Volume 63, Part 3, November 1970

# The Journal of General Microbiology



CAMBRIDGE UNIVERSITY PRESS
For the Society for General Microbiology

# THE JOURNAL OF GENERAL MICROBIOLOGY

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# THE JOURNAL OF GENERAL MICROBIOLOGY

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JEREMY BENTHAM (1748–1832)

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

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

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

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*Journal of General Microbiology* (1970), **63**, 269–280
*With 6 plates*
*Printed in Great Britain*



# Nuclear Behaviour and Ascospore Delimitation in *Xylosphaera polymorpha*

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(*Accepted for publication 3 August 1970*)

SUMMARY

Nuclear behaviour and ascospore delimitation in *Xylosphaera polymorpha* was studied by light and electron microscopy. Electron-dense bodies occurred near nuclei at all stages from crozier formation to the development of young ascospores. It is suggested that these bodies be termed archontosomes. Ascospores were delimited by the invagination of an ascus vesicle and the archontosome–astral-ray complex participated in both the delimitation and orientation of the ascospores. The possible role of this complex in determining ascospore form is discussed.

## INTRODUCTION

The cytology of the ascus has been extensively studied with the light microscope but comparatively few investigations have been made with the electron microscope. The presence of nuclear beaks and astral-ray–'centrosome' complexes and their participation in ascospore delimitation was first reported by Harper (1897). He suggested that the delimiting membranes were formed by the lateral and end-to-end fusion of the astral rays which curved around the nuclei. Some of Harper's claims have been refuted and other mechanisms of ascospore delimitation suggested (Beckett, 1966; Carroll, 1966; Reeves, 1967). Recent observations with the electron microscope on sections of potassium permanganate-fixed material have shown that in several genera of Discomycetes ascospores are delimited by the invagination around individual nuclei of an ascus vesicle composed of two unit membranes (Bracker, 1967; Carroll, 1967; Reeves, 1967; Oso, 1969). Schrantz (1967) confirmed the presence of nuclear beaks, 'centriolar plaques' and astral rays in formaldehyde-fixed preparations of the Discomycete *Pustularia cupularis*. He concluded that the spores were delimited by a vesicle formed from endoplasmic reticulum rather than by the fusion of astral-ray microtubules, but otherwise Schrantz made no reference to the possible role of the 'centriolar plaques' and astral rays during spore delimitation. Carroll (1966) suggested that the astral rays around the nuclei might act as physical barriers to the infolding delimiting membranes. Bracker (1967) suggested that astral rays might direct the pattern of invagination by pulling in the vesicle membrane from the ascus periphery. The present paper reports on the behaviour and function of these various structures during ascus development and spore delimitation in the Pyrenomycete fungus *Xylosphaera polymorpha* (Persoon ex Merat) Dumortier (= *Xylaria polymorpha*) and attempts to resolve some of the confusion about the terminology of these organelles.

## METHODS

### *Light microscopy*

Stromata which contained perithecia of various ages were cut into thin slices, immersed in 1:3 (v/v) propionic acid:ethanol solution, and fixed as described by Beckett & Wilson (1968). The slices were then placed in 70 % ethanol for 20 min., rinsed briefly in distilled water, hydrolysed in 1 N-HCl at 60° for 3 to 7 min., washed in several changes of distilled water and finally stained in propiono-carmine (Beckett & Wilson, 1968). Preparations were viewed and photographs taken with a Zeiss photomicroscope.

### *Electron microscopy*

Stromata were cut into slices while immersed in cold fixative. The fixative used was a mixture of equal volumes of 3 % glutaraldehyde and 3 % acrolein both in 0·1 M-sodium cacodylate buffer with sucrose added (final molarity of 0·3), and a final pH of 7·0. Material was fixed for 20 to 60 min., the first 10 min. being under vacuum, washed in several changes of buffer, post-fixed in 2 % osmium tetroxide in 0·1 M-sodium cacodylate buffer, pH 7·0, at 4° for 12 to 16 h., washed again in buffer, dehydrated in a graded ethanol/water series, soaked in propylene oxide and embedded in Epon. Sections were cut on an LKB ultramicrotome, stained with uranyl acetate followed by lead citrate (Reynolds, 1963) and viewed with an AEI EM6B electron microscope.

## RESULTS

### *Light microscopy*

Amorphous structures were seen near the pre-fusion nuclei in the ascogenous hyphae and croziers (Pl. 1, fig. 1, arrows). These structures, which will be called archontosomes, stained less densely than did the nuclei, and one archontosome was associated with each nucleus. After karyogamy the ascus elongated and the fusion nucleus entered meiotic prophase. During pachytene and diplotene the elongated, paired chromosomes became spread out within a large elongated (*c.* 8 to 10 $\mu$m.) nucleus. There was also a corresponding division and migration of the archontosomes resulting in the appearance of one at either end of the nucleus (Pl. 1, fig. 2, arrows). Archontosomes viewed with the light microscope closely resembled the 'centrosomes', or 'centriolar plaques', found in many Ascomycetes (e.g. Singleton, 1953; Carr & Olive, 1958; Robinow & Marak, 1966; Lu, 1967; Beckett & Wilson, 1968). One edge of the archontosome often appeared to be more heavily stained than the rest (Pl. 1, fig. 2, top arrow).

Metaphase I was characterized by the presence of a longitudinal spindle, the poles of which were located at the densely stained, rod-like archontosomes (Pl. 1, fig. 3). Both archontosomes appeared to be orientated transversely with respect to the spindle and the ascus, and their lightly stained, amorphous parts were not visible at this stage. During anaphase I (Pl. 1, fig. 4) there was an indication of the presence of astral rays radiating from the vicinity of the upper archontosome (Pl. 1, fig. 4, arrow). Archontosomes were seen at all stages of divisions II and III in the ascus and were identical with those described above.

Each interphase nucleus at the end of division III possessed a single archontosome

situated beneath the nucleus at the tip of the so-called nuclear beak (Pl. 1, fig. 5). In Pl. 1, fig. 5 the rod-like archontosome is readily seen in the upper two nuclei. Sometimes asci were seen in which the eight nuclei were disposed in a biseriate arrangement (Pl. 1, fig. 6). No delimiting membranes were detected at this stage with the light microscope. Two features (Pl. 1, fig. 7) caused newly delimited spores to be visible with the light microscope: a thin, dense line around each nucleus and a densely stained rod-like structure 2·5 to 3 $\mu$m. long, pointing towards the base of the ascus and extending between the nucleus and the enclosing membrane(s). Later, the outline of the young spores was distinct, the rod-like structure no longer visible and a dense curved archontosome apparent at the base of some of the spores (Pl. 1, fig. 8, arrows).

*Electron microscopy*

Sections through the very young crozier cell showed the pre-fusion nuclei lying side by side, each associated with a single electron-dense amorphous archontosome about 1·4 to 1·6 $\mu$m. long (Pl. 2, fig. 9). At this stage there was no evidence of a differentiation of the nuclear membrane adjacent to the archontosome. The ensuing diploid nucleus was associated with a similar archontosome (Pl. 2, fig. 10), and in some young asci at this stage microtubules were seen between the archontosomal material and the nuclear envelope (Pl. 2, fig. 11).

As expected from light microscopy the meiotic prophase nucleus was elongated parallel to the long axis of the ascus. At pachytene dense synaptinemal complexes occurred (Pl. 2, fig. 12; Pl. 3, fig. 14), the archontosome region often extended for a considerable distance around the nuclear envelope (Pl. 2, fig. 12), and the nuclear membrane adjacent to the archontosome material was differentiated from that elsewhere by an increase in density (Pl. 2, fig. 12, arrows). Large blebs, or blow-outs, of the nuclear envelope were also characteristic of the prophase fusion nucleus. A deposit of electron-dense material was associated with well-developed blebs, around and along the membrane surrounding them (Pl. 2, fig. 13). Strands of endoplasmic reticulum were sometimes seen lying close to a nuclear bleb. At anaphase I the nucleus was irregularly lobed and the poles of the intranuclear spindle were occupied by an archontosome which then appeared as an electron-dense band of material situated within a characteristic indentation of the nuclear membrane (Pl. 3, fig. 15, 16). Spindle microtubules extended through the nucleoplasm to the chromosomes. Plate 3, fig. 16 shows at least three separate tubules ending at what is apparently a 'ball-and-socket type' kinetochore embedded within a chromosome. Longitudinal sections of astral-ray microtubules can be seen radiating into the ascus cytoplasm on the outside of the archontosome.

No signs of the formation of an ascus vesicle were seen either at this stage or at anaphase II (Pl. 3, fig. 17). At all of the three divisions in the ascus the archontosomes were closely associated with astral-ray microtubules, but at division III these microtubules appeared to be developed to a greater extent (Pl. 3, fig. 18). In some sections large bands of ray microtubules about 3 to 4 $\mu$m. long were seen in the ascus cytoplasm. A frequent feature at metaphase III, and illustrated in Pl. 3, fig. 18, was the asymmetrical location of the spindle and metaphase plate with respect to the centre of the nucleus. This asymmetry resulted in a very short spindle, the length of which was almost entirely occupied by the chromosomes. During nuclear division III an ascus vesicle was assembled at the periphery of the ascus. Both nuclear division and

development of this vesicle appeared to proceed in a basipetal sequence. Plate 3, fig. 18 shows a longitudinal section through the apical nucleus of a tetranucleate ascus. Part of the ascus vesicle can be seen on the left immediately inside the plasmalemma, but there was no sign of the vesicle membrane immediately below this region of the ascus. The three lower nuclei in the ascus had yet to enter metaphase of division III.

Plate 4, fig. 19 shows a longitudinal section through another tetranucleate ascus in the region of the two lower nuclei, which appear to be still at interphase following division II. In the basal nucleus of this pair seen in another section (Pl. 4, fig. 20), nuclear blebs were seen separated from the nuclear envelope as well as in a very early stage of formation from it (arrows). Large amounts of endoplasmic reticulum were nearby though assembly of the ascus vesicle membranes had not yet begun. In section the ascus vesicle membranes showed the typical tripartite unit membrane configuration with an asymmetry caused by the inner dense layers of each membrane being thicker than the outer ones (Pl. 4, fig. 21). A narrow band of electron-dense material lined the outer surface of each membrane. Plate 4, fig. 22 shows a tangential longitudinal section of an early stage in the envelopment of nuclei by the ascus vesicle. Astral-ray microtubules in both longitudinal and transverse section show in the cytoplasm. They extend towards the vesicle membranes, which in the upper part of the micrograph have invaginated from the periphery towards the centre of the ascus. In a median longitudinal section (Pl. 4, fig. 23) the nuclear beak can be seen and the archontosome appears as a curved bar of electron-dense material outside the tip of the beak. The archontosome and its astral-ray microtubules lie at the base of the young spore initial, i.e. pointing towards the base of the ascus.

A conspicuous feature at this stage is the electron-dense material surrounding each astral-ray microtubule (Pl. 4, fig. 23; Pl. 5, fig. 24, 25, 26, 27). A longitudinal section through part of an ascus at an early stage in spore delimitation is seen in Pl. 5, fig. 24. In two of the spore initials the archontosome–astral-ray complex can be seen on the upper side of the nucleus, while in the other two the astral rays are seen in cross section on the lower side of each nucleus. Another section of the same ascus (Pl. 5, fig. 25) shows two apparently delimited spores with astral rays near the base of each. Plate 5, fig. 26, shows a longitudinal section through part of an ascus at a later stage when the young spores were fully delimited. The archontosome–astral-ray complex shows at the base of each spore. Since each archontosome was associated with the nuclear beak, clearly seen in the upper two spores, this arrangement resulted in a distinct orientation of both the nucleus and the spore. In many asci the delimited spores were biseriate and the archontosomes then lay side by side at the bases of adjacent spores (Pl. 5, fig. 27). The fibre body, seen within the nucleus of the young spore in Pl. 5, fig. 27, was a common feature. Details of its structure and possible function will be reported elsewhere.

Soon after their delimitation the spores elongated and wall material was laid down between the two membranes, of which the inner formed the plasmalemma of the spore and the outer the investing spore membrane (Pl. 6, fig. 28, 29). At this stage in spore development the base of each spore became characteristically rounded and slightly bulbous as seen in longitudinal section. Within the base an electron-dense archontosome then lay beneath the nucleus and approximately 2 to 5 $\mu$m. from it depending on the degree of spore elongation. Numerous microtubules ran through the spore cytoplasm from the archontosome towards and to the side of the nucleus. These sometimes

measured 3 to 4 $\mu$m. in one plane of section. Neither the archontosome nor the microtubules appeared to be connected to the nuclear membrane and there was no sign of the differentiated indentation of the nuclear envelope at the lower end of the nucleus. An archontosome was, however, apparent towards the upper end of the nucleus (Pl. 6, fig. 28, arrow), but no microtubules were present at this point.

## DISCUSSION

### *Archontosomes*

The term archontosome (Greek, meaning 'organizing' or 'controlling body') is proposed for the following structures: (1) the amorphous, electron-dense structure situated near to each nucleus in the young crozier; (2) the amorphous structure associated with the differentiated nuclear envelope at meiotic prophase; (3) the rod-like body, lying in an indentation of the nuclear envelope, which is located at the pole of the spindle at metaphase and anaphase of all nuclear divisions; (4) the curved bar of dense material which lies near the nuclear beak at interphase III and during spore delimitation; (5) the electron-dense structure associated with a band of microtubules at the base of the developing spore.

Our light and electron micrographs establish that all these 'structures' are the same organelle.

There is considerable confusion regarding the terminology of this organelle in Ascomycetes and Basidiomycetes, because the terms used ('centriole', 'centriolar plaque' and 'centrosome') are ones which by definition relate to a body or organelle with a precise structure and function at a specific time in the nuclear cycle of the cell. Went (1966) showed that centrioles and centrosomes could be defined on the basis of their structure and function. Electron-microscope studies have shown that from a structural aspect the term centriole should be used for the tubular organelle composed of a ring of nine triplet microtubules which are radially tilted in a clockwise direction when viewed from the proximal end. On a functional basis the centriole is the polarizing body of the mitotic spindle in aquatic Phycomycetes, and also constitutes at least part of the kinetosome at the base of the flagella in the motile stages of these fungi. Cleveland (1963) appears to consider the term centriole valid for any organelle which forms the achromatic figure (astral-ray–spindle complex), irrespective of its structure. Structurally, a centriole has never been convincingly demonstrated in either the Ascomycetes or Basidiomycetes. The report of a so-called central body in yeast cells by Lindegren, Bang & Osmui (1965) is not conclusive. The association of centrioles with flagella cannot be demonstrated in Ascomycetes or Basidiomycetes since they have no flagellate stages. It seems therefore that the only basis for suggesting that a centriole occurs in the higher fungi is that there is a structure situated at the poles of the spindle during nuclear division in these fungi.

The term centriolar plaque was first used by Robinow & Marak (1966) to describe the bipartite, electron-dense, structure situated within an invagination of the nuclear envelope at the poles of the intranuclear fibre apparatus in Saccharomyces. This is an unfortunate term both with Saccharomyces and with Xylosphaera since the structure is neither associated with a flagellum nor of 'centriolar' form. In addition, in Xylosphaera it is strictly a plaque only when constituting the poles of the spindle at metaphase and anaphase of nuclear division.

Centrosomes have been known (Cleveland, 1963) since the studies of Boveri on animal cells. He defined these organelles as large hyaline bodies situated at the poles of the mitotic spindle. In 1901 Boveri (cited in Cleveland, 1963) redefined centrosomes as hyaline bodies which surround the smaller, deeply staining centriole(s), and modern definitions generally adhere to this later usage (De Robertis, Nowinski & Saez, 1965; Brown & Bertke, 1969).

Both light and electron microscopy reveal in Ascomycetes an amorphous body associated with the nucleus in the croziers and in the developing ascus. When stained, this body is dense and does not contain a centriole at its centre. It does, however, replicate and appears to be intimately involved with the organization of both astral-ray and spindle microtubules and further plays a part in ascospore delimitation and development. Cleveland (1963) states that the centrosome plays no part whatever in the production of the achromatic figure or of any other organelle and is not autonomous. He also states that the centrosome is produced anew by the centriole in each cell generation. If one accepts that centrioles do not exist in Ascomycetes and Basidiomycetes, it is unlikely that a centrosome could occur in their absence.

It is suggested that the dense body found near nuclei in Xylosphaera is not adequately described by any of the above terms, and that a more suitable one is required for this organelle in Ascomycetes and if necessary in Basidiomycetes. It is suggested that the term archontosome be adopted. It is defined as an amorphous, electron-dense body near the nucleus which is associated with the astral-ray and spindle microtubules and later the microtubules concerned with spore delimitation and development. At some stages in the cell it resembles the centriolar plaque (*sensu* Robinow & Marak, 1966) in both form and behaviour and performs the function of a centriole during nuclear division. However, at no stage does the archontosome possess the structure of a centriole. The development and behaviour of the archontosome in Xylosphaera is summarized in Fig. 1.

Differences in the shape of this organelle have been reported for numerous Pyrenomycete fungi (Colson, 1934; Singleton, 1953; Heslot, 1958; Carr & Olive, 1958; Lu, 1967) and have usually been regarded as characteristic for the different genera concerned. Beckett & Wilson (1968) showed that the 'centrosome' in *Podospora anserina* was seen with the light microscope as a disc at division I, a rod at divisions II and III, and as a plate at the nuclear beak and in spore-delimitation stages. The present work confirms the fact that the archontosome does indeed change its shape and further suggests that this is a sequential development linked with its role at the various stages in the cytology of the ascus (Fig. 1). Carr & Olive (1958) noted that one edge of the 'centrosome' in *Sordaria fimicola* was thick and deeply stained. They suggested that this edge represented the 'centriole' proper. Lu (1967) reported a similar dense edge on the 'centriole' in *Gelasinospora calospora* and proposed that this 'end-side' was the one associated with the spindle fibres. In Xylosphaera this asymmetry of the archontosome was seen with the electron microscope to result from its association with a differentiated region of the nuclear envelope, and it was at this point that staining was intense. Electron-microscope observations also confirmed that at metaphase and anaphase of meiosis and mitosis this region, which at these stages was plaque-like, was associated on one side with the spindle microtubules, and that astral ray microtubules lay near the outer side of it.

