarum	54-537	B	1·6865	27·0
M. gallinarum	PG-16	B	1·686	26·5
Mycoplasma sp.	SA	F	1·686	26·5
Mycoplasma sp.	693	J	1·685	25·5
Mycoplasma sp.	R-49	M	1·6845	25·0
Mycoplasma sp.	695	I	1·684	24·5
Mycoplasma sp.	1805	K	1·684	24·5
Mycoplasma sp.	HPR-15	E	1·6835	24·0

* Average of four determinations; all others, average of two determinations.

Table 2. *DNA base compositions of Mycoplasma strains other than avian*

Species	Strain	Origin	CsCl buoyant density (g./cm.^{3})	GC content (mole %)
Mycoplasma sp. (saprophyte)	PG-10	Bovine	1·6935	34·2
Mycoplasma sp. (saprophyte)	C-15	Compost	1·692	32·7
M. arthriditis (*M. hominis*, type 2)	07	Human	1·6915	32·1
M. arthriditis (*M. hominis*, type 2)	39	Human	1·6915	32·1
M. granularum		Porcine	1·6915	32·1
M. maculosum	PG-15	Canine	1·689	29·6
M. spumans	PG-13	Canine	1·6885	29·1
M. canis	PG-14	Canine	1·6885*	29·1
M. bovigenitalium	PG-11	Bovine	1·6875	28·1
M. hominis, type 1	4330	Human	1·686	27·3
M. bovirhinis	PG-43	Bovine	1·684	24·5

* Average of four determinations; all others, average of two determinations.

Roberts, 1967) and strains 07 and 39 are designated *M. arthriditis* (*M. hominis*, type 2) (Edward & Freundt, 1965). All strains were purified as described previously (Kelton & Van Roekel, 1963).

In general, 2 l. batches of broth medium (Kelton & Van Roekel, 1963) or more were inoculated with 10 ml. of 48 hr culture/l. and incubated at 37° on a rotary shaker at approximately 90 rev./min. until visible turbidity developed. The organisms were separated from the medium by centrifugation at 7500 *g*, washed once with 0·15 M-NaCl plus 0·1 M-sodium ethylenediaminetetra-acetate, and resuspended in a small volume of the same buffer. With *Mycoplasma synoviae* the broth medium was supplemented with L-cysteine hydrochloride hydrate (Calbiochem) and nicotinamide adenine dinucleotide (Pabst Laboratories) each in final concentration of 0·01 %.

The organisms were lysed by adding sodium dodecyl sulphate to 1 % (w/v) and the DNA was isolated by the method of Marmur (1961) as modified by Mandel, Bergendahl & Pfennig (1965). The base composition was estimated from buoyant density in CsCl in an analytical centrifuge (Schildkraut, Marmur & Doty, 1961). Buoyant densities were calculated relative to DNA of *Escherichia coli* at a density of 1·710 g./cm.^{3}.

RESULTS

Buoyant densities and base compositions of DNA samples derived from strains of avian origin are shown in Table 1. Strains from other sources were included in this study: results from these strains are shown in Table 2.

The GC content of the DNA samples ranged from 24·0 to 35·7 moles %. There was a small cluster of values at about 35 % and another at 32 %, but the rest of the values formed a continuous gradation from 24·0 to 30·5 %.

DISCUSSION

In grouping micro-organisms the GC content appears to be most useful when the values are different. Similar values were obtained from mycoplasmas that are apparently unrelated. *Mycoplasmas arthriditis* (*M. hominis*, type 2) of human origin had the same GC content as *M. granularum* of porcine origin, and the value for the saprophyte, C-15, was only slightly higher. Likewise, within the avian group, serotypes A and S gave similar values and there were only minor differences among the values for serotypes B, C, D, F, G, and H. In the studies by McGee *et al.* (1967) the relationships of five strains having GC content of 32 % were studied by DNA–DNA hybridization. They found that these five strains fell into three unrelated groups and attributed the similar base ratios to chance.

Strains representing 15 serotypes of avian Mycoplasma were included in this study. Despite repeated attempts, DNA suitable for analysis could not be obtained from strain L 3-10, representing serotype Q.

In previous studies (Kelton & Van Roekel, 1963) serological relatedness was demonstrated between serotype E (strain HPR-15) and serotype G (strains PG-30 and O) and these strains were included in the same serotype. Dierks, Newman & Pomeroy (1967) presented similar results in their serological studies of the avian mycoplasmas. The GC contents of the DNA of these strains, however, do not indicate any relationship between these serotypes.

Table 3. *DNA base compositions of Mycoplasma strains reported in the literature*

Species	Strain and source	GC (mole %)	Method*	Reference
M. pneumoniae	Human	39–41	B, C	Neimark & Pene, 1965
	FH, human	39·0, 40·8	B, C	Neimark, 1967
	ATCC-15377, human	39·3	B	McGee *et al.* 1965
	BRU, ATCC-15377, human	38·6	B	McGee *et al.* 1967
M. gallisepticum	A 5969, avian	35·0	B	Marmur & Doty, 1962
	A 5969, avian	34·0	C	Schildkraut *et al.* 1961
	Avian	33·4, 33·7	B, C	Morowitz *et al.* 1967
	A 5969, avian	33·0–33·5	A, C	Morowitz *et al.* 1962
	ATCC-15302, avian	32·5	B	Rogul *et al.* 1965
	S-6, ATCC-15302, avian	32·0	B	McGee *et al.* 1967
M. agalactiae	Goat milk	33·6, 34·2	B, C	Neimark, 1967
M. hominis	H 39, human	33·7, 31·6	B, C	Morowitz *et al.* 1967
M. hominis, type 2	CAMPO, ATCC-14152, human	31·7	B	McGee *et al.* 1967
M. arthriditis	H 606, ATCC-13988	31·9	B	McGee *et al.* 1967
		30·0, 32·6	B, C	Morowitz *et al.* 1967
Mycoplasma sp.	DONNETTA, bovine	32·9	B	Morowitz *et al.* 1967
M. laidlawii, type A	ATCC-14089	32·5	B	McGee *et al.* 1967
		31·7, 35·7	B, C	Neimark, 1967
		34·1	B	Morowitz *et al.* 1967
	SEWAGE	31·0–35·5	B, C	Neimark & Pene, 1965
M. laidlawii, type B	ATCC-14192	32·3	B	McGee *et al.* 1967
		33·2, 33·7	B, C	Morowitz *et al.* 1967
M. granularum	BTS-39, ATCC-19168, porcine	30·4	B	McGee *et al.* 1967
M. bovigenitalium	PG-11, ATCC-14173, bovine	29·6	B	McGee *et al.* 1967
M. hominis, type 1	H-34, ATCC-15056, human	29·2	B	McGee *et al.* 1967
	PPLO-4387, ATCC-14027, human	28·7	B	McGee *et al.* 1967
M. canis	PG-14, canine	28·4	B	McGee *et al.* 1967
M. spumans	PG-13, canine	28·4	B	McGee *et al.* 1967
Mycoplasma sp.	NEGRONI, human	28·3	B	McGee *et al.* 1967
M. gallinarum	ATCC-15319, avian	28·0	B	Rogul *et al.* 1965
	PG-16, ATCC-15319, avian	27·5	B	McGee *et al.* 1967
Mycoplasma sp.	GDL	27·8	B	McGee *et al.* 1967
M. fermentans	GII, ATCC-15474, human	27·8	B	McGee *et al.* 1967
	G, human	27·6	C	Neimark, 1967
M. pulmonis	KON, ATCC-14267, human	27·5	B	McGee *et al.* 1967
M. hyorhinis	BTS-7, ATCC-17981, porcine	27·3	B	McGee *et al.* 1967
M. pharyngis	LGM, ATCC-19524, human	27·0	B	McGee *et al.* 1967
M. maculosum	PG-15, canine	26·7	B	McGee *et al.* 1967
M. orale, type 1	823 B, ATCC-15539, human	26·6	B	McGee *et al.* 1967
M. orale, type 2	DC-1600, human	26·4	B	McGee *et al.* 1967
M. mycoides var. *mycoides*	bovine	26·8, 26·5	B, C	Neimark, 1967
	V 5, bovine	26·1	B	McGee *et al.* 1967
Mycoplasma sp.	Calf	23·6, 26·5	B, C	Neimark, 1967
	Calf	23·5–26·5	B, C	Neimark & Pene, 1965
M. mycoides var. *capri*	PG-3, goat	24–26	A	Jones & Walker, 1963 Jones *et al.* 1965
Mycoplasma sp.	Kid strain, goat	24·1, 25·5	B, C	Neimark, 1967
	Kid	24·0–25·5	B, C	Neimark & Pene, 1965
M. salivarium	MANIRE, human	23·3	B	McGee *et al.* 1967
M. neurolyticum	KSA, ATCC-15049, mouse	23·0	B	McGee *et al.* 1967
M. neurolyticum	Type A, mouse	22·8	C	Neimark, 1967

* A, by hydrolysis and chromatography; B, by thermal denaturation-absorbance measurement; C, by CsCl density gradient centrifugation.

The reports in the literature (Table 3) indicate a range of values for the Mycoplasma group from 22·8 to 41·0 moles %. *Mycoplasma pneumoniae* sets apart with reported values from 38·6 to 41 % while the others present a continuous gradation from 22·8 % to 35·0 %.

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The Separation of Neuraminidase Activity from other Pathological Activities of a Culture Filtrate of *Clostridium sordellii* CN 3903

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SUMMARY

The neuraminidase activity of a culture filtrate of *Clostridium sordellii* CN 3903 was separated on a Sephadex column from the oedema-producing, haemorrhage-producing and lecithinase activities. It is concluded that the neuraminidase of *C. sordellii* is not able by itself to produce oedema and haemorrhage.

INTRODUCTION

Neuramindases (*N*-acetylneuraminate glycohydrolase, E.C. 3.2.1.18) have been demonstrated in many micro-organisms (see, for example, *Vibrio cholera*, Gottschalk, 1957; *Diplococcus pneumoniae*, Madoff, Eylar & Weinstein, 1960; *Corynebacterium diphtheriae*, Warren & Spearing, 1963; some group K streptococci, Hayano & Tanaka, 1967; several human oral bacteria, Thonard, Hefflin & Steinberg, 1965; some myxoviruses, Rafaelson, Schneir & Wilson, 1963; *Clostridium perfringens*, Popenoe & Drew, 1957; Cassidy, Jourdian & Roseman, 1965). Neuraminidases cleave the glycosidic linkage of neuraminic acid in several types of compounds, including glycoproteins and gangliosides. Since these compounds appear to be components of cell membranes, and the neuraminic acid probably contributes to the charge on the membrane surface, neuraminidases might be expected to produce profound changes when introduced into living material. Although the receptor-destroying activity of viral neuraminidases has been known for a long time, no pathological activity of a micro-organism has yet been established to be due to a neuraminidase. Thus Kelly, Grieff & Farmer, (1966) found no correlation between animal virulence of *D. pneumoniae* and its ability to produce neuraminidase; Burton (1963) reported that the neuraminidase of *C. perfringens* was non-toxic to mice; Collee (1965; Gadalla & Collee 1968) was able to show that the haemagglutinin and neuraminidase of *C. perfringens* and of *C. septicum* were separate proteins; Fuhrman & Fuhrman (1968) reported that a factor in culture filtrates of *Vibrio cholera* which inhibited the sodium pump of frog skin differed from the cholera neuraminidase.

Cell extracts and culture filtrates of *Clostridium sordellii* are toxic, containing factors which produce oedema and haemorrhage when injected into guinea-pig skin. We have shown (Mellanby & White, 1968) that these pathological actions are independent of a lysolecithinase which the organisms produce intracellularly in the early stages of growth. At this early stage the culture filtrates contain about 1000 mouse LD50/ml. and neuraminidase activity of about the same specific activity as found in

C. perfringens culture filtrates (see Cassidy *et al.* 1965). The work reported in the present paper comprises a separation on Sephadex of some of the activities of a *C. sordellii* culture filtrate, in an attempt to assess whether the neuraminidase can be correlated with its pathological activity.

METHODS

Lecithin (33% pure from egg), sodium deoxycholate, laboratory reagent, glycylglycine, laboratory reagent, were obtained from British Drug Houses Ltd., Sephadex G-75 was obtained from Pharmacia (Uppsala, Sweden). The ganglioside was prepared from beef brain by the method of Mellanby, Pope & Ambache (1968).

Preparation of concentrated Clostridium sordellii culture filtrate. The *C. sordellii* (strain CN 3903, kindly provided by the Wellcome Research Laboratories) was grown for 4 hr at 37° in 10 l. bottles in a medium containing 30 g. Oxoid Bacteriological Peptone, 7·5 g. Na-β-glycerophosphate and 0·1 ml. of neutralized thiolacetic acid (approximately 0·3 m-mole) per litre. It was inoculated with an inoculum grown overnight in cooked-meat medium, which had itself been inoculated with 1 drop/10 ml. of a spore suspension (containing 100 mg. dry weight/ml.) stored in 50% glycerol at −40°. The organisms were removed by centrifugation at 10,000 *g* for 10 min. The 10 l. of supernatant fluid were concentrated to 200 ml. by pressure dialysis at 4° during a period of several weeks. The protein in the concentrated liquid was precipitated by adding ammonium sulphate (350 g./l.) at 4°, maintained at pH 7. The mixture was allowed to stand for 30 min. and then centrifuged at 17,000 *g* for 30 min. The precipitate was dissolved in 25 ml. cold distilled water and dialysed overnight against 10 l. distilled water at 4°. The dialysed solution was then freeze-dried; this material was stored at 4° and used for the subsequent work.

Assay of neuraminidase activity. In a total volume of 0·2 ml. 0·75 mg. ganglioside in 0·2 M-acetate buffer (pH 5·5) was incubated with the sample of enzyme for 10 min. at 37° and the free neuraminic acid at the end of the incubation estimated by the method of Warren (1959). One unit of neuraminidase activity was defined as that amount which released 1 μmole neuraminic acid from ganglioside per minute.

Assay of lecithinase-A activity. Lecithin and sodium deoxycholate (10 mg./ml. each) were suspended in 0·1 M-glycylglycine buffer (pH 7·2) and the mixture treated ultrasonically (MSE 100 W ultrasonic disintegrator) for 30 sec. to produce a homogeneous suspension. This mixture (0·3 ml.) was incubated with the enzyme sample in a total volume of 1 ml. at 37° for 6 hr. The reaction was stopped by placing the tubes in ice and adding 5 ml. of extraction mixture (isopropanol + *n*-heptane + 2 N-H_2SO_4; 80 + 20 + 1 by vol.). Free fatty acids were then titrated by using the method described by Kelley (1965). One unit of lecithinase activity was defined as the amount of enzyme that released 5×10^{-3} μmole fatty acid/hr (Mellanby & White, 1968).

Pathological activity of culture filtrate fractions in rabbit skin. Serial dilutions of samples (0·1 ml. in 0·1 M-phosphate buffer, pH 7, containing 0·2% (w/v) gelatin) to be tested were injected intradermally into the shaved back of albino rabbits (3 kg.) anaesthetized with Nembutal. Five and a half hr later the rabbits were injected in the ear vein with 1·5 ml. of Pontamine Sky Blue solution (0·5% in 0·45% NaCl). Half an hour after this the diameters of the blue oedematous areas were measured. One unit of oedema-producing activity was defined as that required to produce 10 mm. diameter oedema in rabbit skin after 6 hr (see Arseculeratne, 1965).

RESULTS

Separation on Sephadex of activities in concentrated culture filtrate

The material prepared from concentrated culture filtrate (60 mg.) dissolved in 2 ml. 0·001 M-phosphate buffer (pH 6·0) was applied to a Sephadex column (2·6 × 28 cm.) and eluted in 2·5 ml. fractions with the same buffer. The fractions were assayed for protein, lecithinase, neuraminidase, lethality in mice and ability to produce oedema and haemorrhage in guinea-pig skin. The results obtained are shown in Fig. 1. It can

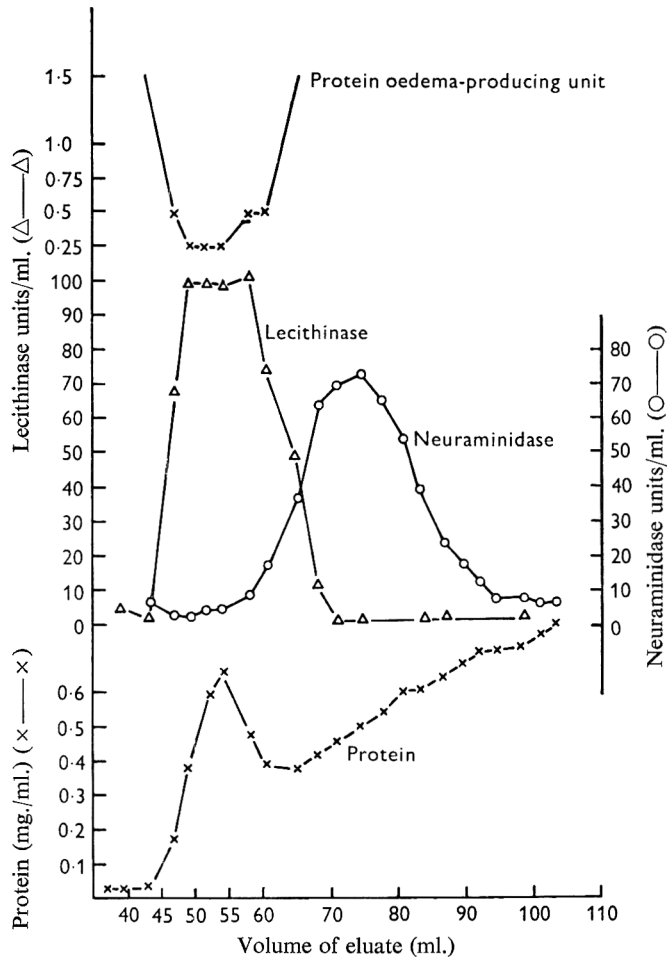

Fig. 1. Separation of activities of *Clostridium sordellii* CN 3903 culture filtrate on Sephadex. × —— ×, Upper curve: mg. needed to give 1 unit oedema-producing activity; lowest curve: protein mg./ml.

be seen that protein was eluted in a steep initial peak followed by a gradual slope. The initial protein peak contained the lecithinase activity and the pathological activity, as manifested in the guinea-pig skin and lethal-toxicity to mice. The material which emerged after the initial protein peak was non-toxic to mice. The early part of the second increase in protein content contained a fraction with the neuraminidase

activity. The tube which contained the greatest amount of neuraminidase activity contained no detectable pathologically active material. It could therefore be concluded that the neuraminidase of *Clostridium sordellii* is not able by itself to produce oedema and haemorrhage. The lecithinase activity paralleled the pathological activity and therefore possible may be responsible for it. Table 1 shows that the procedure resulted in a modest purification of the neuraminidase (about 2½-fold) and of the lecithinase (about 3-fold) activities. The separated neuraminidase had an optimum activity at pH 5·0. while the lecithinase had an optimum activity at pH 7·2.

Table 1. *Recovery and specific activity of neuraminidase, lecithinase and oedema-producing activity on passing a preparation of Clostridium sordellii culture filtrate through Sephadex*

		Neuraminidase* activity		Lecithinase† activity		Oedema-producing activity‡	
Fraction	Total protein (mg.)	Total units	Units/mg. protein	Total units	Units/mg. protein	Total units	Units/mg. protein
Original	60	2·5	0·04	1440	24	36	0·6
Neuraminidase peak (tubes 17–23 inc.)	11·3	1·3	0·11	0	—	0	—
Lecithinase and toxic peak (tubes 11–15 inc.)	7·4	0	—	510	69	24	3·4

* One unit of neuraminidase activity released 1 μmole of neuraminic acid per minute (see Methods).
† One unit of lecithinase activity released 5×10^{-3} μmole fatty acid per hr (see Methods).
‡ One unit of oedema-producing activity gave a 10 mm. diameter oedematous lesion in rabbit skin after 6 hr (see Methods).

In view of the suggestion of Gadalla & Collee (1968) that neuraminidase might increase the pathological activity of other necrotic toxins without itself being pathologically active, the oedema-producing activity of the most active fractions obtained above was compared, with and without addition of the neuraminidase. However, up to 0·01 units of neuraminidase activity had no effect on the oedema-producing or haemorrhagic activity.

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Toxicity of Hyperbaric Oxygen to Yeasts Displaying Periodic Enzyme Synthesis

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SUMMARY

Exponential cultures of *Saccharomyces cerevisiae* and *Candida utilis* exposed to oxygen at ten atmospheres did not undergo any further cell division during treatment. All cells ultimately died when the treatment was prolonged for several days. Populations survived considerably longer with ethanol as a carbon source than with glucose. Stationary-phase populations were more resistant than exponentially-growing populations. The cell cycle of *S. cerevisiae* shows two points of resistance to oxygen toxicity which corresponded to two periods of catalase synthesis in air. These results are attributed to periodic synthesis of protective enzymes.

INTRODUCTION

Bacillus subtilis and *Escherichia coli* continue growth under oxygen at 10 atmospheres and survival depends on the physiological state of the organisms at the time of pressure increase (Gifford, 1968). It was suggested that only cells in which there is an active synthesis of catalase at the time of pressure increase survive: in such cells a high catalase level will be maintained throughout the cell cycle since, in bacteria, induced enzyme synthesis can occur throughout the cycle (Masters, Kuempel & Pardee, 1964; Kuempel, Masters & Pardee, 1965; Masters & Donachie, 1966). This paper describes the effect of high pressure oxygen (HPO) on cultures of *Saccharomyces cerevisiae*, an organism known to display periodic enzyme synthesis (Halvorson *et al.* 1966) and on *Candida utilis*.

METHODS

Yeast strains. Saccharomyces cerevisiae E 8 and *Candida utilis* E 135 were obtained from the culture collection of the Department.

Media and cultivation. Cultures were maintained on malt agar at 25°. Cultures for HPO treatment were grown at 25° on either liquid glucose + peptone medium (1 % (w/v) glucose, 0·5 % (w/v) peptone, mineral salts) or, in the case of *Candida utilis*, glucose + nitrate medium (1 % (w/v) glucose, 0·5 % (w/v) sodium nitrate, mineral salts). In experiments using various carbon sources the cells were sedimented by centrifugation after an appropriate growth period and resuspended in fresh medium containing 1 % or 3 % (w/v) glucose or 1 % (v/v) ethanol or glycerol as the carbon source. Media used in the synchronization procedure were the starvation medium of Williamson & Scopes (1960) and the normal growth medium without glucose.

Catalase determination. Cells for catalase determination were sedimented by centrifugation, resuspended in 3 ml. of acetone for 30 min., then centrifuged down and

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resuspended in 3 ml. of 0·05 M-phosphate buffer (pH 6·8). The catalase activity of this suspension was determined by the method of Herbert (1954).

Synchronization procedure. Saccharomyces cerevisiae was grown in 300 ml. of medium, containing glucose as carbon source, and harvested in the late exponential phase of growth. The larger cells were selected from the population by centrifugation in 15% (w/v) mannitol, resuspended in 25 ml. of starvation medium (Williamson & Scopes, 1960) and shaken at 25° for 12 hr. The cells were then sedimented by centrifugation, washed and resuspended in 25 ml. of growth medium with no added carbon source and placed under oxygen at 5 atm. for 12 hr. It will become evident from the ensuing results and discussion that the deep stationary-phase culture obtained from the starvation procedure was almost entirely resistant to an HPO treatment of this duration. The oxygen treatment provided a relatively rapid and specific means of removing any cells which were out of phase with the majority of the population since only these cells would be sensitive to HPO treatment. Following release of pressure the cells and medium were inoculated into 300 ml. of fresh medium, with glucose as carbon source, in a batch culture vessel. To eliminate, as far as possible, any after effects of the HPO treatment, synchronized cultures were allowed to grow for at least 5 hr before taking samples for the study of enzyme activity or survival under HPO.

Cell counts. Total counts were made with a Helber counting chamber 0·02 mm. deep, with Thoma ruling. Cells were counted according to the criteria of Williamson & Scopes (1960).

Viable counts. Survival, following HPO treatment, was determined by dispensing, in triplicate, 0·2 ml. samples of serially diluted culture on malt agar plates. Colonies were counted after 36 hr incubation at 25°.

Pressure vessels. Cultures were subjected to pressure in steel pressure vessels similar to those described by Caldwell (1956).

RESULTS

Survival of Saccharomyces cerevisiae and Candida utilis cultures subjected to HPO. Exponentially-growing cultures of both organisms were sedimented by centrifugation, resuspended at a suitable concentration in media containing different carbon sources and placed on a shaker at 25° for 2 hr to allow the cells to adapt to the new media. Samples (3 ml.) of cell suspension were then dispensed into 100 ml. conical flasks, placed in pressure vessels and subjected to oxygen at 10 atm. Survival was determined after various times of treatment (Fig. 1, 2). In no case was there any increase in cell numbers under HPO. Death of the populations occurred gradually, over a number of days in some cases. Both yeast populations survived for a considerably longer period with ethanol than with glucose. In the case of *Candida utilis* survival with glycerol was similar to that with glucose.

Resistance of various stages of the cell cycle of Saccharomyces cerevisiae to HPO. Samples (5 ml.) were taken at intervals during the growth of a synchronous culture, dispensed into 100 ml. Erlenmeyer flasks and rapidly exposed to oxygen at 10 atm. In one experiment (Fig. 3*a*) samples were taken at 20 min. intervals and subjected to HPO for 16 hr. In the second experiment (Fig. 3*b*) samples were taken at 12 min. intervals and subjected to HPO for 24 hr. After release of pressure survival was determined by plate counts.

The greatest capacity for survival was found in cells which had just completed division, virtually all the cells from a population at this stage of the cell cycle surviving the 24 hr exposure. A second, much smaller, survival peak was observed to occur just before, or concurrent with, the onset of budding. A maximum survival of 17% was recorded for samples taken from this region though it was generally much lower.

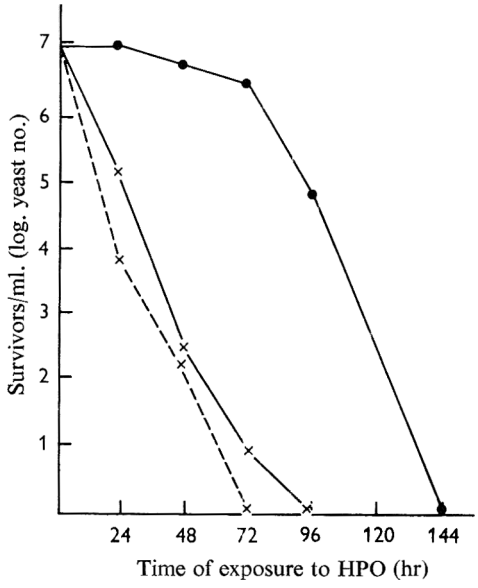

Fig. 1

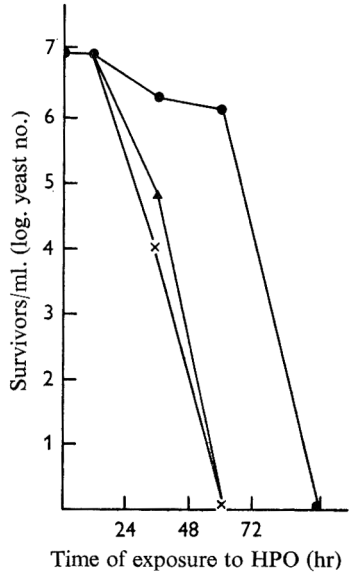

Fig. 2

Fig. 1. Survival of *Saccharomyces cerevisiae* exposed to high pressure oxygen on media containing various carbon sources. × ——×, 1% (w/v) glucose; × – – ×, 3% (w/v) glucose; ●——●, 1% (v/v) ethanol.

Fig. 2. Survival of *Candida utilis* exposed to high pressure oxygen on media containing various carbon sources. ×——×, 1% (w/v) glucose; ▲——▲, 1% (v/v) glycerol; ●——●, 1% (v/v) ethanol.

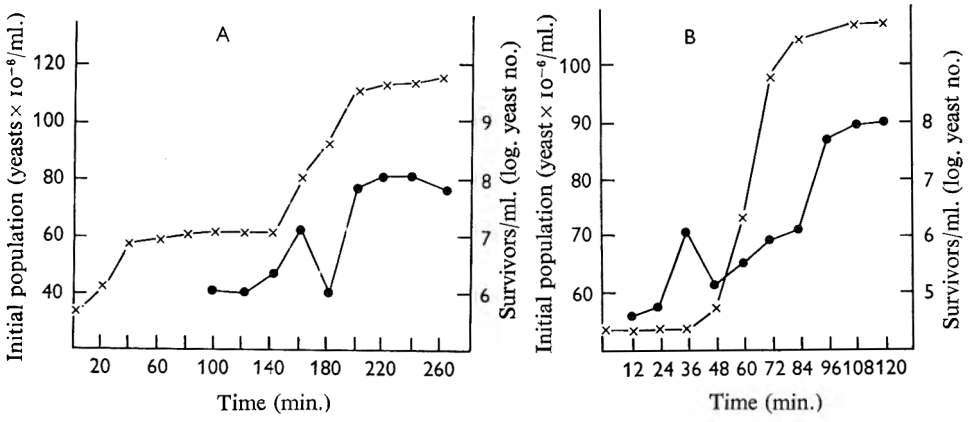

Fig. 3. Survival of *Saccharomyces cerevisiae* exposed to high pressure oxygen at different stages of the cell cycle. (*a*) Samples taken at 20 min. intervals and exposed to HPO for 16 hr. (*b*) Samples taken at 12 min. intervals and exposed to HPO for 24 hr. × ——×, Cell numbers of initial population; ●——●, survival following HPO treatment.

It should be noted that survival is plotted on a log. scale so that the pre-division increase in survival, relative to the samples on either side, is considerable. This pre-division survival peak was noted in three other experiments.

Catalase synthesis in Saccharomyces cerevisiae in relation to the cell cycle. Samples were taken at 10 min. intervals during the growth of a synchronous culture of *Saccharomyces cerevisiae* and the catalase activity determined (Fig. 4). Two peaks of activity were observed, one immediately before division, the other soon after. A considerable decrease in activity was observed during division and there was little increase in the total activity with increasing cell numbers.

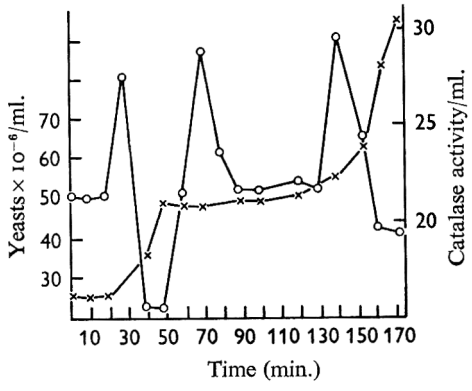

Fig. 4

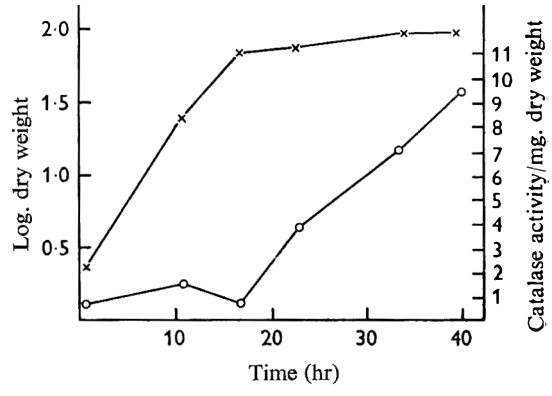

Fig. 5

Fig. 4. Catalase activity of *Saccharomyces cerevisiae* in relation to the cell cycle. O——O, Catalase activity; ×——×, cell numbers.

Fig. 5. Changes in catalase activity during the growth of *Candida utilis*. ×——×, Log. dry weight; O——O, catalase activity.

Effect of cell age on survival and catalase activity of Candida utilis subjected to HPO. The catalase activity, per cell, of stationary-phase cultures was many times higher than that of exponential-phase cultures (Fig. 5). Therefore it was of interest to determine the relative length of time of survival of cultures from these two stages when subjected to HPO.

Exponential-phase cultures of *Candida utilis* were harvested after 6 hr growth at 25°; stationary-phase cultures after 27 hr growth. The cells were sedimented by centrifugation and resuspended in either 1% (w/v) glucose+salts or 1% (v/v) ethanol+salts medium. The concentration of the stationary-phase cultures was adjusted by dilution to that of the exponential-phase cultures since it has been found, during the course of these studies, that the duration of survival of cells in liquid suspension is affected by population density. The rate of build-up of dissolved oxygen in the culture will be slower at a high population density than at a low density.

Samples (5 ml.) of each of the cell suspensions were pipetted into sterile Petri dishes and placed in pressure vessels. They were subjected to 10 atm. oxygen and opened at various intervals to determine survival and catalase activity (Fig. 6). Stationary-phase cultures survived longer than exponential cultures on both media and survival on ethanol was greater than on glucose. Although catalase activity in exponential-phase cultures was much lower than that of stationary-phase cultures, in both cases there was an increase in catalase activity of cells on the ethanol medium in

response to HPO treatment during the first 4 hr. On the glucose medium there was no such increase and catalase activity declined immediately.

Similar results were obtained with *Saccharomyces cerevisiae.*

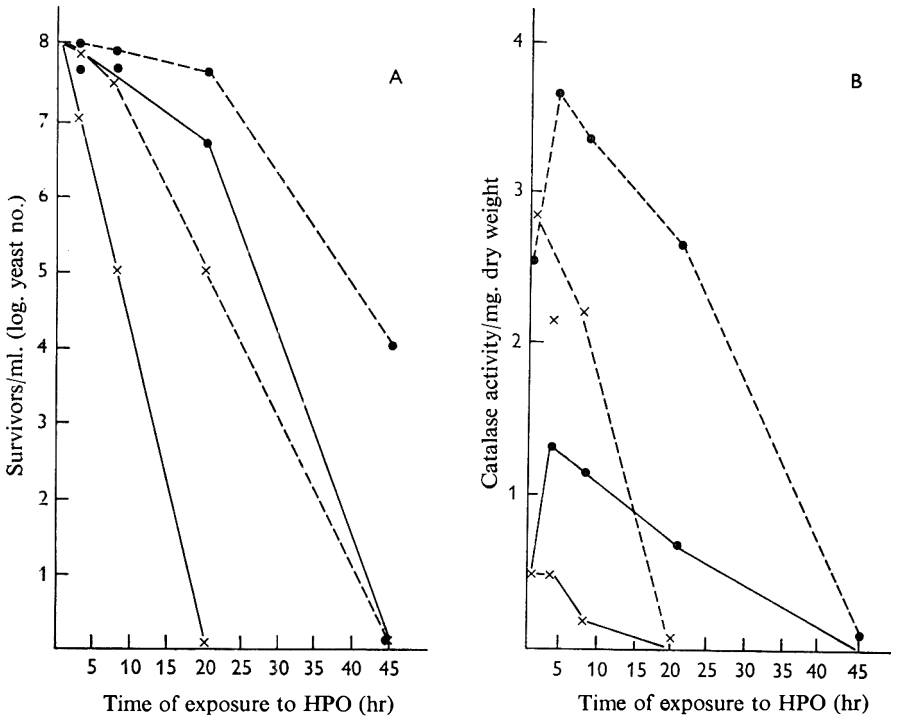

Fig. 6. (*a*) Survival, (*b*) catalase activities of exponential- and stationary-phase cultures of *Candida utilis* exposed to high pressure oxygen on media containing different carbon sources. Exponential phase on: ×——×, 1% (w/v) glucose; ●——●, 1% (v/v) ethanol. Stationary phase on: × – – – ×, 1% (w/v) glucose; ● – – – ●, 1% (v/v) ethanol.

DISCUSSION

The results show that there was no continuing division of yeasts under HPO, the death of an asynchronous population occurring progressively over a period of days at a rate which was influenced by the carbon source to which the cells were exposed. Exposure of samples of synchronized cultures of *Saccharomyces cerevisiae* to HPO has showed that there appear to be two points in the cell cycle at which this yeast showed resistance to HPO toxicity. These correspond to two periods of catalase synthesis in the cell cycle thus implicating this enzyme in the resistance mechanism (Pritchard & Hudson, 1967; Gifford, 1968). All the cells taken just after division were capable of surviving exposure to HPO for 24 hr, whereas a maximum survival of 17% was recorded for cells taken during the pre-division period of survival. This suggests that the two periods of catalase synthesis are of unequal length. Further evidence for the existence of two periods of resistance comes from the morphology of cells in a population which had been killed by exposure to HPO. Predominantly two types of cell may be seen, one bearing small buds, which represents cells which were in, or had reached, the pre-division period of catalase synthesis at the time of exposure,

the other bearing well-developed buds. The morphology of oxygen-killed cells of *Candida utilis* also suggests that there are two periods of resistance in the cell cycle of this organism.

The apparently greater resistance of stationary-phase cells to HPO toxicity may be because virtually all the cells in such a population are in a state in which catalase is being synthesized, as evidenced by the marked increase in catalase at the end of the exponential phase of growth. In exponential populations a much smaller proportion of the cells will be in this condition.

The observation that yeasts die under HPO with no further division was in marked contrast to the continuing division of bacteria under HPO (Gifford, 1968), though it confirmed the fungal results of Caldwell (1964) and Robb (1966). This differing response may be related to a difference in the mechanism of control of protein synthesis. In bacteria, although basal enzyme synthesis is ordered, induced enzyme synthesis may occur at any stage of the cell cycle (Masters *et al.* 1964; Kuempel *et al.* 1965; Masters & Donachie, 1966). In contrast, induced enzyme synthesis in yeasts shows a marked periodicity which, it has been suggested, may be due to variations in the accessibility of genes to transcription during the cell cycle (Halvorson *et al.* 1966). Thus it follows that, in yeasts, the synthesis of catalase will be subject to this periodic inducibility. In both bacteria and yeasts exposure of a random population to HPO results in the survival of those cells possessing a high level of, or are actively synthesizing, catalase. In bacteria the continuous accessibility of genes to transcription enables those cells which survive the initial exposure to maintain a high level of catalase by further induced synthesis and hence to continue division. In yeasts the survivors of the initial exposure will also continue to progress through the cell cycle but are unable to maintain a high level of catalase once they have passed beyond the period of the cell cycle in which catalase can be synthesized. These cells will survive until the catalase activity falls to a value which permits the formation of a toxic lesion. If this lesion is established before the next period of catalase synthesis the cells will die.

Therefore, on the available evidence, it appears that the survival of yeasts under HPO is related to the state of enzyme synthesis at the time of exposure. Cells entering a major period of catalase synthesis will survive for the longest period, those between periods of synthesis will be the first to die and there will be a series of conditions in between. Death of an asynchronous population will occur over a period of days, reflecting the random physiological states of the cells at the time of exposure.

The effect of carbon source on the length of time of survival under HPO may be related to the rate of decline in catalase level during treatment with HPO. Cells die most rapidly on those substrates which allow a rapid rate of growth in air, i.e. more rapid progress through the cell cycle. Cells on such substrates will remain for a short period of time within the period of catalase synthesis, whereas on substrates supporting a slower rate of growth, such as ethanol, the cells may remain within the synthetic period sufficiently long to allow some further synthesis of catalase, thus enhancing survival. The immediate decline of catalase activity of asynchronous cells on glucose and the brief rise in catalase activity of cells on ethanol support this suggestion. A similar effect of carbon source upon survival has been observed in other fungi (Dr S. Ahmed, personal communication).

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Degradation of Ursolic Acid, a Major Component of Apple Wax, by a Pseudomonad Isolated from Soil

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SUMMARY

A *Pseudomonas* sp. isolated from orchard soil utilized ursolic acid ($C_{30}H_{48}O_3$), a major component of apple wax, as a sole source of carbon. It evolved $^{14}CO_2$ from [^{14}C]ursolic acid and incorporated the label into all usual cell constituents; it formed a labelled polar metabolite of ursolic acid. Orchard soil thus contains at least one organism which is able to degrade the terpenes of the cuticular wax of plants.

INTRODUCTION

In the general ecological system of orchard land large amounts of plant materials such as leaves and fruits reach the soil and are degraded by soil organisms. These plant materials are covered with a complex mixture of hydrophobic chemical compounds usually referred to as plant waxes. Two major types of compounds in plant waxes are aliphatic hydrocarbons (and derivatives) of chain length C_{20} to C_{35}, and alicyclic compounds such as triterpenes. Very little is known about the fate of these individual lipids in soil. We have already reported on the utilization of *n*-nonacosane, probably the most common paraffin in plant waxes, by animals (Kolattukudy & Hankin, 1966) and by a soil bacterium (Hankin & Kolattukudy, 1968).

Wax from fruits such as apple (Huelin & Gallop, 1951), grape (Radler, 1965; Radler & Horn, 1965), pomegranate (Brieskorn & Keskin, 1954) and many Japanese plants (Kariyini & Hashimoto, 1953) contain large proportions of terpenes such as ursolic and oleanolic acids. The terpenes are considered to be relatively stable. However, hardly any evidence of significant accumulation of these 'difficultly decomposable' lipids in productive agricultural soil has been noted (Stevenson, 1966). It appears, therefore, that most agricultural soils contain micro-organisms able to utilize these materials. In this communication we describe the isolation of a bacterium from orchard soil, and the utilization by this organism of ursolic acid, a major component of apple wax.

METHODS

Isolation of ursolic acid fraction from apples

Apples collected from the Station experimental orchard were submerged for 1 min. in a large volume of boiling chloroform. The chloroform extract was concentrated *in vacuo* and hexane was added to precipitate the ursolic acid fraction. The precipitate was washed several times with hexane to remove as much of the aliphatic lipids as possible, dissolved in a minimum amount of hot isopropyl ether+acetone mixture (7+3, v/v) and reprecipitated with hexane. Thin-layer chromatography (t.l.c.) on

silica gel G with isopropyl ether + acetone (7 + 3, v/v) showed a single spot (R_F 0·44) detected by stannic chloride spray (Waldi, 1965). Gas–liquid chromatography of the ursolic acid fraction (as the silyl ether) showed this material to be predominantly ursolic acid, but with a smaller quantity of a companion terpene which could not be resolved fully by t.l.c. The ursolic acid fraction was used for both isolation of the test organism and for growth studies. The procedure described yielded 4·5 g. ursolic acid fraction from 4 kg. of apples.

Preparation of [^{14}C]*ursolic acid.* Apple skin sections, 13 mm. diam. and 1 mm. thick (cut from cork-borer plugs), were incubated with sodium [^{14}C]acetate. A ratio of 43 apple skin slices to 1 m.c. of sodium [^{14}C]acetate was used. During incubation the slices were exposed to 800 ft-candles of light at 29·5° for 48 hr. At the end of the incubation period they were placed in a mixture of chloroform and methanol 2 + 1 (v/v). The lipid material was recovered (Folch, Lees & Sloane-Stanley, 1957) and cold hexane added to precipitate the ursolic acid fraction. The precipitate was collected, washed with hexane, redissolved and reprecipitated. This crude ursolic acid fraction was placed on a silica gel column (30 cm. × 1 cm.) and the column was washed first with hexane and then with a mixture of isopropyl ether and acetone 7 + 3 (v/v). The ether + acetone eluate was concentrated and ursolic acid precipitated with hexane. The precipitate was collected and washed with hexane. This fraction was further purified by repeated preparative t.l.c. on silica gel G plates with isopropyl ether + acetone as the developing solvent. The radioactive area corresponding to ursolic acid was scraped off and the acid eluted with the same solvent. This [^{14}C]ursolic acid was used for the metabolic studies.