Heath & Greenwood (1970) have shown that in *Saprolegnia ferax* the poles of the

mitotic spindle are marked by the presence of a pair of true centrioles which lie in an indentation of differentiated nuclear envelope, termed a pocket by these authors. They showed that after division of the centrioles daughter pairs separated by migrating around the nuclear envelope, each pair being located within its pocket. Intranuclear spindle microtubules were formed from the inner side of each pocket. In Xylosphaera the centrioles were represented by the amorphous material of the archontosome which similarly became associated with the nuclear envelope, migrated around it and polarized the spindle. Doguet (1960) presented diagrammatically a similar behaviour pattern for the 'centrosome' in *Sordaria fimicola.*

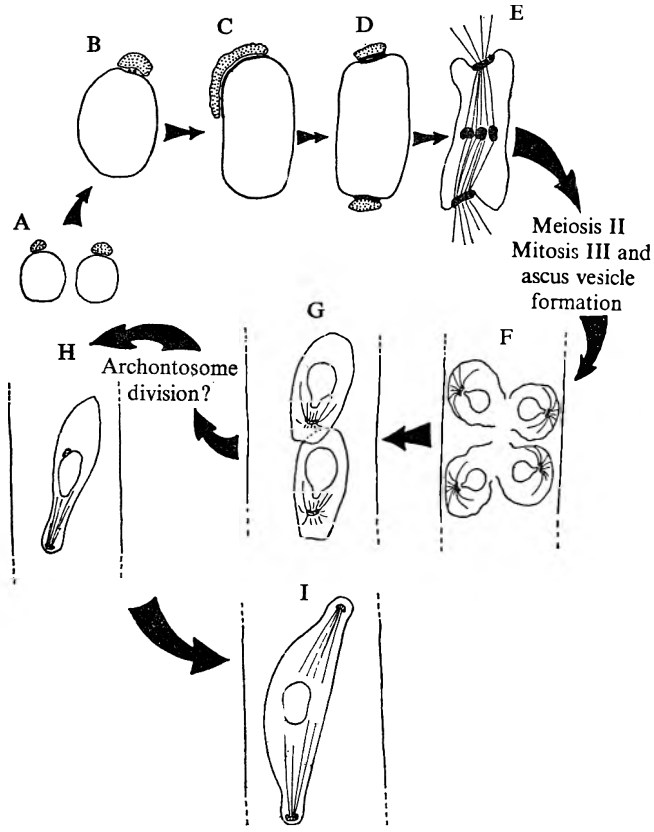



Fig. 1. Semi-diagrammatic representation of the development and behaviour of archontosomes in *Xylosphaera polymorpha.* A, Pre-fusion nuclei with their associated archontosomes. B, Fusion nucleus with one amorphous archontosome. Microtubules occur between the archontosome and the nuclear envelope. C, Pachytene nucleus: migration of archontosome material around the differentiated nuclear envelope. D, Diplotene nucleus: division of the archontosome to form a distinct body at each end of the elongated nucleus. E, Metaphase I: plaque-like archontosomes located at the poles of the intranuclear spindle. Astral rays occur near the outside edge of the archontosomes. F, Ascospore delimitation by the inpulling of an ascus vesicle. G, Reorientation of archontosomes and astral rays within the spore initials. H, Participation of archontosome and microtubules in determining ascospore form. I, Possible configuration of archontosomes and microtubules in the mature spore.

*Astral rays*

Astral-ray microtubules occurred near the archontosome in Xylosphaera. The microtubules between the nuclear envelope and the archontosome in the young ascus before it elongated may have been at an early stage in their development. As the ascus developed, the number of astral rays and the size of the archontosome–astral-ray complex increased, reaching a maximum at metaphase–anaphase III and at spore delimitation. Similar observations were reported for *Neurospora crassa* (Singleton, 1953) and *Gelasinospora calospora* (Lu, 1967). It is likely that this reflects the increasing involvement of the complex with the initiation and organization of spore delimitation, an interpretation supported by the observation of Wells (1969) on the cytology of the ascus of *Ascobolus stercorarius*. When the astral-ray complex was fully developed, the microtubules were associated with a deposit of electron-dense material. This might have been an artefact caused by fixation at a low temperature, but Schrantz (1967) found similar material associated with astral rays in *Pustularia cupularis*, his micrographs showing the dense material to consist of granules not unlike ribosomes. Asymmetric spindles, as well as being frequent at metaphase III in Xylosphaera, are apparently common in *Saprolegnia ferax* (Heath & Greenwood, 1970).

*Ascus vesicle*

The process of nuclear blebbing and ascus-vesicle formation in Xylosphaera confirms the original observations by Carroll (1966) on *Saccobolus kerverni* and *Ascodesmis sphaerospora*. The ascus vesicle in Xylosphaera did not appear to be assembled until the prophase III stage had been reached in the ascus, a later stage than that reported for vesicle formation in *Pyronema domesticum* (Reeves, 1967). The basipetal sequence of both ascus-vesicle assembly and nuclear division in Xylosphaera paralleled the sequence in spore maturation in other Pyrenomycete fungi (Beckett, 1966). Bracker (1967) suggested that the ascus vesicle was formed by invagination of the plasmalemma of the ascus. High magnification electron microscopy of the vesicle membranes in Xylosphaera did not support this view. The plasmalemma in Xylosphaera was asymmetrical, the inner of the two osmiophilic layers being denser. An invagination of the plasmalemma would therefore have resulted in the denser of the two osmiophilic layers lying on the outside of the double membrane so formed and not on the inside, as our results showed. The problem of determining the precise origin of membrane systems is a difficult one, and is made more so by the apparent readiness with which membranes change their form according to function. It is possible that the membrane system which delimited ascopores might have been formed from the nuclear envelope, plasmalemma and endoplasmic reticulum, and that membranes from each source underwent changes to become ascus-vesicle membranes.

*The delimitation process*

The involvement of 'centrioles' in ascospore delimitation has been reported by a number of workers but the precise mechanism is uncertain. In Xylosphaera the astral-ray microtubules acted as links between the ascus vesicle and the archontosomes. If these microtubules contracted or depolymerized at the archontosome in much the same way as certain spindle microtubules appear to depolymerize at the spindle pole,

the result might be a pulling in of the vesicle at points coinciding with the nuclei since the archontosomes were at this stage intimately associated with the nuclear beak. This would be consistent with the mechanism suggested by Bracker (1967). The curving of astral-ray microtubules around the nuclei might also have controlled the initial path of invagination and ensured the inclusion of each nucleus within a spore initial.

Correlation of light and electron microscope observations is difficult due to the low resolution of the light microscope and its inability to resolve the membranes at early stages of ascospore delimitation. In Xylosphaera the nuclear-beak stage (Pl. 1, fig. 5, 6) probably represented a stage at which the archontosome–astral-ray complex had undergone reorientation within spore initials which had already been delimited by the double membrane system and was equivalent to the stages shown in Pl. 4, fig. 23; Pl. 5, fig. 26, 27. Reorientation of the archontosome–microtubules complex at the base of young spores is understandable in view of its participation in determining ascospore shape. Several light microscopists have claimed that 'centriole'–astral-ray complexes do not exist and that spore delimitation occurs by autonomous cleavage of the ascus cytoplasm. This interpretation may be due to the apparent absence of 'centriole'–astral-ray complexes from unsuitably stained material, or, as suggested by Reeves (1967), ascospores may already have been delimited at the time of observation.

*Ascospore shape*

The densely stained band of material seen with the light microscope in ascospores at the stage at which the spore outline became detectable (Pl. 1, fig. 7) could be seen with the electron microscope to consist of a number of microtubules. These microtubules (*c.* 3 to 5 $\mu$m. in length) were closely associated with an archontosome at the base of the spore and extended from it past the lower end of the nucleus. Spores at this stage had elongated considerably to approximately 10 to 11 $\mu$m. and the nuclei occupied the middle portions. This system of microtubules may have played a part in shaping the spore. The synthesis or polymerization of sub-units to form microtubules at the archontosome might conceivably have pushed the base of the spore initial outwards. Such a mechanism would, however, have required the microtubules either to have been anchored at their upper ends or to have continued right through the spore to the tip. This has not been confirmed but microtubules have been seen to pass on either side of the nucleus. In the related fungus *Rosellinia aquila* microtubules extend for a considerable distance past the nucleus (Beckett & Crawford, unpublished). It is not known whether these microtubules exist merely by persistence of the astral-ray microtubules from an earlier stage in development or whether they are formed anew. The absence of electron-dense material normally associated with astral-ray microtubules is, however, notable.

Sections showing a microtubule system at the apex of the spore have not been obtained, but a slightly constricted projection of the spore similar to that seen at the base of the spore (Pl. 6, fig. 28) is known to occur at a later stage of development. It is possible that the archontosome seen associated with the nuclear envelope on the upper end of the nucleus (Pl. 6, fig. 28) might later have been involved in shaping the apical region of the spore. A similar role was implied by Wells (1956) for what she termed the centriole in ascospores of *Sporormia obliquisepta*. Jones (1926) and Jenkins (1934) noted the sudden appearance of the 'centriole' at one end of the spore initial in

*Ophiobolus graminis* and *Cordyceps agariciformia* respectively, both of which form filiform ascospores. It now seems likely that here also spore shape is determined by a microtubule–archontosome complex.

The authors wish to thank Professor L. E. Hawker, Dr M. F. Madelin and Dr R. Campbell for their helpful advice and discussion during the preparation of this paper. Thanks are also due to Mr M. R. Curnock for technical assistance.

## REFERENCES

BECKETT, A. (1966). *Observation on the structure of hyphae, asci and ascospores, with special reference to Pleurage anserina, using light, phase contrast and electron microscope techniques.* Ph.D. Thesis, University College of Wales, Aberystwyth.

BECKETT, A. & WILSON, I. M. (1968). Ascus cytology of *Podospora anserina. Journal of General Microbiology* **53**, 81–87.

BRACKER, C. E. (1967). Ultrastructure of fungi. *Annual Review of Phytopathology* **5**, 343–374.

BROWN, W. V. & BERTKE, E. M. (1969). *Textbook of Cytology*, p. 565. St Louis: Mosby.

CARR, A. J. H. & OLIVE, L. S. (1958). Genetics of *Sordaria fimicola*. II. Cytology. *American Journal of Botany* **45**, 142–150.

CARROLL, G. C. (1966). *A study of the fine structure of ascosporogenesis in Saccobolus kerverni and Ascodesmis sphaerospora.* Ph.D. Thesis, University of Texas.

CARROLL, G. C. (1967). The ultrastructure of ascospore delimitation in *Saccobolus kerverni. Journal of Cell Biology* **33**, 218–224.

CLEVELAND, L. R. (1963). Function of flagellate and other centrioles in cell reproduction. In *The Cell in Mitosis*, pp. 3–31. Edited by L. Levine. New York: Academic Press.

COLSON, B. (1934). The cytology and morphology of *Neurospora tetrasperma* Dodge. *Annals of Botany* **48**, 211–224.

DE ROBERTIS, E. D. P., NOWINSKI, W. W. & SAEZ, F. A. (1965). *Cell Biology*, p. 229. Philadelphia: Saunders.

DOGUET, G. (1960). Contribution a l'étude du noyau du *Sordaria fimicola. Revue de Cytologie et de Biologie Vegetale* **22**, 109–127.

HARPER, R. A. (1897). Kerntheilung und freie Zellbildung im Ascus. *Jahrbuch für Wissenschaftliche Botanik* **30**, 249–284.

HEATH, I. B. & GREENWOOD, A. D. (1970). Centriole replication and nuclear division in Saprolegnia. *Journal of General Microbiology* **62**, 139–148.

HESLOT, H. (1958). Contribution a l'étude cytogénétique et génétique des Sordariacées. *Revue de Cytologie et de Biologie Vegetale* **19**, 1–209.

JENKINS, W. A. (1934). The development of *Cordyceps agariciformia. Mycologia* **26**, 220–243.

JONES, S. G. (1926). The development of the perithecium of *Ophiobolus graminis* Sacc. *Annals of Botany* **40**, 607–629.

LINDEGREN, G. C., BANG, Y. N. & OSMUI, M. (1965). The central body of the ascomycetes. *Canadian Journal of Genetics and Cytology* **7**, 37–39.

LU, B. C. (1967). The course of meiosis and centriole behaviour during the ascus development of the ascomycete *Gelasinospora calospora. Chromosoma, Berlin* **22**, 210–226.

OSO, B. A. (1969). Electron microscopy of ascus development in Ascobolus. *Annals of Botany* **33**, 205–209.

REEVES, F. (1967). The fine structure of ascospore formation in *Pyronema domesticum. Mycologia* **59**, 1018–1033.

REYNOLDS, E. S. (1963). The use of lead citrate at a high pH as an electron-opaque stain in electron microscopy. *Journal of Cell Biology* **17**, 208–212.

ROBINOW, C. E. & MARAK, J. (1966). A fibre apparatus in the nucleus of the yeast cell. *Journal of Cell Biology* **29**, 129–151.

SCHRANTZ, J. P. (1967). Présence d'un aster au cours des mitoses de l'asque et de la formation des ascospores chez l'Ascomycète *Pustularia cupularis* (L.) Fuck. *Compte Rendu Hebdomadaire des Séances de l'Académie des Sciences* **264**, 1274–1277.

SINGLETON, J. R. (1953). Chromosome morphology and the chromosome cycle in the ascus of *Neurospora crassa*. *American Journal of Botany* **40**, 124–144.

WELLS, D. (1956). Nuclear changes accompanying ascus and ascospore development in *Sporormia obliquisepta*. *American Journal of Botany* **43**, 761–768.

WELLS, K. (1969). Nuclear division in Ascomyetes. *International Botanical Congress* **11**, 235.

WENT, H. A. (1966). The behaviour of centrioles and the structure and formation of the achromatic figure. *Protoplasmatologia VI* G 1, 1–109.

## EXPLANATION OF PLATES

Key to lettering: A = archontosome, AV = ascus vesicle, B = bleb in nuclear envelope, DM = delimiting membrane, ER = endoplasmic reticulum, F = fibre body, K = kinetochore, MT = microtubule(s), MTa = astral-ray microtubules, MTs = spindle microtubules, N = nucleus, NB = nuclear beak, NE = nuclear envelope, SC = synaptinemal complex.

### PLATE 1

Fig. 1 to 8. Light micrographs (× 2000) of *Xylosphaera polymorpha*.

Fig. 1. Crozier cells with pre-fusion nuclei and archontosomes (arrows).

Fig. 2. Meiotic nucleus at diplotene. Archontosomes are seen at the poles of the nucleus (arrows).

Fig. 3. Metaphase–anaphase I. Rod-like archontosomes are at the poles of the spindle.

Fig. 4. Late anaphase I. Radiating astral rays can be detected at the upper pole (arrow).

Fig. 5. Interphase III—ascospore delimitation. Archontosome–astral-ray complexes (arrows) can be seen at the bases of their respective nuclei. No membranes can be detected around the spores at this stage.

Fig. 6. Same stage as fig. 5 but with a biseriate arrangement of spores.

Fig. 7. Post-delimitation. Bands of dense material can be seen in the lower part of several spores (single arrows). The outline of the delimited spore can be determined (double arrow).

Fig. 8. A later stage in spore development. Densely stained archontosomes can be seen at the base of several spores (arrows).

### PLATE 2

Fig. 9. A section through part of a crozier cell. The pre-fusion nuclei can be seen with their associated archontosomes. × 10,000.

Fig. 10. A section through a young ascus showing the fusion nucleus and one archontosome. × 12,000.

Fig. 11. Part of a young ascus. Four microtubules in cross-section lie between the archontosome and the nuclear envelope. × 46,000.

Fig. 12. A longitudinal section through a meiotic nucleus at pachytene. The replication of the archontosome material, its association with, and presumed migration around, the nuclear envelope can be seen at the points marked by arrows. Synaptinemal complexes, in cross-section, are also seen. × 11,250.

Fig. 13. A section through part of a meiotic nucleus showing a well-developed nuclear bleb and what appears to be the initiation of a bleb (arrow). × 30,000.

### PLATE 3

Fig. 14. A longitudinal section through a synaptinemal complex from a pachytene nucleus. × 30,000.

Fig. 15. A longitudinal section through a nucleus at anaphase I. The upper end of the obliquely sectioned spindle is visible. × 10,000.

Fig. 16. Part of the spindle region of the nucleus shown in fig. 15. The archontosome is associated with an indentation of the nuclear envelope. Astral-ray and spindle microtubules are seen to the outside and inside of the archontosome respectively. × 30,000.

Fig. 17. A longitudinal section through the upper nucleus at anaphase II. × 12,950.

Fig. 18. A longitudinal section through the upper nucleus at metaphase III. The ascus vesicle is present to the left of the nucleus. × 12,950.

PLATE 4

Fig. 19. A longitudinal section through part of an ascus in the region of the two lower nuclei at prophase III. Endoplasmic reticulum is abundant but no ascus vesicle can be seen. × 6000.

Fig. 20. A serial section through the lower nucleus shown in fig. 19. One nuclear bleb has separated and another one is being formed (arrows). × 18,000.

Fig. 21. A section through a small portion of the double delimiting membrane. The osmiophilic layers can be seen on the inside of each unit membrane, and electron-dense material is associated with both sides of the double membrane. × 80,000.

Fig. 22. A longitudinal section through the basal region of a delimiting spore. The archontosome is closely associated with the nuclear beak and the astral-ray microtubules indicate the plane of the delimiting membranes. × 32,000.

Fig. 23. A tangential longitudinal section through part of an ascus taken during the initial stages of spore delimitation. Astral-ray microtubules are closely associated with the invaginating ascus vesicle. × 18,000.

PLATE 5

Fig. 24. A longitudinal section through the middle region of an ascus at an early stage of ascospore delimitation. The ascus vesicle invaginates at the points marked by the archontosome–astral-ray complex. × 6000.

Fig. 25. A serial section through the ascus shown in fig. 24. × 7500.

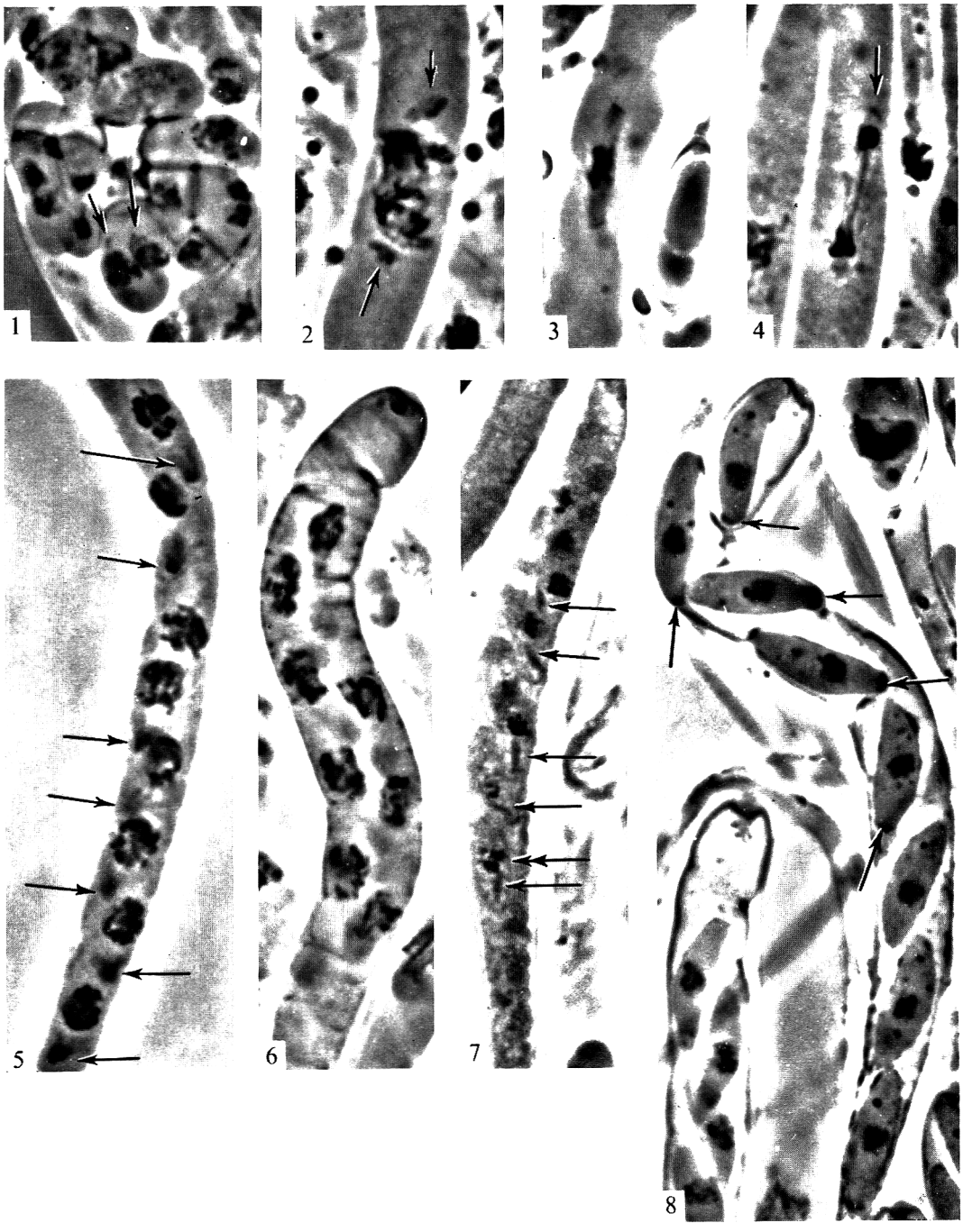
Fig. 26. A longitudinal section through part of an ascus showing four delimited ascospores. Each spore has become orientated so that the archontosome–astral-ray complex lies beneath the nucleus at the base of the spore. × 7500.

Fig. 27. A similar section to that shown in fig. 26 but with biseriate spores. × 15,000.

PLATE 6

Fig. 28. A longitudinal section through a developing ascospore. The archontosome lies at the base of spore and microtubules occur in the cytoplasm between the archontosome and the nucleus. × 10,000.

Fig. 29. A micrograph taken at higher magnification of the region shown within the rectangle in fig. 28. × 36,000.

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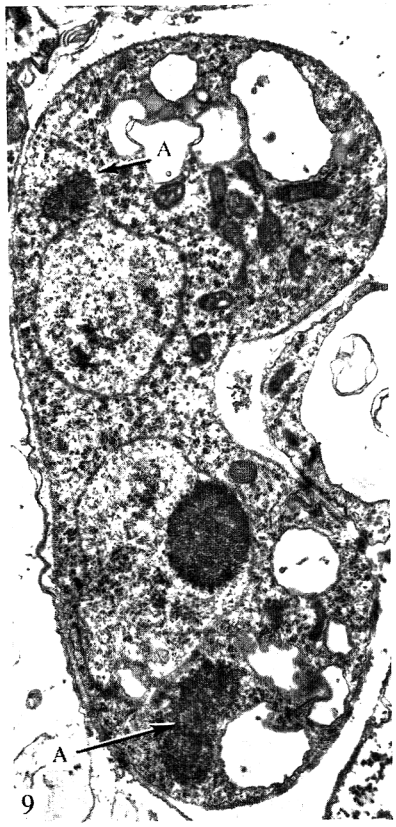

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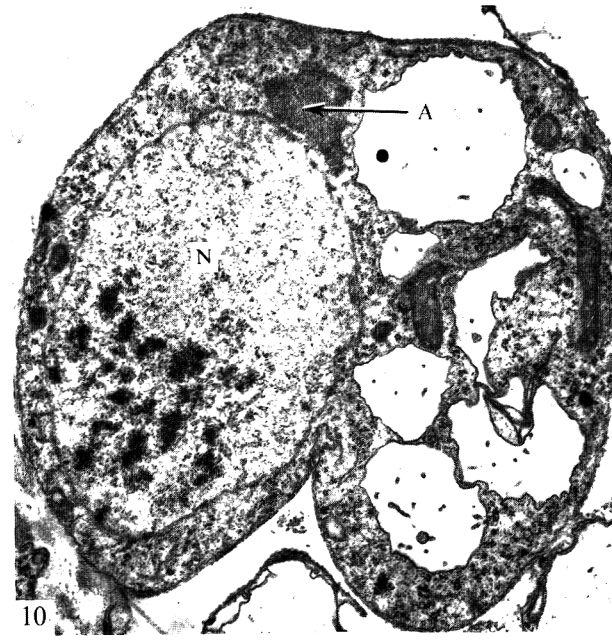

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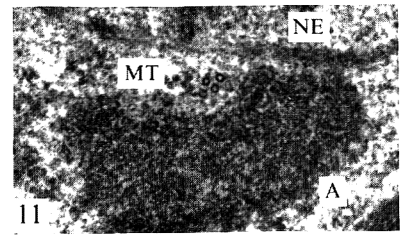

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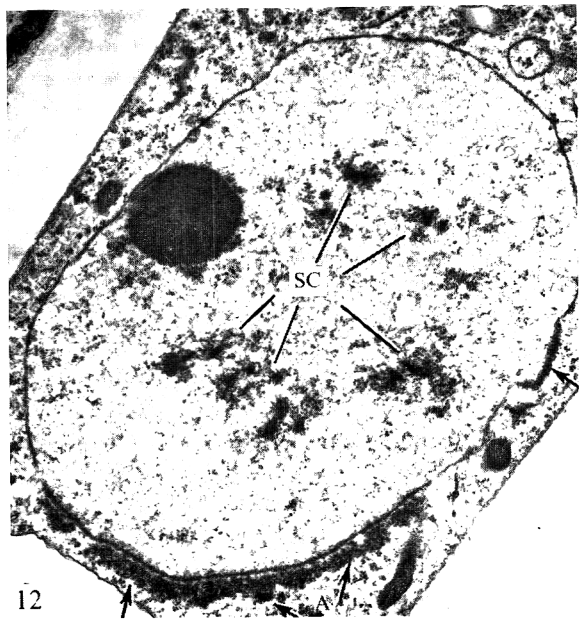

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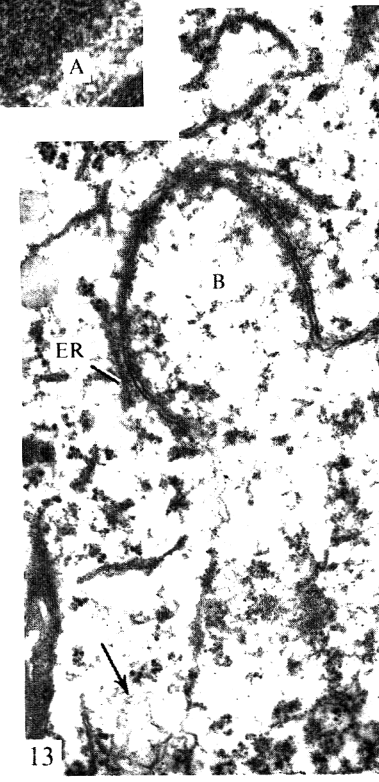

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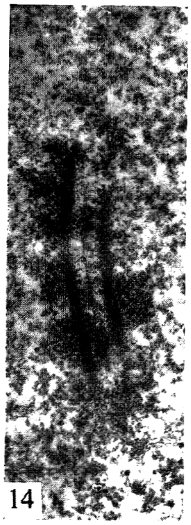
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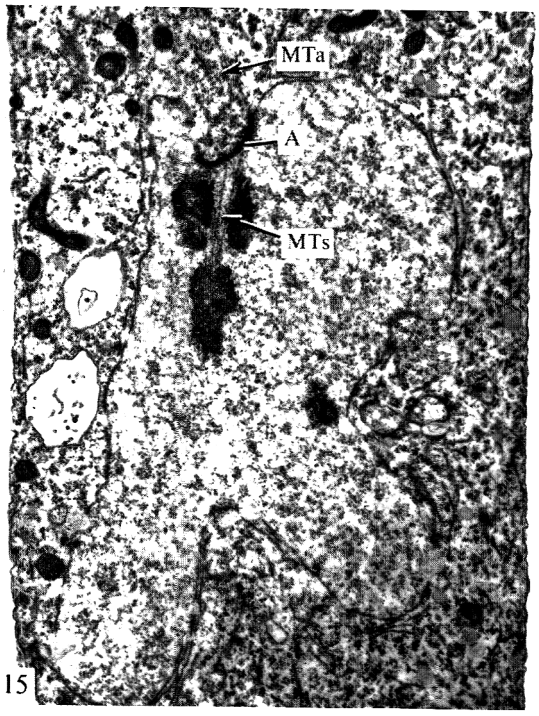

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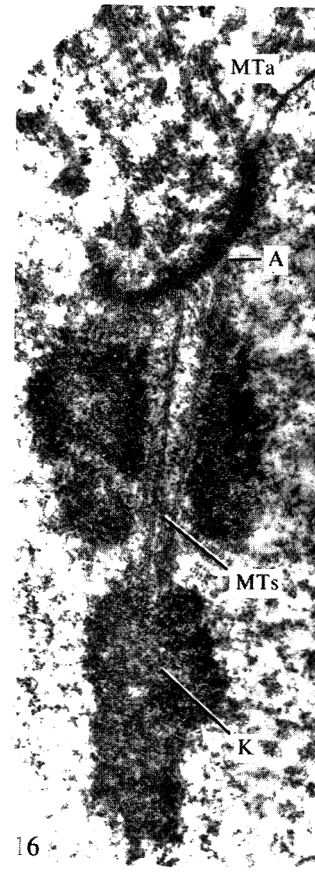

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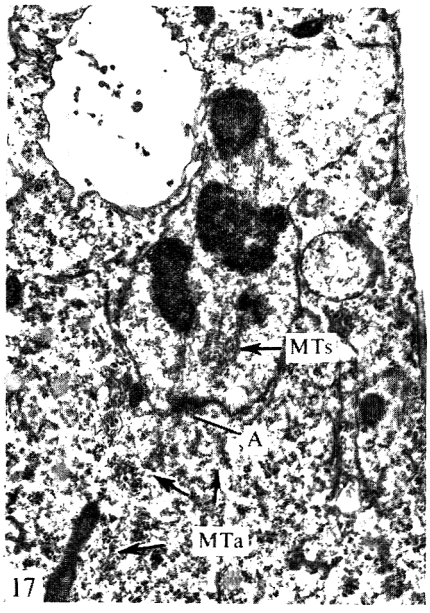

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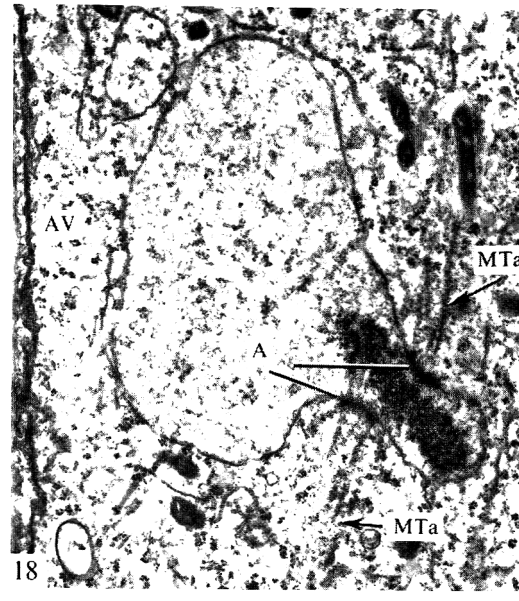

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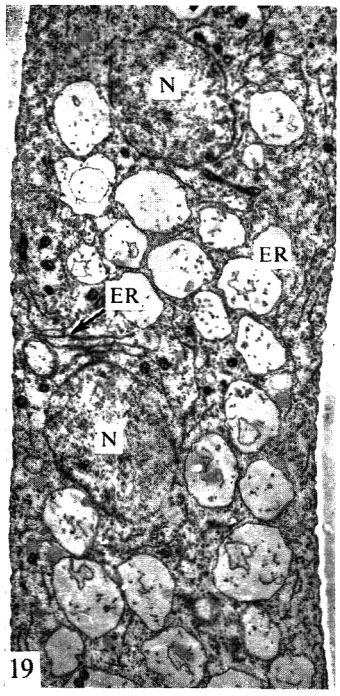


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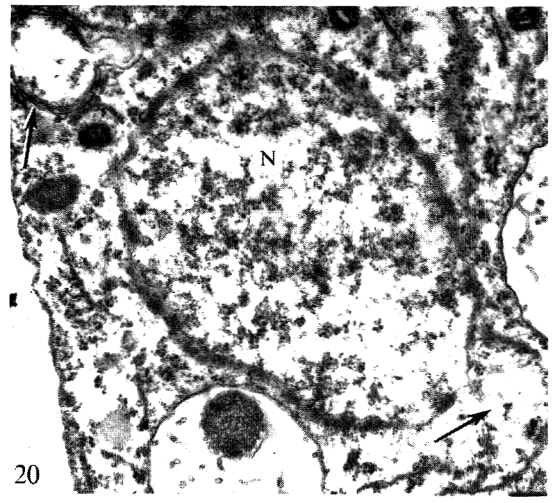


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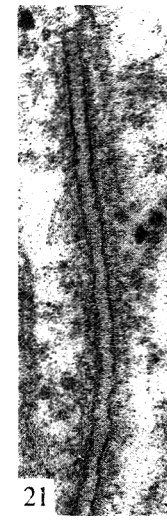

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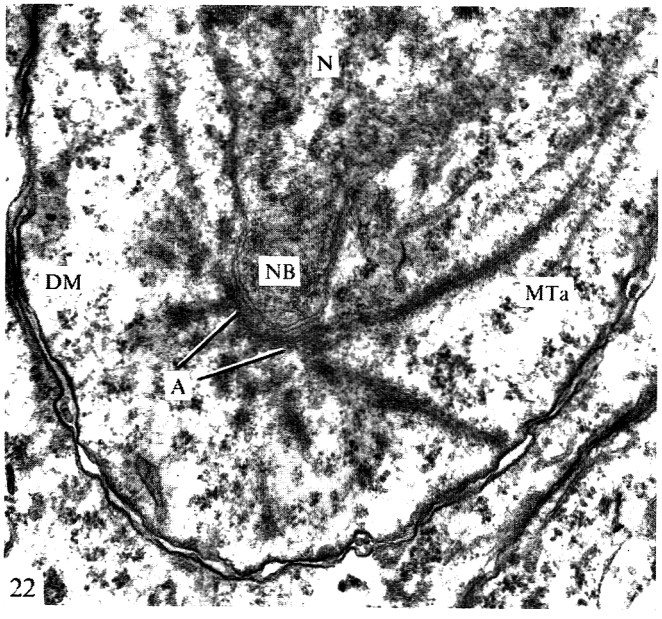


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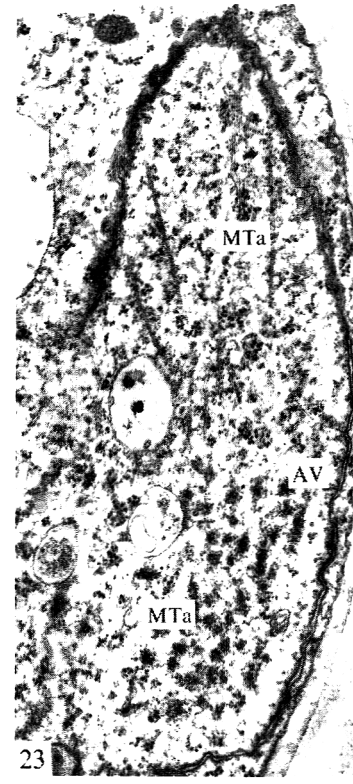


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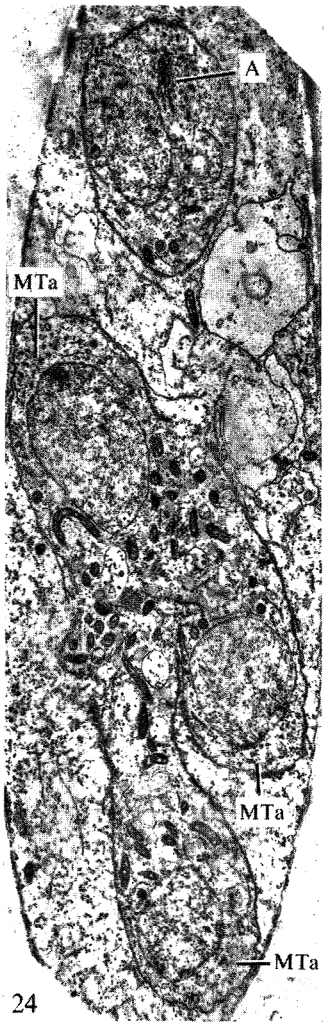


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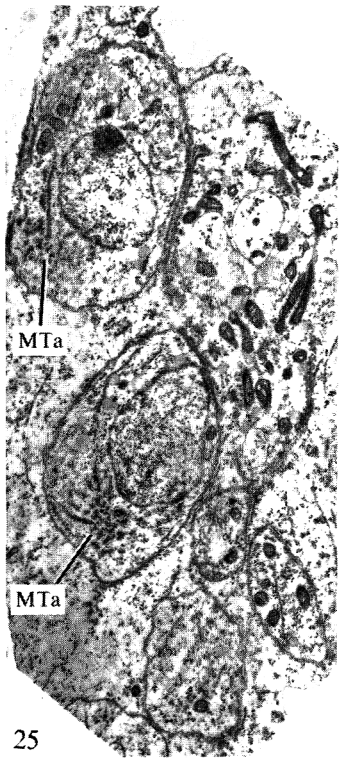


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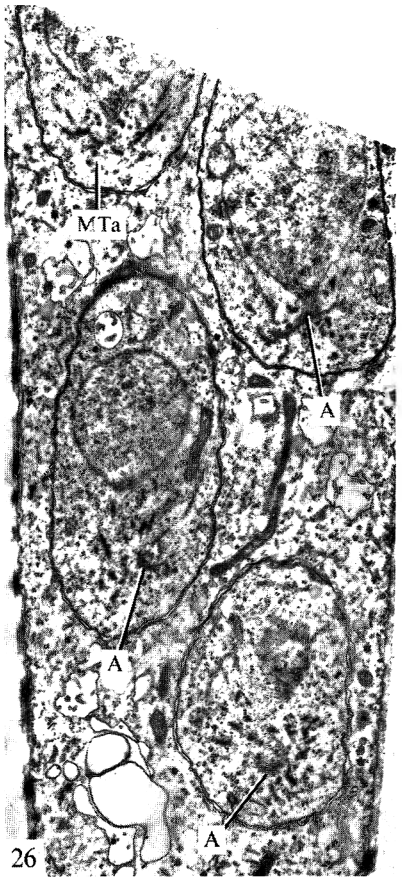