Mineral medium. The basal medium used has previously been described (Hankin & Kolattukudy, 1968).

Ursolic acid medium. Ursolic acid (200 mg.) was dissolved in 100 ml. of hot acetone and kept warm on a steam bath. Mineral medium (200 ml.) was added slowly with continual stirring in a stream of nitrogen until all acetone was removed. The medium was refrigerated until used. Ursolic acid was added at the rate of 0·1%, w/v. in growth studies.

Isolation of test organism. To 50 ml. of sterile ursolic acid medium in 250 ml. Erlenmeyer flasks was added about a spoonful of either soil, leaves, or both, collected from the Station experimental orchard. The flasks were shaken at room temperature (22 to 23°) and each 24 hr a loopful of culture fluid was examined for growth of organisms by staining. From flasks showing growth, 0·1 ml. of fluid was aseptically transferred to another flask of ursolic acid medium and incubated. Such transfers were repeated at least five times. The culture was then streaked on Trypticase Soy Agar plates (Baltimore Biological Laboratory, Baltimore, Md). After growth at room temperature, selected colonies were transferred into fresh medium. Upon evidence of growth, 0·1 ml. of culture was transferred to fresh medium. At least nine such transfers were made. Conventional methods were used to determine purity of culture. The identification of the organism is described under results.

Determination of radioactivity. All radioactivity was determined as previously described (Kolattukudy, 1965). Counting was done with a standard deviation of less than 3%. Internal standards of radioactive toluene were used to determine the efficiency of counting, which was usually 60%.

Experiments with labelled ursolic acid. The test organism was grown for 24 hr on

agar slants (Trypticase Soy Agar) and the organisms harvested by centrifugation. The organisms were washed four times with 0·85% saline and finally suspended in 1 ml. of mineral medium. These organisms were then used as inoculum for 25 ml. of mineral medium containing labelled [^{14}C]ursolic acid (2×10^7 c.p.m.). The contents of the flask were shaken at room temperature (22 to 23°). Air was bubbled through the incubation mixture to remove $^{14}CO_2$ which was collected in a 10% (v/v) solution of ethylenediamine in ethanol.

Since the evolution of $^{14}CO_2$ began to diminish after 11 hr, the experiment was terminated and the entire incubation mixture frozen and lyophilized *in vacuo*. The dried material was refluxed under nitrogen with a mixture of chloroform + methanol (2+1, v/v) for 8 hr. The chloroform-soluble material was recovered after acidifying the mixture with HCl according to the method of Folch *et al.* (1957). The extracts obtained were concentrated *in vacuo*, made to volume and the radioactivity determined.

The chloroform-soluble material was dried *in vacuo* and applied to a preparative silica gel G thin layer plate which was developed with a mixture of isopropyl ether and acetone (7+3; v/v).

The water-soluble portion of the chloroform + methanol extract and the hydrolysed cell residue were separated into acidic, basic and neutral portions with Dowex 50 WX 8 resin and Dowex 1 C 8 resin. Hydrolysis of the cell residue was done with 5 N-HCl in a sealed tube for 24 hr at 110°. The complete hydrolysis of the neutral fractions was done with 6 N-HCl in a sealed tube at 121° for 4 hr.

Paper chromatography. Descending paper chromatography of hydrolysed and unhydrolysed neutral fractions was done on Whatman no. 1 paper with butanol + pyridine + water (6+4+3) as the solvent system. Cationic fractions were analysed by chromatography on Chromar sheet (Mallinckrodt Chemical Co., St. Louis, Mo.) with butanol + acetic acid + water (4+1+5).

RESULTS

Identification of test organism. The bacterium isolated from orchard soil which was able to utilize triterpenoid acids as a sole source of carbon was a Gram-negative motile rod with 1 to 3 polar flagella. It has been classified as a *Pseudomonas* sp. in the fluorescent group according to methods described by Stanier, Palleroni & Doudoroff (1966). It showed the usual biochemical reactions described for this group.

Growth of Pseudomonas sp. The test organism was grown in 25 ml. of ursolic acid medium at 23, 32, and 35° in shake culture. Initial count was $2{\cdot}8 \times 10^{-5}$ organisms per ml. Total counts were made at selected intervals on Plate Count Agar (Difco). The growth curves obtained are shown in Fig. 1. Since 23° was found to be near the optimum, subsequent experiments were carried out at room temperature. We have also obtained good growth up to 30°.

Utilization of [^{14}C]*ursolic acid by Pseudomonas sp.* To demonstrate that this pseudomonad was able to catabolize ursolic acid ($C_{30}H_{48}O_3$), labelled ursolic acid was provided to the organism and the metabolic products were examined. As shown in Fig. 2, after a lag of several hours a linear rate of $^{14}CO_2$ evolution was observed. After 11 hr the rate of $^{14}CO_2$ evolution decreased. About 11% of the administered [^{14}C] appeared in respiratory CO_2 at the end of the experimental period (Table 1).

Water-soluble metabolites comprised about 8% of the administered radioactivity (Table 1). The cell residue contained about 9% of the administered radioactivity and about 64% was in the chloroform-soluble material (Table 1).

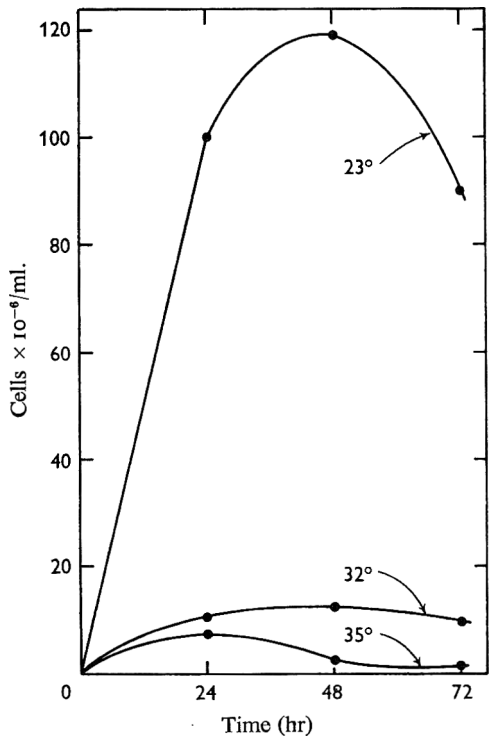

Fig. 1

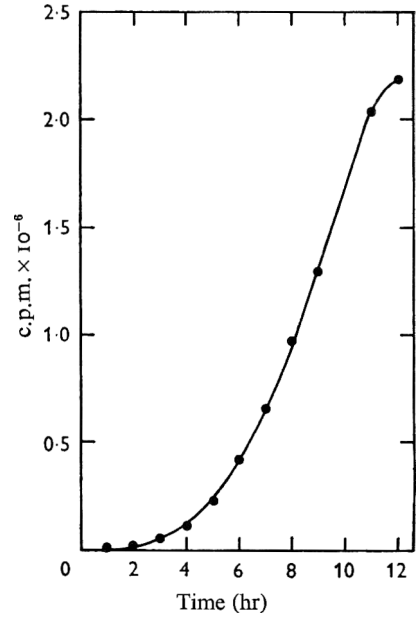

Fig. 2

Fig. 1. Growth of a *Pseudomonas* sp. with ursolic acid as sole source of carbon.

Fig. 2. Production of $^{14}CO_2$ by a *Pseudomonas* sp. growing on [^{14}C]ursolic acid.

Table 1. *Distribution of radioactivity* [^{14}C] *in fractions from incubation mixture when the Pseudomonas sp. was grown with* [^{14}C]*ursolic acid as sole source of carbon*

Fraction	Radioactivity (c.p.m. × 10^{-6})	% of administered
Chloroform soluble	12·84	64·2
Water soluble	1·58	7·9
Respiratory CO_2	2·17	10·9
Cell residue		
Acid hydrolysate*	1·22	6·1
Non-hydrolyzable	0·50	2·5
Recovery	18·31	91·6

* Hydrolysed with 5 N-HCl; sealed tube, 24 hr at 110°.

The water-soluble metabolites and the acid hydrolysate of the cell residue were fractionated into cationic, anionic and neutral fractions, all of which contained significant radioactivity (Table 2). Among the water-soluble metabolites, neutral and anionic fractions accounted for the major part of the radioactivity. Paper chromato-

graphy of the neutral fraction showed that it contained some labelled glucose, but the major part of the [^{14}C] appeared to be in compounds with higher molecular weight. After acid hydrolysis most of the radioactivity was found in the region of glucose. In the cell-residue hydrolysate, on the other hand, the major part of the radioactivity was in the cationic fraction. The cationic material from the water-soluble fraction and the cell-residue hydrolysate were examined by paper electrophoresis and t.l.c. The usual spectrum of amino acids was found to be labelled.

Analysis of the chloroform fraction by t.l.c. showed that most of the radioactivity of this fraction (89%) moved with the substrate. However, about 11% remained at the origin and are designated as polar lipids. Analysis of the substrate showed no radioactivity in the polar lipid region.

Table 2. *Fractionation of water-soluble materials from incubation mixture when the Pseudomonas sp. was grown with* [^{14}C] *ursolic acid as sole source of carbon*

	Radioactivity (c.p.m. × 10^{-6})	
Fraction	Water soluble	Cell residue
Cationic material	0·20	0·93
Anionic material	0·50	0·12
Neutral material	0·87	0·12

Analysis of polar lipids. Refluxing the polar lipid fraction with ethanolic KOH under nitrogen and subsequent analysis by t.l.c. with a developing solvent composed of a mixture of hexane, chloroform, methanol and formic acid (60+35+10+2, v/v) indicated that the material is unchanged by this treatment (R_F 0·2). In this same solvent system the methylated and acetylated polar lipids showed a slightly higher R_F (0·28), indicating that the polar lipid fraction is presumably a metabolic intermediate containing both hydroxyl and carboxyl groups.

DISCUSSION

The only organic material other than ursolic acid in the mineral medium was calcium pantothenate (200 mg./l.) and this was insufficient to sustain growth of the test organism at the levels observed. Furthermore, almost half the [^{14}C] of the administered labelled ursolic acid appeared in the various metabolites. In view of the control experiments and the analysis of the substrate itself, the amounts of radioactivity found in the metabolites cannot possibly be attributed to an impurity that might have been present in the substrate. These results therefore prove that the pseudomonad degraded ursolic acid, the major terpene of apple wax.

It is generally held that the cuticular waxes from plants accumulate in the soil. Although the biodegradability of these materials is undoubtedly less than that of other plant constituents, they must undergo some degradation by soil organisms to account for the relatively small amounts of such compounds found in orchard soil (Stevenson, 1966). The pseudomonad we isolated grew well on the ursolic acid fraction of the apple wax. Probably the organism we have isolated is only one of many present in soil which can degrade the terpene fraction of cuticular lipids.

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Immunochemical Analysis of *Streptococcus bovis*, Strain s 19, Cell Walls

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SUMMARY

Two antigens, a glucose-rich capsular polymer and a cell wall carbohydrate consisting of *N*-acetylglucosamine, glucose and rhamnose, were isolated from *Streptococcus bovis*, strain s 19. *N*-acetylglucosamine and a haptenic fraction rich in glucosamine inhibited approximately 56 and 63% of the quantitative precipitin reaction between the s 19 cell wall carbohydrate and its homologous antiserum, respectively. A cross-reactivity between a preparation of azo-protein, which consisted of terminal *β*-*N*-acetylglucosaminide residues, and s 19 antiserum substantiated the fact that *N*-acetylglycosamine is one of the major antigenic determinants of the s 19 cell-wall carbohydrate.

Haptenic inhibition studies have not revealed any structural feature of the antigenic determinant of the glucose-rich capsular polymer.

INTRODUCTION

Medrek & Barnes (1962) have shown that *Streptococcus bovis*, a member of the group D streptococci, can be separated into at least 12 serological types on the basis of precipitin reactions. The type-specificity of 75 strains, in most instances, was attributed to a capsular-like material. However, in view of the fact that the immunochemical basis for the antigenic specificity of these type-specific substances has not been elucidated, it is possible that the observed type-specific reactions may also be dependent upon components of the cell wall other than the capsular material.

Elliott (1960) reported the isolation of type-specific carbohydrates consisting of glucosamine, rhamnose and glucose from four serological types of group D streptococci. These type-specific carbohydrates were shown to be components of the cell wall and, therefore, were considered to be the structural counterpart of the group-specific carbohydrates of groups A, B, C, and G hemolytic streptococci.

This report describes the isolation, purification and chemical properties of the type-specific carbohydrates of *Streptococcus bovis* (Medreks strain s 19 type 10), and defines in immunochemical terms the basis for the antigenic specificity of the cell-wall carbohydrate.

METHODS

Streptococcal strains. Streptococcus bovis strains s 19 (Medrek's type 10), c 101 (type 5) and c 33 (type 2) were obtained from Dr R. C. Lancefield of the Rockefeller University. Strains TMC 3 (type 1), TMC 36 (type 3), TMC 48 (type 4) and TMC 106 (type 6)

were kindly supplied by Dr E. M. Barnes, Agricultural Research Council, Food Research Institute, Norwich, England.

Preparation of cell walls. Cell walls were prepared from *Streptococcus bovis* according to the method of Bleiweis, Karakawa & Krause (1964).

Analytical methods. Amino acids, glucosamine and muramic acid were determined by the method of Moore, Spackman & Stein (1958) as described by Karakawa & Krause (1966) and employed the Beckman, Spinco model 120 C, amino acid analyser. Total hexosamine was determined by the method described by Rondle & Morgan (1955). The method of Dische & Shettles (1948) was used for the quantitation of rhamnose. Glucose was determined by the method described by Curtis & Krause (1964) which employed the glucostat reagent (Worthington Biochemical Corporation, Freehold, New Jersey).

Sephadex gel filtration. Partially purified cell-wall carbohydrates and capsular material were subjected to Sephadex gel filtration according to the method described by Karakawa & Krause (1966).

Ion exchange chromatography. Crude capsular carbohydrate was purified on diethylaminoethylcellulose (DEAE-cellulose, Gallard-Schlesinger Chemical Manufacturing Corp., N.Y., 0·68 m-equiv./g.) according to the method described by Young (1966). The column was eluted with 0·02 M-$(NH_4)_2CO_3$ buffer at pH 8·6 and the absorbance of the 5 ml. fractions was read at 220 mμ in a Beckman DU-2 spectrophotometer.

Streptococcal vaccine. The vaccine was prepared from *Streptococcus bovis*, strain S 19, according to the method described by McCarty & Lancefield (1955). The bacteria were grown in 500 ml. of Todd-Hewitt broth at 37° and collected by centrifugation. The bacterial sediment was resuspended in 20 ml. of 0·2% formalized saline and stored at 4°.

Detection of antibodies. Group D type-specific antibodies were detected by the capillary precipitin test employed by Swift, Wilson & Lancefield (1943).

Quantitative precipitin analyses were performed according to the method of McCarty (1952).

Zone electrophoresis. Zone electrophoresis of serum proteins employed a Beckman model R-101 Microzone cell according to the method described by Osterland, Miller, Karakawa & Krause (1966).

RESULTS

Previous studies have shown that *Streptococcus bovis* can be separated into at least 12 serological types on the basis of type-specific carbohydrates (Medrek & Barnes, 1962). These type-specific reactions were considered, in most instances, to be due to a capsular material. However, definitive data on the isolation and analysis of these substances are not available and, therefore, the immunochemical nature of the type-specific substances has not be established. The following studies were designed in an attempt to determine the immunochemical basis for the antigenic specificity of *S. bovis* strain S 19, type 10.

Preparaton of specific carbohydrates. Specific carbohydrates were extracted from the cell walls of *Streptococcus bovis* strains S 19, C 101, C 33, TMC 3, TMC 36, TMC 48 and TMC 106 by the hot formamide method of Fuller (1938) as modified by Krause & McCarty (1961). In this procedure the cell walls, which are composed primarily of a

carbohydrate fraction and a rigid mucopeptide matrix, were treated with formamide at 170° for 15 min. The proteins were precipitated from the extract by the addition of acid ethanol; after centrifugation the crude polysaccharide was precipitated with 5 vol. of acetone. The polysaccharide was then subjected to gel filtration on a Sephadex G-100 column (90 × 15 mm.). The chemical composition of *S. bovis* (strain s 19) cell walls, the carbohydrate extracted with hot formamide and the mucopeptide residue is shown in Table 1. As in the case of the group D streptococci previously examined (Elliott, 1960), the bulk of the rhamnose is associated with the soluble carbohydrate; whereas the mucopeptide residue is essentially devoid of this sugar. The other sugars associated with the carbohydrate are glucosamine and glucose. It should be noted that the concentration of threonine is noticeably high and suggests that this amino acid might be a structural component of the *S. bovis* mucopeptide. This observation was a consistent finding in all seven strains tested. Work is now in progress to determine the value of threonine as a taxonomic tool in the identification of *S. bovis*.

Table 1. *Chemical composition of Streptococcus bovis, strain S19, cell walls and the soluble carbohydrate (CHO) and the insoluble residue after hot formamide extraction*

		Formamide treatment	
Constituent	Cell wall (μmole/mg.)	Extracted CHO (μmole/mg.)	Formamide residue (μmole/mg.)
Glucosamine	0·41	0·55	0·39
Rhamnose	0·46	1·41	< 0·01
Glucose	0·70	1·55	< 0·01
Muramic acid	0·25	< 0·01	0·41
Alanine	0·79	< 0·01	1·15
Glutamic acid	0·44	< 0·01	0·52
Lysine	0·33	< 0·01	0·41
Glycine	0·13	< 0·01	0·04
Threonine	0·18	< 0·01	0·24
Ammonia	0·33	0·16	0·24

Table 2. *Chemical composition of formamide-extracted cell-wall carbohydrate of Streptococcus bovis*

	Strains		
Constituent	S 19 (μmole/mg.)	C 101 (μmole/mg.)	C 33 (μmole/mg.)
Glucose	1·55	1·10	1·15
Glucosamine	0·55	0·26	0·94
Rhamnose	1·41	1·97	0·98

Chemical analysis of the cell-wall carbohydrates isolated from strains C 101 and C 33 revealed chemical constituents similar to strain S19. These results are shown in Table 2. Note that glucose, rhamnose and glucosamine are significant components of the cell-wall carbohydrates of all three strains; however, there is considerable variation in their relative concentrations. Strain C 33 carbohydrate contains a higher concentration of glucosamine than do strains S 19 and C 101. Rhamnose, on the other hand, is more abundant in strain C 101 than in the other strains.

Isolation of the capsular material from strain S 19. Medrek & Barnes (1962) suggested that the type-specific reaction of 11 of the 12 types of *Streptococcus bovis* was dependent upon a capsular-like material. In the present studies, washed *S. bovis* (strain S 19), grown in beef-heart infusion broth containing 3% sucrose, were suspended in saline and homogenized at low speed in a Waring blendor for 5 min. The cocci were removed by centrifugation; and the clear supernatant, which contained the capsular material, was subjected to DEAE-cellulose chromatography and Sephadex G-75 gel-filtration as described in Methods. The results of the DEAE-cellulose chromatography of the crude capsular material are shown in Fig. 1. It should be noted that only a single major fraction was eluted from the DEAE-cellulose column. This substance after further purification by gel filtration, consisted of 3·95 μmole glucose/mg., 0·09 μmole glucosamine/mg., and 0·01 μmole rhamnose/mg. Only minimal amounts

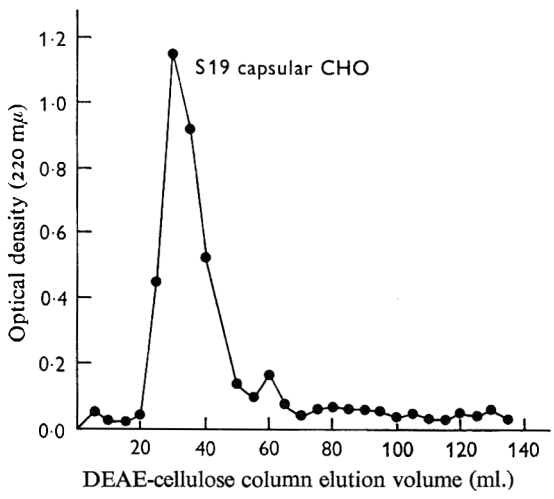

Fig. 1

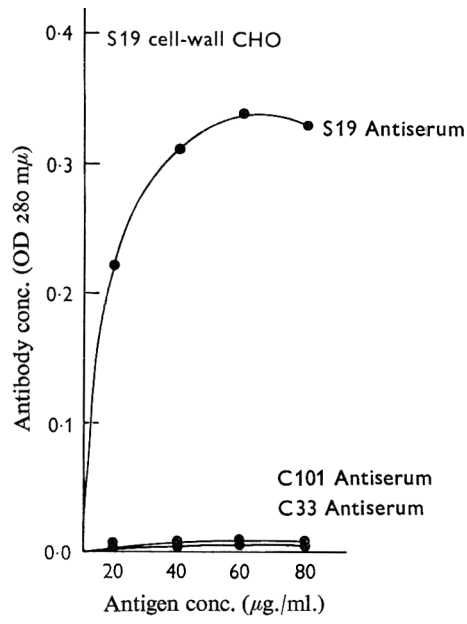

Fig. 2

Fig. 1. DEAE-cellulose column chromatography of the capsular material (CHO) of *Streptococcus bovis*, strain S19. Column dimensions: 300 × 30 mm. Buffer: 0·02 M-$(NH_4)_2CO_3$, pH 8·4 at 4°.

Fig. 2. Quantitative precipitin reaction between the purified cell-wall carbohydrate of *Streptococcus bosi* (strain S 19) and anti-S 19, C 101 and C 33 sera.

of phosphorus were detected, which suggest that the glucose polymer was not a teichoic acid. An abundance of the glucose-rich polymer was also isolated from the growth medium by the method described by Michel & Krause (1967). These results and those presented in Table 2 clearly suggest that *S. bovis*, strain S 19, contains two chemically distinct carbohydrates: a glucose-rich polymer and a cell-wall carbohydrate.

Absorption of serum with Streptococcus bovis, strains S 19, C 101, and C 33, cell walls. The following serological studies were designed to establish the fact that the antibodies present in the S 19 antiserum are elicited by type-specific cell-wall carbohydrates and are

γ-globulins. Strain S 19 antiserum was absorbed with capsular-free cell walls of strains S 19, C 101, and C 33 (1 mg./ml.) at 37° for 30 min. Following absorption with S 19 cell walls, no cell-wall carbohydrate antibodies were detected in the absorbed S 19 antiserum by the capillary precipitin test; whereas, antiserum S 19 absorbed with C 101 and C 33 cell walls gave strong precipitin reactions with S 19 cell-wall carbohydrate. When antibodies recovered from the S 19 cell wall/antibody complex by the acid dissociation technique of Osterland *et al.* (1966) were subjected to zone electrophoresis, the electrophoretic patterns revealed a single band with a mobility similar to that of the γ-globulin peak of the unabsorbed S 19 antiserum. This isolated antibody preparation was identified as IgG-globulin by means of immunoelectrophoresis.

Antigenic properties of carbohydrates extracted from Streptococcus bovis, strain S 19. The results of the quantitative precipitin tests between S 19 cell-wall carbohydrate and antisera S 19, C 101 and C 33 are shown in Fig. 2. The S 19 cell-wall carbohydrate gave a significant quantitative precipitin reaction with its homologous antiserum; whereas the reactions with the heterologous antisera were less reactive in this respect. Quantitative precipitin tests between the carbohydrates of strains TMC 3, TMC 36, TMC 48 and TMC 106 and S 19 antiserum were negative. These results clearly substantiate the fact that the reaction between strain S 19 cell-wall carbohydrate and its homologous antiserum is type-specific.

Table 3. *Chemical composition of partially acid-hydrolysed cell-wall carbohydrate of Streptococcus bovis, strain S 19*

	Carbohydrate (μmole/mg.)			
Constituent	Untreated	Mole ratio	Treated	Mole ratio
Glucosamine	0·55	1·0	0·32	1·0
Glucose	1·55	2·8	1·63	5·1
Rhamnose	1·41	2·5	1·54	4·8

The glucose-rich capsular material isolated from a culture of strain S 19 gave a very weak precipitin reaction with homologous antiserum. Work is now in progress to obtain precipitating antibodies against this glucose polymer from swine and horses.

Characterization of the type-specific determinant of strain S 19 cell wall carbohydrate. Results showed that the cell-wall carbohydrate of various strains of *Streptococcus bovis* possess similar chemical constituents but are immunologically distinct antigens. Theoretically, glucose, rhamnose or glucosamine could contribute to the antigenic specificity of the S 19 carbohydrate. In an attempt to determine the immunochemical basis for this antigenic specificity, purified S 19 carbohydrate was partially hydrolysed with N HCl for 15 min. at 100°. The treated carbohydrate was subjected to Sephadex G-75 gel filtration and lyophilized. The results of the chemical analysis of this acid-treated carbohydrate are shown in Table 3. The treated carbohydrate contained less glucosamine than the untreated carbohydrate. Further, the acid-treated carbohydrate, when employed in a quantitative precipitin test, gave a markedly weaker reaction than the untreated carbohydrate. These results are depicted in Fig. 3. Note that the quantitative precipitin reaction between the treated carbohydrate and antiserum S 19 was more than 50% less than the precipitin reaction between the untreated carbohydrate and antiserum S 19.

Monosaccharides and haptenic inhibitors were used in the quantitative precipitin inhibition studies between S 19 carbohydrate and S 19 antiserum to test the view that glucosamine is the major antigenic determinant of the S 19 cell-wall carbohydrate. Among the inhibitors employed in these studies was a haptenic fraction consisting of glucosamine, glucose and rhamnose in a mole ratio of 1·0:0·5:0·1 which was isolated from an acid-hydrolysate of the S 19 carbohydrate as described by Michel & Willers (1964). The results of the quantitative precipitin inhibition studies are shown in Fig. 4. As depicted in this figure, 63% of the quantitative precipitin reaction between the S 19 carbohydrate and its homologous antiserum was inhibited by the glucosamine-rich hapten. Further, *N*-acetylglucosamine inhibited approximately 56% of this quantitative precipitin reaction at a concentration of 10 mg./ml.; whereas glucose and rhamnose were less reactive at the same concentration.

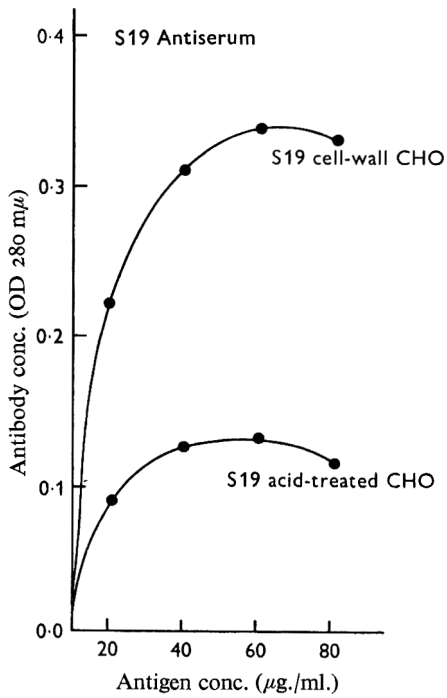

Fig. 3

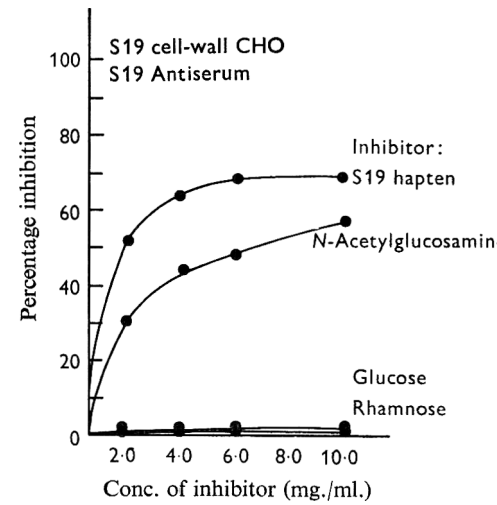

Fig. 4

Fig. 3. Quantitative precipitin reaction between the purified S 19 cell-wall carbohydrate, purified S 19 acid-treated cell-wall carbohydrate and S 19 antiserum.

Fig. 4. Inhibition of the quantitative precipitin reaction between S 19 carbohydrate and S 19 antiserum with monosaccharides and a glucosamine-rich haptenic fraction.

Further evidence to support the view that *N*-acetylglucosamine is the immunodominant determinant of the S 19 carbohydrate was obtained from studies on the cross-reactivity between S 19 antiserum and ovalbumin-azo-phenyl-β-*N*-acetylglucosamine. This azoprotein was kindly supplied by Dr M. McCarty, Rockefeller University. In these studies it was shown that the azoprotein cross reacted with horse antiserum S 19 and this precipitin reaction was specifically inhibited by

N-acetylglucosamine at a concentration of 10 mg./ml. These results suggest that β-*N*-acetylglucosamine could be the immunodominant determinant of the s 19 cell-wall carbohydrate.

DISCUSSION

Evidence has been presented to indicate that strain s 19 contains at least two distinct carbohydrates: a glucose-rich polymer and a cell-wall carbohydrate consisting of *N*-acetylglucosamine, rhamnose and glucose. The capsular carbohydrate was shown to consist of glucose units with minimal concentrations of rhamnose and *N*-acetylglucosamine. This finding is consistent with the observations of other investigators (Duguid, 1951; Dain, Seely & Neal, 1956; Bailey & Oxford, 1958). Bailey & Oxford (1958), for example, consistently observed a dextran-like polysaccharide in certain strains of *Streptococcus bovis* which were grown in sucrose-enriched media. Haptenic inhibition studies, employing glucose, maltose, cellobiose and gentiobiose, have not revealed any of the structural features of the antigenic determinant of the capsular material.

The cell-wall carbohydrate, which is a structural analogue of the group-specific antigens of most haemolytic streptococci, was successfully isolated from a hot formamide extract of *Streptococcus bovis*. Purification was achieved by DEAE-cellulose chromatography and gel filtration. Quantitative precipitin inhibition studies, employing haptenic fractions isolated from partial acid hydrolysates of s 19 carbohydrate, indicated that *N*-acetylglucosamine comprised a portion of the antigenic determinant of the s 19 carbohydrate.

McCarty (1956) has shown that terminal *N*-acetylglucosaminide residues are the major antigenic determinants of the group-specific carbohydrate of group A haemolytic streptococci. A cross-reaction between the group A and s 19 carbohydrates was observed in this study and suggests that *N*-acetylglucosamine, a common feature of both carbohydrates, could possibly be responsible for this cross-reactivity.

The existence of two major carbohydrate antigens in the cell walls is not uncommon in streptococci, Michel & Krause (1967) isolated two antigens, a group-specific and a type-specific carbohydrate, from a hot formamide extract of group F streptococci. The type-specific antigen was composed of rhamnose, glucose, galactose and galactosamine; whereas the group-specific antigen was composed of rhamnose, glucose and galactosamine. Unlike *Streptococcus bovis*, strain s 19, both antigens were components of the cell walls. Group B streptococci also contain two carbohydrate antigens: a type-specific capsular material and a group-specific cell-wall carbohydrate (Lancefield, 1934).

The work reported here supports the view that *Streptococcus bovis* can be separated into distinct serological types (Medrek & Barnes, 1962). Eventually, with greater knowledge of the immunochemistry of the carbohydrates associated with *S. bovis*, it seems feasible to assume that a useful system for the classification of these streptococci may be obtained, which will compare favourably with that employed for the haemolytic streptococci.

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Behaviour of Streptomycetes in Soil

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SUMMARY

Most of the conidia from eight different streptomycetes did not germinate in natural soil under conditions apparently suitable for their germination. When germinated in nutrient solution and then added to soil, they grew for a limited period, sporulated and then the vegetative hyphae disappeared, leaving behind only spores.

Streptomycete conidia produced naturally in garden soil decreased in number by about a half after 12 days. Nevertheless, the conidia are the main means of survival and it appears that streptomycetes exist in soil mainly as spores. Mycelia also existed, but in the main were restricted to micro-sites where nutrient was available.

INTRODUCTION

Streptomycetes are widely distributed in soils, especially in those which are dry and not too acid, and those rich in organic matter; here, in propagule numbers, they frequently exceed the combined counts of all other bacteria (Alexander, 1961; Waksman, 1959). They decompose plant and animal residues, for the addition of these residues to soil greatly increases the activity and size of the streptomycete population (Alexander, 1961; Waksman, 1959).

It is still debatable whether streptomycetes occur in soils mainly as vegetative mycelia or as spores (Waksman, 1959). Lutman, Livingston & Schmidt (1936) concluded from soil smears and from heating soil to kill vegetative mycelia that streptomycetes occur mainly as mycelia. On the other hand, Subrahmanyan (1929), from observations on the origin of colonies arising on dilution plates, and Pfennig (1958), from glass slides buried in soil, concluded that they exist mainly as spores; mycelium was restricted to colonization of plant and animal residues but was found in the soil itself.

Skinner (1951) attempted to solve the problem of how streptomycetes exist in soil by shaking soil samples with sand particles, reasoning that this would increase the propagule numbers by breaking the mycelium into many viable fragments if the streptomycetes were present mainly as mycelium. However, no increase in propagule numbers occurred, and Skinner therefore concluded that the streptomycetes were present, and presumably persisted in soil, as spores rather than as mycelium.

In this paper I have attempted to answer the same question, but by using a more direct method than that employed by Skinner.

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METHODS

Soil. The soil used (Urrbrae loam) was a red-brown earth with a silty loam texture and a pronounced fine sand fraction (Piper, 1938). Red-brown earths occur throughout the wheat-growing areas of South Australia as well as in other parts of Australia. Soil was collected from three sites, all within the grounds of the Waite Agricultural Research Institute: (1) a field in pasture for the previous five years (pasture soil); (2) an experimental plot sown continuously with wheat without nitrogen fertilizer since 1929 (wheat-field soil); (3) an ornamental flower garden with a dark-coloured soil of high organic content (garden soil).

Soil samples collected from these sites were passed through a sieve (2 mm. mesh) and then stored for a short period in plastic bags at room temperature. The approximate numbers of streptomycete propagules/g. dry weight of the stored soils ranged from $9{\cdot}2 \times 10^5$ to $3{\cdot}3 \times 10^6$, based on counts made from dilution plates with chitin agar (Lingappa & Lockwood, 1962; Lloyd, Noveroske & Lockwood, 1965).

When required, garden and wheat-field soils were sterilized by γ-irradiation, the exposure dosage being 5 Mrad. from a ^{60}Co source.

Streptomyces species. Isolates of *Streptomyces* spp. were obtained from garden soil by spraying a diluted soil suspension over the surface of chitin agar (Lingappa & Lockwood, 1962), and subsequently transferring single colonies to agar slopes. From these isolates a few were selected which obviously differed from one another in appearance and in the morphology of their sporophores (Pridham, Hesseltine & Benedict, 1958).

Spore germination, germling (germinated conidia) growth and conidial survival on soil were assayed by a direct method (Lingappa & Lockwood, 1963; Bumbieris & Lloyd, 1967). In brief, either conidia or germlings from different isolates of *Streptomyces* spp. were washed thoroughly in 1/10 mineral solution (Pridham & Gottlieb, 1948) and then 3 or 4 drops of a conidial or germling suspension spread over the surface of moist soil (18 to 20% soil moisture) in a Petri dish and incubated at 27°. At different time intervals the streptomycetes were stained on the soil surface with 1% rose bengal and then recovered in a plastic film made from a solution of polystyrene. Conidia or germlings recovered from soil immediately after deposition served as controls (Pl. 1, fig. 1).

RESULTS

Disappearance of hyphae in soil. The germlings (Pl. 1, fig. 1) of eight different Streptomyces isolates added to unsterilized garden soil grew into hyphae during the first 1 to 2 days, sporulated abundantly by the third day, and then most vegetative hyphae disappeared. Occasionally a germtube failed to grow and instead fragmented into rectangular arthrospores whose linear pattern preserved the general shape of the original germling. Thus in unsterilized garden soil by 3 days there existed arthrospores, sporophores (Pl. 1, fig. 2), abundant conidial chains and individual scattered conidia (Pl. 1, fig. 3), and an increased bacterial population, but few or no vegetative hyphae in the region of soil once occupied by the germlings. The sequence was similar in unsterilized wheat-field soil, except that the process was slower with fewer conidia formed.

With sterilized soils the picture was different. Both sterilized garden and wheat-field

soils supported abundant vegetative growth of mycelia (Pl. 1, fig. 4), and sporulation was so dense that spores were visible in mass on the soil surface.

Inhibition of spore germination by soil. Germinated conidia, when added to unsterilized soil, grew for a short period, sporulated and then the hyphae disappeared, leaving mainly conidia behind in the soil. Now, if these conidia are the survival propagules of streptomycetes, then, presumably, they do not all germinate spontaneously in soil. This hypothesis was next tested.

Table 1. *Germination of streptomycete spores on natural soils, irradiation-sterilized soil and water agar*

	Percentage spore germination*				
Strepto-mycete isolate	Garden soil	Pasture soil	Wheat-field soil	Sterilized soil	Water agar
100	1	13	4	77	94
102	3	7	11	75	95
105	5	9	7	66	94
111	7	4	11	65	76
112	2	2	3	—	—
113	2	14	2	87	77
117	2	4	2	91	91
118	1	6	2	90	96
Average	3	7	5	79	89

* Values are means from the two separate experiments.

Washed conidia of eight different Streptomyces isolates were added to unsterilized soils and incubated for 2 days: most failed to germinate (Table 1). The range of germination was 1 to 7% on garden soil, 2 to 14% on pasture soil and 2 to 11% on wheat-field soil. Most isolates behaved similarly with lower germination on garden soil than on pasture or wheat-field soil. That inhibition of spore germination was closely associated with the presence of other soil micro-organisms was shown by the high germination rate (65 to 90%) on both garden soil and pasture soil previously sterilized by γ-irradiation (Table 1). Nevertheless, a few conidia from each Streptomyces isolate did germinate on natural soils (Table 1). In most cases the emergent germtube quickly produced a terminal sporophore which subsequently sporulated. In other cases the germtube failed to develop further, and fragmented into arthrospores. Occasionally, when conidia were put on the soil in clumps, several spores germinated from within the same clump (Pl. 1, fig. 5).

Survival of conidia in soil. Two separate methods were used: (*a*) germlings of four different Streptomyces isolates were added to garden soil and incubated for 3 days. During that time they grew and sporulated, leaving behind localized regions of high conidial density (soil-grown conidia). From within these regions, five separate areas (each 600 μ^2) were selected, and the conidia counted. Although this non-random sampling was undesirable, it was necessary because the regions of high conidial density were separated from each other by soil almost devoid of conidia. (*b*) With the second method, culture-grown conidia were added to garden soil and then random areas of soil were examined immediately and at different time intervals.

With soil-grown conidia (Fig. 1*a*) the conidial population of 3 of the 4 isolates

decreased rapidly during the first week, but thereafter the mortality rate declined. The fourth isolate was irregular. By the end of 12 days in soil, the combined conidial populations from all 4 isolates had decreased to about half the original number. Culture-grown conidia behaved similarly except that the mortality rate was accelerated after 12 days with 3 of the 4 isolates (Fig. 1*b*). By 16 days the combined populations had decreased to about one-third the original number. Streptomyces isolate 100 was unique: the conidial population added to soil was small, and this decreased during the first week but thereafter remained relatively stable. Perhaps in this case so few alien spores were added to the soil that their presence disturbed the rest of the soil's natural microflora very little. Hence these few introduced spores encountered less antagonism from the soil's natural microflora than occurred when large numbers of alien spores were added to soil, as was the case with the other 3 isolates.

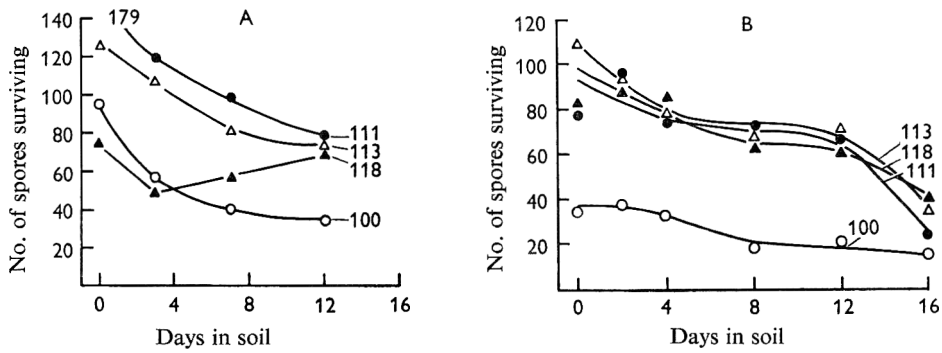

Fig. 1. Survival in soil of conidia from four different Streptomyces isolates.

(*a*) Germinated conidia were added to garden soil, where they grew and sporulated within 3 days. At the end of the 3 days counts were made of the conidia present (0 days), followed by counts every fourth day thereafter.

(*b*) Culture-grown conidia of the same Streptomyces isolates were added directly to soil and conidial counts were made immediately (0 days) and every fourth day thereafter.

The decline in conidial numbers with time may not be as great as suggested here, for the population is dynamic; some spores are destroyed while a few others germinate, grow and sporulate to partly replenish the declining spore population. In this study I counted only those conidia which were originally present at 0 days and ignored any spores formed during the time of the experiment. These newly formed spores could be recognized because they were either attached to sporophores or in chains.

After 16 days drops of 0·1% peptone solution were added to soil containing the culture-grown spores, and the following day the number of spores which germinated was counted. Results were as follows: isolate 100, 63%; isolate 111, 70%; isolate 113, 86%; isolate 118, 61%. Spores from the same isolates, when similarly tested one day after placement on soil, gave results of 71, 79, 76 and 64%, respectively. Thus there was a small reduction in germination of spores kept on soil for 16 days, but, nevertheless, most spores which did survive for 16 days on soil were viable and germinated. When spores from the same isolates were placed on an agar surface the average germination was 89%.

DISCUSSION

Whether streptomycetes exist in soil mainly as spores or mycelia is still debatable. The evidence presented in this paper strongly supports the former view for the following reasons: germinated conidia, when added to unsterilized soil, grew into hyphae, sporulated and then the hyphae disappeared, leaving mainly conidia behind in the soil. Moreover, when conidia of streptomycetes were added to unsterilized soil most failed to germinate. This failure, or inability, to germinate in unsterilized soil does not appear to be an enforced dormancy due to some internal inhibition, for such spores germinated readily on an agar surface, nor is it related to such environmental factors as temperature of the soil or its moisture content, for such spores germinated readily under the same conditions, with the sole difference that the soil had been sterilized previously by irradiation.

Failure to germinate in soil is, however, closely associated with the presence of other soil micro-organisms. Perhaps, as shown by Ko & Lockwood (1967), for many fungal spores in soil, there is a deficiency of the nutrients required for germination of streptomycete spores, imposed by microbial activity in soil. If so, this would explain why streptomycete spores germinated readily in sterile soil. When soil is sterilized by irradiation sufficient nutrients leak from the killed microbial cells to raise the level of exogenous nutrients sufficiently to promote germination of any streptomycete spores added to this soil. Presumably, also, in unsterilized soil the existence of available exogenous nutrients, such as from decomposing plant or animal residues, would also promote germination of streptomycete spores in the vicinity of the residues.

Within the soil there was a continual decline in the size of the original spore population, for streptomycete spores are not especially resistant to destruction in soil. Therefore, if streptomycetes are to survive in soil, there must be replenishment of spores, and this does occur. A few spores within the population do germinate spontaneously, grow into hyphae and then sporulate, but these alone are insufficient to counteract the higher mortality rate.