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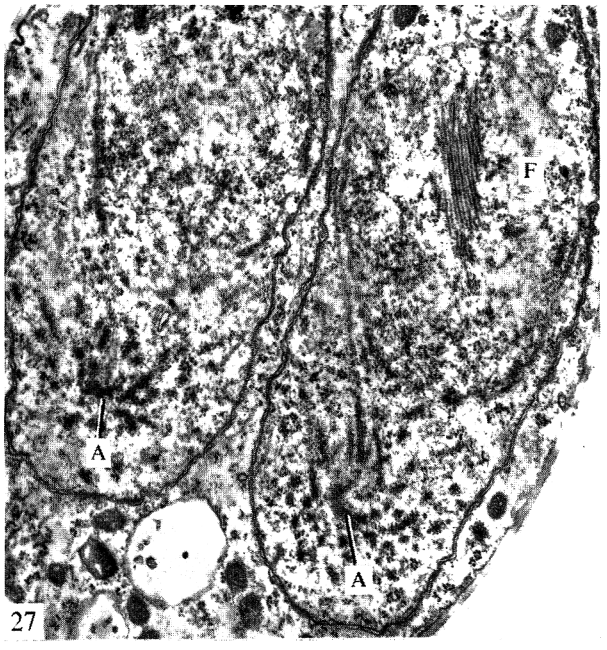


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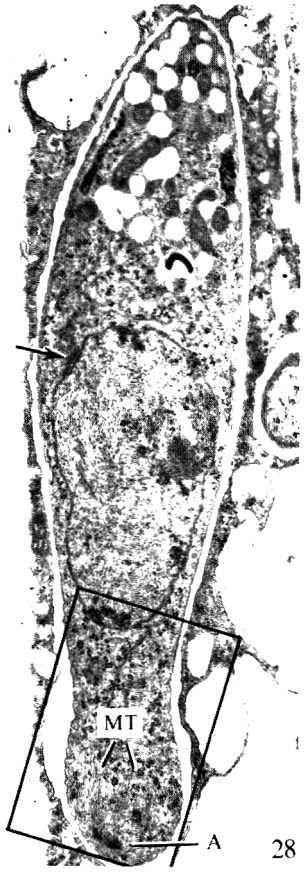


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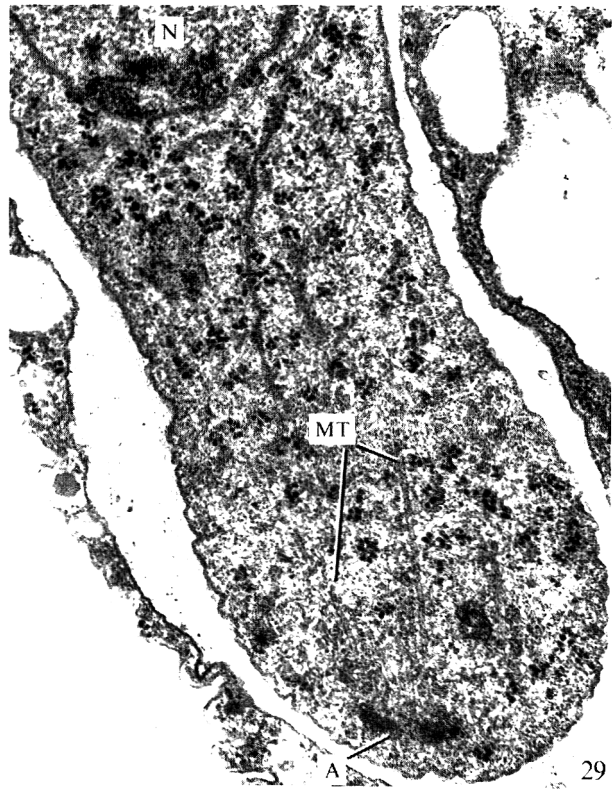


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*Journal of General Microbiology* (1970), **63**, 281–287
*With 2 plates*
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# Electron Microscopic Studies of *Mycoplasma pulmonis* (Negroni Strain)

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(*Accepted for publication 4 August 1970*)

SUMMARY

Human foetal kidney cell cultures and agar and broth media were inoculated with *Mycoplasma pulmonis* (Negroni), a rodent pathogen, and were examined by electron microscopy.

In the tissue cell cultures, abundant extracellular mature mycoplasmas and elementary bodies were seen. The average diameter of elementary bodies was 99 nm. The average long and short axes of mycoplasmas growing on agar measured 800 and 600 nm. respectively.

Whole mounted broth-cultured mycoplasmas consisted of three principal cell types: large pleomorphic cells (up to 1500 nm. in diameter), filamentous forms, and elementary bodies (less than 180 nm. in diameter). Production of elementary bodies by budding from the mature cells was the normal method of multiplication. The life cycle was estimated to be $46 \pm 7$ h. under optimum conditions. Immunoelectron microscopy demonstrated positive reactions only with specific antisera.

## INTRODUCTION

Negroni (1964) reported the isolation of a filterable cytopathic agent from human leukaemic bone marrow. This agent was later proved to be a strain of *Mycoplasma pulmonis*, a rodent pathogen (Grist & Fallon, 1964; Fallon, *et al.* 1965; Butler & Leach, 1966; Sabin, 1967; Somerson, Reich, Chanock & Weissman, 1967). The significance of the relationship between mycoplasma and human leukaemia remains a subject for conjecture (Beale, Christophinis & Negroni, 1968; Fallon & Jackson, 1968).

This report describes the electron microscopic characteristics of the Negroni agent. Fallon & Jackson (1967) noticed serological differences among several strains of *Mycoplasma pulmonis* and emphasized that 'not all strains of *M. pulmonis* are identical, and subtypes of this species exist'. They used ferritin-conjugated anti-Negroni agent antisera, prepared in two separate laboratories, against a conventional strain of *M. pulmonis* and also strain 880, reported to be a strain of *M. pulmonis* (Butler & Leach, 1966; Sabin, 1967; Somerson *et al.* 1967) and a causative agent of human leukaemia (Grace, Horoszewicz, Stim, Mirand & James, 1965).

Mycoplasmas have been considerably confused with certain viruses in the interpretation of electron micrographs, as can be seen in the reports of Negroni (1964) and Inman, Woods & Negroni (1964). This is because of the production by mycoplasmas

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of elementary bodies which closely resemble some virus particles. Greater knowledge of the production of elementary bodies is therefore urgently required, but little work has been done on this. Morowitz & Maniloff (1966) and Furness (1968) have studied the growth cycle of *Mycoplasma gallisepticum* A 5969 and *M. orale* respectively. However, these two species of mycoplasma divide mainly by binary fission, and only one available report, on the development of *M. gallisepticum* S6 (Dutta, Dierks & Pomeroy, 1965), describes reproductive activity similar to that seen in *M. pulmonis* (Negroni).

This paper describes some aspects of the morphology, surface antigenicity and life cycle of the Negroni agent, particularly the mode of production of elementary bodies, as revealed by electron microscopy.

## METHODS

### *Material from tissue culture and agar medium*

Mycoplasma, maintained in broth medium, were inoculated into human foetal kidney cultures. The broth medium used consisted of 70 % (v/v) broth, 20 % (v/v) unheated horse serum, 10 % (w/v) yeast extract, 1 % (w/v) thallium acetate and penicillin (0·1 ml. containing 10,000 units to each ml. medium). Forty-eight h. later any cells remaining on the culture-tube walls were dislodged and the cell suspension was sedimented at 800 *g*. The sediment was fixed in Millonig's buffered phosphate (pH 7·2) containing 2 % (w/v) osmium tetroxide (Millonig, 1962), dehydrated in ethanol, embedded in Maraglas and sectioned. Mycoplasma colonies growing on agar were fixed *in situ*, and thin strips from the surface of the gel were embedded for sectioning. Sections were stained with a 25 % (w/v) solution of uranyl acetate in methanol, followed by Reynolds lead citrate (Reynolds, 1963). The long and short axes of 229 sectioned mature mycoplasma cells in the agar colonies were measured to find the mean cell size.

### *Material from broth medium*

Broth cultures of Negroni strain containing $10^6$ colony-forming units/ml. were incubated at 37°; the number of colony-forming units remained constant until about the fifth day of culture, when it began to decline.

The broth cultures were centrifuged at 26,000 rev./min. in the Spinco rotor 30 for 1 h. at 4°. A 2 % (w/v) aqueous solution of phosphotungstic acid, adjusted to pH 7·1 with potassium hydroxide, was used for negative contrast preparations. In order to spread the phosphotungstate evenly on the grids, approximately 0·05 % (w/v) bovine serum albumin was added to the solution immediately before use. Droplets of the sedimented pellet mixed with phosphotungstate solution were applied to grids bearing carbon support films. The cultures were examined by electron microscopy every day for 10 days. Some specimens were examined without contrast medium.

Some specimens of the mycoplasma were fixed prior to their preparation for negative contrast by a method similar to that employed by De Harven & Friend (1964). Ultracentrifuged pellets from broth culture were resuspended in 3 ml. of 0·12 M-sodium citrate (pH 7·0) which was isotonic with the mycoplasma culture medium. The suspension was squirted into an equal volume of 0·5 % (w/v) osmium tetroxide in Millonig's phosphate buffer (pH 7·2) and resedimented at 25,000 rev./min. in the Spinco rotor 50.

To study the life cycle of Negroni agent, broth cultures containing $10^6$ colony-

forming units/ml. were filtered with a 450 nm. Millipore filter, and then with a 220 nm. filter. The resulting filtrate free of mature mycoplasma was divided into samples for hourly observations by electron microscopy. These filtrates were incubated at 37°. The optimum pH for culture was found to be pH 7·8.

For immunoferritin experiments, a sandwich technique was employed which required only one conjugated antibody. Goat antirabbit globulin was conjugated with ferritin according to the method of Singer & Schick (1961). The test organisms used were *Mycoplasma pneumoniae* (Eaton agent) and the following strains of *M. pulmonis* M1; Cheng; 880; Negroni. Rabbit immune serum globulins were prepared against the above mycoplasmas in the Imperial Cancer Research Fund, except for those against Negroni and 880 strains which were prepared at two different institutes, Imperial Cancer Research Fund, London (L) and Ruchill Hospital, Glasgow (G).

1. Rabbit anti-Negroni (L)
2. Rabbit pre-immunization (L)
3. Rabbit anti-Negroni (G)
4. Rabbit pre-immunization (G)
5. Rabbit anti-880 (G)

Broth cultures of mycoplasma were pelleted and fixed in formal calcium acetate (Douglas, Gottlieb, Strauss & Spicer, 1966). Each of the mycoplasmas listed above was allowed to react with each serum, followed by the conjugate. Controls were set up as follows:

1. Specific sera were followed by unconjugated ferritin alone.
2. Specific serum was omitted and ferritin conjugated antibody was used alone.
3. Unconjugated goat serum was used before the ferritin conjugate, as a blockade.
4. Specific serum was replaced by a saline wash.

Agar and broth cultures of the Negroni strain and human foetal kidney cultures were supplied by Dr G. Negroni, Imperial Cancer Research Fund, London. Rabbit immune sera against mycoplasmas were supplied by Dr Negroni and Dr R. J. Fallon, Ruchill Hospital, Glasgow.

## RESULTS

### *Cultures*

Abundant extracellular mature mycoplasmas were sedimented with the culture cells (Pl. 1, fig. 1). The plasma membrane was approximately 7·5 nm. thick and had a triple-layered unit-membrane structure. It was not possible to decide whether the protein lamellae of the membrane were of equal or unequal thickness. Numerous elementary bodies of average diameter 99 nm. were found scattered among the mature mycoplasmas. They had many of the morphological characteristics of some viruses, namely uniformity of size and shape, a dense core and an outer membrane. The smallest particles among them measured 53 nm. (Pl. 1, fig. 2).

Cells in sectioned, agar-cultivated colonies grew densely in agar and appeared globular in shape. The long and short axes of the organisms had mean lengths of 800 and 600 nm. respectively.

*Examination of whole organisms*

Three principal cell types occurred during the reproductive cycle in broth medium: (*a*) large pleomorphic cells, (*b*) filamentous forms and (*c*) elementary bodies.

Whole organisms, mounted on support films with or without contrast medium, appeared pleomorphic, the largest measuring 1500 nm. in diameter. The organisms exhibited a relatively stout membrane structure, 12·5 nm. maximum thickness, which belied their extreme plasticity. Elementary bodies formed as buds on the surface of mature cells and some began to grow while still attached to the parent cell (Pl. 1, fig. 3). The filamentous forms ramified over areas in excess of 6 $\mu$m. square (Pl. 1, fig. 4). The filaments had an average diameter of 75 nm. and occasionally showed evidence of subdivision into small spheres, believed to be elementary bodies. The elementary bodies measured less than 180 nm. in diameter, the smallest observed being 58 nm. Mycoplasmas which had been exposed to only isotonic conditions and fixed before mounting in negative contrast medium did not differ morphologically from the unfixed specimens. Whereas filaments only occurred intermittently under normal broth culture conditions, exposure to mildly adverse conditions, e.g. 4 to 5° for 16 h. or pH 5 at 37°, resulted exclusively in filament formation.

The large pleomorphic cells with elementary bodies were observed mainly in the stationary phase. Cultures in decline, i.e. older than 5 days, contained increasing numbers of filamentous forms and also particles which were evidently dead mycoplasmas (Pl. 2, fig. 5). These had a shrunken, distorted appearance, an average diameter of 600 nm, and comprised the total population in the 9th and 10th day cultures.

*The life cycle of Negroni agent*

Immediately after the final filtration with 220 nm. Millipore filter, the filtrate was centrifuged at 30,000 rev./min. in the Spinco rotor 50 for 30 min., and the resultant pellet was resuspended in a 2% (w/v) aqueous solution of phosphotungstate for electron microscopy. Numerous elementary bodies were seen but there were no mature organisms. No significant changes were observed up to 34 h. after incubation, but at 39 and 44 h. a few mature mycoplasmas were present, and when the filtrate was examined after 53 h. of incubation many young, large mature cells were recognized. At approximately 72 h. these mature cells became more pleomorphic. The examination of whole organisms suggested that the life cycle of the Negroni mycoplasma was as depicted in Fig. 1.

*Identification of Negroni agent by immunoelectron microscopy*

Positive reactions were obtained with mycoplasmas only when they were reacted with their own specific antisera (Pl. 2, fig. 6, 7); pre-immune sera (Pl. 2, fig. 8) as well as all controls were negative. No cross-reactions occurred between any strains or with the antisera of closely related strains, e.g. between *Mycoplasma pulmonis* (M 1) and *M. pulmonis* (Negroni) (Pl. 2, fig. 9).

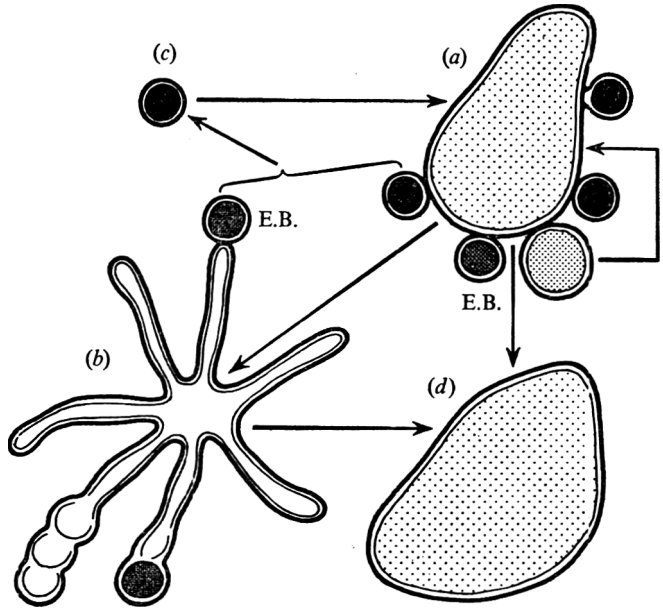



Fig. 1. A suggested scheme of the life cycle of the Negroni strain. (*a*) Large pleomorphic cell; (*b*) filamentous form; (*c*) elementary body (E.B.); (*d*) dead cell.

## DISCUSSION

### *Observations by immunoelectron microscopy*

Fallon *et al.* (1965) first suggested some relationship of Negroni agent to *Mycoplasma pulmonis*. A close relationship of Negroni agent and strain 880 to *M. pulmonis* was confirmed by other laboratories by means of growth inhibition and complement-fixation tests (Butler & Leach, 1966), nucleic acid homology method (Somerson *et al.* 1967) and accessory factor-dependent neutralization test in HEp2 tissue cultures (Sabin, 1967). Nevertheless, a study carried out by Fallon & Jackson (1967) with several strains of *M. pulmonis*, namely Negroni agent, 880, Kon, Cheng, M1 and M2, showed that these strains were, although related, quite distinct from each other. Their conclusions were based mainly on gel diffusion, metabolic inhibition, complement-fixation and growth inhibition tests. Our present observations using immunoelectron microscopy also give support to the opinion of Fallon & Jackson (1967), i.e. no serological cross-reactions were found between any strains of *M. pulmonis*. In contrast to the numerous reports on the use of the immunoferritin technique to identify viruses and bacteria, none has appeared so far concerning the identification of mycoplasma (Andres, Hsu & Seegal, 1967).

### *The life cycle*

Workers on the life cycle of mycoplasmas fall into two principal groups (reviewed by Hayflick & Chanock, 1965). Members of one group support the theory put forward by Freundt (1960) which describes filament growth, the formation of an arborizing

mycelium and its subsequent fragmentation into elementary bodies, whereas members of the other group, e.g. Klieneberger-Nobel (1962), believe that filaments are preparative artefacts resulting from the extreme plasticity of the cell membrane. Though members of the second group accept that filaments grow under certain environmental conditions, they do not consider filament formation to be a major stage in mycoplasma growth. This view has been supported by Reuss working on the Negroni strain (Reuss, 1967). In the present study, the mycoplasma was prepared under conditions isotonic with the culture medium, and the cells were fixed before subjecting them to the rigours of negative contrast, thus preventing gross osmotic distortion. Filaments still occurred under these conditions, and therefore cannot be interpreted as preparative artefacts. This view also agrees well with the work of Hummeler, Tomassini & Hayflick (1965) on the Negroni strain. In cultures at low temperature or low pH, filamentous forms became predominant. It seems, therefore, very likely that filamentary reproduction of elementary bodies is secondary to the budding phenomenon, and the latter should be interpreted as the more normal method of multiplication in *Mycoplasma pulmonis*. According to Dutta *et al.* (1965), *M. gallisepticum* strain S6 appeared to complete its life cycle within 30 to 36 h. The cycle included development of nucleus-like structures in the mother cells, budding from the cell surface, and enlargement of daughter cells, possibly equivalent to elementary bodies, in the final stage. A discrepancy in the cycle time observed in the present report (39 to 53 h.) may be a species difference.