The main means for the survival of the species appears to be the partial resistance of spores coupled with intermittent replenishment of the original spore population by mass germination, which is probably induced by the arrival of a suitable organic substrate. The spores germinate and colonize the new substrate with vegetative hyphae. Eventually, profuse sporulation occurs and then the hyphae disappear, perhaps induced to do so through depletion of available nutrients, accumulation of metabolic waste-products or even increased competition from other micro-organisms. Whatever the inducement, the hyphae disappear either through lysis or through fragmentation into arthrospores. Left behind is a high density of spores, markers of a once flourishing growth of vegetative hyphae. Such mass germination may endanger the survival of the population if the spores germinate in an environment inadequate to sustain vegetative growth. This seems unlikely with streptomycetes, for a sporophore can be formed almost directly from a germtube and frequently before the hypha is lysed or disappears.

Thus it appears that streptomycetes exist in soil mainly as spores. Mycelium also exists, for it is an essential link between one spore generation and the next. But in general mycelium is restricted to those micro-sites in soil where there is available organic matter adequate to promote spore germination and sustain vegetative growth. Also, perhaps, at other micro-sites such as the surface of soil crumbs exposed to the

atmosphere there is sufficient reduction in competition to permit vegetative growth. For the rest of the soil, away from decomposing plant or animal residues, streptomycetes exist mainly as spores.

I would like to thank Mr N. Bumbieris for his technical help.

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EXPLANATION OF PLATE

(All figures approximately × 1600)

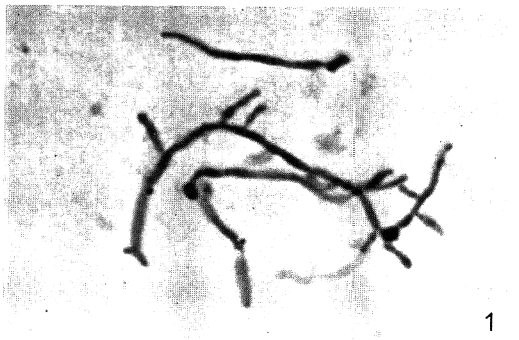
Fig. 1. Germinated conidia of a *Streptomyces* sp. recovered immediately after deposition on soil.

Fig. 2. Spiral sporophores of a *Streptomyces* sp. formed in garden soil 2 to 3 days after addition of germinated conidia to soil.

Fig. 3. Scattered conidia and conidial chains of a *Streptomyces* sp. formed in garden soil 3 to 4 days after addition of germinated conidia to soil.

Fig. 4. Streptomycete hyphae growing on sterilized garden soil 2 days after addition of germinated conidia to soil.

Fig. 5. Several germtubes growing from a clump of streptomycete conidia 1 day after addition of conidia to soil.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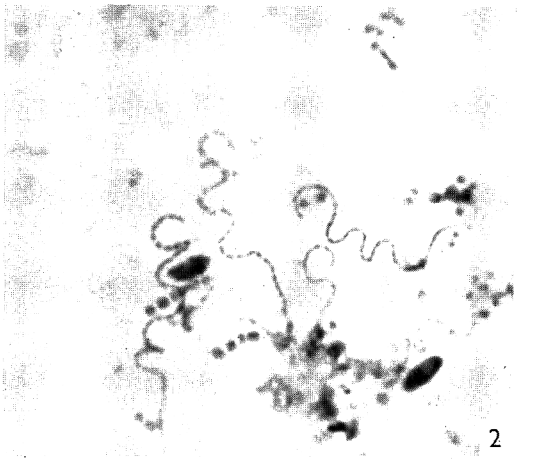
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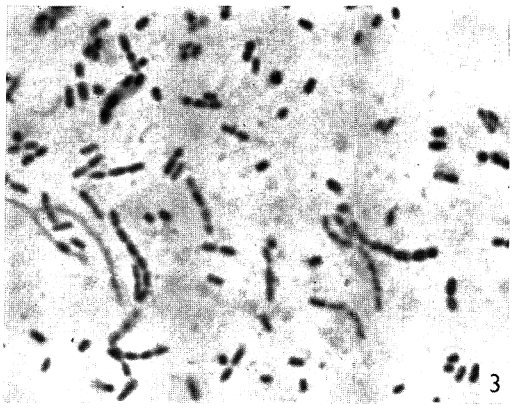
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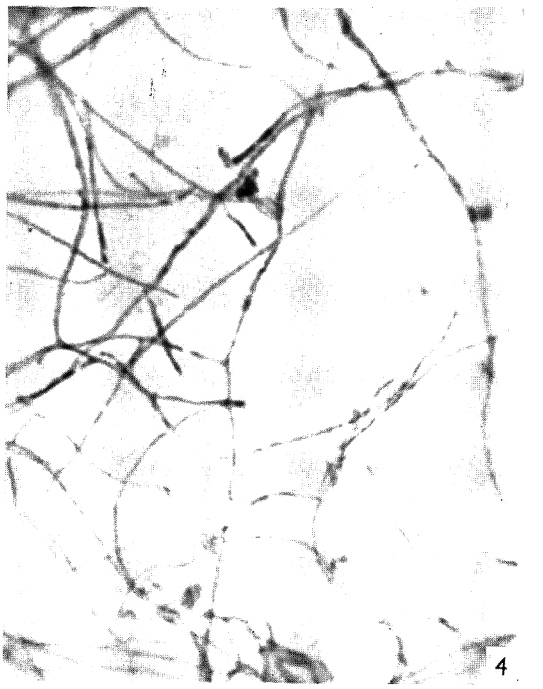
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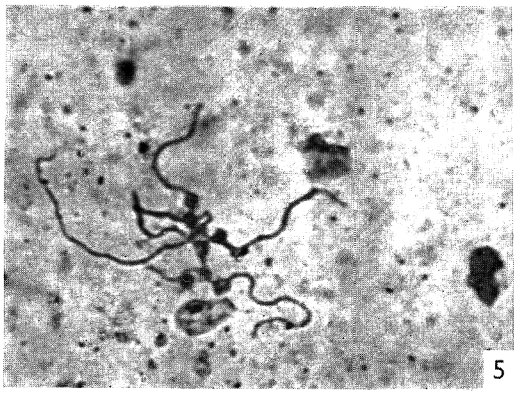
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Sporulation in *Bacillus subtilis*. Establishment of a Time Scale for the Morphological Events

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SUMMARY

Chemostat cultures of *Bacillus subtilis* can be obtained in which a large proportion of the cells are initiated to sporulation. In a steady-state culture the fraction of cells which exhibit any particular stage of spore development is a function of the time taken for a cell to reach that stage after having been initiated to spore formation, and of the time required for the cell to pass through that stage. Equations describing this relationship have been derived. From these equations and from values for the fractions of cells exhibiting the morphological stages of spore development seen in electron micrographs, a time scale for the morphological events occurring during sporulation has been constructed.

By observing the effect of a sudden decrease in flow rate of a continuous flow system initially at steady state it was possible to time the occurrence of specific events, biochemical or physical, which were connected with the formation of spores. By this means, refractility was shown to be associated with stage V.

INTRODUCTION

The sequential nature of the cytological changes which occur during sporulation of bacilli has been demonstrated in *Bacillus cereus* (Young & Fitz-James 1959*a*, *b*; Fitz-James, 1965) and in *Bacillus subtilis* (Schaeffer, Ionesco, Ryter & Balassa, 1965; Szulmajster, 1964; Ryter, 1965; Kay & Warren, 1968). In the latter organisms Schaeffer *et al.* (1965) described eight stages of development (Fig. 1). Starting with the vegetative cell (stage O), in stage I an axial chromatin filament is formed, followed in stage II by the development of a spore septum due to invagination of the cell membrane. During stage III the spore membrane is completed by growth of the spore septum until it encloses the spore chromatin. Stage IV consists of the formation of a cortical layer between the layers of the complete spore membrane; then, in stage V, a spore coat is formed around the cortex. Stage VI is characterized by maturation of the spore inside the mother cell, autolysis of which leads to the free spore, stage VII. Ryter, Ionesco & Schaeffer (1961) and Ryter (1965) isolated mutants blocked at these various stages of spore development; such cells do not reach the morphological stages which occur after the block.

Young & Fitz-James (1959*a*, *b*); Schaeffer *et al.* (1965) and Kay & Warren (1968) have attempted to time the appearance of these morphological stages and to correlate them with the biochemical processes taking place during sporulation. All these experimenters used cells grown in batch culture and sampled at 30 to 90 min. intervals; for this reason and because cells do not sporulate synchronously the times for the appearance of the stages could not be accurately obtained. The sporulation of *B. cereus* is probably the most synchronous that has been reported, but even then the appearance of refractility was spread over a period of 15 to 30 min. (Young & Fitz-James, 1959*a*). In other instances the variability has been greater; for example, in the culture of *B. subtilis* investigated by Kay & Warren (1968) the appearance of refractility was spread over about 120 min. for the whole population. Resuspension experiments with *B. subtilis* give a more defined zero-time but refractility still occurs with a scatter of 90 to 120 min. (Mandelstam & Waites, 1968). It is therefore not surprising that the time scales given by different workers for the morphological events have been inconsistent.

It is shown below that a steady-state continuous culture technique gives more precise values for the time intervals for each of the morphological stages of sporulation to occur. The method depends on the fact that steady-state cultures can be obtained in which a large proportion of the cells are sporulating (Dawes & Mandelstam, 1969). The proportion of such cells exhibiting a particular stage of spore development will increase with the time taken to pass through that stage. By measuring the relative incidence of the stages in a population one can obtain a measure of the duration of each stage. This technique does not depend on synchrony of the sporulation process for accuracy and in principle only one sample is required for examination.

The sequence of morphological events occurring during sporulation can be represented as

$$X_0 \rightarrow X_1 \ldots \rightarrow X_i \rightarrow \ldots \rightarrow X_7,$$

where X_0 to X_7 are the eight stages recognizable by electron microscopy, and X_i is any intermediate stage; $x_0 \ldots x_7$ will be used to denote the concentrations of these species. When vegetative cells are initiated to sporulate at a specific rate k, then the number of cells which are initiated per unit time is kx_0. The cells will then pass through the various stages on their way to becoming mature spores (X_7), and will spend a certain amount of time in each. A time scale for the sporulation process can therefore be constructed and τ_i is defined such that stage X_i takes time τ_{i-1} to τ_i from beginning to end. In the hypothetical case in which there is no washout and k and x_0 are constants

$$x_i = \int_{\tau_{i-1}}^{\tau_i} kx_0 \, dt, \tag{1}$$

giving

$$x_i = kx_0(\tau_i - \tau_{i-1}). \tag{2}$$

The number of cells in any stage is therefore proportional to the time spent in that stage.

In the chemostat, however, the cells exhibiting any particular stage of sporulation become diluted from the culture because of washout. When account is taken of this (Herbert, Elsworth & Telling, 1956), equation (1) becomes

$$x_i = \int_{\tau_{i-1}}^{\tau_i} kx_0 e^{-Dt} \, dt, \tag{3}$$

where D is the rate of dilution of the culture.

In a steady state, x_0 and k are constant, hence

$$x_i = \frac{kx_0}{D}(e^{-D\tau_{i-1}} - e^{-D\tau_i}). \tag{4}$$

If $e^{-D\tau}$ is plotted against τ, the concentration of cells in stage X_i is poportional to the area under the curve between τ_{i-1} and τ_i (see Fig. 1). Moreover, because equation (2) is simply the first approximation of (4), the solution to equation (4) can also be obtained by a method of successive approximation based on the use of equation (2). In this

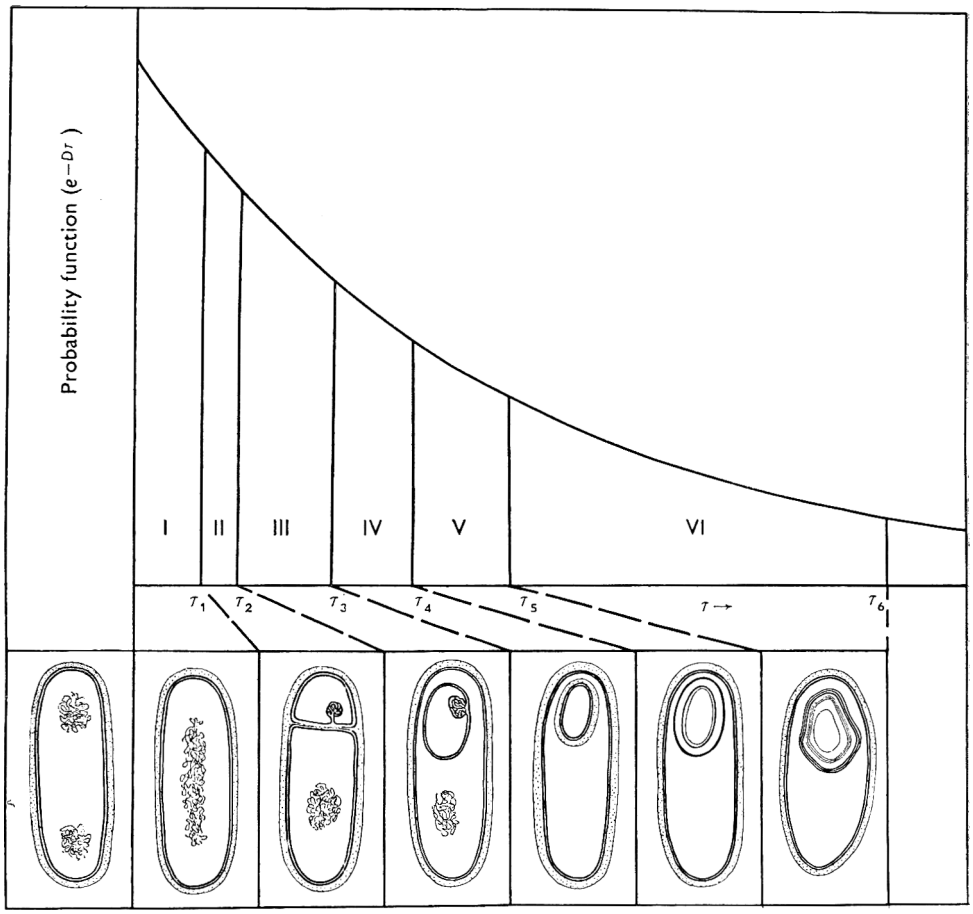

Fig. 1. Theoretical curve describing the probability that a cell initiated to sporulation will remain in the chemostat by the time it reaches a particular stage of spore development. The number of cells in each of the stages is proportional to the area in the appropriate segment of the curve (see text). For example, for stage II the number of cells is proportional to the area under the curve between τ_1 and τ_2. The various stages as defined by Schaeffer *et al.* (1965) are indicated.

method values of τ_i calculated by means of (2) are used to 'correct' the observed values of x_i for washout. These amended data are then used to recalculate a second set of values of τ_i from (2) and the original values of x_i are again amended. This procedure is repeated until the τ_i values no longer undergo significant change.

This method is not readily applicable if stages VI and VII are included, since a

large number of approximation cycles are required to obtain stable values of τ_i. It is less tedious, however, if a reference point can be obtained for the time for the commencement of one of the stages from II to V. This can be done by a second experimental procedure which involves upsetting the steady state of the culture. The probability with which vegetative cells of *Bacillus subtilis* are initiated to sporulation is a function of the growth rate of the bacteria (Dawes & Mandelstam, 1969); hence a sudden decrease in the dilution rate of a continuous culture, and hence a decrease in growth rate, leads to a sudden increase in the incidence of refractile spores at an interval after the change. This interval is the time required for a cell to reach refractility after it is initiated to spore formation. Now, it is known that refractility characterizes the later stages of sporulation but the precise correlation with the morphological changes occurring during sporulation is not known. This correlation can be obtained by comparing the results of electron microscopy with those of phase-contrast microscopy. Once a cell has been initiated to spore formation in a chemostat there is a probability that it will be washed out of the vessel before it has reached a particular stage of development; the later the stage, the greater this probability will be. At any steady state the relationship which holds is

$$y_i = \int_{\tau_{i-1}}^{\infty} kx_0 e^{-Dt}\, dt,$$

where y_i = the fraction of cells which are in stage X_i and all later stages. Therefore

$$y_i = \left(\frac{kx_0}{D}\right) e^{-D\tau_{i-1}}. \tag{5}$$

To correlate refractility with the morphological stages, the fraction of cells which contain refractile spores is determined by direct counting in the phase-contrast microscope; this value is compared with the fractions of cells which have reached each of the morphological stages determined by electron microscopy.

Thus the results in Table 1 can be presented as follows:

Percentage of cells in stage VII	3·7
Percentage of cells in stages VI and VII	18·4
Percentage of cells in stages V to VII (inclusive)	23·0
Percentage of cells in stages IV to VII (inclusive)	27·8

From phase-contrast observations the number of cells exhibiting refractility was 22·0%. Thus refractility covers the last three stages and is developed near the beginning of stage V.

One can now determine the times for the beginning of each stage. Equation (5) is a general equation which holds for refractility as well as for any morphological stage. Hence we can write

$$y_r = \left(\frac{kx_0}{D}\right) e^{-Dt_r}, \tag{6}$$

where y_r is the fraction of cells showing refractility, and t_r is the time required for a cell to reach refractility. From equations (5) and (6) one can show that τ_{i-1}, the time for the beginning of stage X_i is given by

$$\tau_{i-1} = t_r - \frac{1}{D} \ln\left(\frac{y_i}{y_r}\right). \tag{7}$$

It is not necessary to use the method of successive approximation if the above procedure is adopted, since from equation (7) the time at which any intermediate

stage begins (τ_{i-1}) can be determined. The approximation procedure has been presented, however, since it is a method for solving equation (4) in the case of mutants not forming refractile spores.

METHODS

Organism. Bacillus subtilis (Marburg strain 168), auxotrophic for indole or tryptophan, was stored as a spore suspension.

Cultivation. Continuous cultivation was carried out at 37° in a chemostat containing 100 ml. of culture stirred by vigorous aeration (1 l. min.$^{-1}$ of sterile-filtered humidified air). Medium was fed into the vessel by means of a peristaltic pump and the culture maintained at constant volume with a syphon.

The medium used was a modification of that used by Donnellan, Nags & Levinson (1964), with no ammonium ion present and with glutamate at a limiting concentration of 1·36 mM. Growth under these conditions was limited by nitrogen. The concentration of Mg^{2+} was 25 mM; this gave a higher yield of spores.

The spore inoculum, suspended in 10 ml. of 1% hydrolysed casein medium (Mandelstam & Waites, 1968), was added to the culture vessel, which was heated to 80° to decrease contamination by vegetative bacteria that might have been introduced during inoculation. After 10 min. at 80° the vessel was cooled to 37° and aeration and medium flow started. When growth became limited, about 90% of the culture was run out of the vessel and fresh medium added to dilute out residual amino acids. The chemostat was allowed to reach a steady state at a dilution rate of 0·134 hr^{-1}.

Electron microscopy. A sample (5 ml.) was taken from the chemostat, treated with fixative (0·5 ml.), centrifuged and the pellet fixed without resuspension by the method of Kellenberger, Ryter & Séchaud (1958). After staining in uranyl acetate (0·5% in Kellenberger buffer), the cells were dehydrated in graded ethanol and embedded in Araldite. Sections were cut from the Araldite blocks and stained with lead citrate (Reynolds, 1963).

Counting procedures. Counts of the cells exhibiting the eight stages of sporulation were obtained by examination of 43 electron micrographs with a hand lens. Only complete longitudinal sections were scored, since in a transverse section a developing spore might be missed altogether, and the stage of development would have been more difficult to recognize. From the 43 plates, 434 longitudinal cell-sections were counted.

The fraction of the cells in the culture containing refractile spores was estimated by direct counting of a cell sample, fixed in formaldehyde, under the phase-contrast microscope. For study of the effect of dilution rate changes on spore incidence at least 100 spores were counted; for the steady state sample 400 spores were counted. For any particular flow rate the incidence of spores in different cultures was reproducible to within about 1%.

RESULTS

Stages of sporulation in a steady state continuous culture

For the chemostat operating at a dilution rate of 0·134 hr^{-1} (compared with the maximum specific growth rate of 0·467 hr^{-1} obtainable in our conditions) the fraction of the total cell population containing refractile spores was 0·220, obtained by direct counting. The number of cells in the various morphological stages of sporulation obtained from examination of the electron micrographs are given in Table 1, together

with the fraction of the total cell population present in each stage. It was easy to recognize the stages of development in the electron micrographs except for stages V and VI. Distinguishing these stages requires a decision as to whether the spore coat structures are complete, or are still being synthesized: hence the time for completion of stage V given below is approximate.

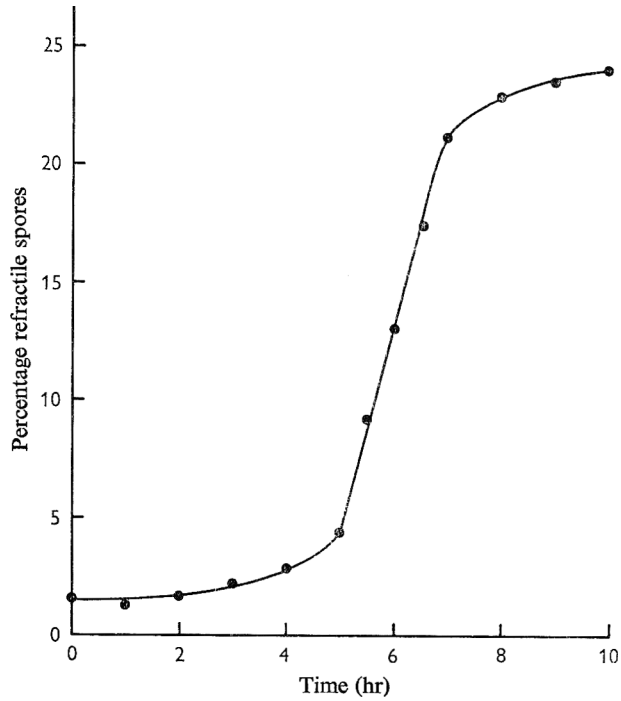

Fig. 2. *Bacillus subtilis* 168. The time taken for the appearance of refractile spores after initiation of cells to spore formation. At zero time the dilution rate of a steady state culture was changed from 0·42 hr^{-1} to 0·13 hr^{-1}. After 6 hr a sudden increase in spore incidence occurs.

Table 1. *Bacillus subtilis 168. Counts of cells in the various morphological stages of sporulation*

A sample was taken from a steady state continuous culture system operating at a dilution rate of 0·134 hr^{-1}. The cells were sectioned and examined by electron microscopy, and those giving complete longitudinal sections were scored for the various stages as defined by Schaeffer *et al.* (1965).

Stage	Number of cells in stage	Fraction of cells in stage
O	235	0·542
I	32	0·074
II	18	0·042
III	28	0·065
IV	21	0·048
V	20	0·046
VI	64	0·147
VII	16	0·037

The time taken for an initiated cell to produce a refractile spore

The time taken for the appearance of refractility was determined as described in the introduction by observing the effect on the incidence of refractile spores of a sudden decrease in the flow rate of the medium. Figure 2 shows the incidence of refractile spores in the culture after a sudden decrease in the dilution rate from a steady state value of 0·42 hr^{-1} to one of 0·13 hr^{-1}. An increase occurred at an average of 6·0 hr after the decrease in flow rate. This has been taken as the time required for the development of a refractile spore from a vegetative cell.

Times for the completion of the morphological stages of sporulation

Table 1 shows that stages V, VI and VII together comprised 23·0% of the total culture population. The percentage of refractile spores in the culture was 22·0%. It therefore appeared that refractility occurred just after the beginning of stage V, since if it began at stage VI, for example, stages VI and VII together would have been more than 22% of the culture population. From these two percentages and the time of 6·0 hr for the appearance of refractility, the washout equation (7) was used to calculate that the average time taken for the cells to reach stage V was 5·7 hr after initiation. This time and the method of successive approximation was then used to calculate the times taken for each of the other stages (Table 2). The results were checked by substitution in equation (4), and the ratios of observed cell counts in the different stages to those calculated by means of equation (4) have been included in Table 2.

Table 2. *Bacillus subtilis 168. Times taken for completion of the morphological stages of sporulation*

These times were calculated as described in the text for counts of the cells present in each of the stages of sporulation from a steady state continuous culture ($D = 0{\cdot}134\ hr^{-1}$). Counts of the cells in each stage were obtained by examination of electron micrographs.

Stage	Time taken for stage (hr)	Ratio of observed to calculated cell count
I	1·4	1·010
II	0·9	0·985
III	1·8	0·990
IV	1·6	1·010
V	2·0	0·990
VI*	7·7	—

* Calculated from equation (4) only.

DISCUSSION

These experiments were done to establish the value of the continuous culture technique in determining the time scale for the morphological changes which take place during sporulation. The procedure described has several advantages over methods using batch cultures for the same purpose. For accuracy, a batch culture experiment depends upon the use of a synchronized culture and the treatment for electron microscopy of a large number of samples taken during the whole period of sporulation. It is difficult to achieve synchrony in cultures of *Bacillus subtilis* 168 for

several reasons. The organisms tend to grow as filaments which often contain as many as 6 cells and occasionally more. This means that young cells cannot be separated from old cells, so that it is not easy to apply the membrane-filter technique of Anderson & Pettijohn (1960). Other procedures for obtaining synchrony, such as starving the organisms of an amino acid, are themselves likely to initiate some of the changes connected with sporulation. By contrast, the continuous culture method avoids the need for synchrony and in principle requires only one sample for examination. In addition, it is intrinsically more exact, since any specified degree of accuracy can be obtained by scoring a sufficient number of sections.

A possible source of error in the continuous culture method arises from spore germination and, in fact, a low percentage of germinating spores was observed in the electron micrographs. A correction for germination can be made by modification of the equations characterizing sporulation in continuous culture, but this was not considered necessary here because of the low percentage of germinating spores.

A check on the accuracy of the data obtained from scoring of the electron micrographs was made by comparing the results of electron microscopy with those of phase-contrast microscopy. The latter suggested that the fraction of cells containing refractile spores was 0·22 and that the time required to reach refractility was 6·0 hr. From these data and equation (5) it can be calculated that the fraction of cells in one or other stage of sporulation should be 0·45. The value from the electron micrographs was 0·46. Moreover, the agreement of these values provides another indication that spore germination is not a significant source of error in this system.

Additional information about the sporulation process can be obtained from continuous cultures by the use of sudden changes in the dilution rate of a culture at steady state. A sudden decrease in the flow rate provides a convenient and general method for investigating the time of appearance of any event connected with sporulation. Its present application to the development of refractility shows that under our conditions this occurred at the beginning of stage V. This agrees with the findings of Warren (1968), who used a batch culture technique with the same strain of *Bacillus subtilis* that we have used, and those of Fitz-James (1965), who worked with *B. cereus*. It contradicts the suggestion (Murrell, 1967) that refractility is associated with stage III. A further extension of this technique could be in the investigation of the successive biochemical events of sporulation. These include, for example, increases in alkaline phosphatase, glucose dehydrogenase and dipicolinic acid (see Schaeffer *et al.* 1965; Szulmajster, 1964; Warren, 1968; Mandelstam, 1969).

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Nitrogen Fixation by Cultures and Cell-free Extracts of *Mycobacterium flavum* 301

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SUMMARY

Growth, nitrogen fixation and acetylene reduction by *Mycobacterium flavum* 301 (NCIB 10,071) were increased with sodium lactate, pyruvate, gluconate or succinate as compared with ethanol, a recommended substrate. Yeast extract could be replaced with $(NH_4)_2SO_4$; in continuous culture a source of fixed nitrogen could be omitted altogether. Growth, nitrogen fixation and acetylene reduction all increased at lowered pO_2 values. Wholly anaerobic conditions did not support growth. Nitrogen fixation was confirmed isotopically.

Cell-free extracts performed the following reductions: N_2 to NH_3, H^+ to H_2, C_2H_2 to C_2H_4, KCN to CH_4, CH_3NC to $CH_4+C_2H_4+C_2H_6$. An ATP-generating system, Mg^{2+}, $Na_2S_2O_4$, and anaerobic conditions during preparation and assay of extracts were required. 3·5 mole ATP were hydrolysed to release 1 mole H_2. Pyruvate, α-ketobutyrate, α-ketoglutarate, succinate, glucose and glucose-6-phosphate did not replace dithionite. ADP, AMP or high concentrations of ATP inhibited reduction. Activity was associated with a particle which sedimented at 145,000*g* over 3½ hr. The nitrogenase system of *M. flavum* thus resembles the particulate system of Azotobacter, rather than the soluble pyruvate-utilizing system of *Clostridium pasteurianum*.

INTRODUCTION

Nitrogen-fixing members of the genus *Mycobacterium* were first isolated from Russian turf-podzol soils by Fedorov & Kalininskaya (1961*a*). They may be the important nitrogen fixers in these soils because Azotobacter is scarce in such relatively acid environments and these mycobacteria are more acid-tolerant than most Azotobacter species (Federov & Kalininskaya, 1959, 1961*a*; L'vov, 1963). Three nitrogen-fixing species of mycobacteria have been reported: *Mycobacterium flavum* 301, *M. roseo-album* 368, *Mycobacterium* sp. 571.

Fedorov & Kalininskaya (1959, 1960; 1961*a*, *b*) originally grew nitrogen-fixing mycobacteria in mixed cultures with other bacteria with which they are probably associated in the soil (Kalininskaya, 1967*a*, *b*, *c*). The associated bacteria stimulated growth and were thought to degrade carbon substrates to an accessible form as well as supply supplementary growth factors, including vitamins.

The particular species used in the present work, *Mycobacterium flavum* 301 (Fedorov & Kalininskaya, 1960; 1961*a*, *b*), is unable to utilize carbohydrates but grows on ethanol or organic acids. Fedorov & Kalininskaya (1961*c*) added yeast extract as a source of growth factors to media based on such carbon sources, in order to maintain the organism in pure culture. Il'ina (1966*a*, *b*; 1967*a*, *b*; 1968) investigated the metal ion requirements of *M. flavum* 301. Molybdenum was specifically required for nitrogen

fixation but at a concentration many times less than those needed by other nitrogen-fixing micro-organisms. This feature may be another reflexion of their acid turf-podzol habitat, which is notably low in available molybdenum (Il'ina, 1966*a*; Mishustin & Krylova, 1965). Cobalt was also specifically required for nitrogen fixation; utilization of ammonium chloride nitrogen did not depend on molybdenum or cobalt. Other trace elements (Cu, Fe, B, Mn, Zn) stimulated nitrogen fixation non-specifically. In this paper cultural procedures for improving growth and nitrogen fixation by *M. flavum* 301 are reported, together with the extraction and some properties of a cell-free nitrogen-fixing system; a preliminary report has appeared (Biggin & Postgate, 1969).

METHODS

Organism and medium. Mycobacterium flavum strain 301 was kindly supplied by Dr T. A. Kalininskaya: a culture is now held by the National Collection of Industrial Bacteria with the catalogue number NCIB 10,071. The organism grew well at 30° on nutrient agar (Oxoid Ltd.) to form homogeneous yellow colonies. For nitrogen-fixation tests a medium based on one prescribed by Dr Kalininskaya (personal communication) was used: K_2HPO_4, 1·67 g.; KH_2PO_4, 0·87 g.; $MgSO_4.7H_2O$, 0·29 g.; $CaCl_2$, 0·07 g.; NaCl, 0·48 g.; $FeCl_3.6H_2O$, 0·01 g.; $Na_2MoO_4.2H_2O$, 0·005 g.; $ZnSO_4.7H_2O$, 0·0002 g.; $MnSO_4.4H_2O$, 0·003 g.; H_3BO_3, 0·005 g.; $CoSO_4.7H_2O$, 2 μg.; biotin, 20 μg.; yeast extract (Oxoid Ltd.), 0·08 g.; carbon source, 4 g.; distilled water, 1 l, pH 6·8.

Culture at various oxygen tensions. To determine the optimum pO_2 for growth and nitrogen fixation, 50 ml. portions of medium in 250 ml. conical flasks were inoculated with 0·4 ml. of a 1/10 dilution in 0·85% NaCl of an actively growing culture. The flasks were flushed with high purity nitrogen and the cotton-wool plugs replaced by sterile 'Suba-seal' rubber stoppers in a stream of nitrogen. Different pO_2 values were established by replacing nitrogen with oxygen by using a syringe inserted through the rubber seal. The cultures were shaken in an orbital shaker at 150 rev./min. at 30° up to 16 days. Samples of the culture fluid were removed aseptically at 2 to 3 day intervals by opening the flask; after sampling the rubber seal was replaced and the appropriate pO_2 re-established. The purity of each culture was tested by plating on nutrient agar at the end of the experiment.

Growth measurements. Extinctions in a 1 cm. cell at 540 mμ were measured in an EEL Spectra spectrophotometer and converted to dry weight organisms/ml. by using a standard curve.

Culture under ^{15}N. Incorporation of ^{15}N by growing cultures was tested by Miss S. Hill in this laboratory by using conventional methods (Burris & Wilson, 1957). Cultures were shaken in 100 ml. of Kalininskaya's medium containing yeast extract and sodium lactate (4 g./l.) in a 250 ml. flask under an atmosphere with $pO_2 = 0·1$, $pN_2 = 0·2$, pAr = 0·7 atm. 7 ml. of 95% enriched $^{15}N_2$ was injected to give a final enrichment of 15 atom % excess. pO_2 was monitored during growth by gas chromatography and oxygen injected aseptically every 1 to 2 days as necessary to maintain a pO_2 of 0·08 to 0·10. Two controls were included: one contained acidified medium, to take up residual $^{15}NH_3$ from the isotopic gas sample; the other was a culture of a non-nitrogen-fixing organism, *Escherichia coli*. After Kjeldahl digestion and Markham distillation of 12-day cultures, the ^{15}N enrichment of samples was determined by mass spectroscopy by Dr C. W. Crane (Queen Elizabeth Hospital, Birmingham 15).

Continuous culture. Mycobacterium flavum 301 was grown continuously in an apparatus of the type described by Baker (1968) in Kalıninskaya's medium with 4 g. sodium lactate/l. under N_2 + air mixtures which flowed over the culture at 490 ml./min.; dilution rate, 0·05 hr^{-1}; temperature, 30°. The culture volume was 580 to 670 ml. according to stirring rate: a low stirring rate (250 rev./min.), which created negligible vortex, provided conditions of relatively low aeration (oxygen solution rate was 0·6 mmole O_2/l./hr); a high stirring rate (1000 rev./min.) resulted in a vortex extending to the bottom of the culture vessel with copious upward bubbling through the medium (oxygen solution rate, 30·4 mmole O_2/l./hr). Oxygen solution rates were measured by sulphite oxidation (Elsworth, Williams & Harris-Smith, 1957) with the vessel open to the atmosphere.

Large scale culture. Large Pyrex bottles (20 l.) containing 18 l. of Kalininskaya's medium with 4 g. sodium lactate/l. were inoculated with about 1 l. of culture from either the overflow of a continuous culture or a 'seed' batch culture in the same medium. During growth the cultures were sparged with air + nitrogen mixture (pO_2 = 0·05 atm.) at approx. 2·5 l./min. and maintained at 30°. After 2 to 3 days, when the yield exceeded equiv. 0·33 mg. dry wt organisms/ml., the bacteria were harvested by batch centrifugation for 8 min. at 40,000*g* in an MSE 'High Speed 18' centrifuge. They were washed in 0·025 M-tris HCl buffer at pH 7·2, and the cell paste stored as pellets in liquid nitrogen.

Acetylene reduction by cultures. Batch cultures (25 ml. in 250 ml. volumetric flasks) were inoculated with 0·2 ml. of a 1/10 dilution in 0·85% NaCl of an actively growing culture and a Suba-seal inserted above the cotton-wool plug. Acetylene was injected to 0·02 atm. and samples were removed for vapour phase chromatography every 2 to 3 days during incubation. Samples from a continuous culture (D = 0·05 hr^{-1}, low stirring rate, pO_2 over culture 0·1 atm., population equiv. 0·26 mg. dry wt organism/ml.) were tested with 0·04 atm. acetylene in a similar way except that 10 ml. portions were incubated for up to 2 hr in sealed 25 ml. conical flasks flushed with nitrogen. Atmospheres of different pO_2 value were obtained by injecting O_2 after an equivalent volume of N_2 had been removed with a syringe.

Preparations of cell extracts. For each experiment, frozen cell paste was thawed at room temperature under argon, 1 to 2 vol. 0·025 M-tris HCl buffer (pH 7·2) was added and the whole stirred to an even consistency. Batches of cell suspension (15 ml.), cooled in ice, were sonicated under a stream of argon by using a Dawe stainless steel Soniprobe at 8 A for 1 min. The sonicate was centrifuged at 38,000*g* for 30 min. under argon and the yellow supernatant solution (4 to 13 mg. protein/ml.) stored in ice under argon for not more than 1 hr before use.

Analyses. Nitrogen contents of growing cultures were determined by Kjeldahl digestion followed by Nesslerization (Umbreit, Burris & Stauffer, 1964), and fixation calculated by difference from acid-killed controls. Nitrogen fixed by cell-free extracts was measured as ammonia (Dilworth, Subramanian, Munson & Burris, 1965). Protein was estimated by the biuret method, as modified by Gornall, Bardawill & David (1949).

Gas samples were analysed for hydrocarbons by using a Pye series 104 gas chromatograph with a 5 ft. (152 cm.) Porapak R column at 45° in a stream of N_2 (50 ml./min.) with a flame-ionization detector. The assay system for cell-free reduction of nitrogen, acetylene, cyanide or methyl isocyanide was based on that of Bulen, Burns & Le

Comte (1965): 12·5 μmole tris HCl buffer (pH 7·2), 5 μmole $MgCl_2$, 20 μmole $Na_2S_2O_4$ and an ATP-generating system (2·5 μmole ATP, 20 μmole creatine phosphate, 0·2 mg. creatine kinase) in a final volume of 1 ml. were contained in 7·5 ml. Suba-sealed ampoules. Reaction was at 30° under argon with shaking, and was started by adding extract (equiv. 2·5 to 5·6 mg. protein). Gas samples for vapour phase chromatography were removed at 15, 30 and 45 min. by using a syringe inserted through the Suba seal. When the ATP-generating system was omitted the $MgCl_2$ was decreased to 2 μmole and ATP increased to 4 μmole. Gas samples were then removed at 6 and 12 min. because the reaction rate fell sharply after 15 min.

ATP-activated hydrogen evolution and reductant-dependent ATP-ase were measured in Warburg vessels (nominally 15 ml.) with a rubber-capped side arm, containing cell-free extract (19·1 to 22·1 mg. protein) in the main compartment; 37·5 μmole tris HCl buffer (pH 7·2), 12·5 μmole $MgCl_2$, 7·5 μmole ATP, 50 μmole creatine phosphate, 0·5 mg. creatine kinase and 50 μmole $Na_2S_2O_4$ in the side arm; 0·1 ml. 40% KOH in the centre well; $V_f = 2·5$ ml. The flasks were flushed with argon for 10 min., extract added to the main compartment and dithionite to the side arm via the rubber cap by using a syringe, and then flushing was continued for a further 5 min. The reaction was started by tipping the contents of the side arm and was at 30° with shaking. After 25 min., while the reaction rate was still linear, it was stopped by adding 0·1 ml. 40% KOH, and inorganic phosphate determined by the method of Taussky & Shoor (1953).

Conventional hydrogenase activity was detected as follows: whole organisms equivalent to 13·8 mg. dry wt organism, or cell-free extract (2·6 to 7·8 mg. protein), were added to an equal volume of 0·07 M-potassium phosphate buffer (pH 7) + 0·1 mM-dye in Thunberg tubes containing H_2. Dye reduction rates were compared with controls under argon.

Reagents. Chemicals used in this work were the best grade commercially available. Isotopic nitrogen as 95% enriched $^{15}N_2$ gas was obtained from M. W. Hardy & Co., Mercantile Ltd., London, E.C. 2. Biochemicals were obtained from Sigma (London) Chemical Co., London, S.W. 6. ATP, ADP and AMP (neutralized 0·1 M solutions) and creatine kinase (2 mg./ml. of 0·025 M-tris HCl buffer, pH 7·2) were stored at −20°. ADP contained approximately 20% AMP as indicated by thin layer chromatography. 0·2 M-$Na_2S_2O_4$ was prepared as required and stored for not more than 2 hr under argon. Methyl isocyanide was prepared by Dr R. L. Richards following the procedure of Shaw & Pritchard (1966), stored at −70° in a solid-CO_2 cabinet, and solutions in water were prepared as required and discarded after 4 hr. Purified acetylene (99·6% minimum) was obtained from Matheson Chemical Co., New Jersey, U.S.A., or produced from calcium carbide and water.

RESULTS

Effect of pO_2 on growth and nitrogen fixation. The data in Table 1 illustrate the conclusion from many samplings: that a pO_2 of 0·1 atm. was optimal for growth and nitrogen fixation in batch culture. The rates of increase of both parameters were double those at normal atmospheric composition. The slight growth and nitrogen fixation which occurred at a pO_2 of zero was attributed to failure to exclude oxygen completely, since they were eliminated in other tests when oxygen was rigorously excluded by the use of a pyrogallol plug.

Table 2 illustrates comparable data for a continuous culture: steady state values for growth and nitrogen fixation increased when the pO_2 of the gas flowing over the culture was decreased from 0·1 to 0·5 atm., provided stirring rate was increased.

These observations indicated that a pO_2 value below atmospheric favoured growth and nitrogen fixation by *Mycobacterium flavum* 301, though wholly anaerobic conditions did not support growth. The difference in the optimal pO_2 between batch and continuous culture is discussed later. For the rest of the work reported here shake-flask cultures were routinely grown at pO_2 = 0·1 atm. and 20-l. batch cultures were sparged with pO_2 = 0·05 atm. air + nitrogen mixtures.

Table 1. *Effect of pO_2 on Mycobacterium flavum 301 in batch culture*

Cultures were shaken at 30°, with lactate medium (see text) under the atmospheres indicated, and sampled at intervals. Twelve-day values are quoted as typical of many.

pO_2 (atm.)	Organism concentration (mg. dry wt/ml.)	Nitrogen fixed (μg. N/ml.)
0·00	0·10*	1·0*
0·01	0·40	9·9
0·02	0·75	17·8
0·05	1·15	31·4
0·10	1·37	60·0
0·20	0·63	29·6

* Marginal growth probably due to incomplete anaerobiosis (see text).

Table 2. *Effect of pO_2 and stirring rate on Mycobacterium flavum 301 in continuous culture*

Steady states were established at $D = 0{\cdot}052 \pm 0{\cdot}003$ hr^{-1} in lactate medium at 30° under air–N_2 mixture at 'low' and 'high' stirring rates (see text).

Stirring rate	pO_2 (atm.)	Organism concentration (mg. dry wt/ml.)	Nitrogen fixed (μg N/ml.)
Low	0·1	0·26	5·7
Low	0·05	0·10	0·9
High	0·05	0·64	12·2

Isotopic nitrogen fixation. The analytical figures from the above experiments leave little doubt that true nitrogen fixation had occurred, not merely scavenging of atmospheric fixed-nitrogen. Nevertheless cultures were tested for incorporation of isotopic nitrogen. Twelve-day-old shake-flask cultures of *M. flavum* 301, grown in an atmosphere with 15 atom % $^{15}N_2$ excess were enriched 9·216 atom % ^{15}N excess compared with enrichments of 0·003 and 0·004 atom % ^{15}N excess for acidified medium and *Escherichia coli* controls respectively.