## REFERENCES


ANDRES, G. A., HSU, K. C. & SEEGAL, B. C. (1967). Immunoferritin technique for the identification of antigens by electron microscopy. In *Handbook of Experimental Immunology*, p. 527. Edited by D. M. Weir. Oxford and Edinburgh: Blackwell Scientific Publications.

BEALE, A. J., CHRISTOPHINIS, G. J. & NEGRONI, G. (1968). Mycoplasma in leukaemia. *British Medical Journal* i, 580–581.

BUTLER, M. & LEACH, R. H. (1966). Mycoplasma associated with tissue cultures, leukaemia and human tumours. *Proceedings of the Royal Society of Medicine* **59**, 1116–1117.

DE HARVEN, E. & FRIEND, C. (1964). Structure of virus particles partially purified from the blood of leukaemic mice. *Virology* **23**, 119–124.

DOUGLAS, S. D., GOTTLIEB, A. J., STRAUSS, A. J. L. & SPICER, S. S. (1966). Selectivity of ferritin–protein conjugates for sites on skeletal muscle. *Experimental and Molecular Pathology* **5** (suppl. 3), 5–20.

DUTTA, S. K., DIERKS, R. E. & POMEROY, B. S. (1965). Electron microscopic studies of the morphology and the stages of development of *Mycoplasma gallisepticum*. *Avian Diseases* **9**, 241–251.

FALLON, R. J., GRIST, N. R., INMAN, D. R., LEMCKE, R. M., NEGRONI, G. & WOODS, D. A. (1965). Further studies of agents isolated from tissue cultures inoculated with leukaemic bone marrow. *British Medical Journal* ii, 388–391.

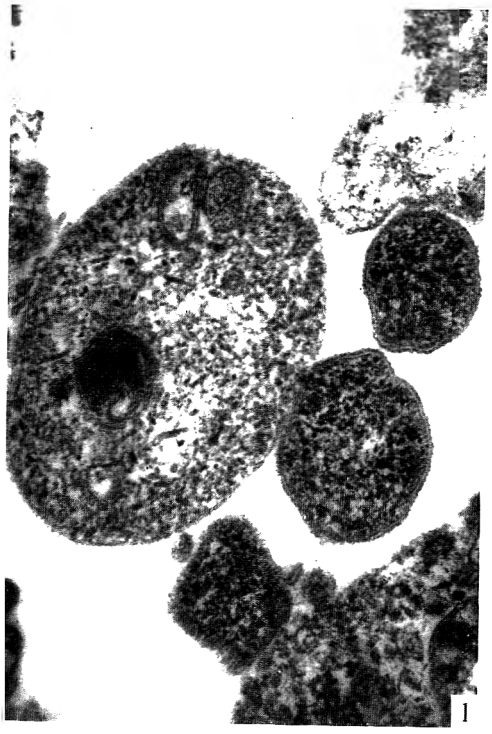
FALLON, R. J. & JACKSON, D. K. (1967). The relationship between a rodent mycoplasma, *Mycoplasma pulmonis*, and certain mycoplasmas isolated from tissue cultures inoculated with material from patients with leukaemia. *Laboratory Animals* **1**, 55–64.

FALLON, R. J. & JACKSON, D. K. (1968). Relation between mycoplasmas and leukaemia and related diseases. *British Medical Journal* iv, 225–228.

FREUNDT, E. A. (1960). Morphology and classification of the PPLO. *Annals of the New York Academy of Sciences* **79**, 312–325.

FURNESS, G. (1968). Analysis of the growth cycle of *Mycoplasma orale* by synchronized division and by ultraviolet irradiation. *Journal of Infectious Diseases* **118**, 436–442.

GRACE, J. T., HOROSZEWICZ, J. S., STIM, T. B., MIRAND, E. A. & JAMES, C. (1965). Mycoplasma (PPLO) and human leukaemia and lymphoma. *Cancer* **18**, 1369–1376.



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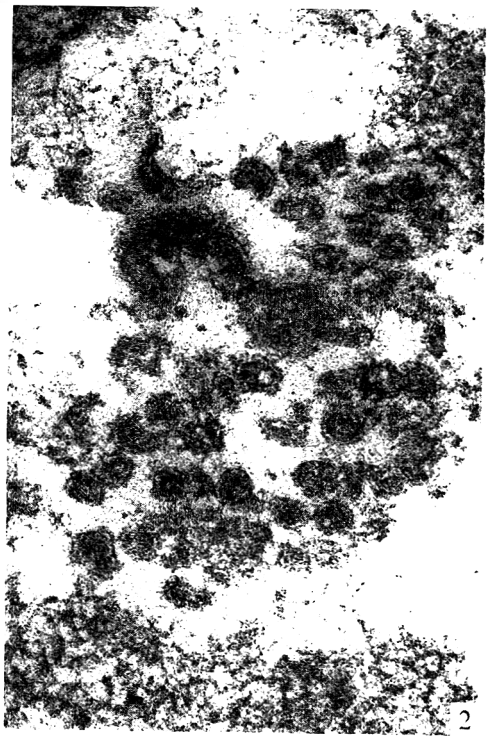

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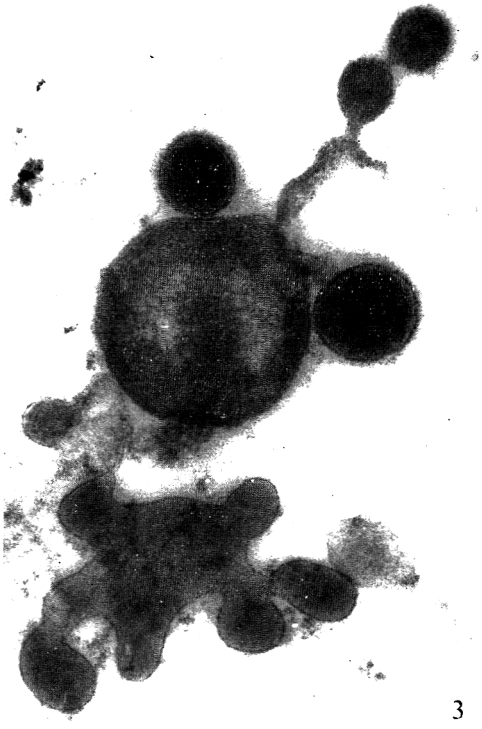

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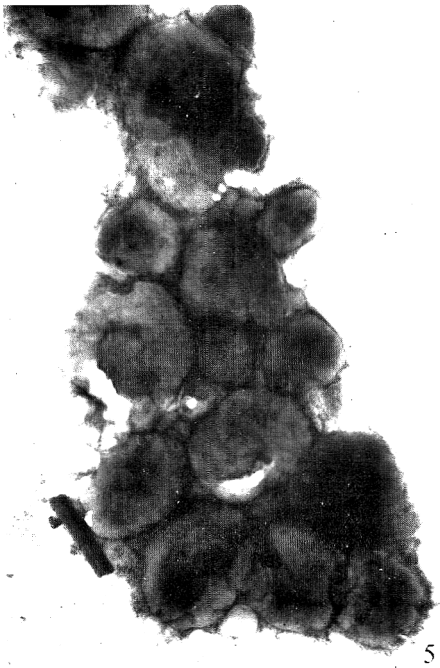

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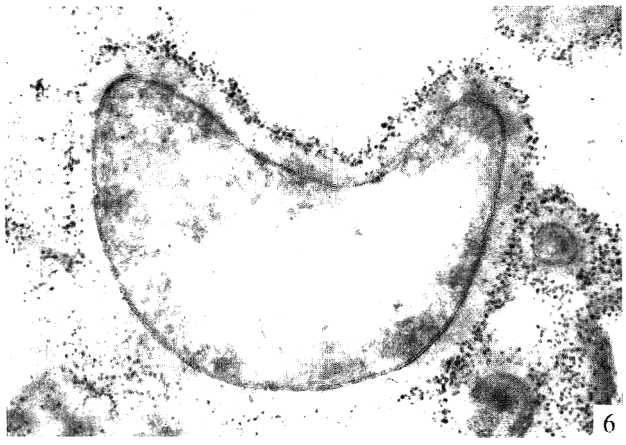

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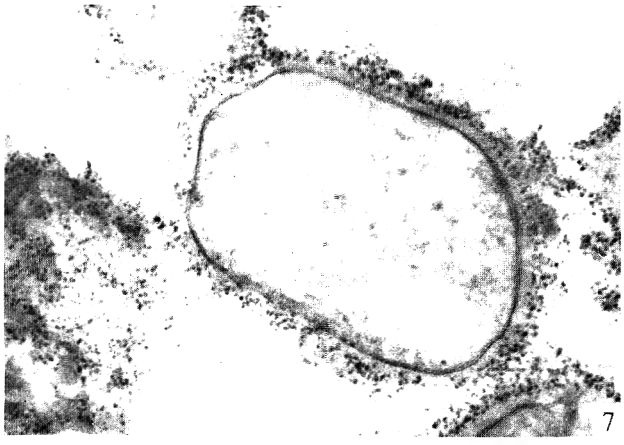

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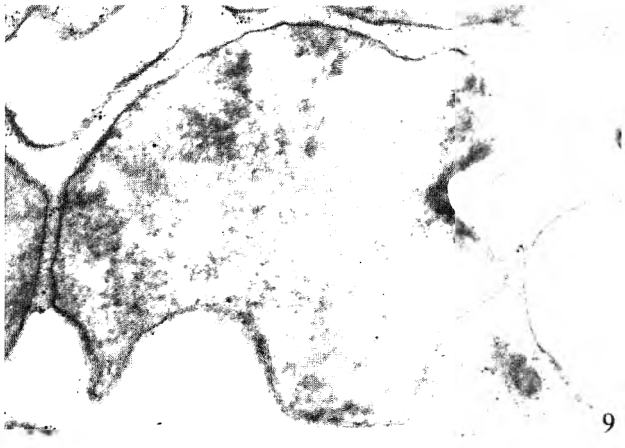

9

GRIST, N. R. & FALLON, R. J. (1964). Isolation of viruses from leukaemic patients. *British Medical Journal* ii, 1263.

HAYFLICK, L. & CHANOCK, R. M. (1965). Mycoplasma species of man. *Bacteriological Reviews* **29**, 185–221.

HUMMELER, K., TOMASSINI, N. & HAYFLICK, L. (1965). Ultrastructure of a mycoplasma (Negroni) isolated from human leukemia. *Journal of Bacteriology* **90**, 517–523.

INMAN, D. R., WOODS, D. A. & NEGRONI, G. (1964). Electron microscopy of virus particles in cell cultures inoculated with passage fluid from human leukaemic bone marrow. *British Medical Journal* i, 929–931.

KLIENEBERGER-NOBEL, E. (1962). *Pleuropneumonia-like Organisms (PPLO) Mycoplasmataceae*. New York: Academic Press.

MILLONIG, G. (1962). Further observations on a phosphate buffer for osmium solutions in fixation. In *Electron Microscopy*, vol. 2, p. 8. Edited by S. E. Breese. New York: Academic Press.

MOROWITZ, H. J. & MANILOFF, J. (1966). Analysis of the life cycle of *Mycoplasma gallisepticum*. *Journal of Bacteriology* **91**, 1638–1644.

NEGRONI, G. (1964). Isolation of viruses from leukaemic patients. *British Medical Journal* i, 927–929.

REUSS, K. (1967). Influence of fixation on gross morphology of Mycoplasma. *Journal of Bacteriology* **93**, 490–492.

REYNOLDS, E. S. (1963). The use of lead citrate at high pH as an electron-opaque stain in electron microscopy. *Journal of Cell Biology* **17**, 208–212.

SABIN, A. B. (1967). Nature and source of Mycoplasma in various tissue cultures. *Annals of the New York Academy of Sciences* **143**, 628–640.

SINGER, S. J. & SCHICK, A. F. (1961). The properties of specific stains for electron microscopy prepared by the conjugation of antibody molecules with ferritin. *Journal of Biophysical and Biochemical Cytology* **9**, 519–537.

SOMERSON, N. L., REICH, P. R., CHANOCK, R. M. & WEISSMAN, S. M. (1967). Genetic differentiation by nucleic acid homology. III. Relationship among Mycoplasma, L-forms and bacteria. *Annals of the New York Academy of Sciences* **143**, 9–20.

## EXPLANATION OF PLATES

### PLATE 1

Fig. 1. Extracellular mycoplasma cells in an infected foetal kidney cell culture. × 55,000.

Fig. 2. An aggregate of extracellular virus-like mycoplasma particles in an infected foetal kidney cell culture. × 150,000.

Fig. 3. Elementary bodies budding from the large pleomorphic mature cells. × 50,000.

Fig. 4. Branching filaments growing from a mycoplasma cell. × 40,000.

### PLATE 2

Fig. 5. Mycoplasma particles from a declining 9 day culture. × 35,000.

Fig. 6. An immunoelectron micrograph of *Mycoplasma pulmonis* (Negroni) reacted with rabbit anti-Negroni agent serum (L). × 130,000.

Fig. 7. An immunoelectron micrograph of *Mycoplasma pulmonis* (strain 880) reacted with rabbit anti-880 serum (G). × 130,000.

Fig. 8. An immunoelectron micrograph of *Mycoplasma pulmonis* (Negroni) reacted with rabbit pre-immune serum (L). × 120,000.

Fig. 9. An immunoelectron micrograph of *Mycoplasma pulmonis* (M 1) reacted with rabbit anti-Negroni agent serum (L). × 120,000.

*Journal of General Microbiology* (1970), **63**, 289–295
*Printed in Great Britain*



# A Study of the Electrokinetic Properties of Some Actinomycete Spores

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SUMMARY

Microelectrophoresis of spores of the actinomycete genera *Micromonospora*, *Nocardia*, *Streptomyces* and *Thermoactinomyces* at various pH values and after various modifying treatments indicated that Micromonospora and Streptomyces spores had amino and carboxyl surface groups. Thermoactinomyces spores and the bacillary or coccoid elements of Nocardia had carboxyl groups but not amino groups; their responses to lipase and sodium dodecyl sulphate suggested presence of lipid. All strains studied showed changes in mobility after treatment with lysozyme.

## INTRODUCTION

Microelectrophoresis has provided information on the surface properties of many types of microbe. The bacteria have received most attention, among those studied being the genera *Aerobacter* (Plummer & James, 1961), *Bacillus* (Douglas & Parker, 1957, 1958*a*, 1958*b*), *Micrococcus* (Dyar, 1948; Dyar & Ordal, 1946), *Rhizobium* (Marshall, 1967) and *Streptococcus* (Hill, James & Maxted, 1963*a*, 1963*b*). A number of fungal spores have also been examined, including those of Penicillium species, Mucor and Fusarium (Douglas, Collins & Parkinson, 1959) and Neurospora (Somers & Fisher, 1967). A wide range of fungal species was studied by Fisher & Richmond (1969).

As far as we know, the technique has not been applied to spores of actinomycetes. Wall composition of many genera of actinomycetes appears to have some taxonomic significance (Becker, Lechevalier & Lechevalier, 1965; Lechevalier & Lechevalier, 1965; Yamaguchi, 1965). Therefore, we studied spores of a number of genera by microelectrophoresis to enable comparison with wall composition data and with the surface properties of bacteria and fungi.

## METHODS

*Strains and their culture.* The organisms used were *Micromonospora* sp., *Nocardia rubra* (CBS), *Streptomyces* sp. (F1), *S. griseus* (Waksman, 3475), *S. finlayi* (ISP 5218), *S. viridochromogenes* (CBS) and *Thermoactinomyces vulgaris* (CBS specifies Centraal-

bureau voor Schimmelcultures, Baarn, Netherlands; ISP denotes code number in International Streptomyces Project).

The Micromonospora and Nocardia had readily wetted spores, and Streptomyces and Thermoactinomyces had relatively hydrophobic spores. Two Streptomyces, strain F1 and *S. griseus*, had unornamented spores, *S. viridochromogenes* had spiny spores and *S. finlayi* had hairy spores.

The streptomycetes and Micromonospora were grown on oatmeal agar (Waksman, 1961). *Nocardia rubra* and *Thermoactinomyces vulgaris* were grown on Oxoid nutrient agar. All were incubated at 25° for 4 weeks except *T. vulgaris*, which was grown at 45° for 4 days.

For certain experiments indicated, *Streptomyces viridochromogenes* was also grown on starch–casein medium (Küster & Williams, 1964) and yeast extract–malt extract agar (Shirling & Gottlieb, 1966). The influence of age on surface properties was studied using 1 to 7 week cultures of *S. griseus* and *S. viridochromogenes*.

*Preparation of suspensions for electrophoresis.* Spores (or bacillary and coccoid elements in the case of *Nocardia rubra*) were removed from plate cultures into deionized water. After homogenization for 15 min. the suspensions were centrifuged and washed five times with deionized water before introduction into prepared buffer solutions. Damage to spores during this procedure was checked by examining samples with a Stereoscan electron microscope (Williams & Davies, 1967).

The main buffer system used was sodium acetate + veronal + HCl (Michaelis, 1930), which covered the pH range 2·5 to 9·0, with NaCl added to adjust the ionic strength ($I$) to 0·05. Lower pH levels were obtained using HCl + NaCl, $I = 0{\cdot}05$. For pH 9·5 to 10·5, glycine + NaOH and for values over 10·5, NaOH + NaCl, both at $I = 0{\cdot}05$, were used.

*Treatment with surface modifying agents.* Sodium dodecyl sulphate (SDS) was used at 1 mM in Michaelis buffer at pH 7·0 ($I = 0{\cdot}05$). Washed spores were mixed with it for 15 min. at 25° before examination. Controls were spores in buffer at pH 7·0. *p*-Toluene sulphonyl chloride (PTSC), recrystallized from ether, was used in saturated aqueous solution; washed spores were left in it for 2 h. at 5° and then removed and washed three times in deionized water. They were then resuspended in Michaelis buffer at pH 7·0 for examination. Controls were spores left at 5° in distilled water and then washed and resuspended in the buffer. Di-sodium tetraborate (DST) was prepared as a 25 mM ($I = 0{\cdot}05$) solution and washed spores were suspended in it for 2 h. at 25°. The solution, buffered at pH 9·2, was used directly for mobility determinations. Controls were obtained by suspending washed spores in Michaelis buffer at the same pH for the same time. Lipase (0·5 mg./ml.) and lysozyme (0·05 mg./ml.) were added to suspensions of washed spores in Michaelis buffer at pH 7·0 which were then incubated for 2 h. at 37°. Controls were comparable suspensions kept at 5° and untreated spores suspended in the buffer for 2 h. at both temperatures.

*Determination of electrophoretic mobilities.* The electrophoresis cells used were of the pattern described by Douglas (1955, 1957), having a channel of rectangular section, 10 mm. wide and 0·2 mm. deep. This was mounted on the stage of a phase contrast microscope capable of 600-fold magnification, illuminated by a 6 V, 100 W ribbon filament lamp with a heat filter between the lamp and the cell. The apparatus was housed in a thermostatically controlled room at 25°.