Acetylene reduction by M. flavum 301. All nitrogen-fixing organisms and extracts so far studied reduce acetylene to ethylene, and this reaction has become a rapid and sensitive assay for nitrogenase activity (Dilworth, 1966; Schöllhorn & Burris, 1966, 1967). Preliminary tests in batch cultures indicated that *M. flavum* 301 cultures reduced

acetylene. Cultures into which 0·02 atm. acetylene were injected were therefore included in our investigations.

Effect of various carbon sources in batch culture. Several different carbon sources supported greater growth, nitrogen fixation and acetylene reduction than did ethanol, the substrate recommended by Dr Kalininskaya (Table 3). Lactate was chosen for routine use because it was readily available and could be autoclaved.

Table 3. *Effect of various carbon sources on growth, nitrogen fixation and acetylene reduction by Mycobacterium flavum 301 in batch cultures*

Cultures were shaken at 30° with 0·4% (w/v) substrate (anions as the sodium salt) in sealed flasks with the atmospheres indicated. The first two columns record data from the same cultures, the third from a parallel series with 0·02 atm. C_2H_2 above the culture. Data are representative of several samplings.

	Incubation periods		
	12 days		3 days
Carbon source	Growth (mg. dry wt/ml.)	Nitrogen fixed (μg. N./ml.)	Ethylene produced (μmole)
pO_2 = 0·1 atm.			
Pyruvate	0·56	25·9	23·8
Lactate	0·46	16·8	38·1
Gluconate	0·45	19·2	4·6
Succinate	0·42	15·4	19·6
Ethanol	0·29	11·5	15·8
pO_2 = 0·2 atm.			
Pyruvate	0·16	2·7	0·04
Lactate	0·17	9·9	0·04

Table 4. *Growth, nitrogen fixation and acetylene reduction by Mycobacterium flavum 301 in batch culture with* $(NH_4)_2SO_4$

Duplicate cultures were grown at pO_2 = 0·1 with 4 g. carbon source/l. and with $(NH_4)_2SO_4$ 40 mg./l. replacing yeast extract 80 mg./l. in Kalininskaya's medium. Acetylene was at 0·02 atm. and gas samples were removed at intervals. Extinction coefficients and nitrogen contents were measured at the end of the experiment.

	Incubation periods		
	10 days		3 days
Carbon source	Growth (mg. dry wt/ml.)	Nitrogen fixed (μg. N/ml.)	Ethylene produced (μmole)
Pyruvate	0·72	26·1	26·8
Lactate	0·38	19·7	34·6
Succinate	0·22	12·5	16·8
Ethanol	0·38	13·9	5·1

Requirement for yeast extract. Though yeast extract is often used in media for nitrogen-fixing mycobacteria (L'vov, 1963), $(NH_4)_2SO_4$ at equivalent nitrogen concentration supported growth, nitrogen fixation and acetylene reduction in batch cultures with a variety of carbon substrates (Table 4). This observation confirmed the personal communication from Dr Kalininskaya that yeast extract was not essential. In

continuous culture a source of fixed nitrogen could be dispensed with altogether: a steady state at $D = 0{\cdot}05$ hr^{-1}, $pO_2 = 0{\cdot}05$ atm., high stirring rate, was maintained in a medium containing 80 mg. yeast extract/l. for eight days. When the medium was changed to one containing neither yeast extract nor $(NH_4)_2SO_4$, the steady state was maintained for a further 9 days; growth and nitrogen fixation continued undiminished when the dilution rate was increased to 0·1 hr^{-1} for another 12 days.

Effect of pO_2 on acetylene reduction in short-term experiments. The conclusion that a low pO_2 favoured growth and nitrogen fixation was tested by using the acetylene-reduction assay system in short-term experiments. Actively growing suspensions of organisms from a continuous culture were incubated up to 2 hr at various pO_2 values and under 0·04 atm. acetylene as described in Methods. The optimum pO_2 for acetylene reduction was 0·05 atm. (Fig. 1); activity at normal atmospheric pO_2 was very low. The slight activity in the anaerobic treatment was probably due to oxygen dissolved in the culture fluid.

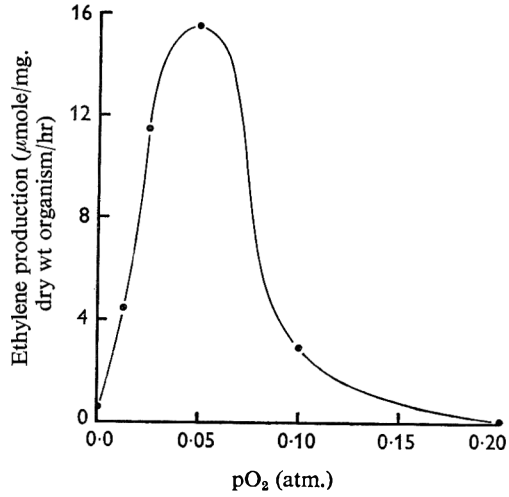

Fig. 1. Effect of pO_2 on acetylene reduction by *Mycobacterium flavum* 301. Samples from a continuous culture were incubated at 30° with shaking under a range of partial pressures of oxygen and with 0·04 atm. acetylene.

Acetylene reduction by cell-free extracts. Cell-free extracts of *Mycobacterium flavum* 301, prepared and tested as described in Methods, catalysed the reduction of acetylene to ethylene; the reaction continued up to 45 min. at 30°. There was practically no activity in the cell debris remaining after centrifugation; cell debris resuspended in 0·025 M-tris HCl buffer (pH 7·2) and added to the extract inhibited acetylene reduction. Typical figures for ethylene production after 30 min. were: extract alone, 3·97 n-mole/mg. protein/min.; cell debris alone, 0·02 n-mole/mg. protein/min.; extract and cell debris together 0·66 n-mole/mg. protein/min. Sodium dithionite and an ATP-generating system were required and $MgCl_2$ had a pronounced stimulatory effect (Table 5). Presumably endogenous Mg^{2+} in the extract was insufficient to support full activity. Table 5 includes evidence that the extract was highly sensitive to air: activity fell by over 90% after 15 min. exposure to air.

Pyruvate, and to a lesser extent α-ketobutyrate, supply both electrons and energy-rich phosphate for reductions catalysed by nitrogenase in cell-free preparations from

Clostridium pasteurianum (Carnahan, Mortenson, Mower & Castle, 1960) and *Bacillus polymyxa* (Grau & Wilson, 1961, 1963). The following substrates, with the co-factors indicated, did not replace $Na_2S_2O_4$ as electron donors for acetylene reduction by *Mycobacterium flavum* 301 extracts + the ATP-generating system: pyruvate (+ thiamine pyrophosphate, coenzyme A), α-ketobutyrate (+ thiamine pyrophosphate, coenzyme A, NAD), α-ketoglutarate (+ coenzyme A, NAD), succinate (+ coenzyme A, NAD), glucose (+ NAD) and glucose-6-phosphate (+ NAD). Cofactors were added in the following quantities: thiamine pyrophosphate, 100 μg.; coenzyme A, 0·05 μmole; NAD, 1 μmole.

Reduction of nitrogen, cyanide and methyl isocyanide by cell-free extracts. Cell-free extracts catalysed the reduction of nitrogen to ammonia (Table 6). Nitrogenase systems from other organisms catalyse reduction of a variety of substrates besides acetylene and nitrogen: cyanide (Hardy & Knight, 1967) and methyl isocyanide (Kelly, Postgate & Richards, 1967) were chosen as representative alternative substrates. Both were reduced (Table 6). Potassium cyanide yielded methane and traces of acetylene, but no detectable ethane or ethylene. Methyl isocyanide was reduced to methane + traces of

Table 5. *Requirements for acetylene reduction by cell-free extracts of Mycobacterium flavum 301*

For preparation of cell-free extracts and test conditions, see text.

	Ethylene produced (% activity)
Complete system	100
Complete system − $Na_2S_2O_4$	5
Complete system − ATP-generating system	5
Complete system − $MgCl_2$	30
Complete system but extract exposed to air 5 min.	6

Table 6. *Reduction of nitrogen, cyanide and methyl isocyanide by cell-free extracts of Mycobacterium flavum 301*

For experimental details see text; . signifies product not tested for.

	Products (n-mole/mg. protein/min.)				
Substrate (conc.)	NH_3	CH_4	C_2H_4	C_2H_6	C_2H_2
Nitrogen (1 atm.)	3·44	.	.	.	.
Acetylene (0·025 atm.)	.	0	5·20	0	0
KCN (2 mM)	.	3·73	0	0	trace
Methyl isocyanide (10 mM)	.	5·52	trace	trace	trace

all three C_2 compounds. Work by Dr M. Kelly in this laboratory suggests that the acetylene observed was a product of non-enzymic reductive breakdown of the substrate. Ethylene and ethane, however, were formed enzymically; they were produced in equivalent amounts up to 45 min., but in double-scale incubation for 90 min. production of ethane was 50% greater than that of ethylene. Kelly *et al.* (1967) and Kelly (1968*a*) reported that the ratios of C_2 products, and the proportions of C_1 to C_2 products, formed from methyl isocyanide by nitrogenase preparations from *Azotobacter chroococcum* depend on test conditions.

Hydrogenase activity in Mycobacterium flavum 301. All intact nitrogen-fixing systems have hydrogenases (Wilson, 1958). Whole organisms of *M. flavum* 301 were

tested and conformed to this pattern: they reduced benzylviologen, methylene blue or tetrazolium after 20 to 30 min. under H_2 but not under argon. Benzylviologen, methylene blue and tetrazolium were reduced by cell-free extracts after 5 to 10 min. under H_2.

Cell-free nitrogen-fixing preparations from a variety of organisms show ATP-dependent H_2 evolution from dithionite (Burns, 1965). Cell-free extracts from *Mycobacterium flavum* 301 also evolved H_2 (6·89 n-mole H_2/mg. protein/min.) from dithionite when supplied with an ATP-generating system, but H_2 evolution was not observed in the absence of ATP. Reductant-dependent ATP-ase activity was measured and, by comparison with a control vessel lacking reductant, the ratio mole ATP hydrolysed to mole H_2 evolved (ATP/$2e^-$) was 3·5.

Pyruvate (250 μmole+coenzyme A, 0·125 μmole; thiamine pyrophosphate, 0·25 μg.; ADP, 6·25 μmole; and Na phosphate, 7·5 μmole; V_f = 2·5 ml.) did not support H_2 evolution by cell-free estracts when added in place of sodium dithionite.

Solubility of cell-free extracts. Crude nitrogenase preparations from *Azotobacter vinelandii* (Bulen *et al.* 1964) and *A. chroococcum* (Kelly, 1966) are particulate: they sediment after about 4 hr at 200,000*g*. This property distinguishes them from the wholly soluble preparations obtained from *Clostridium pasteurianum* (Carnahan *et al.* 1960). Ultracentrifugation of cell-free extracts of *Mycobacterium flavum* 301 for 3·5 hr at 145,000*g* yielded a pellet which contained all the acetylene-reducing activity. The supernatant fluid had virtually no activity, nor did it stimulate activity when added to the resuspended pellet (Table 7).

Table 7. *Acetylene reduction by ultracentrifuged extracts of Mycobacterium flavum 301*

Cell-free extract was centrifuged at 145,000 *g* for 3·5 hr under argon and the pellet resuspended in an equal volume of 0·025 M-tris HCl buffer (pH 7·2). For test conditions see text.

	Ethylene produced (n-mole/mg. protein/min.)
Cell-free extract	0·52
Pellet from ultracentrifugation	0·54
Supernatant fluid from ultracentrifugation	0·01
Supernatant fluid + pellet	0·24

Acetylene reduction without an ATP-generating system. An ATP-generating system is conventionally used in nitrogenase tests because the system requires ATP but is inhibited by high ATP concentrations. Moustafa & Mortenson (1967) showed that the reduction of acetylene by cell-free extracts of *Clostridium pasteurianum* was supported directly by ATP without an ATP-generating system. ATP alone also supported acetylene reduction by extracts of *Mycobacterium flavum* 301 when the ATP-generating system was omitted (Table 8). The reaction continued for 12 to 15 min. and required ATP, $Na_2S_2O_4$ and $MgCl_2$; ATP could not be replaced by ADP or AMP. The optimal concentration of ATP was 4 to 8 mM (Fig. 2) and the inhibitory effect of high ATP concentrations found in other systems was confirmed. Table 8 includes data showing that, with optimal ATP, both ADP and AMP were strongly inhibitory; as mentioned under Methods, the ADP used contained some AMP as impurity. These results parallel those obtained with the clostridial system.

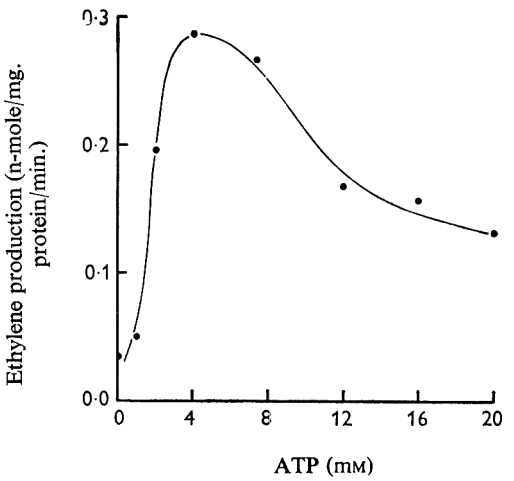

Fig. 2. Effect of ATP concentration on acetylene reduction by extracts of *Mycobacterium flavum* 301. ATP and Mg^{2+} (as $MgCl_2$) in molar ratio 2:1; for other conditions see text.

Table 8. *Acetylene reduction by cell-free extracts of Mycobacterium flavum 301 without an ATP-generating system*

Adenine nucleotide: Mg^{2+} ratio of 2:1 except in blank where Mg^{2+} was 5 mM

Adenosine phosphates (conc.)	Ethylene produced (n-mole/mg. protein/min.)	Adenosine phosphates (conc.)	Ethylene produced (n-mole/mg. protein/min.)
None	< 0·005	ATP (4 mM) + ADP (4 mM)	0·005
ATP (4 mM)	0·29	ATP (4 mM) + ADP (2 mM)	0·005
ATP (4 mM) − $Na_2S_2O_4$	< 0·005	ATP (4 mM) + ADP (1 mM)	0·025
ATP (4 mM) − $MgCl_2$	< 0·005	ATP (4 mM) + AMP (4 mM)	0·005
ADP (4 mM)	0	ATP (4 mM) + AMP (2 mM)	0·005
AMP (4 mM)	0	ATP (6 mM)	0·27
		ATP (8 mM)	0·27

DISCUSSION

Oxygen-sensitivity of nitrogen-fixing Mycobacterium flavum 301. Different optimum pO_2 values for growth and nitrogen fixation as between batch or continuous cultures, and for acetylene reduction by whole organisms in short-term experiments, merely reflect differences in availability of oxygen to the population being studied, which is determined by many factors, including the geometry of the experimental vessels, the shaking or stirring rates and population densities at any given time. Nevertheless the results reported here clearly indicate that nitrogen-fixing cultures of *M. flavum* 301 tend to be microaerophilic. This conclusion agrees with evidence of numerous other workers, cited by Dalton & Postgate (1969), that oxygen inhibits nitrogen fixation in aerobic organisms. The facultative aerobes, *Bacillus polymyxa* (Hino & Wilson, 1958; Grau & Wilson, 1962) and *Klebsiella pneumoniae* (Pengra & Wilson, 1958), only fix nitrogen anaerobically. *M. flavum* 301 appears to be intermediate in oxygen sensitivity between such organisms and better adapted aerobes such as Azotobacter. Kalininskaya

(1967*a*) obtained evidence for an inhibitory effect of oxygen on nitrogen-fixing symbiotic microbial associations similar to those from which *M. flavum* 301 was isolated. Nitrogen fixation was stimulated with the development of the symbiotic association, which Kalininskaya (1967*c*) attributed, at least in part, to protection of the nitrogen fixers from oxygen by the symbionts.

Dalton & Postgate (1969) showed that the sensitivity of Azotobacter species to oxygen inhibition depended on nutritional status. They attributed this phenomenon to the need to protect nitrogenase from damage or interference by oxygen, and postulated two mechanisms of protection: a 'respiratory protection', whereby respiration prevented access of oxygen to the nitrogen-fixing site, and a 'conformational protection'. The latter was reflected by the relative stability to air of the particulate nitrogen-fixing systems extracted from azotobacters (Bulen, Burns & Le Comte, 1964; Kelly, 1966), compared with the soluble, oxygen-sensitive enzyme fractions obtained on fractionating such particles (Bulen & Le Comte, 1966; Kelly, Klucas & Burris, 1967; Kelly, 1968*b*). They suggested that the oxygen-insensitive particle represented a gross manifestation of the 'conformational protection' mechanism against oxygen in Azotobacter. However, nitrogenase from *Mycobacterium flavum* 301 provides an example of a system which is oxygen-sensitive in the particulate form, which suggests an inefficient or totally absent system of 'conformational protection' in this organism. The microaerophilic character of nitrogen-fixing *M. flavum* 301 may reflect a greater dependence on 'respiratory protection'.

Enzymology of nitrogenase from Mycobacterium flavum 301. The cell-free nitrogen-fixing system from *M. flavum* 301 is markedly similar to those from other nitrogen-fixing organisms. It requires: (i) ATP; ADP, AMP or high concentrations of ATP are inhibitory; (ii) a source of low potential reducing power ($Na_2S_2O_4$); (iii) Mg^{2+} ions; (iv) anaerobic conditions during preparation and assay. Analogous substrates such as cyanide, isocyanide or acetylene are reduced; ATP-dependent hydrogen evolution and reductant-dependent ATP-ase activity occur, and conventional hydrogenase activity is present.

The ATP/$2e^-$ ratio of 3·5 observed with *Mycobacterium flavum* 301 extracts is lower than the values of 4 to 5 generally obtained with Azotobacter and *Clostridium pasteurianum* (Bulen & Le Comte, 1966; Mortenson, 1966; Silver, 1967; Winter & Burris, 1968). The system in *M. flavum* 301 may be more efficient, but not only is the validity of the assumptions made in this kind of calculation questionable (Hardy & Burns, 1968), but Kelly (1968*b*) has shown that the ratio varies greatly according to the experimental conditions and the substrate being reduced. Hence this study only shows that the behaviour of nitrogenase from *M. flavum* 301 with respect to ATP is quantitatively of the same order as the behaviour of extracts of other organisms.

The particulate character of the system from *Mycobacterium flavum* 301 and its failure to utilize pyruvate, recall the system of Azotobacter (Bulen *et al.* 1964; Kelly, 1966) rather than the soluble pyruvate-utilizing system of *Clostridium pasteurianum* (Carnahan *et al.* 1960).

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Numerical Taxonomy of Some Named Coryneform Bacteria

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SUMMARY

Numerical analysis of data for seventy coryneform bacteria bearing the following generic names, *Mycobacterium*, *Nocardia*, *Jensenia*, *Listeria*, *Erysipelothrix*, *Kurthia*, *Brevibacterium*, *Arthrobacter*, *Cellulomonas*, *Microbacterium* and *Corynebacterium*, was done by using 70 coded features and both single and complete linkage clustering programs.

Three major phenons were detected, probably corresponding taxonomically to the present family level in the taxonomic hierarchy. The first included *Listeria*, *Kurthia*, *Erysipelothrix*, certain *Microbacterium* strains and *Corynebacterium* species of animal origin. The second included rapid-growing *Mycobacterium* species, *Nocardia* and *Jensenia*. The third was a more ill-defined group of *Arthrobacter*, *Brevibacterium*, *Cellulomonas*, *Microbacterium* and certain other species. We suggest that all three phenons belong in the order Eubacteriales under the possible family names of Corynebacteriaceae, Mycobacteriaceae and Arthrobacteriaceae respectively.

INTRODUCTION

Gram-positive bacteria are currently classified in five major groups. Four of these, the micrococci, sporing bacilli, lactic-acid bacteria and the coryneforms, are considered members of the order Eubacteriales, whilst overtly acid-fast coryneforms and the actinomycetes are placed in a separate order, Actinomycetales. The problems inherent in dividing Gram-positive bacteria into taxa at the generic, familial and ordinal level have been remarked by various workers and the reader is referred to Davis & Freer (1960), Gordon (1966) and Jensen (1966) for material relevant to the coryneform problem in particular. Some of the difficulty stems from the fact that taxonomy of bacteria at the 'alpha' level is based on morphology and, as the name implies, coryneforms are irregular in this respect. More subtle problems are also encountered, however, such as gradation in acid-fastness (Harrington, 1966) and the taxonomic interpretation of cell-wall analysis (Cummins, 1962) and nutritional needs (Keddie, Leask & Grainger, 1966). In attempting to classify bacteria, or anything else for that matter, we assume that natural discontinuities or groupings exist in the material studied and can be detected if looked for. As the amount of information upon which groupings may be made increases, the detection of groups becomes more difficult. Numerical taxonomy (Sokal & Sneath, 1963) offers a practical means of reducing this difficulty by impartial mechanical data analysis.

The present study was an attempt to detect similarity groupings in 70 strains of 'coryneform' bacteria, all of which have been maintained for varying periods in this and other laboratories under the names shown in Table 1. Eleven generic names occur in this list and the strains represent isolates from diverse habitats. An investigation of basically similar design was reported by Da Silva & Holt in 1965.

Table 1. *Strains examined*

Study no.	Name on receipt	Donor*	Donor number
107	*Brevibacterium saperdae*	A	CCEB 366
12	*B. linens*	B	25
13	*B. linens*	B	26
92	*B. insectiphilium*	C	146
91	*B. sociovivum*	C	135
94	*B. imperiale*	C	—
106	*B. imperiale*	A	CCEB 497
104	*B. ammoniagenes*	A	CCEB 364
93	*B. ammoniagenes*	C	—
14	*B. helvolum*	B	67
108	*B. protophormiae*	A	CCEB 282
105	*B. fuscum*	A	CCEB 277
109	*B. vitarumen*	A	CCEB 252
15	*Mycobacterium hyalinum*	B	119
16	*M. phlei*	B	120
66	*M. fortuitum*	—	NCTC 8573
69	*M. rhodochrous*	—	NCTC 8139
70	*M. lacticola*	C	011
71	*M. lacticola*	C	012
134	*M. ranae*	D	—
5	*Nocardia salmonicolor*	B	121
6	*N. opaca*	B	133
8	*N. rubropertincra*	B	134
9	*N. coeliaca*	B	132
76	*N. rubra*	C	—
77	*N. corallina*	C	NOC 19
101	*N. salivae*	—	—
132	*N. asteroides*	B	131
135	*Nocardia* sp.	E	KARLSON
137	*Corynebacterium diphtheriae*	F	2650
140	*C. renale*	G	NCTC 7448
173	*C. ovis*	G	NCTC 3450
179	*C. equi*	G	NCTC 1621
180	*C. ulcerans*	G	48
194	*C. murium*	G	NCTC 949
217	*C. murium*	J	115
200	*C. xerosis*	H	—
237	*C. michiganense*	I	WU 116
34	*Erysipelothrix insidiosa*	K	V 535
208	*E. insidiosa*	F	2682
209	*Listeria monocytogenes*	F	2681
58	*Microbacterium thermosphactum*	L	SULZBACHER SW 26
10	*M. lacticum*	B	275
11	*M. lacticum*	B	101
59	*Microbacterium* sp.	L	9
60	*Microbacterium* sp.	L	10
61	*Microbacterium* sp.	L	11
62	*Microbacterium* sp.	L	15
63	*Microbacterium* sp.	L	22
64	*Microbacterium* sp.	L	27
65	*Microbacterium* sp.	L	119
28	*Kurthia zopfii*	B	92
29	*K. zopfii*	B	93
99	*Jensenia canicruria*	—	—
17	*Arthrobacter terregens*	B	6
242	*A. terregens*	I	NCIB 8909
18	*A. tumescens*	B	7
19	*A. pascens*	B	4

Table 1 (*cont.*)

Study no.	Name on receipt	Donor*	Donor number
20	*A. simplex*	B	5
240	*A. simplex*	I	NCIB 8929
241	*A. citreus*	I	NCIB 8908
244	*A. citreus*	I	WU 484
21	*A. citreus*	B	2
22	*A. globiformis*	B	3
238	*A. globiformis*	I	WU 477
239	*A. globiformis*	I	NCIB 8907
24	*A. ureafaciens*	B	8
23	*A. aurescens*	B	1
96	*Cellulomonas flavigena*	—	NCIB 8073
110	*C. biazotea*	—	NCIB 8077

* *Donors.* A, O. Lysenko, Czechoslovak Acad. Sci., Prague; B, E. Szabo, Microbiology Culture Collection, University of Queensland; C, J. V. Bhat, Indian Inst. of Science, Bangalore; D, M. R. McLean, CSR, Roseville, N.S.W.; E, R. Harris, Inst. of Dental Research, Sydney; F, E. F. Crowder, Inst. of Medical and Veterinary Science, Adelaide; G, R. W. Cobb, I.C.I. Research Division, Macclesfield; H, J. C. Keast, Veterinary Research Station, Glenfield, N.S.W.; I, C. A. Parker, Inst. of Agriculture of West Australia, Nedlands; J, R. F. Jones, Dept. of Veterinary Pathology, University of Sydney, N.S.W.; K, G. Simmonds, Animal Research Institute, Brisbane; L, L. Brownlie, C.S.I.R.O. Meat Preservation Laboratory, Brisbane.

METHODS

(*a*) *Strains studied.* These are listed in Table 1. In view of the possibility of misrepresentation we wish to point out that none of the strains bearing internationally recognized culture collection labels was obtained direct from those collections.

(*b*) *Strain growth and maintenance.* Initial examination of all strains for purity by multiple subculture on solid media was routinely done. All grew aerobically at 28° on the following medium, subsequently referred to as P.Y.E. medium: peptone, 1% (w/v), yeast extract, 0·5% (w/v), NaCl, 0·5% (w/v); for solid media 1·5% (w/v) Oxoid Agar No. 3 was added. During the test period strains were stored at 4° on P.Y.E. slopes. Inocula for tests were grown from these in P.Y.E. broth incubated at 28°. All strains were lyophilized for long-term storage.

(*c*) *Data collection.* Unless otherwise indicated all characters were determined at 28° on P.Y.E. medium or with P.Y.E. medium as the nutrient base. All other procedures and timings were standardized to enable direct strain-to-strain comparison. In the following brief descriptions the numbers in parentheses indicate the number of features coded from an observed character.

Forty-eight-hour discrete colonies were observed for colony diameter as under 1 mm. or over 2 mm. (2); margin entire or irregular (1); surface smooth or otherwise (1); elevation convex or flat (1); consistency butyrous, friable or solid (3). Cell morphology determined by Gram-stained smears (Jensen's modification; Cruickshank, 1965), as rods present (1); regular rods (1); irregular rods (1); cocci (1); branching (1). Gram reaction (1). Acid-fast cells present (1) using 20% (v/v) sulphuric acid for 3 min. as decolorising agent. Catalase activity (1) by adding '10 vol.' H_2O_2 to growth smeared on a clean slide. Oxidase reaction (1) by Kovacs' (1956) method.

Duplicate plate cultures incubated in the light and dark were observed at 48 hr for pigmentation in the light, present (1); pink (1); yellow/red (1); yellow/green (1). Dark-

incubated plates were exposed to light for 1 hr, re-incubated in darkness for 24 hr and observed for photochromogenesis (1), i.e. appearance or enhancement of colour.

Growth in the absence of oxygen (1); at 37° (1); on Hoyle medium (Cruickshank, 1965) (1); on MacConkey agar (Oxoid CM5 broth plus 1·5% (w/v) agar) (1); on Levine E.M.B. agar (Oxoid CM69) (1); all recorded after 48 hr.

Sensitivity to penicillin, 15 I.U. (1), streptomycin, 10 μg. (1), tetracycline, 10 μg. (1), chloramphenicol, 10 μg. (1), novobiocin, 5 μg. (1), oleandomycin, 5 μg (1), erythromycin, 10 μg. (1) was determined after 48 hr with Oxoid Multodisk no. 11–14C.

Growth at 5° (1) was determined on plates incubated for 28 days.

Oxidation or fermentation of glucose (2) was tested by using Baird-Parker (1963) base with 1% (w/v) glucose, 0·003% (w/v) bromcresol purple and 1·5% (w/v) agar by Mossel's (1962) single tube method. Acetoin production (1) was detected by Barritt's method (Cruickshank, 1965) after 6 days in P.Y.E. broth plus 1% (w/v) glucose. Final pH below 5·0 (1) was recorded at 6 days in the acetoin tests above.

The neutral red test (1) of Cann & Wilcox (1965) was modified to use P.Y.E. agar and read at 6 days. Clearing of 5% (v/v) horse blood agar (1) was read at 48 hr. Citrate utilization (1) using Simmons' method, and aldehyde formation from glycerol (1) using Stern's method, were read after 14 days (Cruickshank, 1965). Hydrolysis of salicylate (1) (Tsukamura, 1966) and cellulose (1) (Skerman, 1967) were observed for 4 weeks. Hydrolysis of tributyrin (1) (Willis, 1960); Tweens 20, 40, 60 and 80 (4) (Sierra, 1957); gelatin (1) (Frazier, 1926); casein (1) (Davis & Park, 1962); starch (1) (Davis & Park, 1962); DNA (1) (Jeffries, Holtman & Guse, 1957, using Difco DNA-ase test agar) were read after 6 days. Hydrolysis of xanthine and tyrosine (2) (Gordon & Smith, 1955) observed after 14 days. Liquefaction of Loeffler serum slopes (1) after 3 weeks.

Aesculin hydrolysis (1), method of Davis (1955) using P.Y.E. base; arbutin hydrolysis (1) as for aesculin, both read at 14 days. Hippurate hydrolysis (1) (Davis, 1955) read at 6 days. Ammonia from arginine (1) (Davis, 1955) read at 6 days. Thornley's (1960) arginine test (1) was modified to use P.Y.E. base and read at 6 days. Nitrate and nitrite reduction (2) were tested separately as recommended by ZoBell (1932) after 6 days. Indole production (1) was tested for in P.Y.E. broth plus 1% (w/v) Oxoid acid-hydrolysed casein after 6 days by using the Ehrlich–Bohme reagent. Urease (1) was detected in a modified Christensen test (Cruickshank, 1965) at 6 days. A modified gluconate test (1) (Cruickshank, 1965) was read at 6 days.

Diffusible brown pigment on P.Y.E. agar plus 0·1% (w/v) sterile catechol and in the tyrosine test referred to above was recorded at 6 and 14 days respectively (2).

Motility (1) was observed in hanging drop preparations of 48 hr P.Y.E. broth cultures.

A total of 70 features were coded.

(*d*) *Data analysis.* The simple matching coefficient of Sokal & Sneath (1963) was used for similarity assessment. Two clustering programs were tested, one relying on single linkage analysis and the other on complete (or multiple) linkage. The two programs were prepared by Miss E. Szabo of this department (to be published) and run on a GE 225 computer. The complete linkage analysis was stopped at the 60% *S* level after 21 min. on the computer. The single linkage analysis took 7 min. of computer time and ran to completion.

RESULTS

Several strains showed colonial variation during preliminary culture and were separated into discrete cultures. Three examples of this (strains 61A and B, 63A and B, 92A and B) were included in the study, bringing the total strains studied to 73. Our

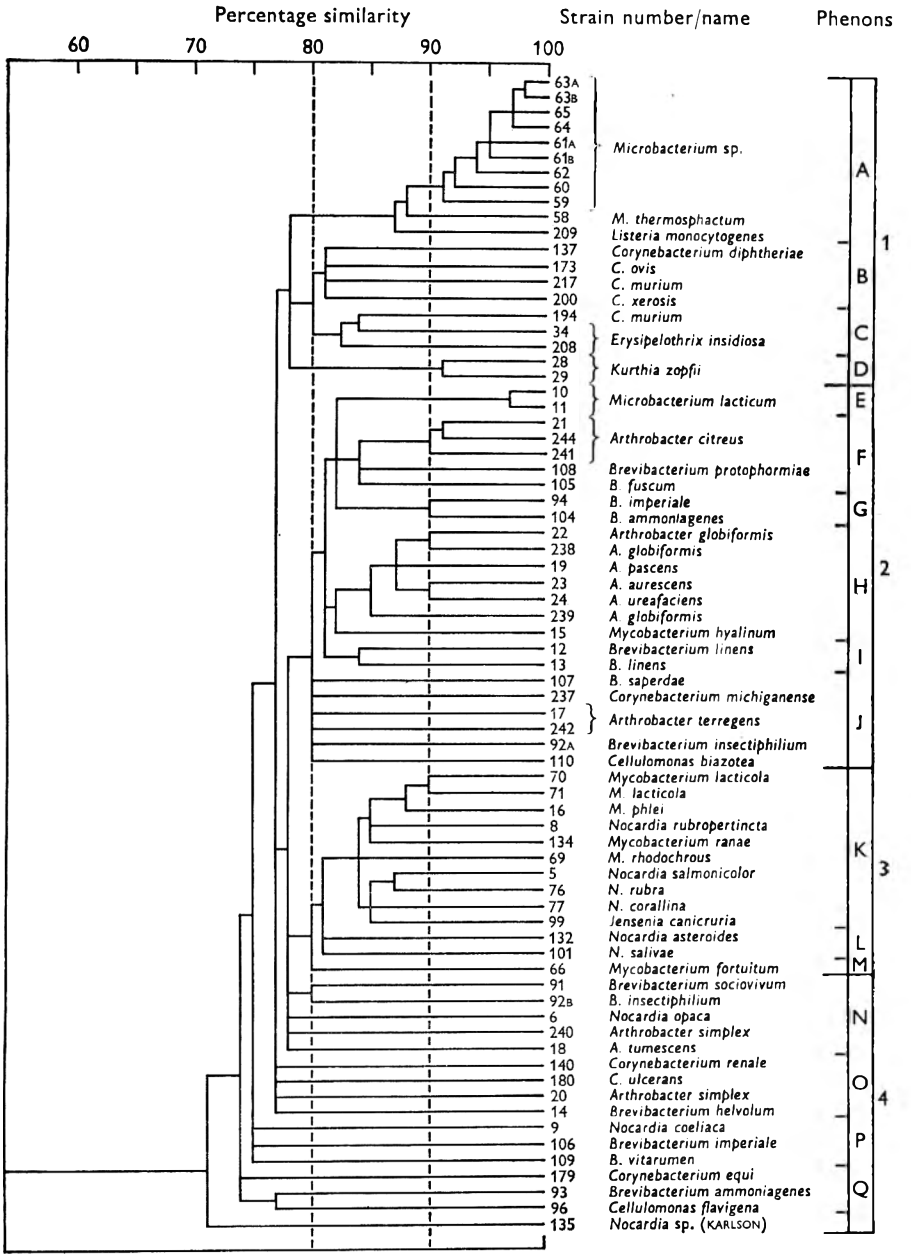

Fig. 1. Dendrogram derived from single linkage analysis.

results indicate that in the case of strains 61 and 63 the cultures separated were essentially the same but in the case of 92 the two cultures differed considerably and neither appeared to fit the description of *Brevibacterium insectiphilium*. Similar deviations from published descriptions were found in a number of the strains studied, e.g. strains 109 and 106 were both Gram-negative and motile. Most of the characters

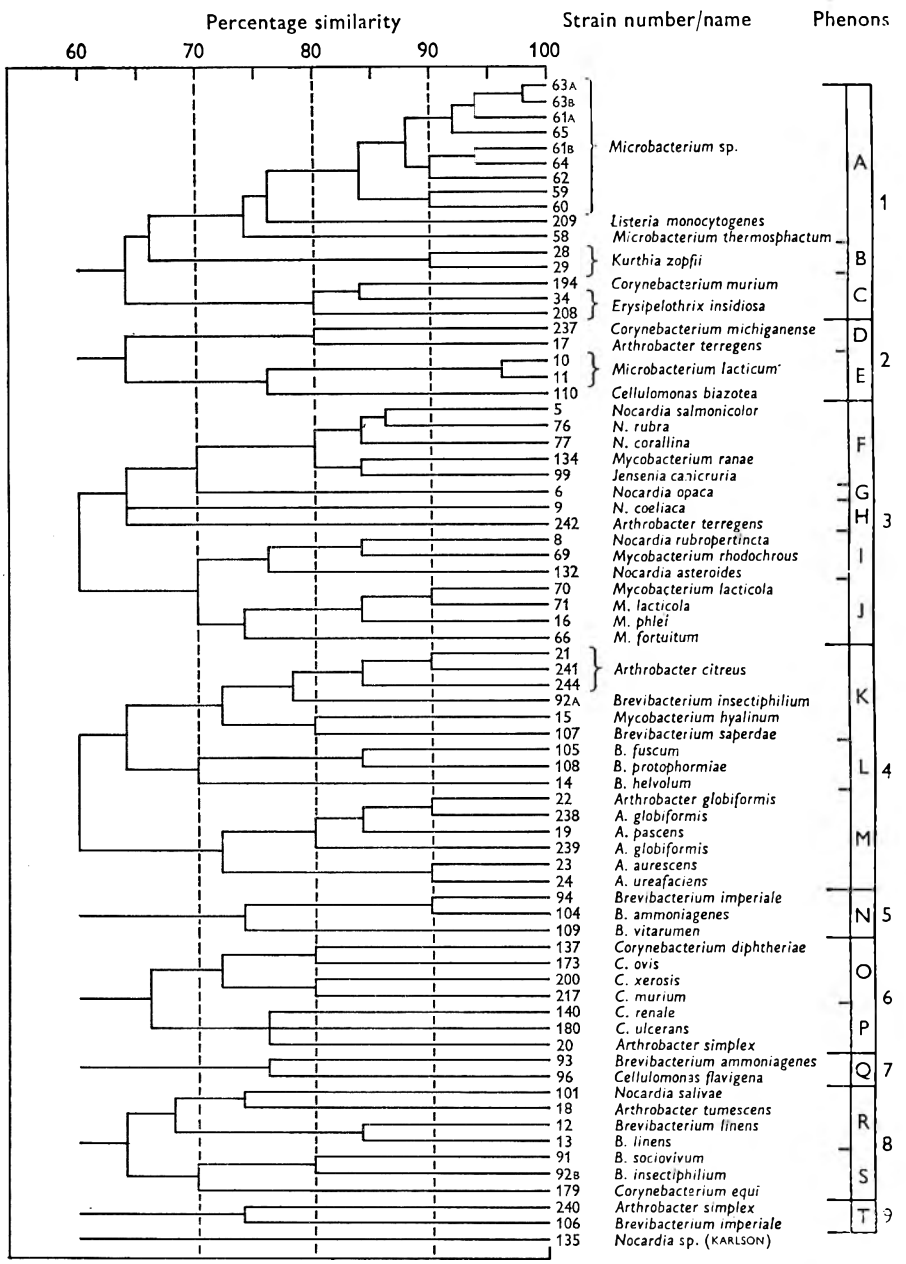

Fig. 2. Dendrogram derived from complete linkage analysis.

recorded in this study have either been tested for in a non-comparable manner or not tested for at all in previous reports. Our objective was not identification but taxonomy and we have used the results obtained without prejudice.

Dendrograms based on the computer outputs using single and complete linkage

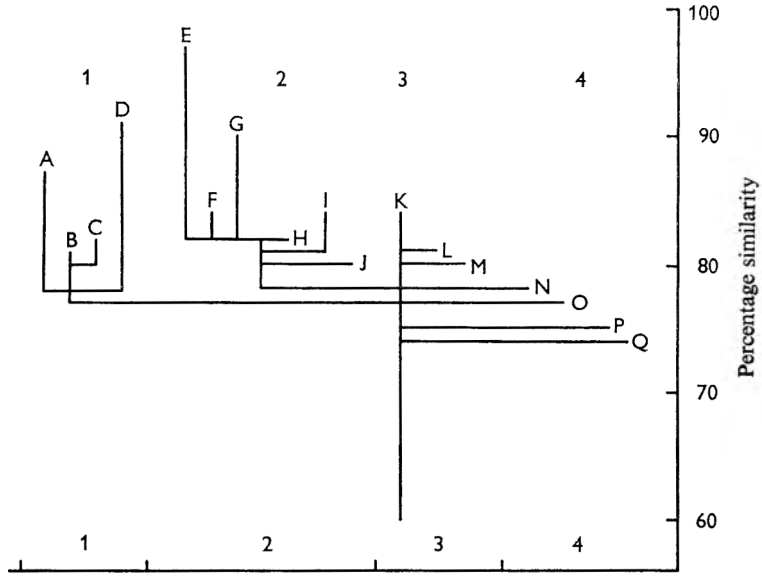

Fig. 3. Schematic representation of single linkage clustering.

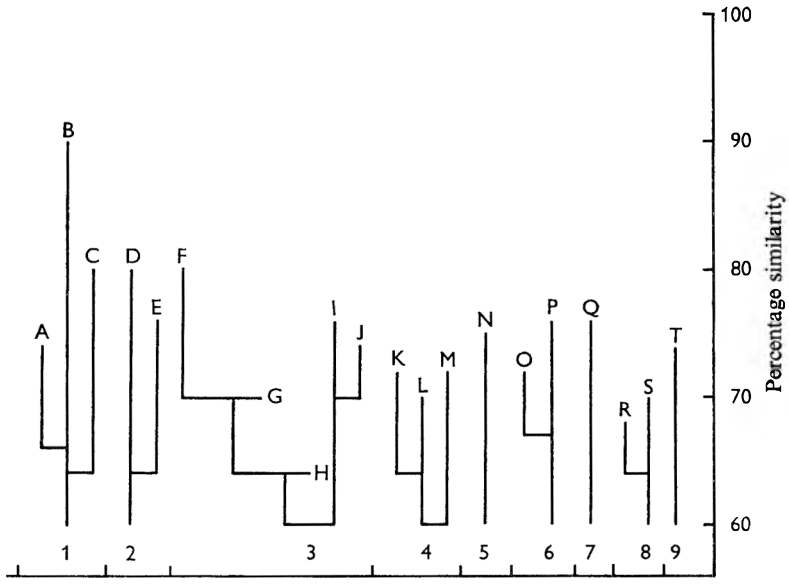

Fig. 4. Schematic representation of complete linkage clustering.

clustering programs are compared in Figs. 1 and 2. Figures 3 and 4 show these in simplified forms. The unsorted similarity matrix for all the strains with the *S* values shown as percentages is given in Fig. 5.

The numbers of clusters (phenons) formed and the numbers of strains clustered by the two methods at descending levels of similarity are shown in Table 2.

The complete linkage analysis to the 60% *S* level resulted in an incomplete sort and in comparison with the single linkage analysis led to differences in cluster (phenon) formation, particularly at lower *S* levels. One strain, *Nocardia* sp. (KARLSON) 135, stood out from the rest of the strains in both analyses. The single linkage analysis indicated three large clusters at *c.* 80% *S* covering 57 of the 73 strains. Thirteen subclusters were indicated in these strains (A–M in Figs. 1 and 3). By complete linkage three large clusters were formed at the 60–65% *S* level (1, 3 and 4 in Figs. 2 and 4), accounting for 46 strains and subdivisible into 11 subclusters (A–C, F–J, K–M in Figs. 2 and 4). Groups 6 and 8 in Figs. 2 and 4 included seven strains each, divisible in each case into two subclusters.