The cell, after cleaning with permanganic acid, distilled water and ethanol, was

filled with octadecyl alcohol in ethanol for about 10 min. The solvent was then evaporated off in a gentle air current, leaving a layer of octadecyl alcohol over the glass surfaces, which ensured an electrochemically symmetrical channel. After filling with the appropriate buffered suspension the cell was mounted vertically on the microscope stage, the instrument being used horizontally.

The channel depth was determined microscopically using a micrometre gauge fixed to the barrel with its probe bearing against the stage. The two stationary levels (i.e. the levels in the channel where electro-osmotic and return flows negate each other's effects) were calculated as fractions of the total depth from the formula of van Gils & Kruyt (1936).

Movement of the spores over a distance of 78 $\mu$m. was timed with a stop-watch reading to 0·1 sec. The current applied was adjusted to give a timing of 8 to 11 sec. and was measured with a Cambridge Unipivot d.c. microammeter. Spores were timed at both stationary levels and travelling in both directions by current reversal. At least 32 observations were made to determine the mean velocity of a spore population (standard error of the mean being no more than 5 %).

The specific conductivities of suspensions were determined with a Mullard conductivity cell and a Wayne–Kerr B 221 bridge; mobilities were calculated and expressed as $\mu$m./sec./V/cm.

## RESULTS

*Effects of homogenization on spores.* No damage to spore surfaces was detected when suspensions were examined with the scanning electron microscope after homogenization and washing. Ornaments were still present on spores of *Streptomyces finlayi* and *S. viridochromogenes*, indicating that the outer sheath was still present.

*Effect of media and culture age on electrophoretic mobility.* Use of the three different media to produce spores of *Streptomyces viridochromogenes* had no significant effect on their pH mobility pattern, which ranged from about 0·4 at pH 3·0 to 1·8 at pH 10·0, despite wide differences in the composition of these media.

The pH mobility patterns of spores from 1 and 7 week cultures of *Streptomyces viridochromogenes* were similar, a decrease in net negative charge occurred with age (Fig. 1). Comparison of the mobilities at pH 7·0 of *S. griseus* and *S. viridochromogenes* spores from cultures aged between 1 and 7 weeks showed no decrease in net negative charge in the former species.

*pH mobility of different species.* These results are given in Fig. 2. Patterns for the variously ornamented spores of Streptomyces species and Micromonospora were the same: after a progressive rise in mobility up to about pH 6·0, the net negative charge remained constant between pH 6·0 and 8·0. Above 8·0, a slight increase in mobility occurred in Micromonospora which was barely detectable in Streptomyces species. Isoelectric points occurred at about pH 2·5 with a net positive charge at pH 2·0. These patterns indicated mixed carboxyl–amino surfaces.

The patterns for Nocardia and Thermoactinomyces were somewhat different. Although mobility increased up to pH 6·0, a gradual rise continued above 6·0. Spores had a small net positive charge at pH 2·0, but no steepening of the curves occurred above 8·0. These patterns indicated the presence of carboxyl groups but few or no amino groups on the surface.

*Effect of surface-modifying treatments on mobility.* These results are summarized

in Table 1. Again, responses of all Streptomyces species and Micromonospora were similar. Treatment with PTSC, which reacts with primary and secondary amines (Cohen, 1945), produced small increases in net negative charge at pH 7·0, again suggesting the presence of amino groups. None of these strains responded to treatment

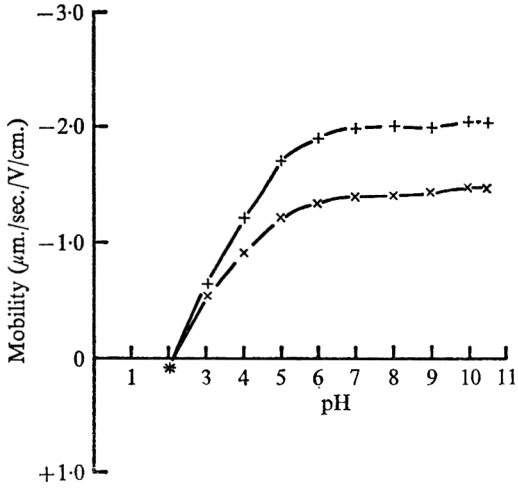


Fig. 1. pH mobility curves of *Streptomyces viridochromogenes* spores from 1 week (+——+) and 7 week (×——×) cultures.

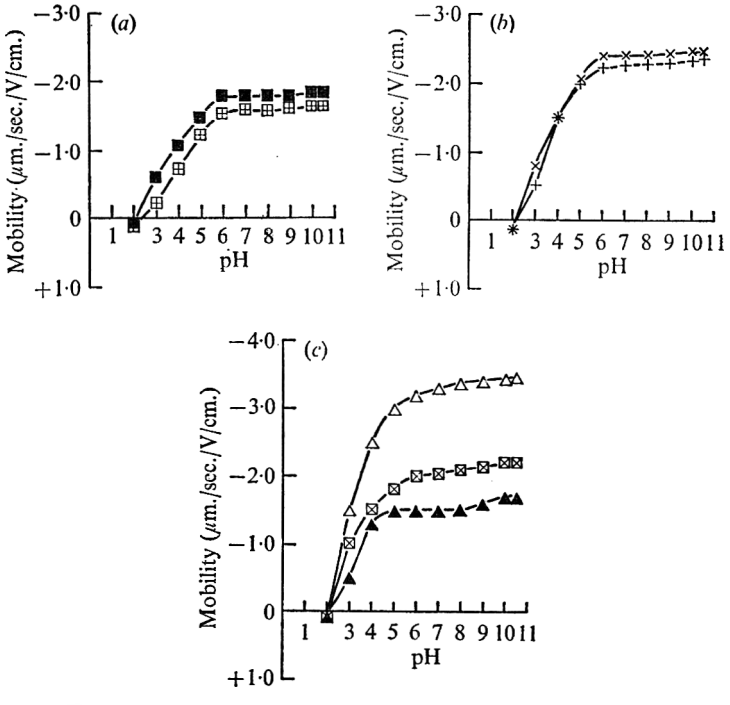


Fig. 2. pH mobility curves for spores of *Streptomyces* sp. (F 1) (⊞——⊞) ,*S. viridochromogenes* (■——■), *S. griseus* (+——+), *S. finlayi* (×——×), *Micromonospora* sp. (▲——▲), *Nocardia rubra* (△——△) and *Thermoactinomyces vulgaris* (⊠——⊠).

with SDS, which reacts rapidly with lipid alkyl chains (Schulman, Pethica, Few & Salton, 1965); consistent with absence of accessible lipid was their lack of response to lipase. No changes was induced by DST, which reacts with 1·2 *cis*-hydroxyl groups associated with most monosaccharide subunits; this indicated that the outer layers were not composed of carbohydrate. However, amino sugars, which have trans-hydroxyl groups, would not react with DST. Treatment with lysozyme produced quite large decreases in net negative charge at pH 7·0, suggesting the presence of glycosidic bonds between the amino sugars acetyl glucosamine and acetyl muramic acid.

Table 1. *The influence of various surface-modifying treatments on the electrophoretic mobility of actinomycete spores*

| | % Changes in mobility to anode after treatment as compared with controls | | | | |
|---|---|---|---|---|---|
| Strains | PTSC at pH 7·0 | SDS at pH 7·0 | DST at pH 9·2 | Lysozyme pH 7·0, 37° | Lipase at pH 7·0, 37° |
| *Streptomyces finlayi* | +10% | 0 | 0 | −43% | 0 |
| *S. griseus* | +9% | 0 | 0 | −22% | 0 |
| *S. viridochromogenes* | +15% | 0 | 0 | −29% | 0 |
| *Streptomyces* sp. (F1) | +7% | 0 | 0 | −21% | 0 |
| *Micromonospora* sp. | +10% | 0 | 0 | −20% | 0 |
| *Nocardia rubra* | 0 | +9% | 0 | −38% | −12% |
| *Thermoactinomyces vulgaris* | 0 | +16% | 0 | −40% | −27% |

For code initials of surface modifying agents, see Methods.

Responses of Nocardia and Thermoactinomyces were again different from those of the above strains. No reaction occurred with PTSC, suggesting that amino groups were absent from the surface; lysozyme produced a decrease in net negative charge, indicating the presence of amino sugars. Again no reaction with DST occurred. Increases in net negative charge were produced by SDS, and the presence of lipid was further suggested by a reduction in mobility after lipase treatment.

## DISCUSSION

The actinomycetes studied here were grown on solid medium and spores examined were produced above this. Presumably this is why we observed no effects of medium and culture age. Such effects among bacteria have usually been detected in liquid cultures, where the cells are more directly influenced by the composition of the medium (Douglas & Parker, 1958*b*; Plummer & James, 1961; Plummer, James, Gooder & Maxted, 1962).

Our results suggest the presence of hexosamine-peptide polymers in all strains. Although amino groups were not detected on the surface of Nocardia bodies or Thermoactinomyces spores, they reacted to lysozyme like the other strains; all had surface carboxyl groups. Becker *et al.* (1965) and Yamaguchi (1965) studied walls of vegetative organisms of these genera, and DeJong & McCoy (1966) showed that the composition of spore walls was very similar to that of vegetative hyphae in Streptomyces species. All genera studied contained glucosamine, muramic acid, alanine and

glutamic acid in their wall hydrolysates, along with other amino acids and sugars, suggesting the hexosamine–peptide configuration of Gram-positive bacteria.

Past results on the effect of lysozyme on actinomycetes vary. Using optical density measurements to detect changes, Sohler, Romano & Nickerson (1958) obtained a positive response with *Streptomyces griseus* but none with *S. viridochromogenes* or *Nocardia rubra*; no effects on *S. viridochromogenes* spores were detected by DeJong & McCoy (1966) using optical density measurements and electron microscopy. Differences between these results and ours are probably due to differences in the sensitivity of the detection methods.

Many Nocardia species have a relatively high content of lipid, and the presence of lipid in cells of *Nocardia rubra* was detected by Adams & McClung (1962). The presence of lipid in Thermoactinomyces spores has not been previously reported. Several workers have detected lipid on the surface of other Gram-positive bacteria using electrophoretic techniques. Lipid in *Micrococcus aureus* was detected by changes in mobility after anionic detergent and lipase treatments (Dyar, 1948; Dyar & Ordal, 1946); SDS treatment detected lipid on Streptococcus cells (Hill *et al.* 1963*b*).

The genera studied here included two with relatively hydrophobic spores (*Streptomyces* and *Thermoactinomyces*) and two with readily wetted cells (*Micromonospora* and *Nocardia*). The hydrophobic properties of Streptomyces may be associated with the presence of lipid (Erikson, 1947) and Kalakoutski & Sokolov (1961) reported the presence of lipids in *Streptomyces violaceus* aerial mycelium. Our results did not indicate the presence of lipid on the surface of any of the Streptomyces spores. Of the genera which had surface lipid, one (*Thermoactinomyces*) had hydrophobic spores, while the other (*Nocardia*) was easily wetted. A similar lack of correlation between water-repellant properties and occurrence of surface lipid was noted in spores of fungi (Fisher & Richmond, 1969). It therefore seems that other explanations for the hydrophobic properties of Streptomyces spores must be sought.

## REFERENCES

ADAMS, J. N. & MCCLUNG, N. M. (1962). On the nature of cytoplasmic inclusions of *Nocardia rubra*. *Journal of General Microbiology* **28**, 231–241.

BECKER, B., LECHEVALIER, M. P. & LECHEVALIER, H. A. (1965). Chemical composition of cell wall preparations from strains of various form-genera of aerobic actinomycetes. *Applied Microbiology* **13**, 236–243.

COHEN, S. S. (1945). The chemical alteration of a bacterial surface with special reference to the agglutination of *B. subtilis* OX-19. *Journal of Experimental Medicine* **82**, 133–142.

DEJONG, P. J. & MCCOY, E. (1966). Qualitative analyses of vegetative cell walls and spore walls of some representative species of Streptomyces. *Canadian Journal of Microbiology* **12**, 985–994.

DOUGLAS, H. W. (1955). Electrophoretic studies on bacterial spores. *Transactions of the Faraday Society* **51**, 146.

DOUGLAS, H. W. (1957). Electrophoretic studies on spores and vegetative cells of certain strains of *B. megaterium*, *B. subtilis* and *B. cereus*. *Journal of Applied Bacteriology* **20**, 390.

DOUGLAS, H. W. & PARKER, F. (1957). Electrophoretic studies on bacteria. IV. The cation charge reversal spectrum of spores of *B. megaterium*, *B. cereus* and *B. subtilis*. *Transactions of the Faraday Society* **53**, 1494–1499.

DOUGLAS, H. W. & PARKER, F. (1958*a*). Electrophoretic studies on bacteria. II. The effects of enzymes on resting spores of *B. megaterium*, *B. subtilis* and *B. cereus*. *Biochemical Journal* **68**, 94–97.

DOUGLAS, H. W. & PARKER, F. (1958*b*). Electrophoretic studies on bacteria. III. The growth cycle of *Bacillus megaterium*, the behaviour of cells and the changes produced by lysozyme. *Biochemical Journal* **68**, 99–105.

DOUGLAS, H. W., COLLIN, A. E. & PARKINSON, D. (1959). Electric charge and other surface properties of some fungal spores. *Biochimica et Biophysica Acta* **33**, 535–538.

DYAR, M. T. (1948). Electrokinetic studies on bacterial surfaces. II. Studies on surface lipids, amphoteric material and some other surface properties. *Journal of Bacteriology* **56**, 821–834.

DYAR, M. T. & ORDAL, E. J. (1946). Electrokinetic studies on bacterial surfaces. I. The effects of surface active agents on the electrophoretic mobility of bacteria. *Journal of Bacteriology* **51**, 149–167.

ERIKSON, D. (1947). Differentiation of the vegetative and sporogenous phases of the actinomycetes. I. The lipid nature of the outer wall of the aerial mycelium. *Journal of General Microbiology* **1**, 39–44.

FISHER, D. J. & RICHMOND, D. V. (1969). The electrokinetic properties of some fungal spores. *Journal of General Microbiology* **57**, 51–60.

VAN GILS, G. E. & KRUYT, H. R. (1936). Zur Methodik der Ultramikroelectrophorese. *Kolloid-beihefte* **45**, 60–98.

HILL, M. J., JAMES, A. M. & MAXTED, W. R. (1963*a*). Some physical investigations of the behaviour of bacterial surfaces. IX. Studies on the streptococcal cell wall. *Biochimica et Biophysica Acta* **75**, 402–413.

HILL, M. J., JAMES, A. M. & MAXTED, W. R. (1963*b*). Some physical investigations of the behaviour of bacterial surfaces. X. The occurrence of lipid in the streptococcal cell wall. *Biochimica et Biophysica Acta* **75**, 414–424.

KALAKOUTSKI, L. V. & SOKOLOV, A. A. (1961). Heterogeneity of the cell walls of *Actinomyces violaceus* aerial mycelium. *Mikrobiologiya* **30**, 67–71.

KÜSTER, E. & WILLIAMS, S. T. (1964). Selection of media for isolation of streptomycetes. *Nature, London* **202**, 928–929.

LECHEVALIER, H. & LECHEVALIER, M. P. (1965). Classification des actinomycètes aérobies basée sur leur morphologie et leur composition chimique. *Annales de l'Institut Pasteur* **108**, 662–673.

MARSHALL, K. C. (1967). Electrophoretic properties of fast and slow-growing species of Rhizobium. *Australian Journal of Biological Science* **20**, 429–439.

MICHAELIS, L. (1930). Der Acetat-Veronal Buffer. *Beiträge zur chemischen Physiologie und Pathologie* **234**, 139–141.

PLUMMER, D. T. & JAMES, A. M. (1961). Some physical investigations of the behaviour of bacterial surfaces. III. The variation of electrophoretic mobility and capsule size of *Aerobacter aerogenes* with age. *Biochimica et Biophysica Acta* **53**, 453–460.

PLUMMER, D. T., JAMES, A. M., GOODER, H. & MAXTED, W. R. (1962). Some physical investigations of the behaviour of bacterial surfaces. V. The variation of the surface structure of *Streptococcus pyogenes* during growth. *Biochimica et Biophysica Acta* **60**, 595–603.

SCHULMAN, J. H., PETHICA, B. A., FEW, A. V. & SALTON, M. R. J. (1965). The physical chemistry of haemolysis and bacteriolysis by surface active agents. *Progress in Biophysics* **5**, 41–71.

SHIRLING, E. B. & GOTTLIEB, D. (1966). Methods for characterization of Streptomyces species. *International Journal of Systematic Bacteriology* **16**, 313–340.

SOHLER, A., ROMANO, A. H. & NICKERSON, W. (1958). Action of lysozyme on isolated cell walls of *Streptomyces fradiae*. *Journal of Bacteriology* **75**, 283–290.

SOMERS, E. & FISHER, D. J. (1967). Effect of dodine acetate on the electrophoretic mobility of *Neurospora crassa* conidia. *Journal of General Microbiology* **48**, 147–154.

WAKSMAN, S. A. (1961). *The Actinomycetes*. Vol. 2. *Classification, Identification and Description of Genera and Species*. Baltimore: The Williams and Wilkins Co.

WILLIAMS, S. T. & DAVIES, F. L. (1967). Use of scanning electron microscope for the examination of actinomycetes. *Journal of General Microbiology* **46**, 171–177.

YAMAGUCHI, T. (1965). Comparison of the cell wall composition of morphologically distinct actinomycetes. *Journal of Bacteriology* **89**, 444–453.

*Journal of General Microbiology* (1970), **63**, 297–302
*Printed in Great Britain*



# The Mode of Action of Metronidazole against *Trichomonas vaginalis*

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(*Accepted for publication 18 August 1970*)

SUMMARY

Metronidazole (1-β-hydroxyethyl-2-methyl-5-nitroimidazole) inhibited the evolution of hydrogen gas in *Trichomonas vaginalis* before it inhibited carbon dioxide evolution. Evidence is presented that the phosphoroclastic reaction of the clostridial type was the major mechanism by which both gases were evolved, and it is postulated that metronidazole inhibits, directly or indirectly, the hydrogenase component of the system. A possible mechanism of action is discussed.

## INTRODUCTION

The recognition that vaginitis caused by the protozoan *Trichomonas vaginalis* was a sexually transmitted disease led to intensive efforts to develop a drug which would prove systemically effective against the organism. Previous treatments involving topical therapy, including vinegar douches and methyl violet impregnated tampons, were unsatisfactory, and reinfection of the sexual partners was frequent. The compound 1-β-hydroxyethyl-2-methyl-5-nitroimidazole (metronidazole) (May and Baker Ltd, Dagenham, Essex) proved to be an extremely effective agent, against not only *T. vaginalis* but also a very wide range of other organisms. These include both Gram-positive and Gram-negative bacteria, spirochaetes, protozoa, and even certain nematodes. Metronidazole's activity, however, is limited to anaerobic or facultatively anaerobic organisms—a fact which prompted the question: does the drug exert its effect by inhibiting a reaction of fundamental importance to anaerobes? In this communication we present evidence showing the probable site of action of the drug, and postulate the mechanism of action.

## METHODS

*Organism. Trichomonas vaginalis* strain F. 1295 was obtained from an infected patient.

*Medium.* A modified Bushby's medium (Bushby & Copp, 1955) at 37° was used. It was prepared by dissolving 18 g. dehydrated liver infusion (Difco) in 1 l. tryptone soya broth (Oxoid) and filtering. To the filtrate was added 20 g. glucose and 1 ml. aqueous 5% calcium pantothenate. The pH was adjusted to 6·0 if necessary, and the medium sterilized at 5 lb./in.$^2$ at 109° for 15 min. Before use, sterile horse serum was added at 20% (v/v) to the medium. Subculturing was carried out every 48 h.

*Identification of hydrogen gas.* This was done chromatographically (Edwards & Corbett, unpublished).

*Manometric measurements.* These were carried out with conventional techniques (Umbreit, Burris & Stauffer, 1964). Hydrogen evolution was measured by means of paired flasks containing 3 ml. liquid with 20% potassium hydroxide in the centre well of one. Carbon dioxide was determined from total gas evolved minus hydrogen gas evolved. The gas phase was nitrogen, shaking rate 72 strokes/min., and incubation temperature 37°.

*Spectrophotometric measurements.* These were made with a Unicam SP-600 or Bausch and Lomb Spectronic-20.

*Disruption of organisms.* This was performed either by grinding packed frozen cells in a Potter–Elvejhem homogenizer or by using a Hughes press (Shandon Scientific Co., London N.W. 10) at −20°. Debris and unbroken cells were removed by centrifugation at 2000*g* for 5 min.

*Phosphoroclastic assays.* The increased evolution of hydrogen caused by the addition of Na pyruvate (Koepsell, 1955) was measured; acetyl phosphate production was measured as the hydroxamate (Lipmann & Tuttle, 1945); the formate hydrogenlyase reaction was measured by monitoring hydrogen evolution after addition of Na formate (Stephenson & Stickland, 1932).

## RESULTS AND DISCUSSION

Metronidazole at 200 μg./ml. (minimum inhibitory concentration, 1·0 μg./ml.) added to growing *Trichomonas vaginalis* in culture resulted in extensive disruption of the organisms. The nucleus, axostyle, and undulating membrane were destroyed within 60 to 75 min.; death occurred at 60 min. and lysis at 75 min. The organisms were judged to be dead if there was no movement of the anterior flagella and undulating membrane, and no growth after 48 h. in fresh medium.

The effect of the drug on evolution of gas from *Trichomonas vaginalis* in Bushby's medium (Fig. 1) was totally to abolish $H_2$ evolution in 15 to 20 min. and $CO_2$ evolution in 60 min, which coincided with death of the organism. The differing sensitivities of the $H_2$ and $CO_2$ evolution to the drug indicated that these gases were not produced by the same reaction. Since the drug affected evolution of the $H_2$ first, we investigated the mechanism of its production in *T. vaginalis*.

There are no data at present available on the mechanisms by which protozoa produce molecular $H_2$ (Review: Baernstein, 1963), but bacterial mechanisms of $H_2$ evolution are well documented (Reviews: Gest, 1954; Kornberg, 1959). Of these, the formate hydrogen–lyase reaction, ferredoxin-linked hydrogenases, and the phosphoroclastic reaction of the coliform and clostridial types form the major mechanisms. The pyruvate phosphoroclastic reaction in which pyruvate is converted to acetyl phosphate, $CO_2$ and $H_2$, was investigated by measuring the increased amount of $H_2$ liberated after adding Na pyruvate (0·02 M final concentration) to washed flagellates in phosphate buffer pH 6·0 (Fig. 2). Metronidazole (200 μg./ml.) halved the increase in $H_2$ evolution. Since the coliform type of phosphoroclastic reaction involves formate decomposition, we attempted to identify this reaction in *Trichomonas vaginalis*. Addition of Na formate (0·01 to 0·2 M) to resting-cell suspensions in phosphate buffer, pH 6·0, produced no significant effect on $H_2$ evolution. Indeed, at the higher concentration there was a slight inhibitory effect (Fig. 3). This corroborated the findings of Ninomiya & Suzuoki-Ziro (1952) and suggested the absence of a formate hydrogenlyase system in *T. vaginalis*.

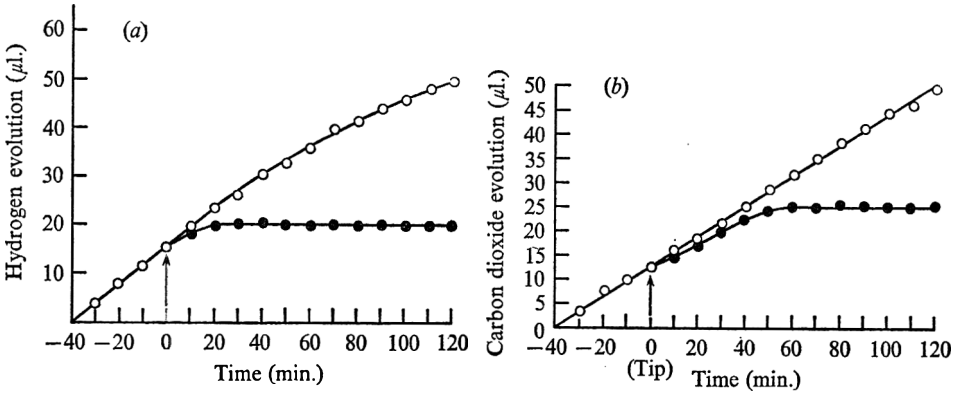


Fig. 1(*a*) The effect of metronidazole on $H_2$ evolution from *Trichomonas vaginalis* strain F. 1295. Main flask contained 3 ml. of a cell suspension ($5 \times 10^7$ organisms/ml.) harvested at 40 h. and resuspended in Bushby's medium at pH 6·0. The side-arm contained either metronidazole in Bushby's medium, to give a final concentration of 200 μg./ml., or medium only. The drug was added from the side-arm at 0 min. ○——○, $H_2$ evolution in absence of drug; ●——●, $H_2$ evolution in presence of metronidazole. (*b*) The effect of metronidazole on carbon dioxide evolution from *Trichomonas vaginalis* strain F. 1295. Data as for Fig. 1(*a*).

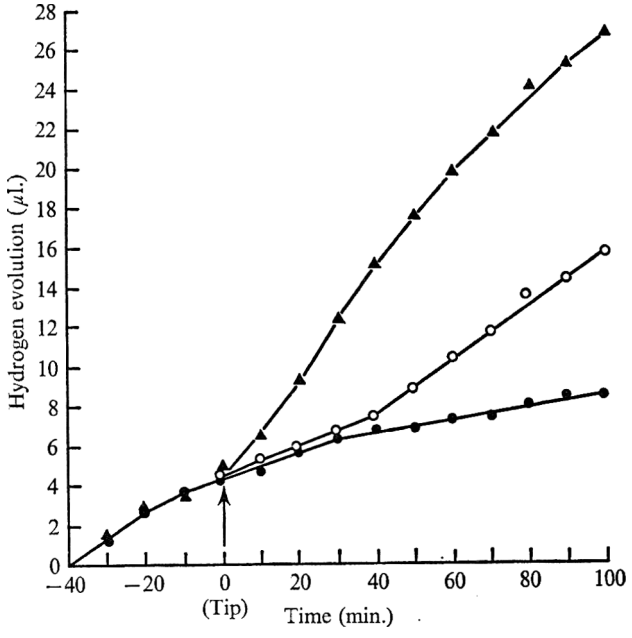


Fig. 2. Effect of metronidazole on the pyruvate phosphoroclastic reaction in *Trichomonas vaginalis* F. 1295. The main flask contained 3 ml. of a cell suspension ($5 \times 10^7$ organisms/ml.) harvested at 40 h., and resuspended in 0·1 M-phosphate buffer, pH 6·0. The side-arm contained either phosphate buffer (pH 6·0) alone or sodium pyruvate in phosphate buffer, pH 6·0, to give a final concentration of 0·02 M, or pyruvate and metronidazole in phosphate buffer to give final concentrations of 0·02 M and 200 μg./ml. respectively. The contents of the side-arm were added at 0 min. ●——●, Endogenous $H_2$ evolution; ▲——▲, $H_2$ evolution as result of pyruvate addition; ○——○, $H_2$ evolution as a result of pyruvate and metronidazole addition.

We also measured the rate of acetyl phosphate synthesis caused by adding Na pyruvate to resting-organism suspensions and organism-free extracts. In both we found a fivefold increase in the synthesis of acetyl phosphate from an endogenous level of 0·5 $\mu$moles occurred in 5 min. Metronidazole had no effect on acetyl phosphate synthesis even at concentrations of 800 $\mu$g./ml.

This evidence indicated that the phosphoroclastic reaction in *Trichomonas vaginalis* was of the clostridial rather than the coliform type. Moreover, since the drug inhibited

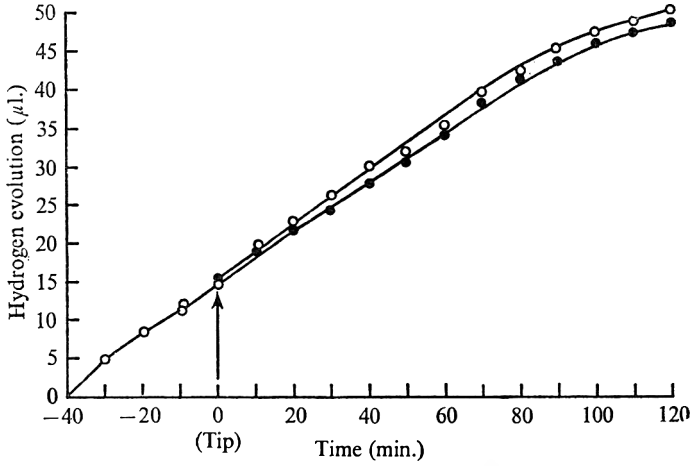


Fig. 3. The effect of formate on hydrogen gas evolution from *Trichomonas vaginalis* strain F. 1295. Each flask contained 3 ml. of $5 \times 10^7$ cells/ml. in 0·1 M-phosphate buffer, pH 6·0. Na formate (0·2 M) was added from the side-arm at 0 min. ○——○, Control; ●——●, effect of addition of formate.

the evolution of hydrogen, but not the synthesis of acetyl-phosphate, the reaction appeared to proceed by way of at least two enzyme systems similar to the ones postulated for the clostridial system. These are:

$$(a)\quad \text{Pyruvate} + \text{phosphate} \rightarrow \text{acetyl phosphate} + CO_2 + 2H^+$$
$$(b)\quad 2H^+ + 2e \rightarrow H_2.$$

The first reaction would involve a pyruvate dehydrogenase complex, and the second, a hydrogenase enzyme. The above results also indicated that the site of action of the drug involved the hydrogenase system rather than the dehydrogenase complex.

Hydrogen evolution in micro-organisms is intimately concerned with electron transfer (Gest, 1954). If a hydrogenase type of system was being inhibited in *Trichomonas vaginalis* by metronidazole then one would expect the drug to affect electron transfer. We therefore investigated the effect of the drug on the ability of the organism to transfer electrons to artificial electron-acceptor dyes.

Methylene blue (0·48 $\mu$moles in phosphate buffer, pH 6·0) in a suspension of *Trichomonas vaginalis* (5 ml. containing $10^8$ organisms/ml.) in Thunberg tubes was rapidly decolorized to the leuco form in 4·5 min. Metronidazole (200 $\mu$g./ml. final concentration) slowed decolorization to 12·5 min. Further experimentation showed that there was a linear correlation between drug concentration and the time taken to decolorize the dye (Fig. 4).

In an organism-free system corresponding to $10^8$ flagellates in phosphate buffer, with dichlorphenolindophenol (DCPIP) at 100 $\mu$g./ml. in place of methylene blue, and with metronidazole at 647 or 323 $\mu$g./ml., dye reduction was halted, in 15 and 17 min. respectively (Fig. 5).

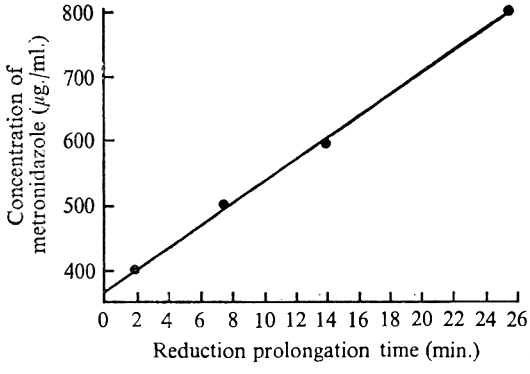


Fig. 4

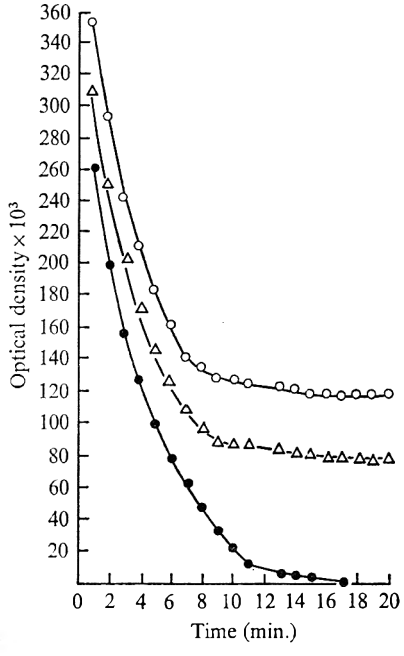


Fig. 5

Fig. 4. The effect of metronidazole on the inhibition of reduction of methylene blue. 0·5 ml. *Trichomonas vaginalis* suspension ($1 \times 10^8$ organisms/ml.) in phosphate buffer, pH 6·0, were placed in the stopper of a Thunberg tube. The tube contained 1·0 ml. 240 $\mu$M-methylene blue in buffer, and 1·0 ml. metronidazole to give final concentrations of 400, 500, 600 and 800 $\mu$g./ml. All tubes were evacuated and flushed with nitrogen several times. After incubation at 37° for 30 min. the contents of stopper and tube were mixed and the time taken for decolorization compared with samples containing no drug.

Fig. 5. The effect of metronidazole on the inhibition of DCPIP reduction by *Trichomonas vaginalis* organism-free extracts. The system comprised an extract corresponding to $10^8$ organisms in 0·1 M-phosphate buffer, pH 6·5; the concentration of metronidazole (where added) was 647 or 323 $\mu$g./ml. DCPIP (100 $\mu$g.) was added last and rapidly mixed in a stream of nitrogen. Decolorization of DCPIP was measured at 600 nm. ●——●, Control; △——△, with metronidazole (323 $\mu$g./ml); ○——○, with metronidazole (647 $\mu$g./ml.)

In view of this evidence, we postulated that the drug exerted its effect on the hydrogenase component of the phosphoroclastic reaction. This presumably would be either the enzyme itself, or the electron transfer protein ferredoxin. Since 'one might expect ferredoxin to be present in all organisms that evolve hydrogen in their metabolism' (Mortenson, 1963), and since ferredoxin is only formed in anaerobic organisms (photosynthetic organisms excepted), we suspected a mechanism of action based on the supposed presence of ferredoxin in *Trichomonas vaginalis*. Metronidazole has a redox (half-wave) potential of $-0{\cdot}56$ V as measured polarographically, and is therefore more negative than ferredoxin ($E_0' = -0{\cdot}46$ V). The drug can therefore act as a better electron acceptor than ferredoxin, both the nitro group and the imidazole ring con-

ferring on the drug the properties of an efficient electron 'sink'. Competition for electrons generated by the phosphoroclastic system would thus ensue between the two, and the drug would, as a result, inhibit not only the hydrogenase system, but also other electron transfer mechanisms.

To test this hypothesis, we investigated whether metronidazole had any effect on photosynthesis. Ferredoxin has the role of an electron acceptor and carrier in photosystem I of the photosynthetic reaction, and the drug should inhibit this if it is competing with ferredoxin. We found (Edwards & Schoolar, 1970) that the drug inhibited sugar synthesis, and also increased the rate of chlorophyll degradation in sugar cane leaf discs. It had no effect on the Hill reaction (photosystem II), and therefore we presume that its site of action was photosystem I.

The only report to date concerning the mode of action of metronidazole is that of Samuels (1962) who found that the action of the drug on *Trichomonas vaginalis* was reversed by adding liver infusion or a mixture containing adenine, guanine, hypoxanthine, xanthine and inosine to the medium. He postulated that the drug inhibits nucleic acid synthesis. We find it difficult to reconcile this theory with our data or with the fact that metronidazole affects only anaerobes. Samuels gave no indication of how rapidly the action of the drug was reversed. It seems likely that the effect he observed was not a primary one.

We thank M. J. Parnell of the Applied Physical Chemistry Laboratory, May and Baker Ltd, Dagenham, for the polarographic analysis, and May and Baker Ltd for financial assistance.

## REFERENCES

BAERNSTEIN, H. D. (1963). A review of electron transport mechanisms in parasitic protozoa. *Journal of Parasitology* **49**, 12–21.

BUSHBY, S. R. M. & COPP, F. C. (1955). The antitrichomonal activity of amido-nitrothiazoles. *Journal of Pharmacy and Pharmacology* **7**, 112–117.

COSAR, C. & JULON, L. (1959). Activité de L'(hydroxy-2 éthyl)1-méthyl-2 nitro-5-imidazole (8.823 R.P.) vis-à-vis des infections expérimentales à *Trichomonas vaginalis*. *Annales de l'Institut Pasteur* **96**, 238–241.

EDWARDS, D. I. & SCHOOLAR, A. (1970). Inhibition of sugar synthesis, and potentiation of chlorophyll degradation by metronidazole in sugar-cane leaf discs. *Zeitschrift für Pflanzenphysiologie* (In Press).

GEST, H. (1954). Oxidation and evolution of molecular hydrogen by micro-organisms. *Bacteriological Reviews* **18**, 43–73.

KOEPSELL, H. J. (1955). In *Methods in Enzymology*, vol. 1, pp. 479–488. Edited S. P. Colowick and N. O. Kaplan. New York: Academic Press.

KORNBERG, H. L. (1959). Aspects of terminal respiration in micro-organisms. *Annual Reviews of Microbiology* **13**, 49–77.

LIPMANN, F. & TUTTLE, L. C. (1945). A specific micromethod for the determination of acyl phosphates. *Journal of Biological Chemistry* **159**, 21–28.

MORTENSON, L. E. (1963). Nitrogen fixation: role of ferredoxin in anaerobic metabolism. *Annual Reviews of Microbiology* **17**, 115–138.

NINOMIYA, H. & SUZUOKI-ZIRO, (1952). The metabolism of *Trichomonas vaginalis* with comparative aspects of trichomonads. *Journal of Biochemistry (Tokyo)* **39**, 321–331.