Table 2. *Comparison of strain clustering and phenon formation levels in single and complete linkage analyses*

	Single linkage		Complete linkage	
S (%)	No. of phenons	No. of strains	No. of phenons	No. of strains
95	2	8	2	4
90	8	24	10	23
85	8	35	10	25
80	6	59	18	51
75	2	69	19	60
70	1	73	18	70
65	—	—	15	72
60	—	—	9	72

Similarities between the two cluster analyses were as follows: Nine strains labelled *Microbacterium* sp. clustered with one named *Microbacterium thermosphactum* (McLean & Sulzbacher, 1953) and the single strain of *Listeria monocytogenes* (Cluster 1A in both analyses). Two strains of *Microbacterium lacticum* were well separated from this cluster and in both analyses they fell into the same major group as a strain of *Corynebacterium michiganense* and one of *Cellulomonas biazotea*. Two strains of *Erysipelothrix* clustered with one labelled *Corynebacterium murium* (194). They eventually linked with the *Microbacterium*/*Listeria* and the *Kurthia* subclusters in both analyses. A second strain of *Corynebacterium murium* (217) clustered with three other corynebacteria of animal origin (*xerosis*, *ovis*, *diphtheriae*) in both analyses.

Cluster 3 in both analyses represented a clear group of *Mycobacterium* and *Nocardia* species although the exact composition and subclustering varied. *Jensenia canicruria* was included in this group.

Three strains of *Arthrobacter citreus* showed close similarity as also did three strains of *A. globiformis; A. pascens* (19), *A. ureafaciens* (23) and *A. aurescens* (24) were linked to the latter strains.

One strain of *Arthrobacter simplex* (20) showed relationship to *Corynebacterium renale* and *C. ulcerans*, which clustered together. *Corynebacterium equi* (179) was well separated from other corynebacteria.

Cellulomonas flavigena (96) associated with a strain of *Brevibacterium ammoniagenes* (93); *Cellulomonas biazotea* (110) was well separated. A second strain of *B. ammoniagenes* (104) linked with a strain of *B. imperiale* (94) which in turn was well separated from the second *B. imperiale* strain (106). The two strains of *B. linens* clustered together. *Brevibacterium fuscum* (105) and *B. protophormiae* (108) were linked and clustered with the *A. citreus* and *A. globiformis* strains; *B. saperdae* (107) and *Mycobacterium hyalinum* (15) fell into the same cluster.

Differences noticeable in the two cluster analyses were the separation of the animal corynebacteria from cluster 1 in the multiple linkage analysis; the breakdown and redistribution of single linkage cluster 2 in the multiple linkage analysis and a similar redistribution of the poorly clustered strains in the lower part of Fig. 1 by the more searching analysis.

DISCUSSION

Apart from the absence of 'sugar fermentation' tests the characters used in this study resemble in general terms those commonly used in recently published papers on bacterial taxonomy by numerical analysis, e.g. Goodfellow (1967); Splittstoesser, Wexler, White & Colwell (1967). Photochromogenesis is a characteristic used in Runyon's diagnostic scheme for anonymous mycobacteria (Runyon, 1955), and depends upon adaptive carotenoid production. It is a factor which needs to be considered in assessing cultures for pigment formation in taxonomic work. The following strains showed this character, *Nocardia rubropertincta* and *Nocardia* sp. (KARLSON), *Mycobacterium hyalinum* and *M. rhodochrous*, *Brevibacterium aurescens*, *B. ureafaciens*, *B. insectiphilium* (92A) and *B. saperdae*. Except for *Nocardia* sp. (KARLSON) these strains clustered in two separate positions, some in the *Nocardia/Mycobacterium* cluster and some (including *M. hyalinum*) in the *Arthrobacter globiformis/citreus* cluster. Photo-induced carotenogenesis was discussed by Burchard & Dworkin (1966) and Mathews (1966).

The *Mycobacterium/Nocardia* cluster of strains is well defined in our results and there is little evidence of linkage between this group and any of the corynebacteria examined. This reinforces Gordon's (1966) conclusion that the genus *Corynebacterium* is not a suitable repository for her *Mycobacterium rhodochrous*. Although there is good evidence against the present division of *Corynebacterium*, *Mycobacterium* and *Nocardia* at the ordinal level we cannot support Harrington's (1966) plea for a common genus. Recent studies on *Mycobacterium* taxonomy by Tsukamura (1966) and Bogdanescu & Racotta (1967) did not cover the problems raised by Gordon's 1966 work although Tsukamura's separation of the rapidly growing and slowly growing species into two groups suggests that it may be possible to recognize a separate taxon composed of rapidly growing mycobacteria and related forms. This would include Gordon's *Mycobacterium rhodochrous* group and resemble the *Mycobacterium/Nocardia* group detected in the present study. In view of the fact that the type species of *Nocardia*, *N. asteroides*, would probably be included it seems reasonable to suggest that the generic name *Nocardia* be retained for this taxon in spite of Gordon's desire to restrict it to strains capable of aerial mycelium formation. Such a taxon would clearly be heterogeneous in certain characters, particularly morphology, but others (e.g. DNA base ratios, Hill, 1966, and lipid analysis, Lanéele, Asselineau & Castelnuovo, 1965) would probably uphold it, particularly if strains of *Streptomyces*

currently masquerading as nocardias were eliminated. *Corynebacterium equi* (NCTC 1621), which was included by Gordon in an atypical *M. rhodochrous* group and clustered with mycobacteria in Harrington's work, was not well clustered in the present study. Our strains of *Nocardia opaca*, *N. salivae* (Davis & Freer, 1960, renamed *Rothia dentocariosus* by Georg & Brown, 1967) and *N. coeliaca* were also imperfectly clustered in one or other of the two programs used. Current evidence for the grouping of *Corynebacterium* species into sub-generic taxa is conflicting. Apart from the probable exclusion of *C. haemolyticum* and *C. pyogenes* (Cummins & Harris, 1956) and a possible division into animal and plant corynebacteria (Da Silva & Holt, 1965) opinions differ on what should be done with the present system (see Harrington, 1966, and Lazar, 1968). Our results support the recognition of a taxon for the animal corynebacteria (Fig. 2, Group 6) and although only one plant corynebacterium (*C. michiganense*) was used the resulting clusters support Da Silva & Holt's grouping of this bacterium with strains labelled *Microbacterium lacticum* (Fig. 2, Group 2; Fig. 1, Group 2) and their separation from the *C. diphtheriae* group.

Recent studies of *Microbacterium* taxonomy by Robinson (1966*a*, *b*) did not include strains of the organism named *Microbacterium thermosphactum* by McLean & Sulzbacher (1953). Robinson recognized the three species *M. flavum*, *M. lacticum* and *M. liquefaciens* but recommended that the first of these be reallocated to the genus *Corynebacterium* on cell-wall and other evidence. He also noted close relationship between *M. lacticum* and *M. liquefaciens*. In our work two strains of *M. lacticum* associated in both cluster analyses with strains labelled *Corynebacterium michiganense*, *Arthrobacter terregens* and *Cellulomonas biazotea*. In Fig. 1 these bacteria fall into the large cluster including *Arthrobacter globiformis* and *A. citreus*. Keddie *et al.* (1966) could not classify a strain of *M. lacticum* with *Arthrobacter*, *Corynebacterium* or *Cellulomonas* on the basis of cell-wall composition but examination of their and other results suggests that the species shows cell-wall similarities with *Arthrobacter terregens* and *A. flavescens*, and inclusion in the Arthrobacter group in the present study is not unreasonable.

As pointed out by Barlow & Kitchell (1966), the systematic position of *Microbacterium thermosphactum* is uneasy. It is more similar in certain respects to the lactobacilli than the coryneform microbacteria. The nine strains labelled '*Microbacterium* sp.' in this work were obtained in the study reported by Weidemann (1965) from beef stored in nitrogen at 0°. They clustered well with a strain of *M. thermosphactum* and one of *Listeria monocytogenes* (Group 1A, Figs. 1 and 2). Relationship with certain animal corynebacteria, *Kurthia* and *Erysipelothrix* is also indicated. This group (1 in Figs. 1 and 2) differs from the other large clusters in Fig. 1 and all except Group 6 in Fig. 2 in being largely composed of non-pigmented, regular rods, showing anaerobic glucose metabolism, no reduction of nitrate and with no acid-fast members. The inclusion in this group of representative strains of four generic type species suggests that the subclusters discernible within Group 1 in Figs. 1 and 2 correspond to current genera. With the possible exception of the animal corynebacteria, most of these bacteria would probably be better accommodated with the lactic-acid bacteria than the coryneforms.

Brevibacterium is acknowledged to be a taxon of convenience erected by Breed in 1953. Its heterogeneity is confirmed by the distribution of strains so named in Figs. 1 and 2. Da Silva & Holt (1965) recommend that the type species *B. linens* be reallocated

as *Arthrobacter linens*. Such a move would invalidate the present genus. In our results *B. linens* clustered closely with *Arthrobacter globiformis* upon single linkage analysis but was separated from the *A. citreus/globiformis* group by complete linkage analysis. In the latter analysis three possibly related groups of *Brevibacterium* strains occur, in Groups 4, 5 and 8. In Group 4, five *Brevibacterium* strains associate with the *A. citreus/globiformis* cluster. In Group 8, four strains cluster with *A. tumescens*. No association with the arthrobacters is seen in Group 5. These results suggest, however, that it may be possible to re-allocate most of the strains currently named *Brevibacterium* in generic taxa emerging from the *Arthrobacter* complex. Goodfellow (1967) found that his own results agreed with previous evidence for the separation of *A. simplex* and *A. tumescens* from a group of arthrobacters associated with *A. globiformis*. This subdivision is supported by our results, *A. simplex* and *tumescens* being separated in

Table 3. *Percentage incidence of potentially diagnostic characters in the three major groups discernible by single linkage clustering (57 strains)*

Character	*C. diphtheriae/ Listeria* group (20 strains)	*Arthrobacter/ Brevibacterium* group (24 strains)	*Mycobacterium/ Nocardia* group (13 strains)
Irregular rods	20	90	100
Colony convex	40	96	80
Margin entire	20	100	33
Acid-fast	0	0	72
Pigmented	0	75	88
Catechol-induced pigment	5	67	0
Growth anaerobically	90	42	16
Growth at 5°	45	90	8
Growth on MacConkey	75	96	64
Sensitivity to penicillin	95	90	40
Glucose oxidized	15	75	88
Glucose fermented	65	20	0
Aesculin hydrolysed	60	80	24
Starch hydrolysed	20	80	0
Tyrosine hydrolysed	0	80	32
Casein hydrolysed	5	84	0
Gelatin hydrolysed	5	84	8
DNA hydrolysed	5	71	0
Tributyrin hydrolysed	60	42	100
Tween 20 hydrolysed	30	62	88
Tween 40 hydrolysed	15	67	100
Tween 60 hydrolysed	15	62	100
Tween 80 hydrolysed	15	25	100
Nitrate reduced	10	54	100
Motile	20	33	0

both analyses from the main group of arthrobacters (cf. Cummins & Harris, 1959). If, as seems likely, the genus *Brevibacterium* is dispersed in reclassification of the arthrobacters, the family Brevibacteriaceae will lose its type genus and its validity in nomenclature. Our results indicate that *Kurthia zopfii* may represent a valid taxon related to the *Listeria/Erysipelothrix/Microbacterium thermosphactum* complex, with probable relationship also to animal corynebacteria. The displacement of *Kurthia*

from the present family Brevibacteriaceae and inclusion at family level (Corynebacteriaceae?) with the above genera would not be unreasonable.

In conclusion we suggest that the present exploratory study indicates the existence of three major phenons in the strains studied. These are:

(*a*) Animal corynebacteria and other bacteria showing metabolic and other characters of possible relationship to the family Lactobacillaceae (Group 1, Fig. 1; Groups 1 and 6, Fig. 2).

(*b*) Rapid-growing mycobacteria and *Nocardia* sp.; Family Mycobacteriaceae Order Eubacteriales. (Group 3 in Figs. 1 and 2.)

(*c*) *Arthrobacter* complex, requiring new family affiliation (Arthrobacteriaceae?) (Groups 2 and 4 (?), Fig. 1; Groups 2, 4, 5, 7, 8 and 9, Fig. 2.)

The two Tables 3 and 4 have been included in an attempt to show the over-all characters of these three phenons based only on features that showed uneven distribution among the three groups.

Table 4. *Percentage incidence of potentially diagnostic characters in the three major groups discernible by complete linkage clustering (70 strains)*

Character	*C. diphtheriae*/ *Listeria* group (23 strains)	*Arthrobacter*/ *Brevibacteria* group (32 strains)	*Mycobacterium*/ *Nocardia* group (15 strains)
Regular rods	64	27	33
Irregular rods	17	72	92
Colonies convex	39	84	80
Acid-fast	0	0	60
Pigmented	4	68	86
Growth anaerobically	90	59	13
Growth at 37°	51	84	86
Growth at 5°	59	77	26
Growth on MacConkey	77	96	53
Growth on EMB	0	53	26
Sensitive to penicillin	94	71	33
Growth on Hoyle	30	22	80
Glucose oxidized	21	65	86
Glucose fermented	64	27	0
Acetoin produced	69	34	0
Aesculin hydrolysed	51	71	24
Starch hydrolysed	17	62	0
Tyrosine hydrolysed	0	71	33
Casein hydrolysed	17	71	0
Gelatin hydrolysed	17	84	0
DNA hydrolysed	4	62	0
Tributyrin hydrolysed	60	46	92
Tween 20 hydrolysed	43	56	86
Tween 40 hydrolysed	17	71	100
Tween 60 hydrolysed	17	62	100
Tween 80 hydrolysed	21	27	92
Oxidase	9	62	7
Nitrate reduced	13	62	86
Motile	17	28	13

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APPENDIX

Table A shows the coded features and the numbers used to indicate them. Table B lists the results for these features for all the strains used.

Table A

Feature no. in Table B	Coded feature	Feature no. in Table B	Coded feature
1	Colony under 1 mm. in diameter	36	Chloramphenicol sensitive
2	Colony over 2 mm. in diameter	37	Novobiocin sensitive
3	Margin entire	38	Oleandomycin sensitive
4	Surface smooth	39	Erythromycin sensitive
5	Consistency butyrous	40	Growth on Hoyle's medium
6	Colony adherent	41	Growth anaerobically
7	Colony friable	42	Growth at 5°
8	Colony convex	43	Growth at 37°
9	Colony pink	44	Cellulose hydrolysed
10	Colony yellow/green	45	Aesculin hydrolysed
11	Colony yellow/red	46	Arbutin hydrolysed
12	Regular rod forms seen	47	Glucose oxidised
13	Irregular rod forms seen	48	Glucose fermented
14	Branching rod forms seen	49	Acetoin from glucose
15	Cocci seen	50	Stern's test positive
16	Motile	51	Hippurate hydrolysed
17	Acid-fast cells present	52	Nitrate reduced
18	Catalase positive	53	Nitrite reduced
19	Oxidase positive	54	Indole produced
20	Tween 20 hydrolysed	55	Urea hydrolysed
21	Tween 40 hydrolysed	56	Ammonia from arginine
22	Tween 60 hydrolysed	57	Thornley's test positive
23	Tween 80 hydrolysed	58	Citrate utilized
24	Gelatin hydrolysed	59	Gram positive
25	Casein hydrolysed	60	Photochromogenic
26	Starch hydrolysed	61	Tributyrin hydrolysed
27	Tyrosine hydrolysed	62	Horse blood cleared
28	Melanin from tyrosine	63	Neutral red test
29	Xanthine hydrolysed	64	Gluconate test
30	Salicylate hydrolysed	65	Pigment on catechol
31	Growth on MacConkey	66	Serum hydrolysed
32	Growth on EMB	67	DNA hydrolysed
33	Penicillin sensitive	68	Pigment produced
34	Streptomycin sensitive	69	Rod forms seen
35	Tetracycline sensitive	70	Final pH under 5·0

Table B. *Complete data for 73 strains of coryneform bacteria relating to 70 features* (*See Table A*)

No.	Name	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35
5	*Nocardia salmonicolor*	−	−	−	−	+	+	−	+	−	−	+	−	+	+	+	−	+	+	−	+	+	+	+	−	−	−	+	−	−	−	+	−	+	+	+
6	*N. opaca*	+	−	+	−	+	+	−	+	−	−	−	−	+	+	−	+	−	+	−	+	+	+	−	−	−	−	+	−	−	+	−	−	−	+	+
8	*N. rubropertincta*	+	−	−	−	+	−	−	+	+	−	−	−	+	−	−	−	+	+	−	+	+	+	+	−	−	−	−	−	−	−	−	−	−	+	+
9	*N. coeliaca*	+	−	−	−	+	−	−	+	−	−	+	−	+	−	−	−	−	+	+	+	+	+	+	−	−	−	−	−	−	+	−	−	+	+	+
76	*N. rubra*	−	−	+	+	+	−	−	+	−	−	+	−	+	+	+	−	−	+	−	−	+	+	+	−	−	−	+	−	−	−	−	−	+	+	+
77	*N. coralina*	−	−	+	+	+	−	−	+	−	−	+	−	+	+	−	−	+	+	−	+	+	+	+	−	−	−	+	−	−	−	+	−	+	+	+
101	*N. salivae*	−	−	+	+	+	−	−	+	−	−	−	−	−	+	+	−	−	+	−	+	+	+	+	+	−	−	−	−	−	−	−	−	+	+	+
132	*N. asteroides*	+	−	−	−	+	+	+	−	+	−	−	+	+	+	−	−	+	+	−	+	+	+	+	−	−	−	−	−	−	−	−	−	−	−	−
135	*N.* sp. (KARLSON)	+	−	−	−	−	+	+	+	−	−	+	−	+	+	−	−	−	+	−	−	+	−	−	+	+	−	−	−	−	+	−	+	−	−	−
15	*Mycobacterium hyalinum*	−	+	+	+	+	−	−	+	−	+	−	−	+	−	−	+	−	+	+	−	−	−	−	+	+	+	+	−	−	−	+	+	−	−	−
16	*M. phlei*	+	−	−	−	−	−	+	−	−	−	+	−	+	−	−	−	+	+	−	+	+	+	+	−	−	−	−	−	−	−	−	−	−	+	+
66	*M. fortuitum*	+	−	−	+	+	−	−	+	−	−	−	+	+	−	−	−	+	+	−	+	+	+	+	−	−	−	−	−	−	+	+	+	−	−	+
69	*M. rhodochrous*	−	−	−	−	+	−	−	−	+	−	−	+	+	−	−	−	−	+	−	+	+	+	+	−	−	−	−	+	−	−	+	−	−	+	−
70	*M. lacticola*	+	−	−	−	−	+	+	+	−	−	+	+	+	−	−	−	+	+	−	−	+	+	+	−	−	−	−	−	−	+	+	−	+	+	+
71	*M. lacticola*	+	−	−	−	−	−	+	+	−	−	+	−	+	−	−	−	+	+	−	+	+	+	+	−	−	−	−	−	−	+	+	−	+	+	+

No.	Name	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50	51	52	53	54	55	56	57	58	59	60	61	62	63	64	65	66	67	68	69	70
5	*Nocardia salmonicolor*	+	+	+	+	+	+	−	+	−	−	−	+	−	−	−	−	+	−	+	−	−	−	+	+	−	+	−	−	−	−	−	−	+	+	−
6	*N. opaca*	+	+	+	+	+	−	+	+	−	−	−	+	−	−	−	+	+	+	−	+	−	−	+	+	−	+	+	+	+	−	−	−	−	+	−
8	*N. rubropertincta*	+	+	+	+	+	−	−	+	−	−	−	+	−	−	−	−	+	+	−	−	−	−	−	+	+	+	−	−	−	−	−	−	+	+	−
9	*N. coeliaca*	+	+	+	+	−	−	+	−	−	−	+	−	−	−	−	−	−	−	+	+	−	+	+	+	−	+	+	−	−	−	+	−	+	+	−
76	*N. rubra*	+	+	+	+	+	−	−	+	−	−	−	+	−	−	−	−	+	−	−	−	−	−	+	+	−	+	+	−	−	−	−	−	+	+	−
77	*N. coralina*	+	+	+	+	+	+	−	+	−	−	−	+	−	−	−	+	+	−	−	+	+	+	+	+	−	+	+	−	−	−	−	−	+	+	+
101	*N. salivae*	+	+	+	+	−	−	−	+	−	+	+	+	−	+	+	−	+	−	+	−	−	−	−	+	−	+	+	−	+	−	−	−	−	+	−
132	*N. asteroides*	−	−	+	+	+	−	−	+	−	+	+	+	−	−	−	−	+	+	−	−	−	−	−	+	−	+	−	−	−	−	−	−	+	+	−
135	*N.* sp. (KARLSON)	−	−	+	+	−	−	−	+	−	+	−	−	−	−	+	−	−	+	+	−	−	−	−	+	+	−	−	−	−	−	−	−	+	+	−
15	*Mycobacterium hyalinum*	+	+	+	+	−	−	+	+	−	+	−	+	−	−	−	−	−	+	−	−	−	−	−	+	+	+	+	−	+	+	−	+	+	+	−
16	*M. phlei*	+	+	−	+	−	−	−	+	−	−	−	−	−	−	−	−	+	−	+	−	−	−	−	+	−	+	−	−	+	−	−	−	+	+	−
66	*M. fortuitum*	−	−	−	+	+	−	−	+	−	−	−	−	−	−	−	−	+	−	−	−	−	−	−	+	−	+	−	+	−	−	−	−	−	+	−
69	*M. rhodochrous*	+	+	−	+	+	−	−	+	−	−	+	+	−	−	−	−	+	+	−	−	−	−	+	+	+	+	−	−	+	−	−	−	+	+	−
70	*M. lacticola*	+	+	+	+	+	−	−	+	−	−	−	+	−	−	−	+	+	−	+	−	−	−	−	+	−	+	−	−	−	−	−	−	+	+	−
71	*M. lacticola*	+	+	+	+	+	−	−	+	−	+	−	+	−	−	−	−	+	−	−	−	−	−	−	+	−	+	−	−	+	−	−	−	+	+	−

Table B (*cont.*)

No.	Name	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35
134	*M. ranae*	+	−	−	+	+	−	−	+	−	−	+	−	+	+	−	−	−	+	−	+	+	+	+	−	−	−	−	−	−	−	+	−	−	+	+
137	*Corynebacterium diphtheriae*	+	−	−	+	+	−	−	+	−	−	−	−	+	−	−	−	−	+	−	+	−	−	−	−	−	+	−	−	−	−	+	−	+	+	+
140	*C. renale*	+	−	+	+	+	−	−	+	−	+	−	+	−	−	+	−	−	+	−	+	−	−	+	+	+	−	−	−	−	−	+	−	+	+	+
173	*C. ovis*	+	−	−	+	+	−	−	+	−	−	−	−	−	−	+	−	−	+	−	+	−	−	−	+	+	+	−	−	−	−	+	−	+	+	+
179	*C. equi*	−	−	−	+	+	−	−	−	+	−	−	−	−	−	+	−	−	+	−	+	−	−	−	+	−	−	−	−	−	−	+	−	−	+	−
180	*C. ulcerans*	−	+	+	+	+	−	−	−	−	−	−	−	−	−	+	−	−	+	−	+	+	+	+	+	−	+	−	−	−	−	+	−	+	+	+
194	*C. murium*	+	−	−	+	+	−	−	−	−	−	−	+	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−	+	−
200	*C. xerosis*	+	−	−	+	+	+	−	+	−	−	−	−	+	−	−	−	−	+	−	+	−	−	+	−	−	+	−	−	−	−	−	−	+	−	−
217	*C. murium*	+	−	+	+	+	−	−	+	−	−	−	−	+	−	−	−	−	+	−	+	−	−	+	−	−	+	−	−	−	−	−	−	+	−	+
237	*C. michiganense*	+	−	+	+	+	−	−	+	−	+	−	−	+	−	−	−	−	+	−	−	−	−	−	+	−	+	−	−	−	−	+	−	+	+	+
34	*Erysipelothrix insidiosa*	+	−	−	+	+	−	−	−	−	−	−	+	−	−	−	−	−	−	−	−	+	+	+	−	−	−	−	−	−	−	+	−	+	+	+
208	*E. insidiosa*	+	−	+	+	+	−	−	+	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	+	+	+
209	*Listeria monocytogenes*	−	−	−	+	+	−	−	+	−	−	−	−	+	−	−	+	−	+	−	+	+	−	−	−	−		−	−	−	−	+	−	+	+	+
58	*Microbacterium thermosphactum*	−	+	−	+	+	−	−	−	−	−	−	+	−	−	−	−	−	+	−	−	−	−	−	+	+	−	−	−	−	−	+	−	+	−	+
59	*Microbacterium* sp.	−	−	+	+	+	−	−	+	−	−	−	+	−	−	+	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	+

No.	Name	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50	51	52	53	54	55	56	57	58	59	60	61	62	63	64	65	66	67	68	69	70
134	*M. ranae*	+	+	−	+	+	−	−	+	−	−	−	+	−	−	−	+	+	−	−	−	−	−	−	+	−	+	+	−	+	−	−	−	+	+	−
137	*Corynebacterium diphtheriae*	+	+	+	+	−	+	−	+	−	−	−	+	−	−	−	+	−	−	+	−	−	−	−	+	−	−	+	−	−	−	−	−	−	+	+
140	*C. renale*	+	+	+	+	+	+	−	+	−	−	−	−	+	+	−	+	−	+	−	+	−	−	−	+	−	+	−	−	+	+	−	−	+	+	+
173	*C. ovis*	+	−	+	+	−	+	−	+	−	−	−	−	+	−	+	−	−	−	+	+	−	−	−	+	−	−	+	−	+	−	−	−	−	−	+
179	*C. equi*	+	−	+	+	+	+	−	+	−	−	−	−	+	−	−	+	+	−	+	−	−	−	−	+	−	+	−	−	−	−	−	−	+	−	−
180	*C. ulcerans*	+	+	+	+	+	+	−	+	−	−	−	−	+	+	+	−	−	+	−	+	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−
194	*C. murium*	+	−	+	+	+	+	−	+	−	−	+	−	−	−	+	−	−	−	−	−	−	+	−	+	−	+	−	−	+	−	−	+	−	−	+
200	*C. xerosis*	−	+	+	+	+	+	−	+	−	−	−	−	−	−	+	−	−	−	−	−	−	−	−	+	−	+	−	−	+	−	−	−	−	−	+
217	*C. murium*	+	+	+	+	+	+	−	+	−	+	+	−	+	−	−	+	+	−	−	+	−	−	−	+	−	+	−	−	+	−	−	−	−	+	−
237	*C. michiganense*	+	+	+	+	−	−	+	−	−	+	+	+	−	+	+	+	−	+	−	−	−	−	−	+	−	+	−	−	+	−	−	+	+	+	−
34	*Erysipelothrix insidiosa*	+	−	+	+	+	+	−	−	−	−	−	−	−	−	+	−	−	+	+	−	+	+	−	+	−	−	−	−	+	−	−	−	−	+	−
208	*E. insidiosa*	+	+	+	+	−	+	−	+	−	−	−	−	−	−	+	−	+	−	−	−	+	+	−	+	−	−	+	−	−	−	NC	−	−	+	−
209	*Listeria monocytogenes*	+	+	+	+	+	+	+	+	−	+	+	−	+	+	+	+	−	+	+	−	−	−	−	+	−	+	+	−	+	−	−	−	−	+	+
58	*Microbacterium thermosphactum*	+	+	+	+	−	+	+	+	−	+	+	−	+	+	−	+	−	+	−	+	+	−	−	+	−	−	−	−	−	−	−	−	−	+	+
59	*Microbacterium* sp.	+	+	+	+	−	−	+	−	−	+	+	−	+	+	+	−	−	+	+	−	−	−	−	+	−	+	+	−	+	−	−	−	−	+	+

Table B (*cont.*)

No.	Name	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35
60	*Microbacterium* sp.	−	−	−	+	+	−	−	+	−	−	−	+	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	+	−	+	+	+
61 A	*Microbacterium* sp.	−	+	−	+	+	−	−	−	−	−	−	+	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	+
61 B	*Microbacterium* sp.	−	+	−	+	+	−	−	−	−	−	−	+	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	+	−	+
62	*Microbacterium* sp.	−	−	−	+	+	−	−	−	−	−	−	−	−	−	+	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	+	−	+	−	+
63 A	*Microbacterium* sp.	−	+	−	+	+	−	−	−	−	−	−	+	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	+	−	+	−	+
63 B	*Microbacterium* sp.	−	+	−	+	+	−	−	−	−	−	−	+	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	+	−	+	−	+
64	*Microbacterium* sp.	−	+	−	+	+	−	−	−	−	−	−	+	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	+	−	+	−	+
65	*Microbacterium* sp.	−	+	−	+	+	−	−	−	−	−	−	+	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	+	−	+	−	+
10	*M. lacticum*	+	−	+	+	+	−	−	+	−	+	−	−	+	−	−	−	−	+	+	+	+	+	−	+	+	+	+	+	−	−	+	−	+	+	+
11	*M. lacticum*	+	−	+	+	+	−	−	+	−	+	−	−	+	−	−	−	−	+	+	+	+	+	−	+	+	+	+	+	−	−	+	−	+	+	+
28	*Kurthia zopfii*	−	+	−	+	+	−	−	−	−	−	−	+	−	−	−	+	−	+	+	+	−	+	−	−	−	−	−	+	−	−	+	−	+	+	+
29	*K. zopfii*	−	+	−	+	+	−	−	−	−	−	−	+	−	−	−	+	−	+	+	+	+	+	−	−	−	−	−	+	−	−	+	−	+	+	+
99	*Jensenia canicruria*	−	−	+	+	+	−	−	+	+	−	−	−	+	+	+	−	+	+	−	+	+	+	+	−	−	−	+	−	−	−	−	−	−	+	+
17	*Arthrobacter terregens*	+	−	+	+	+	−	−	+	−	+	−	−	+	−	+	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	+	−	+	+	−
18	*A. tumescens*																																			
19	*A. pascens*	−	−	+	+	+	−	−	+	−	−	−	−	+	−	−	−	−	+	−	−	+	+	−	+	+	+	+	−	+	−	+	+	+	+	+

No.	Name	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50	51	52	53	54	55	56	57	58	59	60	61	62	63	64	65	66	67	68	79	70
60	*Microbacterium* sp.	+	+	+	+	−	+	+	−	−	+	+	−	+	+	+	−	−	+	+	−	−	−	−	+	−	+	−	−	+	−	−	−	−	+	+
61 A	*Microbacterium* sp.	+	+	+	+	−	+	+	−	−	+	+	−	+	+	+	−	−	+	−	−	−	−	−	+	−	+	−	−	+	−	−	−	−	+	+
61 B	*Microbacterium* sp.	+	+	+	+	−	+	+	−	−	+	+	−	+	+	+	−	−	−	−	−	−	−	−	+	−	+	+	−	+	−	−	−	−	+	+
62	*Microbacterium* sp.	+	+	+	+	−	+	+	−	−	+	+	−	+	+	+	−	−	+	−	−	−	−	−	+	−	+	+	−	+	−	−	−	−	−	+
63 A	*Microbacterium* sp.	+	+	+	+	−	+	+	−	−	+	+	−	+	+	+	−	−	+	−	−	−	−	−	+	−	+	+	−	+	−	−	−	−	+	+
63 B	*Microbacterium* sp.	+	+	+	+	−	+	+	−	−	+	+	−	+	+	+	−	−	+	−	−	−	−	−	+	−	+	+	−	+	−	−	−	−	+	+
64	*Microbacterium* sp.	+	+	+	+	−	+	+	−	−	+	+	−	+	+	+	−	−	+	+	−	−	−	−	+	−	+	+	−	+	−	−	−	−	+	+
65	*Microbacterium* sp.	+	+	+	+	−	+	+	−	−	+	+	−	+	+	+	−	+	+	+	−	−	−	−	+	−	+	+	−	+	−	−	−	−	+	+
10	*M. lacticum*	+	+	+	+	+	−	+	+	−	+	+	−	+	−	−	−	+	−	+	−	−	−	−	+	−	−	+	−	+	−	−	+	+	+	+
11	*M. lacticum*	+	+	+	+	+	−	+	+	−	+	−	−	+	−	−	−	+	−	−	−	−	−	−	+	−	−	+	−	+	−	−	+	+	+	+
28	*Kurthia zopfii*	+	+	+	+	−	+	+	+	−	−	−	+	−	+	−	−	−	+	−	−	−	−	+	+	−	+	+	−	+	+	−	−	−	+	−
29	*K. zopfii*	+	+	+	+	−	−	+	−	−	−	−	+	−	+	−	−	−	+	−	−	−	−	−	+	−	−	+	−	+	−	+	−	−	+	−
99	*Jensenia canicruria*	+	+	+	+	+	−	+	+	−	−	−	+	−	−	−	+	+	−	−	−	−	−	−	+	−	+	−	−	+	−	−	−	+	+	−
17	*Arthrobacter terregens*	−	+	+	−	−	−	+	−	−	−	+	−	−	−	+	+	+	−	−	−	−	−	−	+	−	−	−	−	−	−	−	−	+	+	−
18	*A. tumescens*	+	+	+	+	−	+	+	+	−	+	−	−	−	−	+	+	+	−	−	−	−	−	−	+	−	+	+	−	−	−	−	+	−	+	−
19	*A. pascens*	+	+	+	+	−	−	+	+	−	−	−	+	−	+	−	+	−	+	−	−	−	−	+	+	−	−	+	−	−	−	−	−	−	+	−

Table B (*cont.*)

No.	Name	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35
20	*Arthrobacter simplex*	−	+	+	+	+	−	−	+	−	−	−	−	−	−	+	−	−	+	−	+	−	−	−	−	+	−	−	−	−	−	+	−	+	+	−
21	*A. citreus*	−	+	+	+	+	−	−	+	−	+	−	−	+	−	+	+	−	+	+	+	+	+	+	+	+	+	+	−	−	−	+	+	+	+	+
22	*A. globiformis*	−	−	+	+	+	−	−	+	−	−	−	−	+	−	+	−	−	+	+	+	+	+	−	+	+	+	+	−	+	−	+	+	+	+	+
23	*A. aurescens*	−	−	+	+	+	−	−	+	−	−	−	−	+	−	−	−	−	+	+	−	−	−	−	+	+	+	+	+	+	−	+	+	+	+	+
24	*A. ureafaciens*	−	−	+	+	+	−	−	+	−	−	−	−	+	−	−	+	−	+	+	−	−	−	−	+	+	−	+	+	+	−	+	+	+	+	+
238	*A. globiformis*	+	−	+	+	+	−	−	+	−	−	−	−	+	+	−	−	−	+	+	−	+	+	−	+	+	+	+	−	+	−	+	+	+	+	+
239	*A. globiformis*	+	−	+	+	+	−	−	+	−	−	−	−	+	−	−	−	−	+	−	−	+	+	−	+	+	+	+	−	+	−	+	+	−	+	+
240	*A. simplex*	−	−	+	+	+	−	−	+	−	−	−	−	+	−	−	+	−	+	−	+	+	+	+	+	+	−	+								
241	*A. citreus*	−	−	+	+	+	−	−	+	−	+	−	−	+	+	−	+	−	+	+	+	+	+	+	+	+	+	+	−	−	−	+	−	−	+	+
242	*A. terregens*	+	−	+	+	+	−	−	+	−	+	−	−	+	−	−	+	−	+		+	+	+	+	−	−	+	−	−	−	−	−	−	+	+	+
244	*A. citreus*	−	+	+	+	+	−	−	+	−	+	−	−	+	−	+	+	−	+	+	+	+	+	+	+	+	+	+	−	−	+	+	−	+	+	+
12	*Brevibacterium linens*	+	−	+	+	+	−	−	+	−	−	+	−	+	−	−	−	−	+	−	+	+	+	−	+	+	−	+	−	+	−	+	−	−	+	+
13	*B. linens*	−	+	+	+	+	−	−	+	−	−	+	−	+	−	−	−	−	+	+	+	+	+	+	+	+	−	+	−	+	−	+	−	−	+	−
14	*B. helvolum*	−	−	+	+	+	−	−	+	−	+	−	−	+	−	+	+	−	+	+	+	+	+	+	+	+	+	+	+	+	−	+	+	+	−	+
91	*B. sociovivum*	−	−	+	+	+	−	−	+	−	−	+	+	−	−	+	−	−	+	−	+	+	−	−	−	−	−	−	−	−	−	+	−	+	−	+
92A	*B. insectiphilium*	−	+	+	+	+	−	−	+	−	−	+	−	+	−	−	+	−	+	−	+	+	+	−	+	+	+	+	−	+	−	+	+	+	+	+

No.	Name	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50	51	52	53	54	55	56	57	58	59	60	61	62	63	64	65	66	67	68	69	70
20	*Arthrobacter simplex*	+	+	+	+	−	+	−	+	−	−	−	+	−	+	+	+	+	+	−	+	−	−	−	+	−	+	+	−	+	−	−	−	−	−	+
21	*A. citreus*	+	+	+	+	−	−	+	+	−	+	−	+	−	−	−	−	+	−	−	−	−	−	−	+	−	+	−	−	−	+	−	+	+	+	−
22	*A. globiformis*	+	+	+	+	−	−	+	+	−	+	+	+	−	−	+	−	−	+	+	−	−	−	+	+	−	−	+	−	−	−	−	−	−	+	−
23	*A. aurescens*	+	+	+	+	−	−	+	+	−	+	−	+	−	+	−	+	−	+	−	−	+	−	−	+	+	+	+	−	+	+	−	+	−	+	−
24	*A. ureafaciens*	+	+	+	+	−	−	+	+	−	+	−	+	−	+	−	+	−	+	−	−	−	−	+	+	+	−	+	−	−	−	−	+	−	+	−
238	*A. globiformis*	+	+	+	+	−	−	+	+	−	+	+	+	−	−	+	−	−	+	−	−	−	−	−	+	−	−	+	−	+	−	−	−	−	+	−
239	*A. globiformis*	+	−	+	+	−	+	+	+	−	+	+	+	−	−	+	+	−	+	−	+	+	−	−	+	−	−	−	−	−	−	−	−	−	+	+
240	*A. simplex*	+	+	+	+	−	−	+	+	−	+	−	+	−	−	−	−	−	+	−	−	+	−	−	+	−	+	+	−	−	−	+	+	−	+	−
241	*A. citreus*	+	+	+	+	−	−	+	+	−	+	+	+	−	−	−	−	+	−	−	−	−	−	−	+	−	+	−	−	+	−	−	+	+	+	−
242	*A. terregens*	+	+	+	+	−	−	+	−	−	+	−	+	−	−	+	−	−	−	−	−	+	−	−	+	−	−	−	−	+	−	−	−	+	+	−
244	*A. citreus*	+	+	+	+	−	+	+	+	−	+	−	+	−	−	−	−	+	−	−	−	−	−	−	+	−	−	+	−	−	+	+	+	+	+	−
12	*Brevibacterium linens*	+	+	+	+	−	+	+	−	−	−	−	+	−	+	−	+	+	+	+	−	−	−	−	+	−	+	+	−	+	−	−	−	+	+	−
13	*B. linens*	+	+	+	+	−	+	+	+	−	−	−	+	−	−	−	+	+	−	−	−	−	−	−	+	−	+	+	−	+	+	−	+	+	+	−
14	*B. helvolum*	+	+	+	+	−	+	+	+	−	+	−	−	+	+	−	+	−	−	+	+	+	−	−	+	−	+	+	−	−	−	−	+	+	+	+
91	*B. sociovivum*	+	+	+	+	−	+	−	+	−	+	−	+	+	−	−	−	+	−	−	−	+	−	+	+	−	−	+	−	+	−	−	+	+	+	−
92A	*B. insectiphilium*	+	+	+	+	−	+	−	+	−	+	−	+	−	−	−	−	+	−	−	−	+	−	−	+	+	−	+	−	+	−	−	+	+	+	−

Table B (*cont.*)

No.	Name	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35
92B	*B. insectiphilium*	−	−	+	+	+	−	−	+	−	−	+	+	−	−	+	−	−	+	−	+	+	+	+	−	−	−	−	−	−	−	+	−	+	−	+
93	*B. ammoniagenes*	−	+	−	+	+	−	−	−	−	−	−	+	−	−	+	−	−	+	−	−	+	+	−	+	−	−	+	−	−	−	+	+	+	+	+
94	*B. imperiale*	−	−	+	+	+	−	−	+	−	−	+	−	+	−	−	−	−	+	+	−	−	−	−	+	+	+	+	−	−	−	+	+	+	+	+
104	*B. ammoniagenes*	−	−	+	+	+	−	−	−	−	−	+	+	−	−	−	−	−	+	+	−	−	−	−	+	+	+	+	−	−	−	+	+	+	+	+
105	*B. fuscum*	−	+	+	+	+	−	−	+	−	+	−	+	−	−	+	−	−	+	+	+	−	−	−	+	+	+	+	−	−	−	+	+	+	+	+
106	*B. imperiale*	−	+	−	+	+	−	−	+	−	−	−	+	−	−	−	+	−	+	−	+	+	+	+	+	+	−	+	−	−	+	−	−	−	+	−
107	*B. saperdae*	+	−	+	+	+	−	−	+	−	+	−	−	+	−	−	+	−	+	−	−	+	+	−	−	−	+	+	−	−	−	+	−	−	+	+
108	*B. protophormiae*	−	+	+	+	+	−	−	+	−	+	−	+	−	−	+	−	−	+	+	+	+	−	−	+	+	−	+	−	+	−	+	−	+	+	+
109	*B. vitarumen*	−	+	−	+	+	−	−	+	−	−	+	+	−	−	−	+	−	+	+	+	+	−	−	+	+	−	−	−	−	−	+	+	−	+	+
96	*Cellulomonas flavigena*	−	+	−	+	+	−	−	−	−	−	−	+	−	−	+	−	−	+	+	−	−	−	−	−	−	−	+	−	−	−	+	+	−	+	
110	*C. biazotea*	+	−	+	+	+	−	−	+	−	+	−	−	+	−	−	−	−	+	+	+	+	+	+	+	+	+	−	−	−	−	+	−	−	+	−

No.	Name	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50	51	52	53	54	55	56	57	58	59	60	61	62	63	64	65	66	67	68	69	70
92B	*B. insectiphilium*	+	+	+	+	+	+	+	+	−	+	+	+	−	+	−	+	+	−	+	−	−	−	−	+	−	+	−	−	−	−	−	−	+	+	−
93	*B. ammoniagenes*	+	−	+	+	+	+	+	+	−	−	−	+	−	−	−	+	+	−	+	+	−	+	+	+	−	−	−	−	−	+	−	−	−	+	−
94	*B. imperiale*	+	+	+	+	+	+	+	+	−	+	+	−	+	+	−	−	−	+	+	−	−	−	−	+	−	−	+	−	+	−	−	+	+	+	−
104	*B. ammoniagenes*	+	+	+	+	+	+	−	+	−	+	+	−	+	−	−	−	−	+	−	−	−	−	+	+	−	−	+	−	+	−	−	+	+	+	+
105	*B. fuscum*	+	+	+	+	−	+	+	−	−	+	−	−	+	+	−	+	+	−	−	−	−	−	−	+	−	+	+	−	+	−	−	+	+	+	+
106	*B. imperiale*	+	+	−	−	−	+	−	+	−	−	−	+	−	−	−	+	+	+	+	−	+	+	−	+	−	+	+	−	+	−	−	−	−	+	+
107	*B. saperdae*	+	+	+	+	−	+	+	−	−	+	−	+	−	−	−	−	+	−	−	−	−	+	−	+	−	+	+	−	−	+	−	+	+	+	+
108	*B. protophormiae*	+	+	+	+	−	−	+	+	−	−	−	+	−	−	−	−	+	−	−	−	−	−	−	+	−	+	+	−	+	−	−	+	+	+	+
109	*B. vitarumen*	+	−	−	+	−	+	+	+	−	+	+	−	+	+	−	−	+	+	−	−	−	−	−	+	−	−	+	−	+	−	−	−	−	+	+
96	*Cellulomonas flavigena*	−	−	−	+	−	+	+	+	−	−	−	+	−	−	−	−	+	+	+	−	−	+	+	−	−	−	+	−	+	−	−	−	−	−	
110	*C. biazotea*	+	+	−	+	−	+	−	+	+	+	+	+	−	−	+	−	+	−	−	−	−	−	−	+	−	−	−	−	−	−	−	+	+	+	+

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Carboxymethylcellulase Production by *Verticillium albo-atrum*

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SUMMARY

A strain of *Verticillium albo-atrum* produced CM cellulase in liquid culture containing either cellulose powder or carboxymethyl-cellulose (CM cellulose) as sole carbon source, and when the fungus was growing in its natural host (lucerne). The CM cellulase liberated when the fungus was grown in liquid culture was fractionated on Sephadex G75, giving three major peaks and two minor peaks of cellulase activity. The materials of the three major peaks differed from each other in molecular weight, Michaelis constant and pH/activity curve, but were the same whether induced by cellulose powder or CM cellulose. Incubation of the materials of the three major peaks with CM cellulose resulted in the production of only medium-length polymers and cellobiose. No cellobiase was detected in liquid culture. CM cellulase activity was found to be only slight in the living infected lucerne tissue but was considerable in the dead infected tissue.

INTRODUCTION

The components involved in the enzymic breakdown of cellulose (cotton) by *Trichoderma viride* were separated by Selby & Maitland (1967) into a carboxymethylcellulase (CM cellulase), a cellobiase and a component (C_1) which had no action alone but operated synergistically with the other two components to convert cotton completely into water-soluble products. Previous investigations with *Myrothecium verrucaria* (Selby & Maitland, 1965) had shown that this fungus produces three major cellulolytic components, one having 90% of the total CM cellulase activity, the other two being mainly responsible for the activity of the filtrates towards cotton. Recently cellulases have been separated from the culture medium of *Penicillium notatum*. The larger of the two peaks obtained on DEA Sephadex A25 was further resolved into two components by fractionation on Sephadex G-75 (Pettesson, 1968).

The active role of cellulases during the penetration of plant pathogenic fungi through the cellulose walls of host tissues is in doubt. Talboys (1958) clearly demonstrated cellulase activity in cultures of *Verticillium albo-atrum* causing a vascular wilt disease in hops. Enzyme activity in cultures grown on filter-paper pulp led to the production of a reducing sugar, tentatively identified as cellobiose. Starch, sucrose, glucose and lactose were found to inhibit methylcellulase activity in the presence of cellulose, and from the likely presence of these substances in the host cells Talboys assumed that the passage of the very fine penetration hyphae through cell walls was accomplished by mechanical rather than by chemical means. He suggested that the cellulase system of *V. albo-atrum* involved both an initial cellulase (C 1) and a CM

cellulase (Cx), although no attempt at separation was made. Stoddart & Carr (1966) found that the major wilting activity in *V. albo-atrum* filtrates could be ascribed to a high molecular weight fraction of protein and polysaccharide components, and since Selby & Maitland (1967) found that the C1 component in Trichoderma cultures was a glycoprotein, it seems that protein-carbohydrate complexes are of considerable importance in the extra-cellular products of these fungi. In the present paper we report on the results of an investigation into CM cellulase produced by a strain of *V. albo-atrum* which attacks lucerne.

METHODS

Cultures. A strain of *Verticillium albo-atrum* isolated from diseased lucerne (from Norfolk in 1964) was cultured in 16 oz. medical flats containing 100 ml. of medium at 25° in the dark for 20 days. The medium contained either cellulose powder (Whatman no. 1) 5·0 g./l., or carboxymethylcellulose (CM cellulose) (Na^+ salt, D.S. 0·45 to 0·55, British Drug Houses), 5·0 g./l., and the following mineral salts: KCl, 0·5 g./l.; $MgSO_4 7H_2O$, 0·5 g./l.; $FeSO_4 7H_2O$, 0·01 g./l.; in 0·05 M-sodium phosphate buffer (pH 6·0). The fungus was removed by centrifugation at 3000 *g* for 15 min. in a swing bucket centrifuge. The supernatant fluid was reduced to about 5% of its original volume in a rotary evaporator at 45° and the concentrate sterilized by membrane filtration (Oxoid 'standard' grade) before separation and assay procedures.

Enzyme separation. Approximately 25 ml. of sterile concentrate of culture fluid were applied to a column (95 cm. × 2·5 cm.) of Sephadex G-75 (dry sieved particle size < 75 μ) contained in a water jacket at 25°. The column was eluted with a solution containing NaCl 100 mM, Na_2HPO_4 3·3 mM, NaH_2PO_4 6·7 mM (pH 6·5) (Selby & Maitland, 1965), at a rate of 12 ml./hr., and the eluate collected in 5 ml. fractions. The Sephadex column was calibrated by using blue dextran (MW 2,000,000), bovine serum albumin (MW 64,000), peroxidase (MW 45,000), cytochrome c (MW 12,000) and sucrose (MW 342). The blue dextran, bovine serum albumin and cytochrome c were assayed by measuring the extinction at 625, 230 and 412 mμ, respectively, the peroxidase by the hydrogen peroxide/pyrogallol method (Polis & Shmukler, 1953) and the sucrose estimated by total fructose (Bacon & Tell, 1948).

Assay methods. Eluted fractions of the culture medium were assayed viscometrically in an Ostwald type viscometer (BS/1P/U size D) with 1 ml. samples and 25 ml. 1% carboxymethylcellulose (CM cellulose) (Na^+ salt, D.S. 0·45 to 0·55, B.D.H.; prepared by mechanical stirring at room temperature) in 6 mM-acetate buffer (pH 5·0). Reaction mixtures were incubated at 25° for 30 min.

Michaelis constants of the materials of the three main enzyme peaks, separated by the above method from cultures grown in media containing either cellulose powder or CM cellulose as the sole carbon source, were calculated from results of viscometric assays. The disappearance of substrate is expressed in CM cellulose equivalents, i.e. the concentration of CM cellulose that would have the same viscosity. The molecular weight of the CM cellulose used for Km determinations was calculated from osmotic pressure measurements with a high speed membrane osmometer (Mechrolab model 502) at 25° with concentrations ranging from 0·01 to 0·07%.

The effect of pH value on the activity of the three main enzyme components was investigated viscometrically by using 2 ml. of 0·2 M-citrate phosphate buffer, 0·3 ml.

enzyme solution and 25 ml. 1% CM cellulose. Results are expressed as a percentage of the maximum activity.

The long-term breakdown of CM cellulose by the three main components separated from cultures grown in a medium containing cellulose powder was followed viscometrically over a period of 120 hr (chloroform was added to maintain sterility). At the end of these incubations 5 ml. of each mixture was made up to 70% (v/v) ethanol in water (to precipitate long-chain polymers) and then de-salted on a bio-deminrolit column (12 cm. × 2 cm.) (B.D.H. 1965). The eluate was concentrated under infra-red lamps before paper chromatography. Chromatograms (Whatman no. 1 paper) were run for 20 to 24 hr with solvent *n*-propanol + ethyl acetate + water (13 + 2 + 5, by vol.). Carbohydrates were detected by spraying with trichloracetic acid + benzidine (2 g. trichloracetic acid + 0·24 g. benzidine + 5 ml. water made up to 100 ml. with ethanol (Bacon & Edelman, 1951).

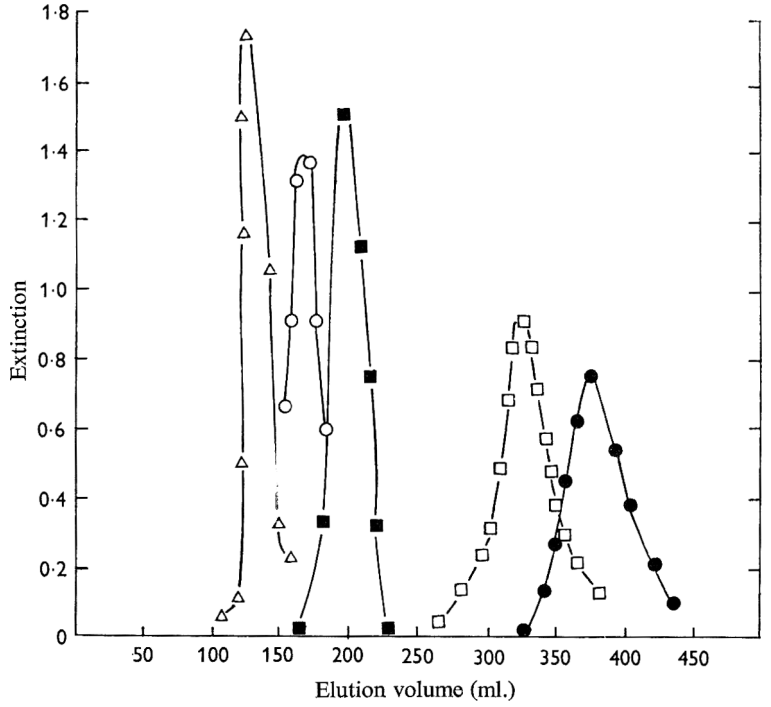

Fig. 1. Elution pattern of standards from Sephadex G-75 column (showing volume of buffer required to elute standards of known molecular weight). △, Blue dextran (M.W. 2×10^6); ○, serum albumin (M.W. $6{\cdot}4 \times 10^4$); ■ peroxidase (M.W. $4{\cdot}5 \times 10^4$); □, cytochrome C (M.W. $1{\cdot}2 \times 10^4$); ●, sucrose (M.W. 342).

Cellobiase activity was estimated (Nelson, 1944; Somogyi, 1952) by measuring the increase in reducing sugar after incubation of cellobiose solution with both the culture filtrate and the three separated CM cellulose peak components.

To investigate the production of CM cellulase in the host, tissue from various parts of healthy and diseased lucerne plants (Du Puit strain) was ground in 0·1 M-phosphate buffer (pH 5·0; 0·5 g. tissue/ml. buffer). The slurry was centrifuged at 3000*g* for 10 min. and 1 ml. of the supernatant fluid assayed viscometrically for CM cellulase activity. The assay was done twice with different samples of lucerne material.

RESULTS

In the Sephadex column calibration (Fig. 1) the blue dextran indicates the void volume since it is totally excluded from the gel because of its high molecular weight (2×10^6). The sucrose was below the lower limit of separation obtainable with G-75, but the other three substances were clearly resolved. The molecular weight estimations based on the physical characteristics of the column (Whitaker, 1963) showed good correlation with the known weights of the standards (Fig. 2).

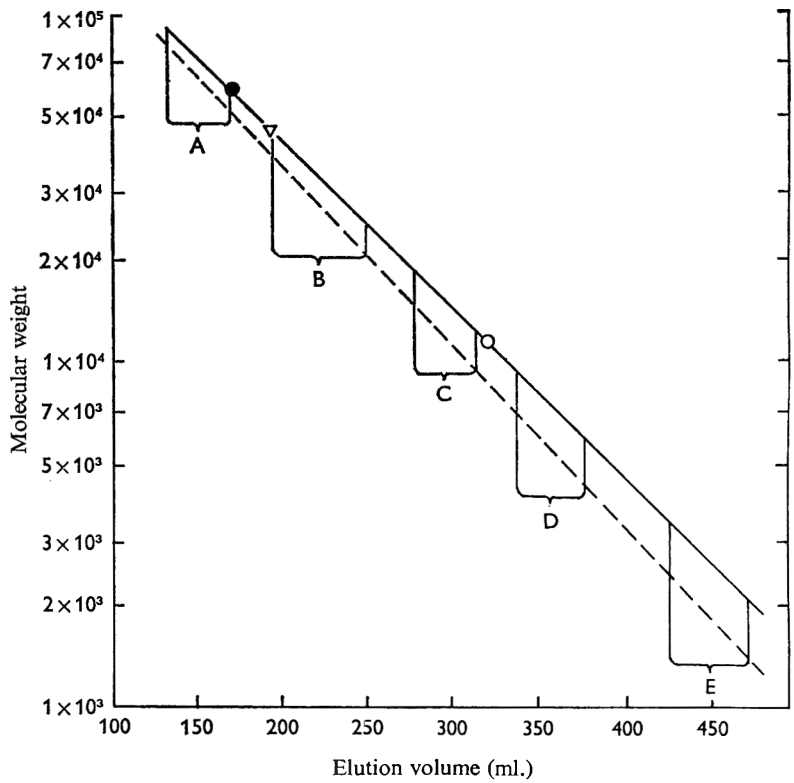

Fig. 2. Estimation of molecular weight by calibration of G-75 column. —, From standards; – – –, theoretical; ●, serum albumin; ▽, peroxidase; ○, cytochrome C.

The separation of CM cellulases from cultures grown in CM cellulose or cellulose powder medium resulted in three main peaks designated A, B and C and two smaller peaks D and E (Fig. 3). The approximate molecular weights of these components estimated from the standard curve (Fig. 2) are A 75,000, B 32,000, C 16,000, D 6500 and E 2200.

K_m determinations of components A, B and C gave values of $1{\cdot}33 \times 10^{-5}$, $2{\cdot}86 \times 10^{-5}$ and $3{\cdot}33 \times 10^{-5}$ at pH 5·0, respectively, i.e. A had the highest affinity and C the lowest affinity for its substrate (Fig. 4*a*, *b*, *c*). High concentrations of substrate inhibited the reaction. The molecular weight of CM cellulose used for these determinations was calculated as 2500 (Fig. 5). These three components all showed activity

over a wide pH range (Fig. 6*a*, *b*, *c*); A and B had two maxima, A at pH 4·5 and 7·5 and B at pH 5·0 and above 8·0, whereas C had a single maximum at pH 8·0. There was no difference in the viscosity of the CM cellulose controls at any pH value used, either initially or after incubation.

Incubation of A, B and C materials (derived from cultures grown in cellulose medium) separately with CM cellulose over an extended period showed that the viscosity of all the mixtures was tending towards the same value (Fig. 7*a*). Component B caused the most rapid breakdown of substrate (Fig. 7*b*). Since its K_m was intermediate between that of components A and C this indicated a greater proportion of the B component in these cultures. At the end of this incubation, paper chromatograms

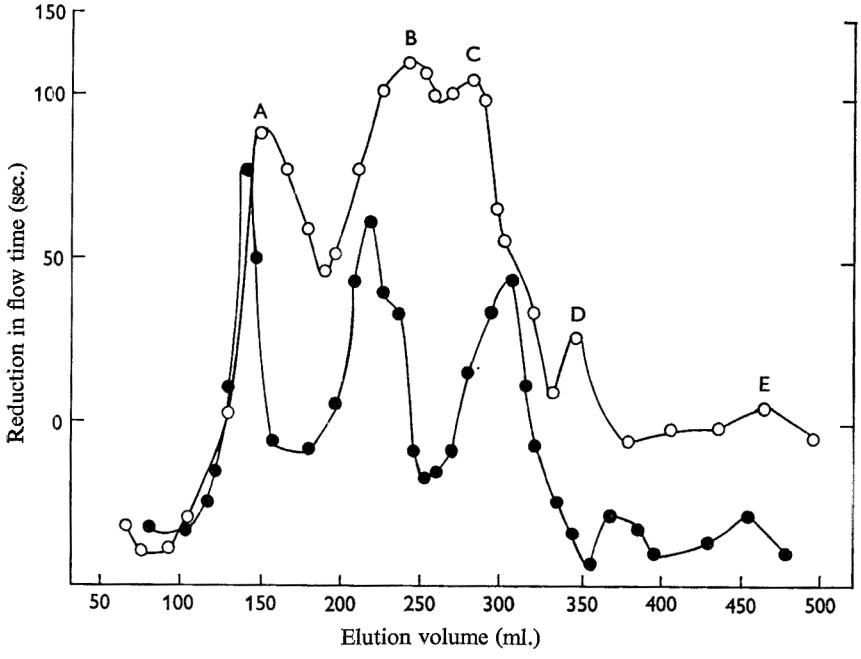

Fig. 3. Elution pattern of CM cellulases from culture filtrates. ●, Cultures grown on cellulose powder; ○, cultures grown on CM cellulose.

of the mixtures were run. These showed only the presence of cellobiose (authenticated by comparison with standard) and medium-length polymers (still at the origin). The ratio of cellobiose to medium-length polymers was much higher in the mixtures resulting from incubation with C than in those with either A or B. There was no indication of short-chain polymers other than cellobiose. None of the three main peak components showed any cellobiase activity. The only glucose detected was a contaminant of the CM cellulose; this was shown by the chromatographic detection of glucose in CM cellulose in the same small quantities before enzymic hydrolysis as were found after reaction with the enzyme components.

There was a greatly increased (approximately 20-fold) degree of CM cellulase activity in the dead infected lucerne stem as compared with the healthy stem (Table 1). The living tissue of infected plants had only a slightly (approximately three-fold)

increased CM cellulase activity as compared with those of healthy plants. The dead root material was at this time extensively invaded by numerous saprophytic soil organisms and so a cellulase assay was not attempted.

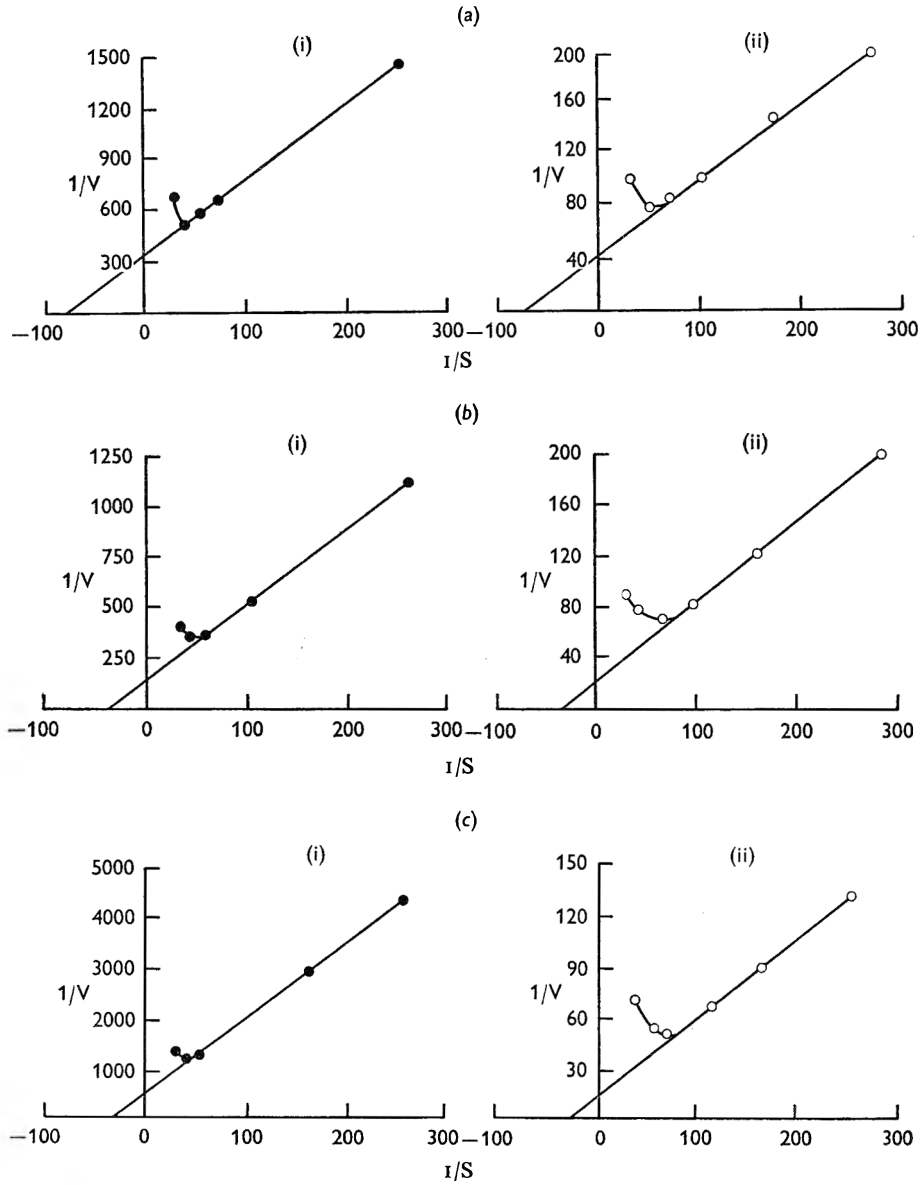

Fig. 4. Lineweaver–Burk reciprocal plots for K_m determinations of enzyme peaks A (*a*), B (*b*) and C (*c*).

$$\frac{I}{S} = \frac{I}{\text{Substrate concentration (mM)}},$$

$$\frac{I}{V} = \frac{I}{\text{initial reaction velocity (decrease in CM cellulose equivs (g./100 ml./sec.))}}.$$

(i) Cultures grown on cellulose powder; (ii) cultures grown on CM cellulose.

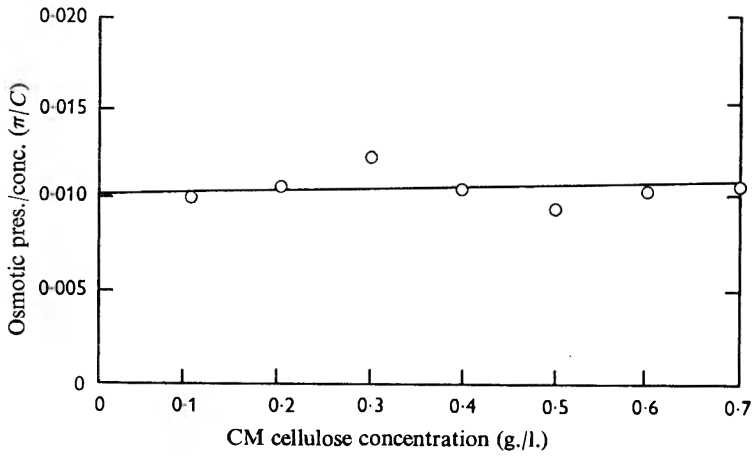

Fig. 5. Calculation of molecular weight of CM cellulose from osmotic pressure measurements. When the concentration tends to zero, $\pi/C = 0{\cdot}01025$.

$$\text{Average molecular weight} = \frac{RT}{\pi/C} = \frac{0{\cdot}082 \times 307}{0{\cdot}01025} = 2456.$$

Table 1. *Production of CM cellulase by Verticillium albo-atrum in lucerne tissue*

	Decrease in CM cellulose equivalents. Assayed viscometrically
Living stem	
Uninfected	0·010
Lightly infected young stem	0·030
Heavily infected old stem	0·025
Dead stem	
Uninfected	0·000
Infected	0·230
Living root	
Uninfected	0·010
Heavily infected old root	0·030

DISCUSSION

These experiments indicate that several distinct CM cellulases are produced extracellularly by *Verticillium albo-atrum*. The data also show that the components possess the same characteristics (molecular weight, Michaelis constant, activity/pH curve) whether induced by CM cellulose or cellulose powder as sole carbon source in the medium. The K_m determinations were not ideal since the calculations assumed the breakdown of a highly viscous substrate to a product of viscosity similar to water though it is obvious that the medium-length polymers would have appreciable viscosity. However, since all the incubation mixtures progressed towards a similar viscosity, a valid comparison could be made between them and with other K_m determinations similarly calculated (Mandels & Reese, 1963). Although inhibition at high substrate concentrations is commonly encountered in hydrolytic enzyme systems it is doubtful whether a similar restricting mechanism was operating here, since we used a relatively low maximum substrate concentration. Any such

inhibition might have been due to an increase in the viscosity of the reaction mixture decreasing molecular movement and hence availability of substrate to enzyme. The double pH optima obtained with components A and B were unexpected, since accord-

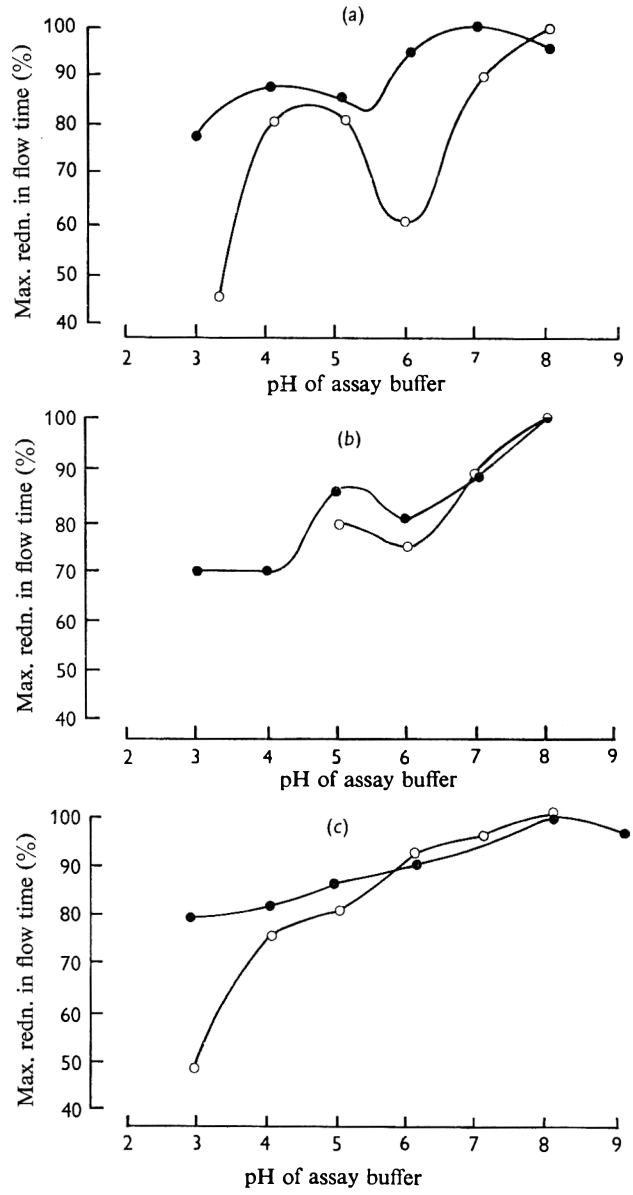

Fig. 6. pH/activity range of CM cellulase peaks A (*a*), B (*b*) and C (*c*). ●, Culture grown on cellulose powder; ○, culture grown on CM cellulose.

ing to Mandels & Reese (1965) most cellulases have a single optimum between pH 4·5 and 5·5. The double optima may indicate that the enzyme enters two active states, or alternatively that the presence of two enzymes of similar molecular weight which we had failed to separate.

Of the five enzyme components separated, only B (MW 32,000) and D (MW 6500) can be correlated with any of those found by Selby & Maitland (1965) in *Myrothecium verrucaria* cultures. The high molecular weights of components C, B and particularly of A suggest that their action is largely restricted to exposed surfaces and loose chain ends, since molecules of this size would have difficulty in penetrating naturally occurring cellulose in the plant cell wall. Because of the difficulties inherent in investigating the initial steps of cellulose degradation no attempt was made to

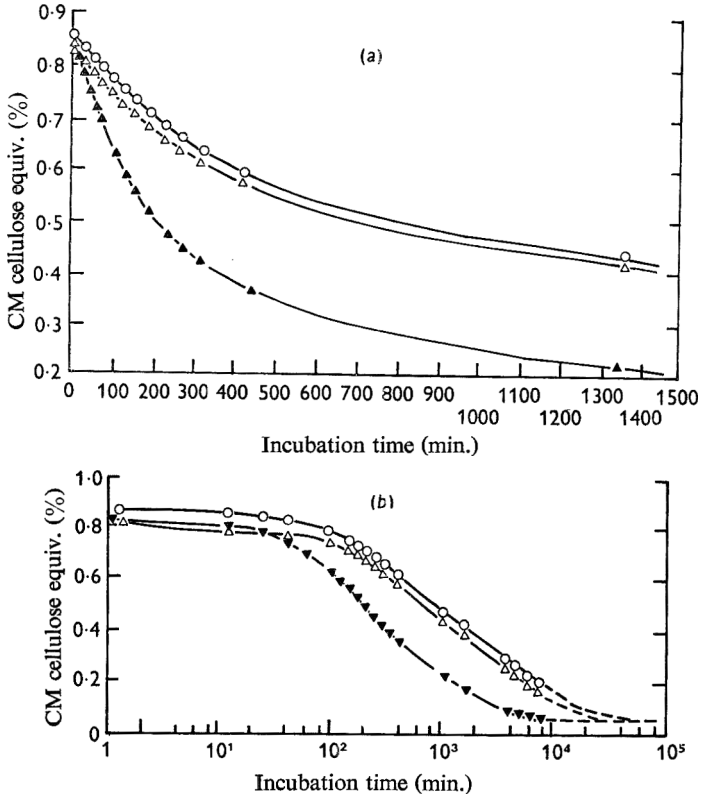

Fig. 7. Viscosity of CM cellulose plotted against time during incubation with enzyme peaks A, B and C.

(*a*) On a linear scale for about 1 day, ○, Enzyme peak A; ▲, enzyme peak B; △, enzyme peak C.

(*b*) On a logarithmic scale for about 7 days.

establish whether a C1 component was produced by *Verticillium albo-atrum*. However, Selby & Maitland (1967) showed that CM cellulases have no detectable effect alone on natural forms of cellulose so the fact that *V. albo-atrum* can utilize these as sole carbon source is circumstantial evidence for the presence of a C1 component. No cellobiase activity was associated with any of the peak components and cellobiose was identified in the CM cellulose incubation mixtures and in harvested culture fluids containing cellulose powder as sole carbon source. This strongly suggests that cellobiose is the end product of cellulose breakdown by *V. albo-atrum* and is presumably taken up in this form by the hyphae.

The simplest hypothesis to explain the different ratio of CM cellulose products shown in the three main peak components is that component C removes a cellobiose unit from the chain when it cleaves β1–4 linkages, whereas components A and B hydrolyse the linkage without removing a cellobiose unit (unless it is at the end of a chain). The degree of substitution controls the hydrolysis of cellulose by cellulases, the enzyme not attacking substituted glucose units (Mandels & Reese, 1965). In the present case the CM cellulose was substituted at 0·45 to 0·55 carboxymethyl groups per glucose unit (i.e. an average of one substituent group per two glucose units). It is likely that in some parts of the chain there would be two adjacent substituted glucose units and in other parts one substituent group per three or four glucose units. This would explain why all the CM cellulose was not hydrolysed to cellobiose and yet some cellobiose was produced.

The high values for CM cellulase in the dead infected stem, compared to living infected stem and healthy stem, indicate that the enzyme was only produced in large quantities after the death of the host tissue. This almost complete repression throughout the parasitic phase can be explained by an inhibition of enzyme production by sugars such as glucose and sucrose, as shown in *in vitro* studies by Talboys (1958) and by D. P. Gupta (personal communication). We cannot at this stage rule out the possibility that CM cellulases might be produced in minute amounts by fine penetration hyphae before and during passage through the host cell walls, although we have not yet successfully designed a technique to detect this. The activity detected in dead and dying tissue is of obvious importance to the pathogen. It enables an otherwise poorly competitive saprophyte (Heale & Isaac, 1963) to utilize at least part of the cellulose fraction of the host (after the more easily assimilable sugars are exhausted) before general saprophytic invasion occurs. The darkly pigmented resting mycelium characteristic of *Verticillium albo-atrum* is formed during this latter stage of the disease and is of some importance in the survival of the fungus in the soil.

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Polarity of Flagellar Growth in Salmonella

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SUMMARY

The phenomenon whereby Salmonella cells produce curly flagella in media containing *p*-fluorophenylalanine was used to investigate the polarity of flagellar growth. *Salmonella typhimurium* LT2 in logarithmic growth in broth was transferred to minimal medium or saline, or their flagella shortened by mechanical breaking. Then, after 2 to 3 hr at 37° in a medium containing *p*-fluorophenylalanine the distributions of number, length and shape of their flagella were observed. Curly waves appeared at the distal portions of flagella. The growth rate of a flagellum decreased as its length increased, reaching zero at approximately five normal wave-numbers (about 15 μ). The growth rate of flagella shortened by mechanical breaking was not less than that of intact ones of similar length. The decline is attributed to decrease in transport efficiency with increase in length rather than to ageing of the flagellum-forming apparatus.

A normal flagellar strain and a curly mutant strain of *Salmonella abortusequi* were grown together in broth. Numbers of both types of rods and flagella increased to 1·5-fold in 3 hr. At this stage, neither heteromorphous rods nor single flagella having both normal and curly waves were detected. Hence, flagellin molecules reach the top of a growing flagellum without being excreted into the culture medium.

INTRODUCTION

Reconstitution experiments *in vitro* with Salmonella flagella have shown that the flagellar filaments have structural polarity and that they grow by a sequential orderly polymerization of flagellin molecules at a structurally defined end, called the T-end, of each flagellar filament (Asakura, Eguchi & Iino, 1968). The T-end corresponds to the distal end of a flagellum attached to the cell. Therefore, if flagellar growth *in vivo* is homologous with that *in vitro*, it must take place at the tip. The present experiments were undertaken to investigate whether or not this is the case. In order to identify newly formed parts of flagella, the phenomenon that the normal Salmonella rods produce curly flagella in the media containing *p*-fluorophenylalanine (Kerridge, 1959*a*) was used. Data on the growth rate of flagellar filaments *in vivo* are reported.

METHODS

Salmonella strains LT2, SJ30 and SJ25 were used. They carry peritrichous flagella in wide range of cultural conditions (Kerridge, 1959*b*; Quadling & Stocker, 1962). Strain LT2 is the diphasic ancestor of *Salmonella typhimurium* SW1061 in which Kerridge (1959*a*, 1960) reported the production of curly flagella in media containing *p*-fluorophenylalanine (FPA) and normal flagella without FPA. SJ30 is a curly

flagellar mutant of *S. abortusequi* (Iino, 1962). SJ25 is a strain in which the chromosomal region carrying flagellin-structure gene *H2-enx* of SJ30 was replaced by the homologous region of LT2 by P22-phage-mediated transduction. SJ30 thus differs from SJ25 only in flagellar characters: the former produces curly flagella of *enx*-antigen type while the latter normal flagella of *1.2*-antigen type.

Minimal medium (MM) consisted of (%, w/v): K_2HPO_4, 0·7; KH_2PO_4, 0·2; sodium citrate. $5H_2O$, 0·05; $MgSO_4.7H_2O$, 0·01; and glucose 0·2 in distilled water. FPA treatment was carried out in MM supplemented with a complete amino acid mixture (0·1 mg of each L-amino acid/ml.) in which phenylalanine was replaced by FPA 1 mg./ml. This medium was termed RM(FPA).

Mechanical separation of flagella from bacterial bodies was performed as follows: $4{\cdot}8 \times 10^9$ of bacteria in 2·5 ml. saline (NaCl, 0·85%, w/v) in a screw-capped glass tube (diameter 10 mm., length 30 mm.) were shaken for 90 min. (750 strokes/min. with 25 mm. amplitude) with fine glass beads. After centrifuging at 1200 *g* for 15 min. the pellet was suspended in an appropriate medium for further experiment.

Bacterial numbers in liquid media were counted in an Elma bacterial counting chamber. Staining and counting of flagella were by the method of Leifson (1951). Wave numbers were recorded by counting decimal fractions as units. Observations were made on 400 bacteria in each experiment.

RESULTS

Formation of curly and heteromorphous flagella by FPA treatment. The cells of *Salmonella typhimurium* strain LT2 in logarithmic growth in 10 ml. broth were centrifuged and suspended in 5 ml. of MM, incubated 1 hr at 37°, centrifuged again

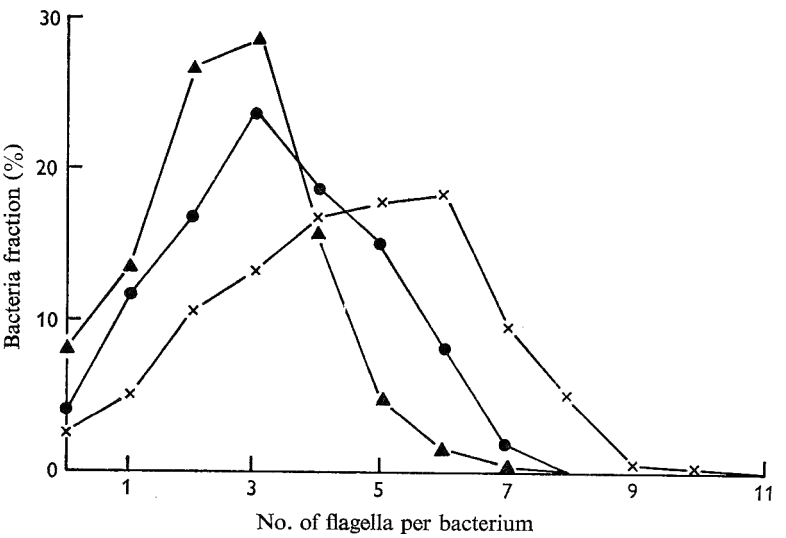

Fig. 1. Changes in the distribution of flagellar number per bacterium during the growth of *Salmonella typhimurium* LT2 in RM (FPA): ×, 0 hr; ●, 2 hr; ▲, 3 hr.

and suspended in 2 ml. of RM(FPA). The bacteria ($2{\cdot}5 \times 10^8$/ml.) were incubated at 37° without aeration. At 0, 2 and 3 hr of incubation, 0·2 ml samples were fixed immediately by 1% (v/v) formalin and the flagella stained.

During incubation in RM(FPA), the bacteria increased to $4{\cdot}1 \times 10^8$ and $6{\cdot}0 \times 10^8$/ml. at 2 and 3 hr, respectively, while the flagella decreased from 4·4/cell at 0 hr to 3·3 and 2·5/cell at 2 and 3 hr respectively. Therefore, flagellar numbers were calculated to have

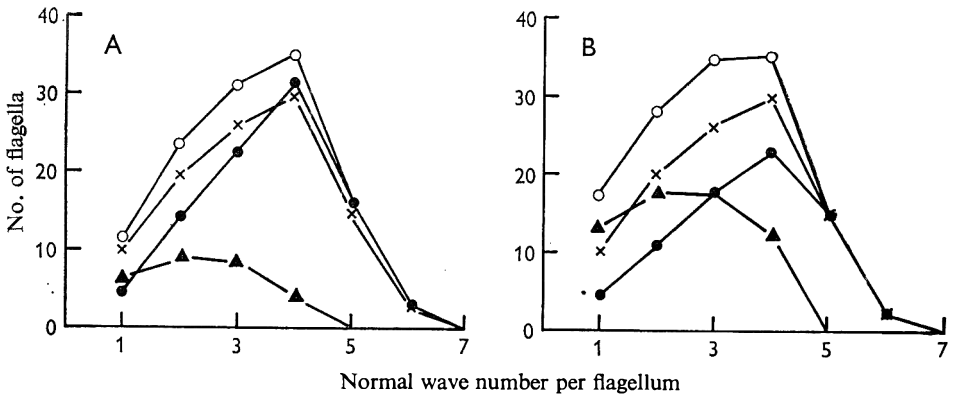

Fig. 2. Distribution of normal wave numbers among flagella of *Salmonella typhimurium* LT2 grown in RM (FPA): ×, normal flagella at 0 hr; ●, normal flagella; ▲, heteromorphous flagella; ○, normal plus heteromorphous flagella; A, at 2 hr, B, at 3 hr. The total number of flagella at 0 hr was taken as 100. The numbers at 2 and 3 hr were adjusted by multiplying the percentage of each fraction by the net increase of flagellar number during incubation; thus, the total numbers of flagella at 2 and 3 hr were taken as 123 and 136 respectively (see Table 1).

Table 1. *Flagellar shape of Salmonella typhimurium LT2 grown in RM(FPA)*

(*a*) Numbers of flagella counted on 400 bacteria; (*b*) percentage of flagella of different shapes at each incubation time; (*c*) numbers of flagella adjusted by multiplying the percentage of each fraction by the net increase of flagellar number during incubation: that is, (*b*) × 1·23 for 2 hr values and × 1·36 for 3 hr.

		Time (hr) incubated in RM (FPA)		
Flagella shape		0	2	3
Normal	(*a*)	1772	935	507
	(*b*)	100	71·2	50·1
	(*c*)	100	87·6	68·1
Heteromorphous	(*a*)	0	321	433
	(*b*)	0	24·5	42·8
	(*c*)	0	30·1	58·2
Curly	(*a*)	0	57	72
	(*b*)	0	4·3	7·1
	(*c*)	0	5·3	9·7
Total	(*a*)	1772	1313	1012
	(*b*)	100	100	100
	(*c*)	100	123	136

increased 1·23-fold at 2 hr and 1·36-fold at 3 hr, and the fractions of the newly formed flagella among them were therefore 18·7% at 2 hr and 26·5% at 3 hr. Changes in distribution of flagellar numbers per bacterium are shown in Fig. 1. The rate of flagellar formation *de novo* in RM(FPA) was considerably lower than the rate of cell multiplication.

After FPA treatment, both curly flagella and heteromorphous ones, having both

normal and curly waves on the same flagellum, appeared together with normal flagella. Heteromorphous flagella were normal at the region nearest the cell and curly at the distal end. Changes in the numbers of normal, curly and heteromorphous flagella during incubation in RM(FPA) are shown in Table 1, and the distributions of normal wave numbers among both normal and heteromorphous flagella are shown in Fig. 2; the number of wholly normal flagella with 1 to 4 waves decreased with passage of time while those of both curly and heteromorphous flagella increased.

Table 2. *Distribution of flagellar shape among Salmonella typhimurium LT2 bacteria grown in RM(FPA)*

Numbers outside parentheses indicate the bacterial number at 2 hr, and inside the parentheses at 3 hr.

		Flagellar number per cell						
		Total (curly, heteromorphous and normal)						
Curly	Hetero-morphous	2	3	4	5	6	7	Total cell number
0	0	23 (19)	36 (13)	23 (6)	19 (0)	9 (0)	2 (1)	112 (39)
	1	26 (52)	27 (38)	24 (17)	13 (5)	9 (0)	1 (0)	100 (112)
	2	9 (20)	11 (28)	9 (14)	13 (7)	8 (1)	1 (0)	51 (70)
	3	—	1 (18)	4 (10)	7 (4)	1 (1)	1 (0)	14 (33)
1	0	4 (8)	5 (4)	4 (7)	2 (0)	1 (0)	0 (0)	16 (19)
	1	4 (6)	13 (4)	8 (3)	3 (0)	2 (1)	1 (0)	31 (14)
	2	—	1 (7)	1 (4)	4 (3)	0 (1)	0 (0)	6 (15)
	3	—	—	0 (0)	0 (0)	0 (2)	0 (0)	0 (2)
	4	—	—	—	0 (0)	0 (0)	1 (0)	1 (0)
2	0	(2)	1 (1)	0 (0)	0 (0)	0 (0)	0 (0)	1 (3)
	1	—	0 (0)	1 (1)	0 (1)	1 (0)	1 (0)	3 (2)
3	1	—	—	0 (0)	0 (1)	c (0)	0 (0)	0 (1)
	Total	66 (107)	95 (113)	74 (62)	61 (21)	31 (6)	8 (1)	335 (310)

As the curly waves were presumed to be newly formed during incubation in RM (FPA), pure curly flagella were formed *de novo* in RM(FPA). The incidence of wholly curly flagella (4·3% at 2 hr and 7·1% at 3 hr) was, however, less than that of the newly-formed fractions (18·7% at 2 hr and 26·5% at 3 hr). Therefore, the remaining fractions of newly-formed flagella must be either normal or heteromorphous. Even though these fractions are all heteromorphous, 41·2% (= $100 \times [24{\cdot}5-(18{\cdot}7-4{\cdot}3)]/24{\cdot}5$) of heteromorphous flagella at 2 hr and 54·7% (= $100 \times [42{\cdot}8-(26{\cdot}5-7{\cdot}1)]/42{\cdot}8$) at 3 hr are estimated to have changed from normal flagella present at 0 hr to heteromorphous ones.

Distribution of curly and heteromorphous flagella among the bacteria grown in RM(FPA). Distributions of the numbers of bacteria with numbers of normal, curly and heteromorphous flagella were scored in the 2 and 3 hr samples. As shown in Table 2, curly and heteromorphous flagella were evenly distributed among the cells with different number of flagella. This may mean that every cell in RM(FPA) had an equal potentiality to produce curly waves.

Distribution of curly wave among heteromorphous flagella. The percentage of flagella with different numbers of curly waves was caluculated for each group having different numbers of normal waves (Fig. 3). All distributions fitted a Poisson distribution. The average numbers of curly waves per flagellum decreased with increase in normal

wave number and reached zero for five to six normal waves (Fig. 4); the lines at 2 and 3 hr were not paralled but convergent. The observed distribution of the pure curly fraction (Fig. 3 A), compared with the theoretical one, indicated that a fraction of potentially active flagellum-forming apparatus did not produce flagella at all. If this

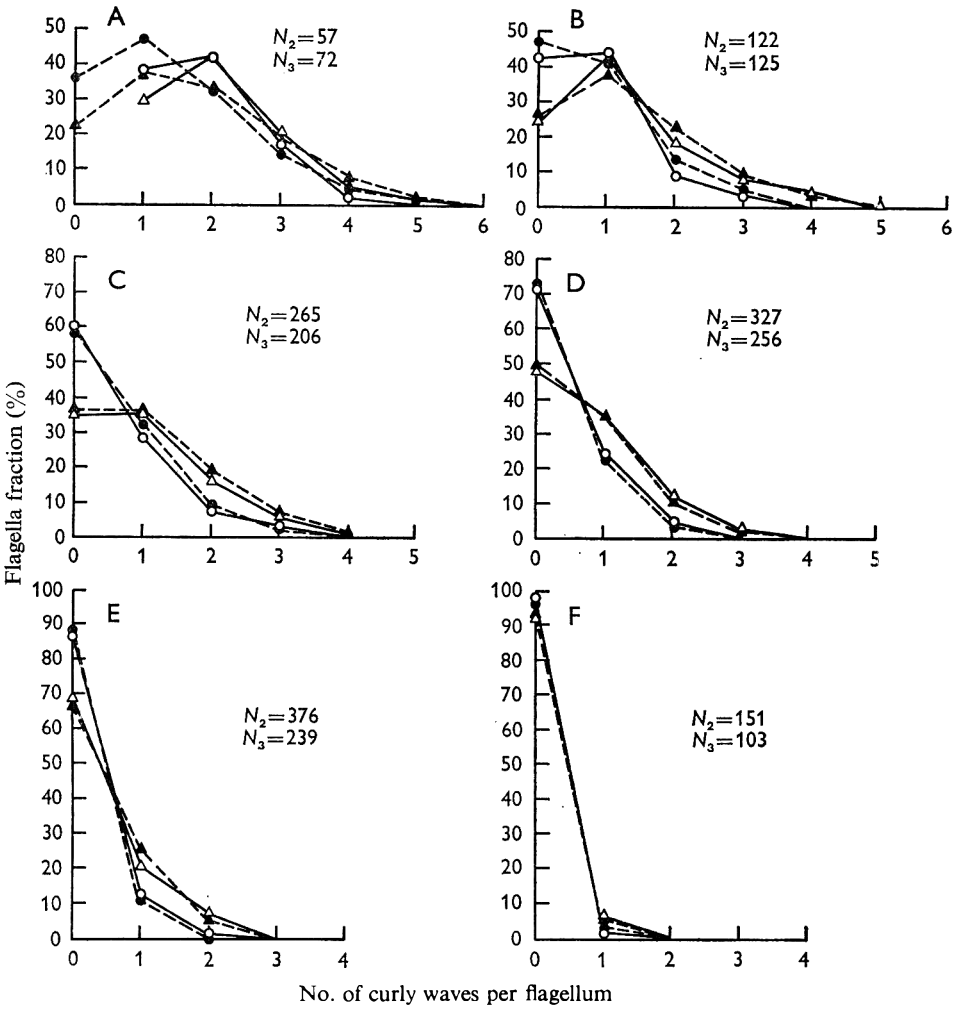

Fig. 3. Distribution of curly wave numbers in flagella with different normal wave number in *Salmonella typhimurium* LT2 grown in RM (FPA): A, pure curly flagella; B, flagella with one normal wave; C, with two normal waves; D, with three normal waves; E, with five normal waves; F, with six normal waves. ○, observed value at 2 hr; △, at 3 hr; ●, Poisson distribution with the mean observed at 2 hr; ▲, at 3 hr. N_2, observed flagellar number at 2 hr; N_3, at 3 hr.

null fraction is included in calculating the mean values, the percentages of pure curly flagella become smaller than those plotted in Fig. 3. A corrected mean (m) was calculated from the mean (n) obtained from the observed wave numbers in pure curly flagella by the following equation: $n = e^{-m}/(1-e^{-m})$ where 2^{-m} represents the null fraction. The m values thus corrected were plotted in Fig. 4 by broken lines.

Formation of curly and heteromorphous flagella in RM(FPA) by bacteria pre-incubated in saline. The foregoing experiment showed that more curly waves were formed in RM(FPA), the smaller the number of normal waves. However, we cannot decide whether the larger curly wave number, observed on the heteromorphous flagella, represents more growth or a tendency to grow curly waves at the tips of shorter flagella, because normal as well as curly waves increased considerably in RM(FPA) and it was impossible to differentiate normal waves newly grown in RM(FPA) from those present before treatment. To decrease the production of normal waves in RM(FPA) as much as possible, several different procedures were tried, and it was found that preincubation of bacteria in saline fulfilled the requirement.

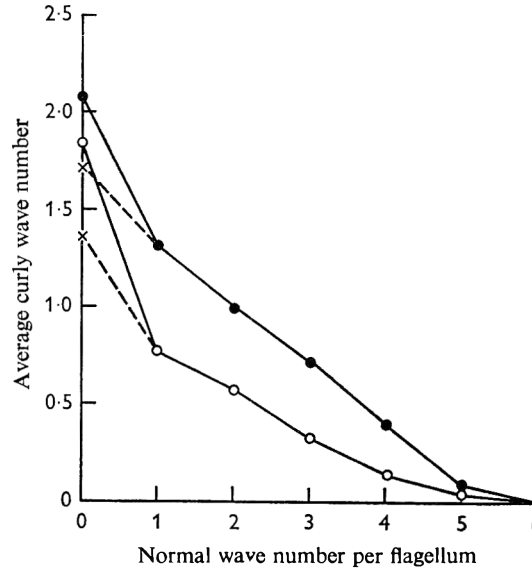

Fig. 4. Average number of curly waves in flagella with different number of normal waves in *Salmonella typhimurium* LT2 grown in RM (FPA). ●, At 2 hr; ○, at 3 hr; – –, corrected values counting the null fraction estimated from the Poisson distribution.

Bacteria of strain LT2 in late logarithmic growth were centrifuged and suspended in 5 ml. saline at $1{\cdot}9 \times 10^9$/ml. 2·5 ml. of the suspension was used for the experiment reported in the next section. The remaining 2·5 ml. was kept for 90 min. at 23°, when the bacterial concentration increased to $2{\cdot}4 \times 10^9$ cells/ml. The number of viable bacteria measured on serial dilution plates was not significantly different from the total number. The suspension was then centrifuged and the pellet of bacteria suspended in 2·5 ml. of RM(FPA), diluted tenfold, and incubated 3 hr at 37°. The flagellar shape and the distribution of flagellar number were examined before and after incubation.

During incubation, the concentration increased from $1{\cdot}8 \times 10^8$ to $2{\cdot}7 \times 10^8$ bacteria/ml. Distributions of flagellar numbers at 0 and 3 hr are shown in Fig. 5 and frequencies of normal, heteromorphous, and curly flagella are listed in Table 3. As in the earlier experiment, heteromorphous flagella were all curly distally and normal at the proximal end. The number of normal flagella at 0 hr was approximately the same as the summed number of normal and heteromorphous flagella at 3 hr. The distribution of normal

wave number among normal and heteromorphous flagella and the fraction of wholly normal flagella among them are shown in Fig. 6. The distribution of normal waves was almost identical between 0 and 3 hr. Thus, both flagella newly formed in RM(FPA) and flagellar parts extended in RM(FPA) were almost all curly and the curly wave number may be regarded as the measure of the growth of flagella during incubation. The number of curly waves in each group of flagella with the same normal waves per flagellum with different normal wave numbers are shown in Fig. 8. As in the earlier

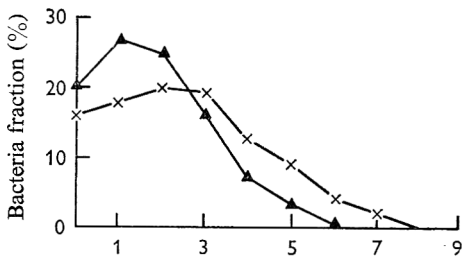

Fig. 5

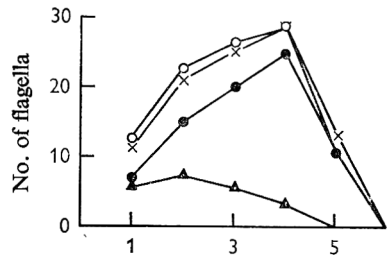

Fig. 6

Fig. 5. Changes in the distribution of flagellar number per bacterium during the growth of *Salmonella typhimurium* LT2 in RM (FPA) after preincubation in saline for 90 min.: ×, at 0 hr; ▲, at 3 hr.

Fig. 6. Distribution of the numbers of normal waves among flagella of *Salmonella typhimurium* LT2 grown in RM (FPA) after preincubation in saline for 90 min.: ×, normal flagella at 0 hr; ●, normal flagella at 3 hr; ▲, heteromorphous flagella at 3 hr; ○, normal plus heteromorphous flagella at 3 hr. The total number of flagella at 0 hr was taken as 100. The number of flagella at 3 hr was adjusted by multiplying the percentage of each fraction by the net increase of flagellar number during incubation; thus, the total number at 3 hr was taken as 106 (see Table 3).

Table 3. *Flagellar shape of Salmonella typhimurium LT2 grown in RM(FPA) after pre-incubation in saline*

(*a*) Numbers of flagella counted on 400 bacteria; (*b*) percentage of flagella of different shape in each incubation time; (*c*) numbers of flagella adjusted by multiplying the percentage of each fraction by the net increase of flagella during the incubation: that is, (*b*) × 1·06 for 3 hr.

		Time (hr) incubated in RM (FPA)	
Flagellar shape		0	3
Normal	(*a*)	992	510
	(*b*)	100	72·5
	(*c*)	100	76·9
Heteromorphous	(*a*)	0	147
	(*b*)	0	20·9
	(*c*)	0	22·2
Curly	(*a*)	0	46
	(*b*)	0	6·5
	(*c*)	0	6·9
Total	(*a*)	992	703
	(*b*)	100	100
	(*c*)	100	106

experiment, the newly-grown curly portion was shorter in flagella with longer normal portions and become zero at around five normal waves.

The difference in growth among different lengths of flagella may occur for three reasons: (1) growth in RM(FPA) initiates later in longer flagella, (2) growth stops earlier in longer flagella in RM(FPA) because they reach their maximal length sooner; (3) growth rate declines with increase in length. If the first possibility held, flagellar growth after initiation would be the same, regardless the length of previously formed flagellum, and the lines at 2 and 3 hr in Fig. 4 would be parallel. The convergence in Fig. 4 therefore excludes this possibility. The second possibility is also less plausible because, in both average and maximal length, the whole length (normal plus curly) of a flagellum is shorter in flagella with smaller number of normal waves (Table 4); that is, neither heteromorphous nor curly flagella had reached their maximal length during the 3 hr incubation in RM(FPA). The remaining possibility, (3), fits the available data and may be accepted as the most reasonable.

Table 4. *Average and maximal length of flagella with different numbers of normal waves in Salmonella typhimurium LT2 grown 3 hr in RM(FPA) after preincubation in saline*

		Normal wave number					
		0	1	2	3	4	5
Average	Curly wave number	1·38	0·73	0·44	0·25	0·08	0·00
	Flagellar length (μ)*	2·6	4·4	6·8	9·5	12·2	15·0
Maximal	Curly wave number	4	3	3	2	1	0
	Flagellar length (μ)*	7·6	8·7	11·7	12·8	13·9	15·0

* Normal wave number × normal spiral unit length (3·0 μ) + curly wave number × curly spiral unit length (1·9 μ)

Growth of flagella shortened by mechanical breakage. The decline in growth rate of flagella as their length increased, may occur (1) because the flagellum-forming apparatus at the base of each flagellum 'ages', or (2) because the growth rate depends on the distance between the flagellin factory, probably at the base of each flagellum, and the site of flagellin polymerization at the tip of each flagellum.

Growth of flagella which had been shortened by mechanical breaking was examined in parallel with the earlier experiments. If hypothesis (1) is correct, mechanically shortened flagella should grow more slowly than intact flagella of the same length; whereas, on hypothesis (2) both flagella should grow at approximately the same speed unless the mechanical shock is unfavourable to the polymerization of flagellin.

Bacterial suspension in saline (2·5 ml.), left aside in the earlier experiment, was deflagellated at 23°. The bacterial concentration increased during shaking from $1{\cdot}9 \times 10^9$ to $2{\cdot}5 \times 10^9$ bacteria/ml.: values not significantly different from those of unshaken bacteria. The bacteria were suspended in RM(FPA), diluted tenfold, and the bacterial concentration rose from $1{\cdot}8 \times 10^8$ to $2{\cdot}4 \times 10^8$ bacteria/ml. in 3 hr. The total number of flagella per 400 bacteria at 0 hr in RM(FPA) was 335; i.e. 66·2% of the 992 flagella in the unshaken control were removed by shaking. Comparison of normal wave numbers at 0 hr in the previous and the present experiments (Fig. 3, 9) indicates that flagella with more than three waves were almost all broken by shaking and converted to zero to two wave flagella.

After 3 hr, heteromorphous and curly flagella appeared; heteromorphous flagella were curly at the distal portions. The normal wave distributions (Fig. 10) were not significantly different at 0 and 3 hr, indicating that flagella formed in RM(FPA) were almost all curly.

The distribution of curly waves on flagella with different numbers of normal waves followed a Poisson distribution (Fig. 7). Among short flagella in shaken bacteria both

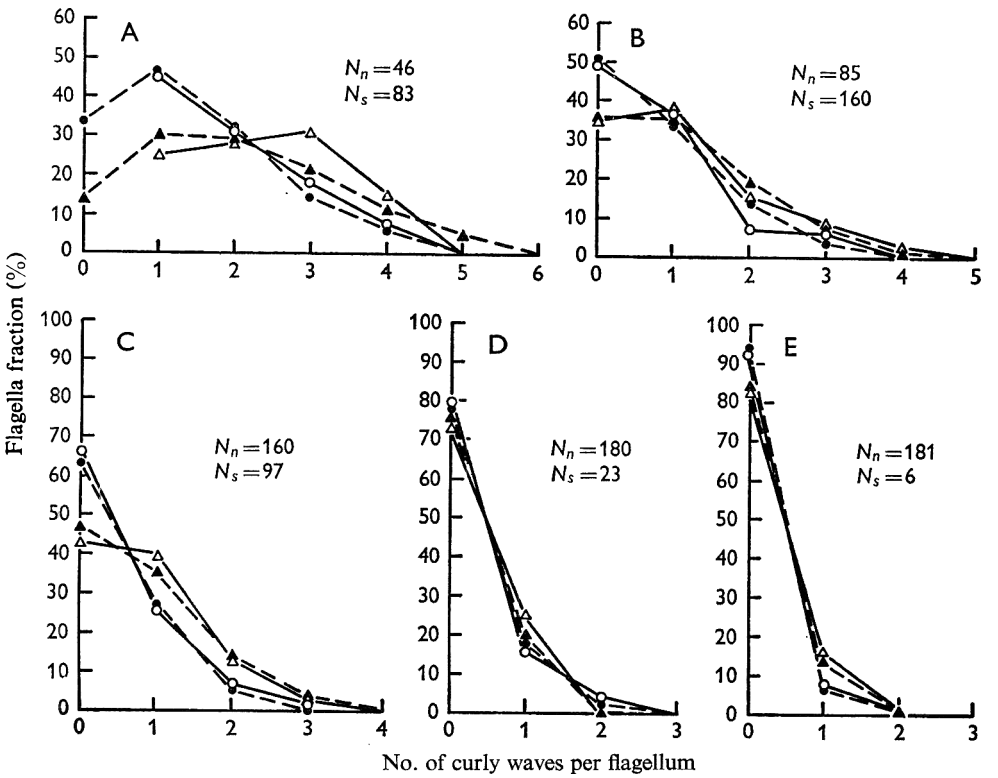

Fig. 7. Distribution of the number of curly waves in flagella with different numbers of normal waves in *Salmonella typhimurium* LT2 grown in RM (FPA) after preincubation in saline or after mechanical shaking for 90 min.: A, pure, curly flagella; B, flagella with one normal wave; C, with two normal waves; D, with three normal waves; E, with four normal waves. ○, observed value at 3 hr in RM (FPA) after preincubation in saline; △, after shaking. ●, Poisson distribution with the same mean as that observed value on unshaken bacteria; ▲, on shaken bacteria. N_n, number of flagella observed on unshaken bacteria; N_s, number of flagella observed on shaken bacteria.

intact and broken flagella are inferred to be mixed. For example, 112/400 bacteria had flagella with one normal wave at 0 hour in the unshaken sample, while the number in the shaken sample was 206/400 bacteria. Therefore, at least 45·6% of the flagella with one normal wave were produced by breaking longer flagella.

The average number of curly waves per flagellum with different number of normal waves is shown in Fig. 8. As in the two earlier experiments, newly grown curly waves were shorter in flagella with more normal waves and reached zero at about five normal waves. In favour of hypothesis (2) the average numbers of curly wave in short flagella

were not smaller, but somewhat larger, in the shaken sample as compared with those in the intact one.

Shapes of flagella grown in mixed culture of normal and curly flagellar strains of Salmonella. For flagella to grow at their tips, flagellin synthesized in the bacterial bodies must be transported to the tip. How is this done? The flagellins of normal and curly flagella can copolymerize and form curly flagella when mixed in the proportions, 1/1 to 1/5 (w/w) (Asakura, Eguchi & Iino, 1966). If flagellin molecules are excreted from the bacterial bodies into the medium, normal bacteria should form curly flagella when grown in mixed culture with an appropriate concentration of curly mutant bacteria.

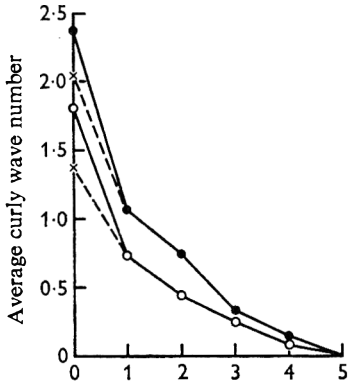

Fig. 8

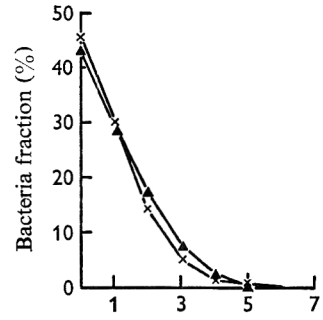

Fig. 9

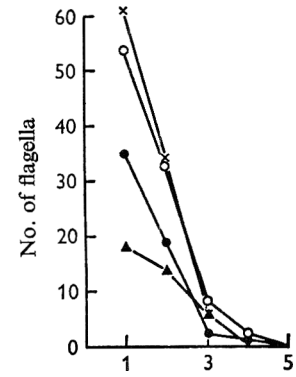

Fig. 10

Fig. 8. Average number of curly waves of a flagellum with different numbers of normal waves in *Salmonella typhimurium* LT2 grown in RM (FPA) after pre-incubation in saline or after mechanical shaking: ○, 3 hr in RM (FPA) after pre-incubation in saline for 90 min.; ●, after shaking in saline for 90 min.; × corrected values counting the null fraction estimated from the Poisson distribution.

Fig. 9. Changes in the distributuion of flagella numbers per bacterium during growth of *Salmonella typhimurium* LT2 in RM (FPA) after mechanical shaking in saline for 90 min.: ×, 0 hr; ▲, 3 hr.

Fig. 10. Distribution of the numbers of normal waves on flagella of *Salmonella typhimurium* LT2 grown in RM (FPA) after mechanical shaking: ×, normal flagella at 0 hr; ●, normal flagella at 3 hr; ▲, heteromorphous flagella at 3 hr; ○, normal plus heteromorphous flagella at 3 hr. Total number at 0 hr was taken as 100. Number at 3 hr was adjusted by multiplying the percentage of each fraction by the net increase of flagella number during incubation. Thus, the total number of flagella at 3 hr was taken as 150.

Two strains were used whose flagellins are known to copolymerize (Asakura *et al.* 1966): SJ25, producing normal flagella, and SJ30, a curly mutant. Equal volumes of these cultures were transferred to fresh broth and cultivated at 37°. At 0 and 3 hr of cultivation, part of each culture was fixed with formalin and the flagella examined. The proportion of normal to curly bacteria in the mixture was also estimated by plating a sample on semisolid media; normal bacteria form swarms while curly ones grow in compact colonies (Iino, 1962). As seen in Table 5, the numbers of both bacteria and flagella increased approximately 1·5-fold. The flagella of salmonellas are

distributed to both daughter bacteria at division (Quadling & Stocker, 1962). Therefore about one-third of the flagella present at 3 hr were presumably produced during growth and distributed among the bacteria, coexisting with ready-made flagella on a cell. However, the flagella carried by a given bacterium were either normal or curly, and no heteromorphous flagella were detected among 400 cells examined.

Table 5. *Shape of flagella grown in mixed culture of normal and curly flagellar strains of Salmonella*

Incubation time (hr)	Type of bacteria	Number of bacteria per ml ($\times 10^8$)*	Number of flagella†	
			Per bacterium	Per ml ($\times 10^8$)
0	Normal	1·4	2·9	4·1
	Curly	1·1	3·0	3·3
	Heteromorphous	0	—	—
3	Normal	2·0	3·1	6·2
	Curly	1·7	3·2	5·4
	Heteromorphous	0	—	—

* Measured on semisolid media. † Average values calculated from the observation of 400 stained cells.

DISCUSSION

The present experiments show that Salmonella flagella grow at their free ends. This agrees with the polarity of flagellar growth *in vitro* (Asakura *et al.* 1968). Thus, the basic molecular mechanism of flagellar growth *in vivo* may be homologous with that of polymerization of flagellin molecules into flagella *in vitro*. As for the initiation of flagellar formation *de novo*, there still exists a gap between flagellar reconstitution *in vitro* and flagellar growth *in vivo*. *In vitro*, a fragment of flagellum is required as a nucleus of polymerization under ordinary physiological conditions; while *in vivo*, flagellar formation *de novo* from the bacteria having no flagellar fragments was demonstrated in phage-mediated transduction of flagellation genes to non-flagellate mutants (Iino & Enomoto, 1966). The identification of the initiator of polymerization *in vivo* which replaces the role of flagellar fragments *in vitro* is essential for a full understanding of flagellar morphogenesis.

The present experiments show that flagellin molecules are unlikely to be excreted into media and used for the growth of flagella. The remaining pathway by which flagellin molecules can reach the tip of a growing flagellum is the tubular hole of the flagellum. A flagellar fibre is a tubular thread consisted of a monolayer of flagellin molecules. Its central hole has a diameter of approximately 60 Å; the space is large enough to pass a flagellin molecule whose diameter is 40 to 45 Å (Kerridge, Horne & Glauert, 1962; Lowy & Hanson, 1965). If this be the case, how and from where do flagellin molecules receive energy to travel from the base of a flagellum all the way to its tip? At present, we know nothing of the mechanism which provides transport energy for flagellin. The proximal part of a flagellum ends as a structure called a 'hook', connected to a spherical granule, called a 'basal granule' (Abram, Koffler & Vatter, 1965; Hoeniger, Iterson & Zanten, 1966; Iterson, Hoeniger & Zanten, 1967; Kerridge *et al.* 1962; Lowy, 1965) These basal structures are presumed to be responsible for the synthesis of flagellin and initiation of flagellar formation as well as flagellar movement. They might also be promoters of flagellin transport; for example, accumulation of

flagellin molecules in the basal structure might cause the efficient diffusion or pushing of the molecules through the hole to the tip of the flagellum.

From the observations of reappearance of flagellate bacteria in deflagellated suspensions of salmonellas, Stocker & Campbell (1959) presented evidence that a flagellum gradually elongates for a limited period and thereafter persists without further elongation, and also that the rate of growth of flagella is unaffected by deflagellation. The present experiments support these conclusions and further show that, during flagellar growth *in vivo*, the speed of growth decreases gradually with increase of the length of flagella, reaching zero at around 15 μ.

As suggested from reconstitution experiments *in vitro*, the distal ends of flagella can grow infinitely as long as the proper concentration of flagellin molecules is present in the reaction mixture (Asakura, Eguchi & Iino, 1964). The decline of the growth rate *in vivo* may therefore be caused by a decrease in the supply of flagellin molecules to the tip, the polymerization site of flagellin. This decline in growth rate depended not on functional decline of the flagellum-forming apparatus by ageing but on the distance between the growing site and the base of the flagellum, which would be consistent with the view that the growth rate depended on the distance to be traversed by flagellin molecules through the tubular hole of a flagellum.

The length of flagella grown on bacterial bodies has an upper limit. In Salmonella, the greatest number of waves seen on the wild-type strain was between five and six normal waves in ordinary cultural conditions. This maximum corresponds to that at which the growth rate of flagella reached zero in the present experiments. It is quite plausible that the mechanism as determining the decline in growth rate of flagella also plays an important role in determining flagellar length in growing bacteria. This postulate does not of course necessarily exclude the possibility of functional ageing of the flagellum-forming apparatus (Stocker & Campbell, 1959) but such ageing might not be the primary cause of the gradual decline of the growth rate of extending flagella.

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Factors Affecting the Toxicity of Oxygen Towards Airborne Coliform Bacteria

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SUMMARY

The maximum bactericidal effect of oxygen on airborne coliform bacteria occurred at the lowest relative humidity tested. *Escherichia coli* COMMUNE and *Klebsiella aerogenes* were very much less sensitive than *E. coli* B and *E. coli* JEPP. Only a small partial pressure of oxygen in a nitrogen-oxygen mixture at atmospheric pressure was required to produce the maximum effect. The oxygen toxicity for each bacterial strain was altered by changing the growth conditions or by including certain chemicals in the spray fluid. These chemicals are protective to freeze-dried bacteria and therefore inactivation of oxidative enzymes in freeze dried and airborne bacteria probably occur by similar mechanisms. The same oxidative enzymes in bacterial colonies were slightly inactivated by hyperbaric oxygen pressures.

INTRODUCTION

Cox (1966) and Cox & Baldwin (1967) showed that *Escherichia coli* sprayed from distilled water died rapidly in air or in oxygen and that death was very marked at low relative humidity (RH) values. At RH values above 70% other death mechanisms prevail (Cox & Baldwin, 1966; Benbough, 1967) which may be associated with rehydration on collecting the partially dried bacteria.

Past investigations into metabolic disturbances caused by oxygen have been hampered by difficulty in recovering adequate quantities of bacteria from laboratory aerosols. However, comparison of the oxygen effects on freeze-dried bacteria (Lion & Bergmann, 1961; Lion & Avi-Dor, 1963) with those on airborne bacteria and simulation of the aerosolized state by drying bacterial suspensions on filter membranes (Benbough, 1967) have been successful in attempts to elucidate the death mechanisms of airborne bacteria. In dried bacteria enzymes and/or coenzymes concerned with pyruvate degradation and with the reduction of tetrazolium salts were affected. The effect on pyruvate degradation recalled the findings of Thomas, Neptune & Sudduth (1963) who attributed the toxicity of hyperbaric oxygen on pyruvic oxidase activity to oxidation of the dithiol moiety of α-lipoic acid, a cofactor of pyruvic oxidase.

Polyhydroxycompounds have been the most widely used protective agents of aerosolized bacteria (Webb, 1960); according to Cox (1966) they protect because they form supersaturated viscous layers around bacteria and thus limit loss of water. An increase in the bacterial polysaccharide content ought then to be beneficial towards aerosol stability by a similar mechanism to the added polyhydroxycompounds; this is reported in this paper.

METHODS

Escherichia coli (strains B, JEPP and COMMUNE) and *Klebsiella aerogenes* (H.8) were either grown in a chemically defined medium (Benbough, 1967) or in a complex tryptone medium (Anderson, 1966).

Aerosol survival estimation. This was described by Benbough (1967). In tests involving the effect of oxygen concentration the quantities of oxygen were admitted to the drum previously containing pure nitrogen. The bacteria were sprayed with pure nitrogen so the quoted oxygen concentrations in the drum were corrected for the volume displaced by that nitrogen.

Chemicals whose protective ability were being tested were added to the spray fluid before aerosolization.

Polysaccharide content of bacteria. This was expressed as a percentage of the dry weight and was measured as follows. To 1·0 ml. suspension containing 2×10^{11} bacteria/ml. 1·0 ml. 5% redistilled Analar grade phenol was added followed by 5·0 ml. concentrated sulphuric acid. These were thoroughly mixed and the red colour compared at 545 mμ with standard glucose solutions. The dry weight was measured as described by Anderson & Dark (1966).

Drying and storage of dried bacteria in a controlled RH. A bacterial suspension was spread evenly over Oxoid membrane filter (diam. 6 cm.). The culture fluid was drained by suction before placing the membrane filter in a desiccator containing 100 ml. of saturated K_2CO_3 solution which kept the RH constant at 44%.

The effect of oxygen on the enzymic activity of dried bacteria. The bacteria-laden membrane filters were stored in either nitrogen, air or oxygen for 1 hr. Then a small volume of phosphate buffer was used to wash bacteria from the membrane. Each suspension was diluted so that the final bacterial count was approximately 2×10^{10} per ml. The utilization of pyruvate was measured as described by Benbough (1967). The rate of reduction of tetrazolium salts was measured by adding to 0·2 ml. suspension, 0·5 ml. of 5% triphenyltetrazolium chloride and 3·0 ml. of a solution of 0·4M-sodium malate (or lactate or succinate) dissolved in 9·0 ml. of 0·1M-tris at pH 8·8 for malate, 7·4 for lactate or 6·8 for succinate. The tubes were incubated at 37° for 15, 30, 45 and 60 min. each and formozan formation measured as described by Benbough (1967).

Very little reduced nicotinamide adenine dinucleotide (NADH) oxidase activity could be detected with whole bacteria. Cell-free extracts were prepared by treating 10 ml. suspension in 50 mM-tris buffer at pH 7·4 by an MSE sonic disintegrator at 0° for 30 min. at maximum power and centrifuging the resulting suspension at 12,000 *g* for 15 min. The NADH oxidase activity of the supernatant fluid was determined as follows: to 0·2 ml. supernatant fluid, 2·8 ml. of 50 μM-tris buffer pH 7·4 (containing 0·1 mM-NADH (B.D.H. Chemicals Ltd., Poole, England) were added and rapidly mixed in a $5 \times 1 \times 1$ cm. fused silica spectrophotometer cell. After mixing, the extinction at 340 mμ was measured as a function of time on a Unicam spectrophotometer at room temperature.

The effect of oxygen concentration on bacterial colony growth and on activity of enzymes extracted from bacterial colonies on agar surface. About 100 organisms were spread evenly over agar plates which were placed in containers whose oxygen concentration in nitrogen ranged from 0 to 100% (v/v) at atmospheric pressure. After

incubation overnight at 37° the visible colonies were counted. Bacterial colonies on agar were also exposed to atmospheres of nitrogen, air or oxygen for about 6 hr, then they were resuspended in phosphate buffer and pyruvate utilization, formozan production and NADH oxidase activities were measured.

The effect of nitric oxide on the oxygen toxicity of dried bacteria. Immediately after placing bacteria-laden membrane filters in a desiccator at 44% RH, the vessel was evacuated, filled with pure nitrogen, evacuated again and finally partially filled with nitrogen. A calculated quantity of nitric oxide, formed by mixing sodium nitrite and potassium ferrocyanide in dilute acetic acid within an air-free container, was drawn into the desiccator by negative pressure *via* a soda lime tube; the pressure was restored to atmospheric by adding more pure nitrogen. After 15 min. the filter was removed from the desiccator into air, was well ventilated to limit contact with nitrogen dioxide, and transferred to another vessel containing air at 44% RH. The survival of dried bacteria which received the above treatment was compared with controls which did not receive the nitric oxide treatment.

RESULTS

Figure 1 shows that at 70% RH similar survivals were obtained for storage in air or nitrogen. At low RH the survival in nitrogen was much greater than in air or in a 20% (v/v) oxygen+80% (v/v) nitrogen mixture at atmospheric pressure in which the survivals were similar. Therefore, oxygen must be the toxic atmospheric component towards airborne coliform bacteria. Figure 2 shows that there was a considerable

Table 1. *The influence of growth media on the survival of coliform bacteria sprayed and stored into air at 40% RH for 30 min.*

	Growth medium	
	Chemically defined	Complex tryptone
	Survival (%)	
Escherichia coli B	1·3	16
E. coli JEPP	2·2	5·1
E. coli COMMUNE	33	55
Klebsiella aerogenes	22	53

variation in the sensitivity of different coliform strains to oxygen. Figure 2 shows that the increase in this sensitivity was directly related to the decrease in the test RH between 70 and 30%.

The results in Figs. 1 and 2 were obtained from tests on washed bacteria which were originally grown on chemically defined media. Bacteria grown in this way survived less well at low relative humidities than corresponding strains grown in complex tryptone media (Table 1).

The maximum death rate occurred within the first second of spraying into either

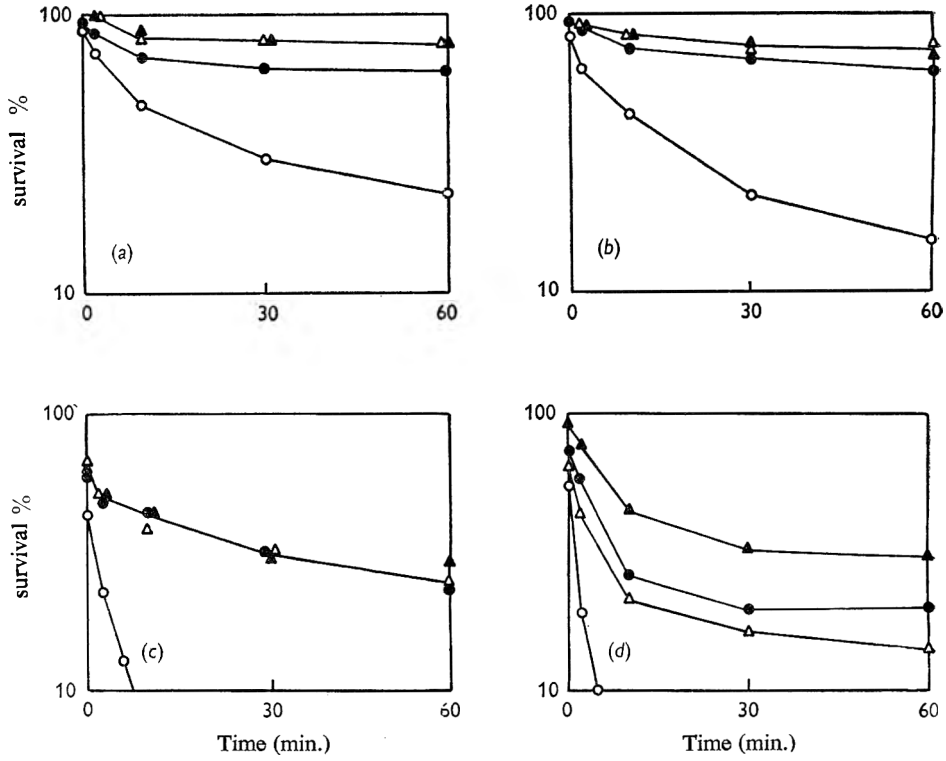

Fig. 1. Death rates of aerosolized: (*a*) *Escherichia coli* COMMUNE, (*b*) *Klebsiella aerogenes*, (*c*) *E. coli* (JEPP), (*d*) *E. coli* B which was sprayed into nitrogen at 70% RH (▲—▲); sprayed into air at 70% RH △—△; sprayed into nitrogen at 40% RH (●—●); sprayed into air at 40% RH (○—○).

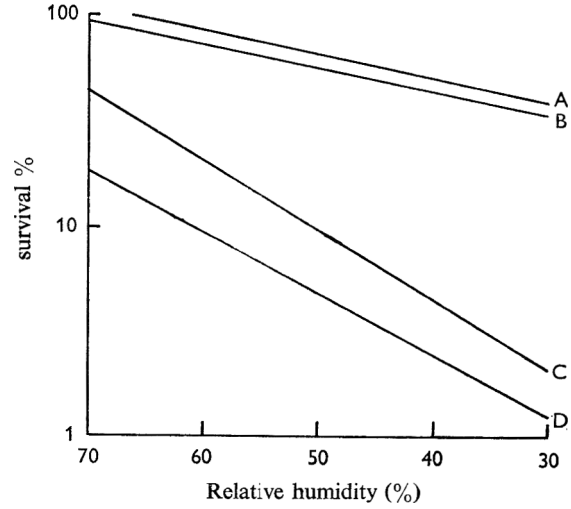

Fig. 2. Survivals of aerosolized bacteria in air as a function of RH (aerosol age 10 min.). A, *Escherichia coli* COMMUNE; B, *Klebsiella aerogenes*; C, *E. coli* (JEPP); D, *E. coli* B. Each of the above straight lines represents between 15 and 25 experimental points.

air or nitrogen but was fastest in air at low RH. Also, at low RH the secondary death rates in air did not tend to become zero as compared to those in nitrogen or air at high RH values (Fig. 1). Figure 3 shows that bacteria sprayed and stored in nitrogen for 0·4 sec. before exposure to air were more sensitive to air than those sprayed directly into air in otherwise similar conditions. Small additions of oxygen to nitrogen atmospheres induced large increases in the death rate; the maximum death rate was reached by fairly low oxygen concentrations in nitrogen (Fig. 4).

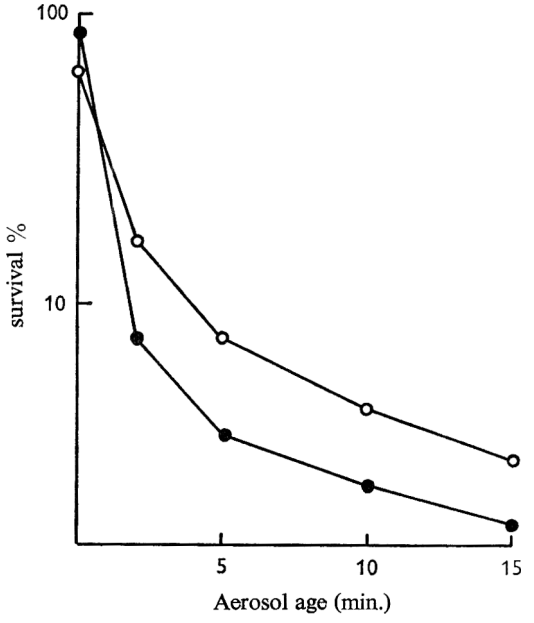

Fig. 3. The deleterious effect of spraying *Escherichia coli* B into nitrogen before being passed into and stored in air at 50% RH: ○—○, bacteria sprayed into *air* and stored in air; ●—●, bacteria sprayed into *nitrogen* and stored in air after aerosol age of 0·4 sec. In the above figure the survival given at 0 min. is in fact the survival at 0·4 sec.

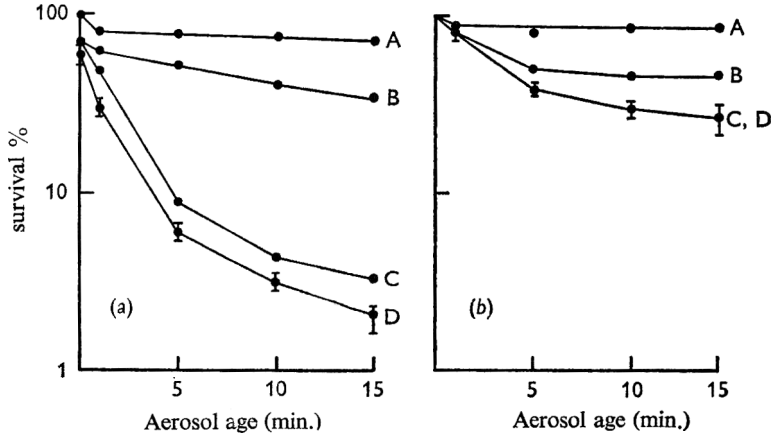

Fig. 4. Effect of oxygen concentration on death rate of aerosolized *Escherichia coli* B sprayed into nitrogen and stored in nitrogen-oxygen mixtures at (*a*) 50% RH; (*b*) 70% RH; A, pO_2 is 0·00 atmosphere; B is 0·03 atm; C is 0·36 atm; D is 0·60, 1·20, 3·00 and 4·50 atm.

Table 2. *The effect of some compounds included in the spray fluid on the survival of aerosolized Escherichia coli* B *at 50% RH*

Compound	Concentration (mM)	Aerosol age (min.) Survival (%) 1	2	5	10
Distilled water*		85	80	80	76
Distilled water*		32	14	6	3
Thiourea	0·7	68	59	51	45
KNO_2	0·5	43	31	24	24
KI	0·3	42	38	35	35
KCNS	1·2	40	32	18	15
2-aminoresorcinol	0·3	58	38	22	20
Methylene blue	0·05–5·0	42	20	6	1
Thymolindophenol	0·05–5·0	29	5	1	—
Ascorbic acid (pH to 7·0 with NaOH)	1·2	45	22	12	9
Catalase	0·01%	38	16	10	5
H_2O_2	2·0	35	13	9	5
Raffinose	300	68	62	40	20
Raffinose	3·0	30	16	4	3
Glucose	2·8	31	10	4	2
Sucrose	2·8	38	20	6	3
Glycerol	2·5	29	12	5	4
Glycerol	250	48	42	26	16
$MnCl_2$	0·4	40	24	19	12
$MnSO_4$	0·5	48	35	24	16
$CoCl_2$	0·2	37	21	15	10
$ZnCl_2$	0·4	30	10	5	1
KCl	0·5	31	10	3	1
$MgCl_2$	0·4	32	18	8	6
$MgSO_4$	0·6	37	23	9	7

* Sprayed into N_2; all others sprayed into air.

Table 3. *Polysaccharide contents of coliform bacteria grown in various chemically defined media*

Strain	Growth medium	Polysaccharide dry weight
Escherichia coli B	*a*	0·13
Escherichia coli COMMUNE	*a*	0·23
Escherichia coli JEPP	*a*	0·10
Klebsiella aerogenes	*a*	0·22
Escherichia coli B	*b*	0·23
	c	0·19
Escherichia coli COMMUNE	*b*	0·38
Klebsiella aerogenes	*b*	0·42

a, denotes the chemically defined medium described in Methods.

b, denotes the above medium except that the ammonium citrate concentration was decreased to 50 μg./ml.

c, denotes the above medium except that the magnesium sulphate concentration was decreased to 0·24 μg./ml.

The death rate of bacteria in the aerosol at low RH is decreased when certain chemicals are included in the spray fluid (Benbough, 1967; Cox, 1966). The protective effects of more chemicals are listed in Table 2; most of these were effective in millimolar quantities. Redox dyes were tested because in the reduced form their ability to combine with oxygen might limit death in the aerosol. Methylene blue decreased the initial death rate substantially, but the secondary death rate was accelerated; thus it appeared to delay the toxic effect of oxygen. Thiourea, ascorbic acid, 2 aminoresorcinol, like cysteamine (Benbough, 1967), protected aerosolized bacteria at low RH only; these compounds were toxic between 60 and 80% RH. In contrast to the protective compounds already described, sugars and other polyhydroxycompounds were needed in high concentrations to protect aerosolized bacteria. Catalase did not protect, hence toxic amounts of H_2O_2 were not produced; H_2O_2 included in the spray fluid was not toxic.

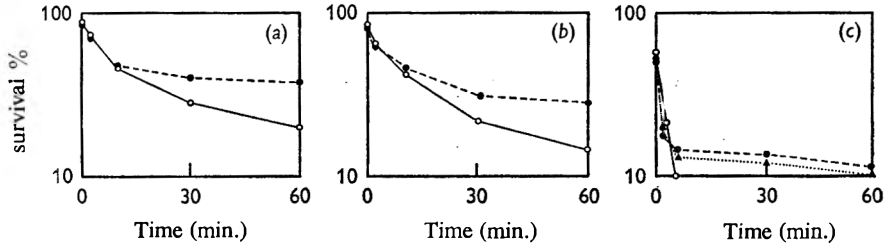

Fig. 5. Death rates of aerosolized: (*a*) *Escherichia coli* COMMUNE; (*b*) *Klebsiella aerogenes*; (*c*) *E. coli* B sprayed into air at 40% RH. ○—○, Bacteria grown on chemically defined media as described in Methods; ●—●, bacteria grown in the same medium except that the ammonium citrate was reduced to 50 μg./ml.; ▲—▲, bacteria grown in the same medium except that the $MgSO_4$ was decreased to 0·24 μg./ml.

Strains of coliform bacteria with the higher polysaccharide content can withstand the oxygen effect much better in the aerosol (Table 3). By increasing the polysaccharide content of each strain by growing and incubating in carbon-rich and nitrogen- or magnesium-limiting media (Wilkinson, 1959), the following result was obtained: the initial death rate was as fast as corresponding strains grown on the complete defined medium but the secondary death rate was considerably slower (Fig. 5).

Enzymes concerned with reduction of 2:4:6-triphenyl tetrazolium chloride, NADH oxidase and pyruvate degradation were inactivated by oxygen in partially dried bacteria (Table 4). Results on Table 4 also show that hyperbaric oxygen inactivated the same enzymes in bacterial colonies. The effects of oxygen on partially dried bacteria and on colonies may therefore be basically the same; however, much higher oxygen concentration was needed to produce the same effect in the latter case. Double atmospheric oxygen pressure produces a threshold bacteriostatic effect on *Escherichia coli* colonies whilst five times atmospheric oxygen pressure causes 50% and 30% inhibition of colony formation from single *E. coli* B and *E. coli* COMMUNE bacteria respectively.

When partially dried bacteria on membrane filters were exposed to nitric oxide for a few minutes before exposure to oxygen the number of bacteria killed was greatly diminished. The maximum protective effect was produced by 50 parts per million nitric oxide in nitrogen (Table 5).

Table 4. *The inactivation of three bacterial enzyme systems by oxygen*

(*a*) Enzymes concerned with the utilization of pyruvate

Treatment	Uptake of pyruvate by suspension μM/min./10^{10} bacteria
E. coli B dried at 44% RH for 1 hr at 1 atm. in	
Nitrogen	31 ± 1
Oxygen	21 ± 1
Air	22 ± 1
E. coli B colonies exposed for 18 hr at 1 atm. in	
Nitrogen	33 ± 1
Oxygen	28 ± 1
Air	33 ± 1

(*b*) Enzymes concerned with the reduction of 2:4:6-triphenyltetrazolium chloride to formozan

	Incubated with		
	Succinate	Malate	Lactate
Treatment	Rate of increase of extinction/hr; *n*-butanol extract		
E. coli B dried at 44% RH for 1 hr at 1 atm. in			
Nitrogen	0·120	0·033	0·042
Oxygen	0·048	0·000	0·015
Air	0·045	0·000	0·019
E. coli B colonies exposed for 18 hr. at 1 atm. in			
Nitrogen	0·132	0·072	0·074
Oxygen	0·061	0·039	0·048
Air	0·140	0·065	0·075

(*c*) NADH oxidase

Treatment	Activity n-mole NADH oxidized/min.
E. coli B dried at 44% RH for 1 hr at 1 atm. in	
Nitrogen	190 ± 10
Oxygen	90 ± 10
Air	110 ± 10
E. coli COMMUNE dried at 44% RH for 1 hr at 1 atm. in	
Nitrogen	140 ± 10
Oxygen	90 ± 10
Air	90 ± 10
E. coli B colonies exposed for 18 hr at 1 atm. in	
Nitrogen	220 ± 10
Oxygen	130 ± 10
Air	230 ± 10

Table 5. *Effect of nitric oxide on the survival of dried Escherichia coli* B *at 44% RH exposed to air*

NO conc. p.p.m. at 1 atm.	Proportion of bacteria surviving after 30 min. exposure to air
0	$0{\cdot}3 \pm 0{\cdot}1$
10	$0{\cdot}5 \pm 0{\cdot}2$
50	$0{\cdot}6 \pm 0{\cdot}2$
60	Less than 0·001
100	0

DISCUSSION

The lethal action of oxygen appears to depend on the loss of water from bacteria. Bateman, Stevens, Mercer & Carstensen (1962) measured the water content of *Serratia marcescens* at many RH values and found that it decreased rapidly between 100% and 70% RH, but below 70% the decrease was very much less. This shows that strongly bound water is lost when bacteria are exposed to RH values of 70% and less. Bound water has a structural significance and its loss may cause changes in accessibility and reactivity of macromolecules. Freeze-dried *Escherichia coli* show electron spin-resonance signals (Dimmick, Heckley & Hollis, 1961; Lion, Kirby-Smith & Randolph, 1961; Lion & Bergmann, 1961); these signals are emitted by free radicals and the signals were greatly enhanced when the freeze-dried bacteria were exposed to oxygen, presumably because paramagnetic molecules such as oxygen react readily with free radicals to form peroxyradicals. Such events probably occur when bacteria are sprayed into air at low RH values. The peroxyradicals formed in this way would inactivate many of the oxidative enzymes. Protection by nitric oxide can be explained by its paramagnetic character: it would react with free radicals formed during bacterial hydration and thus prevent peroxyradical formation.

Airborne bacteria can be protected in two ways. First, polyhydroxy compounds limit the loss of water from bacteria in the same way as those bacterial strains that possess a high polysaccharide content. Secondly, other compounds may act directly on the oxygen inactivating site; these compounds are effective in micromolar quantities and they include paramagnetic ions such as Mn^{2+}, NO_2^-, I^- and compounds with labile hydrogens such as ascorbic acid, aminothiols and reduced dyes.

Comparison of the extent of enzyme inactivation in bacterial colonies by hyperbaric oxygen to inactivation in dried bacteria by air confirms the role of cellular water in limiting oxygen toxicity.

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Phage-induced Alteration of Enzymic Activity in Lysogenic *Mycobacterium smegmatis* Strains

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SUMMARY

Changes in the amidase, nitrate reductase and phenolphthalein sulphatase activity of *Mycobacterium smegmatis* were observed following B 1-lysogeny. These changes were more consistent in lysogenic derivatives of strains SN 2 and SN 14 than in those of strain SN 10. Strains SN 2 and SN 14 appeared to differ from SN 10 only in that the former possessed a functional salicylamidase locus while the latter did not. Emergence of various phenotypes in the same strain and during the same lysogenic event were observed. Multiple sites of phage integration may have accounted for the variation of SN 10 (B 1) phenotypes.

INTRODUCTION

Soon after its discovery d'Herelle suggested that the bacteriophage was not merely a lethal agent but also an important factor of bacterial variability (d'Herelle, 1930). In 1951 Freeman found that toxigenicity in *Corynebacterium diphtheriae* was determined by phage β carried by toxic diphtheria strains (Freeman, 1951). Changes in colonial morphology (Ionesco, 1953) and antigenic composition (Uetake, Nagakawa & Akiba, 1955) due to the interaction of the host and phage genome have been demonstrated subsequently.

Lysogenic conversion resulting in altered enzymic activity has been reported previously in *Mycobacterium smegmatis* (Bönicke & Juhasz, 1965; Bönicke, 1967). In addition to altered α-nicotinic acid oxidase and malachite green reductase activity of *M. smegmatis* SN 2, it has been observed recently that phage infection affected the ability of *M. smegmatis* ATCC 607 to reduce nitrates to nitrites and to hydrolyse Tween 80 (Jones & White, 1968). The present paper describes changes in the amidase, nitrate reductase and phenolphthalein sulphatase activities of *M. smegmatis* strains following lysogenization by phage smegmatis B 1.

METHODS

Bacterial strains. Mycobacterium smegmatis strains SN 2, SN 10 and SN 14 of the Borstel Collection served as hosts in this study. The original designation of strain SN 10 was ATCC 607.

Phage smegmatis B 1 (Juhasz & Bönicke, 1965) was used to infect and lysogenize *Mycobacterium smegmatis* strains.

Media. Bacterial cultures were maintained on Loewenstein–Jensen slopes, phage strains in heart infusion broth (Difco). Lysogenization experiments were performed on

nutrient agar plates (Difco); the agar overlay was prepared from nutrient broth (Difco) by the addition of 0·7% Bacto agar. For the enzymic experiments all the strains were grown on Loewenstein–Jensen plates.

Procedures for the recovery and purification of lysogens through single colony transfers were the same as described for B2-lysogenic *Mycobacterium phlei* (Juhasz & Bönicke, 1966).

Amidase test. Bönicke's amide series (Bönicke, 1960; Juhlin, 1967) was employed to detect amidase activity. Bacteria grown on Loewenstein–Jensen plates for 12 to 15 days were harvested and suspended in physiological NaCl. They were washed by centrifugation for 20 min at 3000 rev./min. and resuspended in pH 7·2 phosphate buffer to give equiv. 10 mg. dry weight/ml. One ml. samples were mixed with 1 ml. each of a 0·00164 M-amide solution. The ten amides used in these experiments were: acetamide, urea, isonicotinamide (Sigma Chemical Company), pyrazinamide, allantoin (courtesy of Dr R. Bönicke), benzamide, nicotinamide, salicylamide, succinamide, malonamide (J. T. Baker Chemical Company). Each amide + bacteria mixture was incubated for 22 hr at 37°. Liberation of ammonia resulting from the hydrolysis of the given amide was detected by the phenol-hypochlorite method (Russell, 1944, Bönicke, 1960). Eau de Javelle obtained from Dr R. Bönicke was used as a source of hypochlorite. The amount of liberated ammonia was determined by comparison of the experimental tubes with standards containing measured amounts of ammonia. The two controls used were bacterial suspensions without amides and amide solutions without bacteria.

The nitrate reductase test of Virtanen (Virtanen, 1960) was used to measure reduction of nitrate to nitrite. Suspensions containing equiv. 0·4 mg. dry weight bacteria were inoculated into nitrate broth (Difco) and incubated at 37° for 2 hr. The colour reaction obtained by Shinn's napthylethylenediamine + sulphanilamide method (Shinn, 1941) was compared with that of standards containing known concentrations of $NaNO_2$. The latter was obtained from Merck, Sharpe and Dohme, Inc. *N*-1-naphthylethylenediamine from Mann Research Laboratories, sulphanilamide from Eastman Organic Chemicals.

Phenolphthalein sulphatase activity was determined as suggested by Wayne (Wayne 1961). Dubos oleic acid agar (Difco) containing 650 mg. phenolphthalein disulphate tripotassium salt (Eastman Organic Chemicals) per litre was inoculated with a suspension containing equiv. 0·4 mg. dry weight bacteria. Readings were made after 14 days of incubation at 37°. The quantity of phenolphthalein produced was measured by comparison with standards containing known amounts of phenolphthalein (J. T. Baker Chemical Company).

RESULTS

The effect of phage B1 on various enzymic activities of *Mycobacterium smegmatis* strains SN2, SN10 and SN14 is shown in Table 1. While the four SN2 (B1) lysogens emerged in the same lysogenic event, SN10 (B1) and SN14 (B1), lysogens were obtained in two different lysogenic events (as indicated by arabic and roman numerals).

A definitive decrease in salicylamidase and nitrate reductase activity characteristic for SN2 and SN14 lysogens was accompanied by a decreased phenolphthalein sulphatase activity in strains SN2 (B1), nos 2, 3 and 4, and in SN14 (B1), nos. 1, 3 and—to a lesser degree—in SN14 (B1), no. I. The decrease in these enzymic activities varied in degree. Nitrate reductase activity seemed to disappear completely in strains SN2 (B1), nos 2,

Table 1 *Amidase activity*

Strains	Aceta-mide	Benza-mide	Urea	Iso-nicotin.	nicoti-namide	Pyrazi-namide	Salicy-lamide	Allan-toin	Succi-namide	Malona-mide	Nitrate reductase activity	Phenolph-thalein sulphatase activity
NS 2	6·3	5·5	7·1	6·8	8·0	6·5	3·8	1·1	8·0	2·0	4·0	8·0
	++	++	++	++	+++	++	+	0	+++	(+)	+	++
NS 2 (B1), no. 1	5·5	3·1	7·3	3·1	6·0	5·5	0·8	0·5	8·0	1·0	1·0	6·0
	++	+	++	+	++	++	0	0	+++	0	+	++
SN 2 (B1), no. 2	7·0	3·5	9·0	6·0	7·5	6·0	1·5	1·0	8·1	0·5	0·0	0·0
	++	+	+++	++	++	++	0	0	+++	0	0	0
SN 2 (B1), no. 3	5·3	1·9	6·8	3·0	4·3	5·5	1·5	0·0	6·8	1·5	0·0	0·7
	++	0	++	+	+	++	0	0	++	0	0	0
SN 2 (B1), no. 4	3·7	3·7	7·0	1·1	6·0	5·7	0·7	0·7	7·7	0·0	0·0	0·7
	+	+	++	0	++	++	0	0	++	0	0	0
SN 10	4·3	6·0	7·3	6·1	6·3	5·5	1·0	1·3	6·7	1·0	10·0	5·0
	+	++	++	++	++	++	0	0	++	0	+++	+
SN 10 (B1), no. 1	6·0	4·5	8·0	5·0	6·0	6·0	0·3	0·0	7·8	0·3	50·0	1·5
	++	+	+++	++	++	++	0	0	++	0	++++	0
SN 10 (B1), no. 2	4·5	4·2	7·5	3·0	4·5	5·5	0·0	0·0	6·2	0·0	5·0	5·0
	+	+	++	+	+	++	0	0	++	0	++	+
SN 10 (B1), no. I	4·7	4·5	8·8	6·0	5·8	5·2	0·0	0·0	6·2	0·0	2·0	20·0
	+	+	+++	++	++	++	0	0	++	0	+	+++
SN 10 (B1), no. II	5·2	4·5	7·5	5·8	6·2	6·5	0·5	0·0	7·2	0·5	10·0	0·0
	++	+	++	++	++	++	0	0	++	0	+++	0
SN 14	7·0	6·8	7·5	7·0	7·5	6·0	3·8	0·3	7·0	1·5	10·0	12·0
	++	++	++	++	++	++	+	0	++	0	+++	++
SN 14 (B1), no. 1	5·7	4·7	7·0	3·7	5·2	5·7	0·0	0·0	6·5	0·0	0·3	0·0
	++	+	++	+	++	++	0	0	++	0	(+)	0
SN 14 (B1), no. 2	2·5	2·5	5·2	2·0	3·2	4·7	0·7	0·0	5·2	0·0	0·0	6·0
	(+)	(+)	++	(+)	+	+	0	0	++	0	0	++
SN 14 (B1), no. 3	4·7	3·1	6·5	4·3	4·8	5·8	1·1	0·0	7·2	0·0	2·0	0·0
	+	+	++	+	+	++	0	0	++	0	+	0
SN 14 (B1), no. I	3·5	4·5	7·5	4·2	5·8	6·0	0·5	0·0	6·0	0·0	0·0	4·0
	+	+	++	+	++	++	0	0	++	0	0	+

All the cultures were grown on Loewenstein–Jensen plates for 12 to 15 days. Arabic and Roman numerals indicate different lysogenic events. Readings are expressed in μg. per ml. of the reaction product, i.e.: ammonia in the amidase test—0 to 2 μg. = 0, 2 to 3 μg. = (+), 3 to 5 μg. = +, 5 to 8 μg. = ++, $\geqslant$ 8 μg = +++ (Juhlin, 1967); nitrate in the nitrate reduction test—0 to 0·1 μg. = 0, 0·1 to 1·0 μg. = (+), 0 to 5·0 μg. = +, 5·0 to 10·0 μg. = ++, 10 to 50 μg. = +++, 50 to 100 μg. = ++++; phenolphthalein in the phenolphthalein sulphatase test—0 to 2 μg. = 0, 2 to 3 μg. = (+), 3 to 6 μg. = +, 6 to 16 μg. = ++, 16 to 40 μg. = +++. Amidase activity was read after 22 hr, nitrate reductase activity after 2 hr and phenolphthalein sulphatase activity after 14 days. All figures are based on at least two experiments.

3, 4 and in SN 14 (B 1), nos. 2 and I, but not in strains SN 2 (B 1) no. 1 and SN 14 (B 1), no. 3. On their part, strains SN 2 (B 1), no. 2 and SN 14 (B 1), nos. 1 and 3 revealed no detectable phenolphthalein sulphatase activity, a residual activity was found in strains SN 2 (B 1), nos. 3, 4, finally SN 2 (B 1), no. 1 and SN 14 (B 1), no. 2 showed a phenolphthalein sulphatase activity resembling that of the parental type. In addition to the enzymic changes already mentioned, SN 2 (B 1) lysogens displayed varying degrees of decrease in benzamidase and, with the exception of SN 2 (B 1), no. 2, in isonicotinamidase activity. Similar, but less significant changes, have also been observed in the amidase spectrum of SN 14 (B 1) lysogens.

In contrast to strains SN 2 and SN 14, B 1-lysogeny did not appear to modify strain SN 10 in a consistent manner. Although nitrate reductase activity decreased in strain SN 10 (B 1), no. I, it increased significantly in strain SN 10 (B 1), no. 1. Phenolphthalein sulphatase activity, undetectable in strain SN 10 (B 1), no. II and decreased in strain SN 10 (B 1), no. I, showed, on the other hand, definitive increase in strain SN 10 (B 1), no. I. The only obvious difference between strain SN 10 and the former strains was the ability of parental type SN 2 and SN 14 to hydrolyse salicylamide.

DISCUSSION

The changes observed in B 1-lysogenic strains of *Mycobacterium smegmatis* SN 2 and SN 14 resulted in a decrease of the salicylamidase, nitrate reductase and to somewhat lesser extent the phenolphthalein sulphatase activity of wild strains. No consistent changes were observed in SN 10 (B 1) lysogens. The different behaviour of *M. smegmatis* strains appeared to be related to the presence of a functional salicylamidase locus in wild SN 2 and SN 14 and to its absence in wild SN 10. It would be premature to speculate whether mutation or repression of the salicylamidase locus at a possible site for phage integration or some other mechanism accounted for the differences.

Various degreees of change were observed, not only when different hosts were lysogenized, but among derivatives of the same strain emerging in the same lysogenic event. (Compare, for example, the nitrate reductase activity of SN 2 (B 1), no. 1 and SN 2 (B 1), no. 2 strains.) Since decrease of the salicylamidase activity appeared to be the primary and most consistent change, multiple sites for the integration of phage B 1 was considered unlikely in SN 2 and SN 14 lysogens. It may have accounted, however, for the diversity of SN 10 (B 1) phenotypes, in which the salicylamidase locus was presumably not available for phage integration. Missing its preferential site the phage may have integrated at alternative sites. This would explain the apparent loss or decrease of phenolphthalein sulphatase activity compensated by an increased nitrate reductase activity in some strains and the converse in others.

Rapidly growing mycobacterial strains which cannot be identified and classified by the available methods are isolated from time to time in various laboratories. Such strains often show: (1) resistance to the known mycobacteriophages, and (2) enzymic activities which are different from that of the known rapidly growing mycobacterial species. Although attempts to isolate phages from them usually fail, the non-availability of susceptible indicator strains or the defectiveness of the prophage they carry may account for this failure. The reported *in vitro* experiments have shown that a considerably wide range of changes may follow lysogeny and alter potentially important taxonomic characters.

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Properties of *Proteus mirabilis* and Providence Spheroplasts

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SUMMARY

Proteus mirabilis strain 13 and Providence strain NCTC 9211 were converted to osmotically sensitive spheroplasts by growth in the presence of penicillin or glycine in sucrose-supplemented or unsupplemented medium. Lysozyme in the presence of tris and ethylenediaminetetra-acetic acid (EDTA) converted both these strains to spheroplasts. Penicillin spheroplasts could be stabilized with sucrose, streptomycin, spermine or di-valent cations. Electron micrographs of these spheroplasts demonstrated that the outer layer of the bacterial cell wall was retained. Penicillin-induced spheroplasts possessed receptor sites for bacteriophages.

INTRODUCTION

Bacterial protoplasts have been reviewed by McQuillen (1956, 1960), Weibull (1958) and Martin (1963). The term protoplast is limited to a cell devoid of cell wall (McQuillen, 1960). Cells converted to osmotic sensitive spherical bodies which retain non-rigid cell walls are called spheroplasts (Tulasne, Minck, Kirn & Krembel, 1960; McQuillen, 1960). The cell walls of *Bacillus megaterium*, *Sarcina lutea* and *Micrococcus lysodeikticus* consist predominantly of mucopolymer (Salton, 1960). The cell wall of *Escherichia coli* consists of a superficial lipoprotein, an intermediate lipopolysaccharide and an innermost rigid mucopolymer layer (Weidel, Frank & Martin, 1960). Salton & Shafa (1958) emphasized that cell walls of Gram-negative bacteria contain the mucopeptide-substrate of lysozyme as a minor component. In *E. coli* and *Proteus mirabilis* the lipoprotein and lipopolysaccharide layers are anchored to the mucopolymer in an intricate mosaic of functional surface sites (Hofschneider & Martin, 1968). Weidel *et al.* (1960) also showed that the receptor sites for bacteriophages were located in the lipoprotein and lipopolysaccharide layers and that phages did not adsorb to the rigid layer. Kellenberger & Ryter (1958) provided microscopic proof for the multi-layered cell wall in *E. coli*. They demonstrated a cell wall in which two electron dense layers were separated by a less dense layer. Depolymerization of the mucopolymer which provides rigidity to the cell wall is necessary for conversion to spheroplasts. This can be accomplished by lysozyme which acts on β-1,4-*N*-acetylglucosamides (Salton & Ghuysen, 1959) or penicillin (Lederberg, 1956, 1957) which inhibits mucopolymer synthesis (Park & Strominger, 1957). To explain the action of penicillin on synthesized mucopolymer the presence of two enzymes in the cell was suggested by Weidel *et al.* (1960). One splits certain bonds in the mucopolymer while the other resynthesizes mucopolymer in these gaps. Bayer (1967) observed the formation of bag-like protrusions of the soft layers and the simultaneous appearance of gaps in the proteinaceous portion of the rigid layer during penicillin treatment of *E. coli*.

Weibull (1953*a*, *b*) converted cells of *Bacillus megaterium* suspended in 0·15 to 0·2M-sucrose to protoplasts by treatment with lysozyme. These protoplasts did not adsorb bacteriophages (Weibull, 1953*a*) and were devoid of the cell-wall component diaminopimelic acid (Weibull & Bergström, 1958). Fukuda (1961), however, described the adsorption of bacteriophages to lysozyme-induced protoplasts of several strains of *B. subtilis*, suggesting incomplete removal of cell-wall components. To enhance the effect of lysozyme on Gram-negative bacteria additional procedures were employed. These included freezing and thawing (Kohn, 1960), incubation at alkaline pH (Zinder & Arndt, 1956) and the addition of the chelating agent ethylenediaminetetra-acetic acid (EDTA) in tris buffer (Repaske, 1956, 1958). Repaske (1958) reported differences in susceptibility of Gram-negative bacteria to lysis by the combined effects of lysozyme, EDTA and tris buffer. *Escherichia coli* strains B and H, *Pseudomonas aeruginosa*, *P. fluorescens* and *Azotobacter vinelandii* were rapidly lysed. *Proteus vulgaris* was slightly lysed while *Aerobacter aerogenes* and *Serratia marcescans* were not affected. Gebicki & James (1958) proved *A. aerogenes* to be resistant to lysozyme even in the presence of EDTA. Voss (1964) showed that lysis of *E. coli* and *P. aeruginosa* by lysozyme in the presence of EDTA and tris buffer was not necessarily preceded by spheroplast formation. These organisms were converted to osmotically fragile rods rather than spheres. Jeynes (1957) prepared protoplasts of *Vibrio cholera*, *Salmonella typhi*, *S. typhimurium* and *S. paratyphi* by growth in liquid medium containing glycine.

Tabor (1961) reported that 10^{-3}M-spermine or spermidine prevented lysis of lysozyme-induced protoplasts of *Escherichia coli* strains B and W and *Micrococcus lysodeikticus* in hypotonic media. Stabilization of penicillin spheroplasts of *E. coli* W was less complete. Streptomycin and Ca^{2+} ions were also effective stabilizers, but Mg^{2+} ions as well as mono-valent cations had no protective properties at 10^{-3}M-concentrations with slight protection at higher concentrations. Indge (1968) found that K^+, Na^+ Mg^{2+} and spermidine inhibited the effect of chelating agents on lysis of yeast protoplasts.

As a preliminary to the study of transfection (Spizizen, Reilly & Evans, 1966) in Proteus and Providence strains the properties of spheroplasts of these organisms were investigated and are reported here.

METHODS

Media. Difco nutrient broth was used. The spheroplasting medium consisted of nutrient broth supplemented with 0·5M-sucrose and 0·2% (w/v) $MgSO_4.7H_2O$. Tris buffer (0·067M, pH 8·0), lysozyme (Sigma) and ethylenediaminetetra-acetic acid (Merck) were used. Incubation was at 37°.

Bacteria and bacteriophages. Providence strain NCTC9211 (Coetzee, 1963*a*), *Proteus mirabilis* strain 13 (Coetzee & Sacks, 1960) and *Escherichia coli* strain B were used. For phage adsorption studies the temperate Providence bacteriophage PL25 (Coetzee, 1963*a*) and the virulent *P. mirabilis* bacteriophage 13*vir* (Prozesky, De Klerk & Coetzee, 1965) were used.

Lysozyme + EDTA spheroplasts. The spheroplasts of *Escherichia coli* B were prepared according to Fraser & Mahler (1957) as modified by Fraser, Mahler, Shug & Thomas (1957). Spheroplasts of *Proteus mirabilis* 13 and Providence NCTC9211 were prepared from overnight cultures in nutrient broth. Organisms were harvested by centrifugation of 10 ml. of the cultures at 2000 *g* for 30 min., washed twice with 0·067M-tris and

resuspended in 1 ml. tris containing 0·5 M-sucrose. To the suspension of the Providence strain 0·2 ml. lysozyme (2 mg./ml.) and after 3 min. 0·05 ml. EDTA (4%, w/v) were added. For the *P. mirabilis* strain 0·4 ml. lysozyme and 0·1 ml. EDTA were used. The mixtures were then incubated for 8 min. followed by a 1/5 dilution with nutrient broth. The preparations were examined in a phase-contrast microscope.

Glycine spheroplasts. These were obtained by adding 5 ml. of an overnight culture to 100 ml. nutrient broth supplemented with 0·5 M-sucrose, 0·2% $MgSO_4 . 7H_2O$ and glycine (3%, w/v). The cultures were aerated. Samples were removed at intervals and examined by phase-contrast microscopy. After incubation for 5 hr the spheroplasts were collected by centrifugation at 2000*g* for 30 min.

Penicillin spheroplasts. Spheroplasts of *Escherichia coli* B were prepared by the method of Lederberg (1956). Penicillin spheroplasts of Providence and *Proteus mirabilis* strains were obtained by diluting 10 ml. of an overnight culture in 100 ml. spheroplasting medium. Penicillin G (100 to 10,000 u./ml.) was added to the medium which was then incubated with aeration. Samples were removed at intervals, examined microscopically and plated on MacConkey agar to determine colony-forming bacteria. The method was varied by substituting unsupplemented nutrient broth for the spheroplasting medium. Spheroplasts were counted in a Petroff–Hauser chamber with the use of a phase-contrast microscope. Spheroplasts were harvested after 3-hr incubation.

Osmotic sensitivity. Duplicate samples of lysozyme, glycine and penicillin spheroplasts were collected by centrifugation at 2000 *g* for 30 min. The pellets were resuspended in equal volumes of 0·5 M-sucrose and deionized water respectively. Extinctions at 650 mμ were determined within 10 min. in a Hitachi Elmer–Perkin spectrophotometer. Stability of spheroplasts prepared in unsupplemented medium were determined by resuspending in deionized water and nutrient broth.

Stabilization of penicillin spheroplasts. After incubation for 3 hr the penicillin spheroplasts were collected by centrifugation at 2000 *g* for 30 min. The pellets were suspended in equal volumes of 0·5 M-sucrose, water, streptomycin sulphate (10^{-3} M), $CaCl_2$ (10^{-2}, 10^{-3} M), spermine (10^{-3}, 10^{-4}, 10^{-5} M), $MgCl_2$ (10^{-2}, 10^{-3} M), NaCl (10^{-2} M) and unsupplemented nutrient broth. Extinctions of the suspensions at 650 and 260 mμ were determined within 10 min. after suspending in a Hitachi Elmer–Perkin spectrophotometer. The suspensions were also examined microscopically.

Electron microscopy. Penicillin spheroplasts of Providence and *Proteus mirabilis* were prepared and collected as above. Fixation was done in osmium tetroxide (Kellenberger & Ryter, 1958). Preparations were stained in uranyl acetate before dehydration through a graded acetone series and embedding in Epon-Araldite. Electron microscopy was performed in a Philips EM 200 electron microscope.

Phage adsorption. Penicillin spheroplasts of *Proteus mirabilis* and Providence were collected by centrifugation and resuspended in 0·5 M-sucrose. Bacteriophages PL25 and 13*vir* at multiplicities of input of 100 to 300 were added to spheroplasts of Providence strain NCTC9211 and *P. mirabilis* strain 13, respectively. After incubation for 10 min. the interaction was stopped by the addition of osmium tetroxide. Staining and embedding were done as before.

RESULTS

Lysozyme + EDTA spheroplasts

At least 99% of the Providence organisms were converted to spherical forms by this method. *Proteus mirabilis* 13 was more resistant to lysozyme-EDTA treatment and higher concentrations of lysozyme (0·4 ml.) and EDTA (0·1 ml.) were necessary for optimum yields. For this strain conversion to spherical forms was never more than 80% and was not improved by further increase in the concentrations of lysozyme or EDTA.

Glycine spheroplasts

Glycine at concentrations of 3 to 5% (w/v) proved an effective inducer of spheroplasts. In both strains the first small spheroplasts were observed at 40 min. At 4 hr all the cells were spherical and incubation for 12 hr yielded a dense-culture of spheroplasts.

Penicillin spheroplasts

Incubation of the *Proteus mirabilis* and Providence strains in the presence of penicillin caused the rod-shaped cells to develop swellings centrally or terminally. The swellings enlarged to form spheres with remnants of rods protruding. The sequence of forms in both *P. mirabilis* and Providence was similar to those described for *P. vulgaris* and *Escherichia coli* (Liebermeister & Kellenberger, 1956; Hahn & Ciak, 1957). The concentration of penicillin was not critical and 500 to 10,000 u./ml. were equally efficient. Incubation of the *P. mirabilis* and Providence strains in unsupported nutrient broth in the presence of high concentrations of penicillin (500 u./ml. for the *P. mirabilis* and 3000 u./ml. for the Providence strain) also resulted in dense cultures of spheroplasts which were identical to those described above. Lower concentrations of penicillin (2000 and 500 u./ml. respectively) caused the formation of spherical as well as filamentous forms in both the organisms (Fleming, Voureka, Kramer, Hughes 1950). The *E. coli* strain treated similarly underwent complete and rapid lysis. Irrespective of whether the spheroplasts were prepared in unsupported nutrient broth or in spheroplasting medium all spheroplasts lysed in water. While the spheroplasts prepared in spheroplasting medium lysed when resuspended in unsupported nutrient broth those obtained in nutrient broth were stable in the latter medium.

Stability of penicillin spheroplasts

On account of the efficiency and reproducibility of the penicillin method of spheroplast formation these spheroplasts prepared in spheroplasting medium were examined in greater detail. As indicated in Table 1 the spheroplasts were stabilized when suspended in 0·5M-sucrose. Streptomycin sulphate (10^{-3}M), $CaCl_2$ (10^{-3}M) and $MgCl_2$ (10^{-3}M) were effective stabilizers while NaCl (10^{-2}M) provided very little support. Spermine at 10^{-3}M was a good stabilizer for penicillin spheroplasts of *Escherichia coli* B. Maximal stabilization of penicillin-induced spheroplasts of *Proteus mirabilis* 13 and Providence NCTC9211 was obtained at a spermine concentration of 10^{-4}M. Higher concentrations caused aggregation of the spheroplasts while low concentrations provided no protection against lysis. Suspension in water caused rapid lysis of the spheroplasts with a drastic decrease in optical density at 650 mμ and the presence of membranous structures detected by phase-contrast microscopy.

Table 1. *Stability of penicillin induced spheroplasts*

Ten ml. of penicillin spheroplasts prepared in sucrose-supplemented medium were centrifuged at 2000 *g* for 30 min. and resuspended in equal volumes of the different solutions.

Suspending medium (M)	Providence NCTC 9211	*Proteus mirabilis* 13	*Escherichia coli* B
Sucrose, 0·5	0·52*	0·48	0·64
Water	0·16	0·14	0·095
Streptomycin sulphate, 10^{-3}	0·49	0·32	0·60
$CaCl_2$	0·34	0·26	0·36
Spermine, 10^{-3}	0·29	0·155	0·45
Spermine, 10^{-4}	0·50	0·42	0·16
Spermine, 10^{-5}	0·24	0·13	0·08
$MgCl_2$, 10^{-3}	0·31	0·24	0·32
NaCl, 10^{-2}	0·26	0·16	0·27
Nutrient broth	0·29	0·14	0·11
Sucrose, 0·5†	0·38	0·36	—
Nutrient broth†	0·38	0·36	—

* Extinction determined at 650 mμ within 10 min. after suspension.
† Spheroplasts produced in unsupplemented nutrient broth.

Electron microscopy

A section of a penicillin-induced spheroplast of Providence strain NCTC9211 is shown in Pl. 1, fig. 1. The cell contents are surrounded by two superimposed integuments identical in appearance. The innermost layer adheres closely to the cytoplasmic mass and has the triple layer structure of a unit membrane (Robertson, 1959). This layer corresponds to the cytoplasmic membrane in *Escherichia coli* (Kellenberger & Ryter, 1958). The outer triple layer is located in a position corresponding to the cell wall of *E. coli* and *Proteus mirabilis* (Hofschneider & Martin, 1968). Areas where the outer triple membrane is stripped from the inner membrane can be seen. No structural differences were detected between penicillin spheroplasts prepared in sucrose-supplemented or unsupplemented medium. In Pl. 1, fig. 2, bacteriophage PL25 is adsorbed to spheroplasts of the Providence strain. Similar results were obtained in the *P. mirabilis* 13—phage 13*vir* system.

DISCUSSION

The spherical bodies of Providence and *Proteus mirabilis* described here correspond to spheroplasts on grounds of morphology and osmotic sensitivity. Lysozyme in the presence of the chelating agent EDTA converted cells of Providence NCTC9211 to spheroplasts but *P. mirabilis* 13 was more resistant to the combined action of these reagents. This suggests differences in the cell walls of these closely related species (Coetzee, 1963*b*). Glycine and penicillin were equally efficient in the production of spheroplasts in the Providence and *P. mirabilis* strains. These results are in agreement with the postulate of Park (1958) that both glycine and penicillin inhibit synthesis of cell-wall material in a manner which causes accumulation of uridine-5′-pyrophosphate derivatives. The reason why Proteus and Providence spheroplasts can be prepared in nutrient broth unsupported by sucrose is obscure. No morphological differences were detected between spheroplasts prepared in sucrose-supplemented or plain nutrient broth. Preparation in the latter medium may select for a different type of cell which is less fragile. The *P. mirabilis* and Providence strains differ in this respect from *Escherichia*

coli and this behaviour may indicate that cell walls of the former organisms are less affected by penicillin than those of *E. coli*.

The mechanism of stabilization of spheroplasts by spermine is not clear, but Mager (1959) suggested that binding of the negatively charged polyamine molecules is a prerequisite for its action.

Electron micrographs did not reveal morphological differences in spheroplasts obtained by the lysozyme-EDTA, penicillin or glycine methods. They demonstrated the presence of two triple layers similar in appearance. The outer layer corresponds in position to the cell wall of normal bacteria. Electron micrographs also demonstrate the adsorption of bacteriophages to penicillin spheroplasts. This indicates that these spheroplasts retain receptor sites for bacteriophages and suggests that only the mucoprotein layer is affected during penicillin treatment. The adsorption of phages to penicillin spheroplasts supports the concept that penicillin prevents synthesis of mucopeptide (Duguid, 1946) but does not affect the lipoprotein and lipopolysaccharide which contain specific receptor sites for phages (Burnet, 1934; Jesaites & Goebel, 1952; Oram & Reiter, 1968). Oram & Reiter (1968) have, however, demonstrated the inactivation of phage *ml3* by the plasma membrane of *Streptococcus lactis* strain ML3 while the cell wall did not possess inactivating properties.

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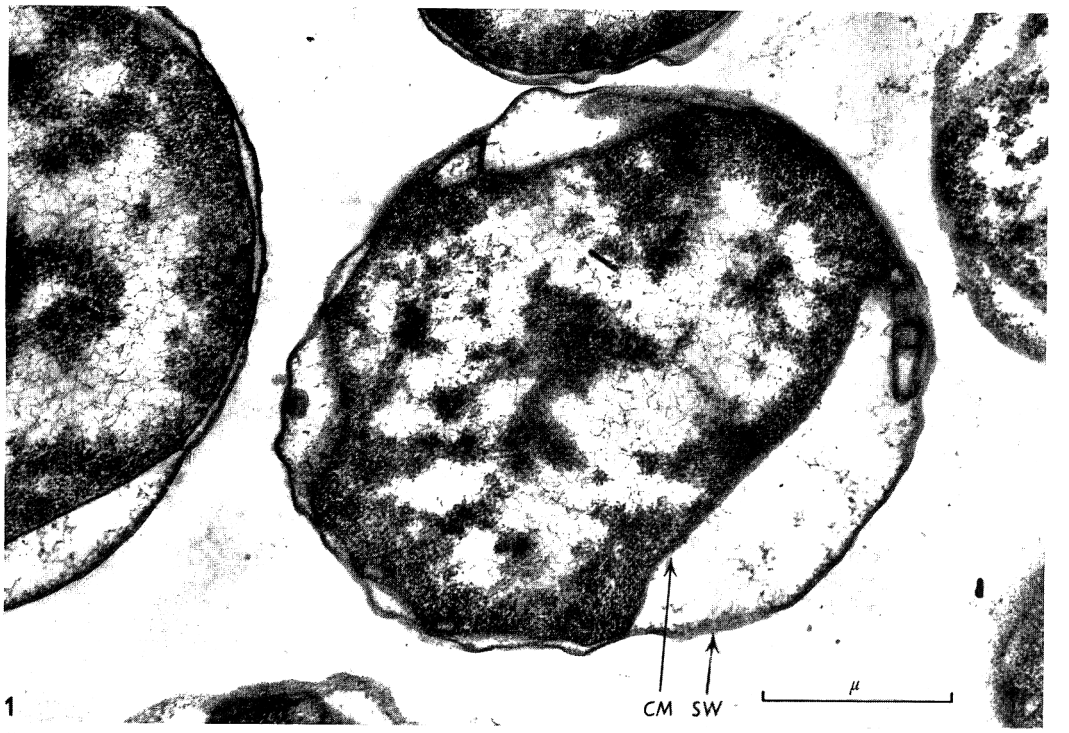
EXPLANATION OF PLATE

Fig. 1. Ultrathin section of a penicillin spheroplast of Providence NCTC 9211. *SW*, Spheroplast wall; CM, cytoplasmic membrane. The bar represents 1 μ.

Fig. 2. Adsorption of phage PL25 to penicillin spheroplasts of Providence NCTC 9211. The bar represents 1 μ.



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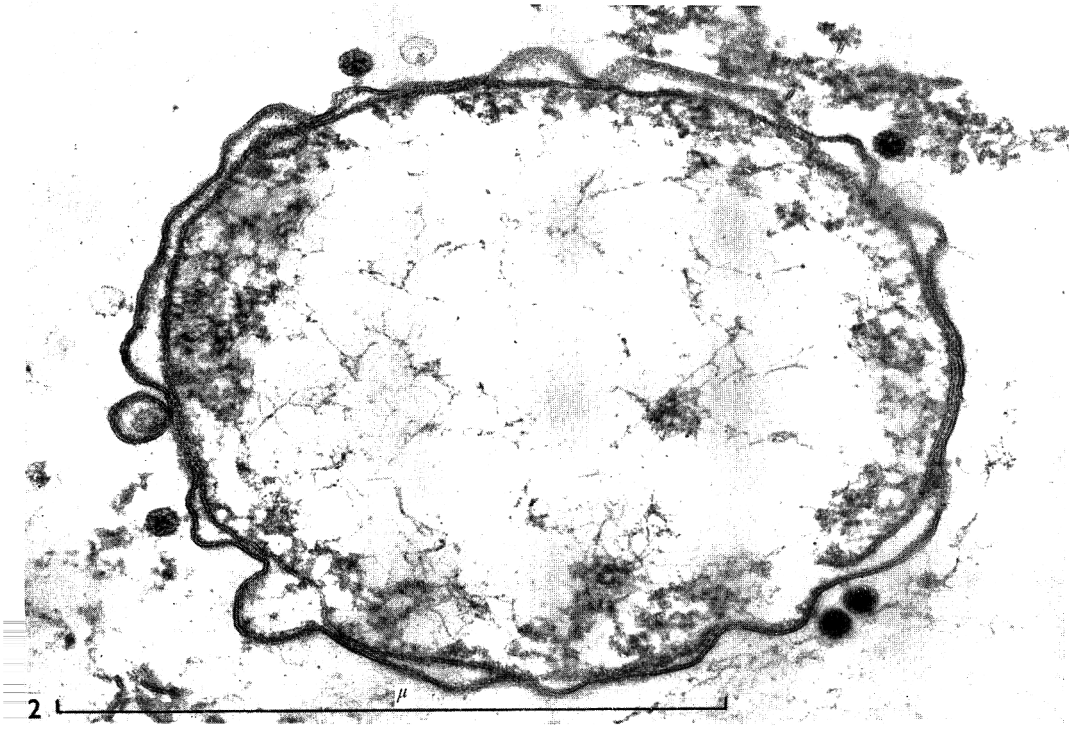

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(*Facing p.* 264)