SAMUELS, R. (1962). Reversal of metronidazole inhibition of *Trichomonas vaginalis*. *Journal of Protozoology* **48**, 30.

STEPHENSON, M. & STICKLAND, L. H. (1932). Hydrogenlyases: bacterial enzymes liberating molecular hydrogen. *Biochemical Journal* **26**, 712–724.

UMBREIT, W. W., BURRIS, R. H. & STAUFFER, J. F. (1964). *Manometric Techniques*. Minneapolis, Minnesota: Burgess Publishing Company.

*Journal of General Microbiology* (1970), **63**, 303–310
*Printed in Great Britain*



# Relation of Dipicolinic Acid to Heat Resistance of Bacterial Spores

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(*Accepted for publication 19 August 1970*)

SUMMARY

Spores of five strains of *Clostridium botulinum* differing widely in their heat resistance contained 7·4 to 13·4 % dipicolinic acid. There appeared to be no correlation between DPA content and heat resistance of the various strains. The rate of loss of DPA during heating at 75 and 100° was consistently slower than the rate of loss of spore viability, though, in general, heat-resistant strains lost DPA less quickly than did heat-sensitive strains. At the instant of thermal death, spores still retained 28 to 99·6 % of their original DPA, though this DPA could be released on continued heating.

## INTRODUCTION

Since its discovery by Powell (1953), dipicolinic acid (DPA, pyridine-2,6-dicarboxylic acid) has in various ways been related to heat resistance of bacterial spores. Spores as a group are exceptional in the biological world in that they contain large amounts of DPA: 5 to 15 % of their dry weight (Church & Halvorson, 1959; Murrell & Warth, 1965; Murrell, Ohye & Gordon, 1969). Appearance of heat resistance during formation and maturation of spores has been correlated with the synthesis of DPA (Perry & Foster, 1955; Collier & Murty, 1957; Hashimoto, Black & Gerhardt, 1960; Wooley & Collier, 1965).

Release of DPA from spores proceeds at a slower rate than loss of viability of spores during heating (Foster, 1959; El-Bisi, Lechowich, Amaha & Ordal, 1962), or loss of heat resistance during spore germination (Wooley & Collier, 1965). However, reports of a quantitative relationship between the spore's content of DPA and its degree of heat resistance are the subject of some controversy (Lund, 1958; Lechowich & Ordal, 1960; Levinson, Hyatt & Moore, 1961; Byrne, Burton & Koch, 1960; Walker, Matches & Ayres, 1961; Murrell & Warth, 1965; Murrell *et al.* 1969).

We have postulated that a relationship between the heat resistance of a bacterial spore and the rate at which its DPA is released on heating would be a reasonable probability in that it would relate the heat resistance to the energy required to remove DPA from any hypothetical protective spore–DPA complex(es) such as have been postulated by various workers (Halvorson, 1958; Riemann, 1963; Tang, Rajan & Grecz, 1968). In order to test this hypothesis, it was desirable to select a taxonomically closely related group of bacteria whose spores demonstrated widely different resistances to heat; *Clostridium botulinum* was selected since the heat resistance of the spores of different strains of this species vary by as much as 1000-fold (Murrell, 1955).

## METHODS

*Test organisms.* The five strains of three serological types (A, B, E) of *Clostridium botulinum* in Table 1 were used.

*Spore production.* The sporulation medium consisted of 5 % trypticase (BBL) and 0·5 % peptone (Difco) to which freshly sterilized 10 % (w/v) sodium thioglycollate was added to 0·1 % just before inoculation. For production of spores of type E, freshly sterilized 10 % glucose was added aseptically to 1 %.

Table 1. *Strains of Clostridium botulinum*

| Strain no. | Serological type | Heat resistance | Source of culture |
|---|---|---|---|
| V.H.* | E | Very low | Food Research Institute, University of Chicago |
| 51B | B | Low | K. F. Meyer, University of California (isolated from toxic cheese) |
| 41B | B | Very high | W. E. Perkins, National Canners Association, Berkeley, California |
| 36A | A | Low | |
| 33A | A | High | |

* Vancouver Herring.

The inoculum was built up by incubating 20 ml. of medium in a screw-cap tube with 2 ml. of the stock culture. To activate the spores for germination they were heat-shocked at 80° for 10 min. (type A and type B strains), or 60° for 13 min. (type E strain). Serial transfers were carried out at daily intervals for 2 to 3 days to obtain an actively growing culture. Aliquots of 10 ml. of this culture were inoculated into 5 oz. prescription bottles each of which contained 100 ml. of freshly prepared sporulation medium. The bottles were inverted several times in a 9 and 17 h. cycle. Incubation was carried out at 30° and the progress of sporulation was followed by phase contrast microscopy. Spores were harvested by continuous centrifugation in a refrigerated Servall RC-2 centrifuge, washed three times in distilled water, resuspended in 0·067 M Sorenson's phosphate buffer (pH 7) and stored at −20° until needed.

*Heat treatment.* Heating was carried out in a 'Hot-Pack' water bath. To eliminate corrections for the time necessary to reach the heating temperature, the suspending buffer was first heated to the desired temperature. The stock spore suspension was added at zero time to give $10^7$ to $10^8$ spores/ml.; samples were withdrawn at intervals, rapidly cooled in ice and stored in the refrigerator. Spore viability was determined by colony counts in oval culture tubes using the medium of Wynne, Schmieding & Daye (1955) solidified with 1·5 % agar.

DPA was assayed by (*a*) the colorimetric method of Janssen, Lund & Anderson (1958) or (*b*) the direct spectrophotometric determination of the extinction at 270 nm. of the ethyl ether fraction obtained by reflux extraction (Perry & Foster, 1955).

## RESULTS

### *Release of DPA during heating*

Fig. 1 compares the rate of loss of DPA during thermal inactivation at 75° of spores of *Clostridium botulinum* strain 33A (type A, heat resistant) *v.* strain VH (type E, heat

sensitive). Strain 33A exhibited a relatively slow decline of the number of viable spores; the time necessary to destroy 90 % of the spores ($D_{75}$) was approximately 150 min. Release of DPA from strain 33A at 75° was barely measurable.

Strain VH.E showed a rather rapid rate of death within the first 10 min. followed by a less pronounced death rate on longer heating. The $D_{75}$ for the initial portion of the survival curve was approximately 9 min. The rate of loss of DPA during the first 10 min. was relatively rapid, i.e. only slightly slower than the rate of loss of viable spores. Furthermore, on heating beyond 10 min. the rate of loss of DPA appeared to exceed the rate of loss of spore viability.

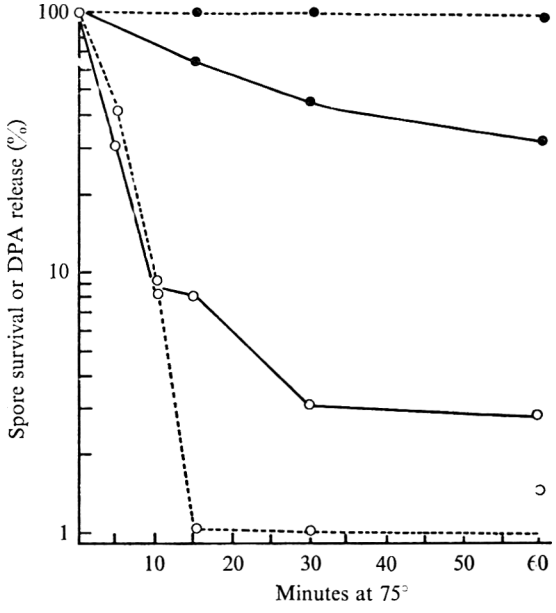


Fig. 1. Relation between thermal loss of dipicolinic acid and loss of viability of spores of *Clostridium botulinum*. The solid lines represent spore survival and the broken lines DPA release; ●, strain 33A; ○, strain VH.E.

Fig. 2 shows that during heating at 100° the initial rate of loss of DPA from four strains of *Clostridium botulinum* was consistently slower than the rate of loss of spore viability. Delay in DPA release such as observed here in *C. botulinum* has been repeatedly reported in studies with spores of other micro-organisms (Lund, 1958; Foster, 1959; El-Bisi *et al.* 1962).

Apparent exceptions were strains VH.E (Fig. 1) and 36A (Fig. 2), which exhibited a rate of release of DPA which after 10 min. of heating seemed to exceed the rate of loss of spore viability. However, unlike the other organisms studied, these two strains had distinctly diphasic survival curves suggesting that their spore populations were heterogeneous. The survival curves indicated that the populations consisted of approximately 90 to 98 % heat-sensitive spores responsible for the steep initial decline, and 2 to 10 % of heat-resistant spores responsible for the characteristic change in slope of the survival curves after 5 to 10 min. of heating. The small number of heat-resistant spores in the residual population would not contribute any measureable amount of

DPA. Under these conditions, one would expect to obtain the results found with strains VH.E and 36A. Similar diphasic survival curves have been reported by Graikoski & Kempe (1964) and Roberts & Ingram (1965) for spores of *Clostridium botulinum* type E.

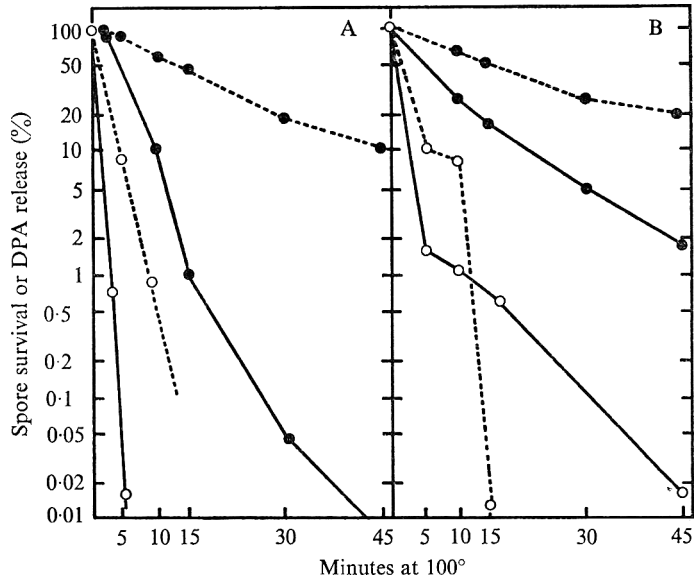


Fig. 2. Relation between thermal loss of dipicolinic acid and loss of viability of spores of *Clostridium botulinum*. The solid lines represent spore survival and the broken lines DPA release. A: ●, strain 33A; ○, strain 51B. B: ●, strain 41B; ○, strain 36A.

Table 2. *Release of DPA from spores of Clostridium botulinum strains of differing heat resistance*

| Strains* | Total DPA (% dry wt) | *D* (spore viability)† 75° | *D* (spore viability)† 100° | *D* (loss of DPA)‡ 75° | *D* (loss of DPA)‡ 100° |
|---|---|---|---|---|---|
| | | min. | min. | min. | min. |
| 33A | 13·4 | 150 | 10 | >23 h. | 42 |
| 36A | 8·7 | — | 3·0; 20 | — | 5 |
| 41B | 8·1 | — | 27 | — | >50 |
| 51B | 7·4 | — | 2·5 | — | 4 |
| VH.E | 9·5 | 9·0 | — | 10 min. | — |

* The letters A, B, and E signify serological types.

† *D* (spore viability) = the decimal reduction time, i.e. the time required for a 90% decrease in viable spores at the specified temperature.

‡ *D* (loss of DPA) = the time required for a 90% loss of DPA at the specified temperature.

There was no detectable correlation between the DPA-content of spores and their heat resistance (Table 1). Thus the most sensitive strain (VH.E) and the most resistant strain (41B) both contained about the same amount of DPA, namely 9·5 and 8·1 % respectively.

However, there seemed to be some correlation between the rate of loss of spore viability (i.e. heat resistance) and the rate of loss of DPA. From Fig. 1 and 2 it was

estimated that the heat-sensitive strains 36A and 51B lost 90 % of their DPA within 4 to 5 min. at 100°, whereas the heat-resistant strains 33A and 41B lost 90 % of their DPA within 42 and > 50 min. respectively.

From the *D* values listed in Table 2, the ratio of the rate of loss of DPA to the rate of loss of viability at 100° was 1·6 to 1·7 for the heat-sensitive strains and 2 to 4 for the heat-resistant strains. These ratios represent the extent by which loss of DPA lagged behind loss of viability. In this sense it may be concluded that in general the more heat-resistant strains retained DPA during heating more tenaciously than did the less heat-resistant strains.

### *Retention of DPA by heat-killed spores*

To determine whether there was any correspondence between the amount of DPA which remained spore-bound after various periods of heating and the number of spores that survived this treatment to retain their viability, the total residual spore-bound DPA was assayed and expressed as a % (w/w) of the calculated dry weight of the

Table 3. *Effects of* (*a*) *viability and* (*b*) *DPA retention of heating spores of Clostridium botulinum 33A at 100°*

The amounts of DPA retained has been calculated as a percentage of the dry weight of the surviving, viable spores.

| | Viable spores/ml. | | DPA retained | |
|---|---|---|---|---|
| Min. at 100° | Number $\times 10^8$ | Dry wt $\mu$g. | Total $\mu$g./ml. | Calculated as % (w/w) of viable spores |
| 0 | 3·5 | 8,225 | 1,102 | 13·40 |
| 2 | 3·2 | 7,520 | 1,048 | 13·94 |
| 5 | 2·3 | 5,405 | 976 | 18·05 |
| 10 | 0·37 | 869·5 | 694 | 79·81 |
| 15 | 0·038 | 89·3 | 526 | 589·02 |
| 30 | 0·0017 | 3·995 | 211 | 5,281·6 |
| 45 | 0·00028 | 0·658 | 137 | 20,820·0 |

surviving, viable spores. This experimentally determined ratio was then compared with the known DPA content of unheated, viable spores of this species (13·4 %, w/w). Three possibilities were envisaged: that this experimental ratio would possess (i) a value of 13·4 %, which would mean that heat-killed spores must be devoid of DPA; or (ii) a value of < 13·4 %, which would indicate total or very extensive loss of DPA from killed spores plus some loss of DPA from surviving, viable spores; or (iii) a value of > 13·4 %, in which case some DPA must have been retained by heat-killed spores irrespective of whether some DPA might simultaneously have been lost by surviving, viable spores.

Table 3 (last column) shows that possibility (iii) was in fact the actual situation. The calculated ratio steadily increased above the initial 13·4 %, reaching over 20,000 % after 45 min. of heating. Apparently, a considerable amount of DPA was retained by heat-killed spores. Thus it may be concluded that the thermal resistance of the spore was broken some time before all its DPA was lost.

The amount of DPA which may be retained in the spore at the instant of its death was estimated by plotting the number of spores killed *v.* amount of DPA released,

expressed as a percentage of the dry weight of killed spores (Fig. 3). During the initial heating, rapid loss of spore viability occurred with relatively little loss of DPA; this is evident from the steeply ascending portions of the plots in Fig. 3. On more prolonged heating, following the death of most of the spore population, additional DPA continued to be released as shown by the horizontal plateau of the plots in Fig. 3. The sharp 'breaking point' at the intersection of the horizontal and ascending portions of a plot represents the point of maximal destruction of spore viability, namely at this point the spore population was inactivated to the extent of 94 to 98 % (at 100°) and 91 to 92 % (at 75°). At the 'breaking point' the amount of DPA which had been

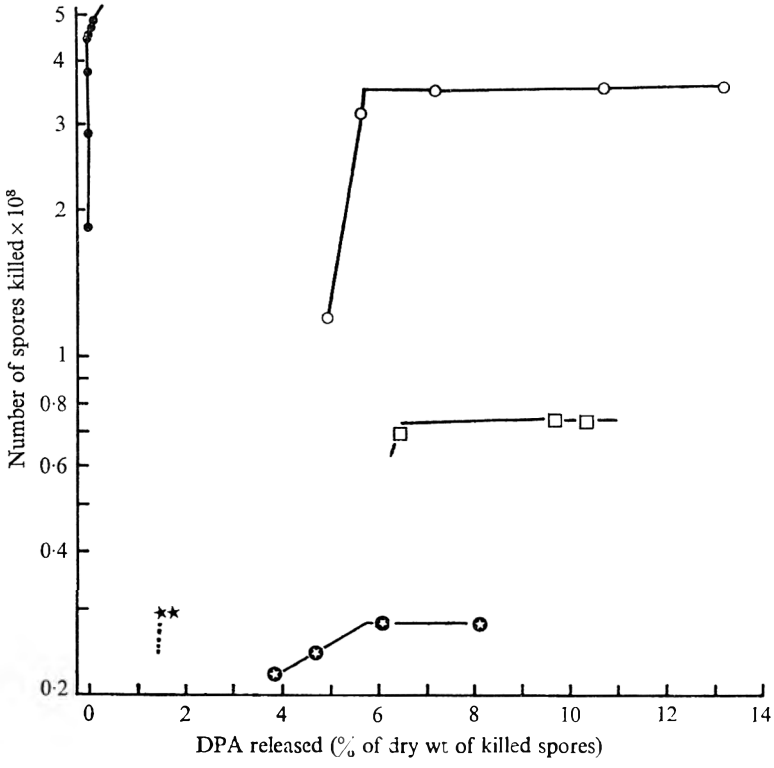


Fig. 3. Relation of released DPA to the number of spores killed during heating. The strain numbers and the temperature at which the spores were heated are as follows: ●, 33A, 75°; ○, 33A, 100°; □, VH.E, 75°; ★, 36A, 100°; and ✪, 41B, 100°.

Table 4. *Consequences of heating spores of Clostridium botulinum strains to the 'breaking points' indicated in Fig. 3*

| Strain | Heating temp. (°C) | Spores killed (% of total) | DPA: % By wt of spore Lost | DPA: % By wt of spore Retained | DPA: % of initial content retained |
|---|---|---|---|---|---|
| 33A | 100 | 97 | 5·8 | 7·6 | 57 |
| 41B | 100 | 94 | 5·8 | 3·2 | 28 |
| 36A | 100 | 98 | 1·56 | 7·14 | 83 |
| 33A | 75 | 91 | 0·05 | 13·35 | 99·6 |
| VH.E | 75 | 92 | 6·44 | 3·06 | 32 |

released from the spores was strain-determined and varied over a range from 0·4 to 72 % of the total initial DPA concentration (Table 4); therefore, at the instant of thermal death, all spores must have retained a considerable amount of their DPA, conceivably as much as 28 to 99·6 %.

## DISCUSSION

Our experiments have established a correlation between the rate of DPA loss during heating and the rate of thermal death of spores. However, the evidence does not enable one to say whether the loss of DPA is intimately connected with the event of thermal death or is merely a consequence of general structural degradation within the spore.

Release of DPA from spores during heating may involve at least two distinct sequential steps: (i) breaking or detachment of hypothetical DPA complexes from attachment sites in the spore (Rieman, 1963; Tang *et al.* 1968); and (ii) leakage of DPA or DPA complex(es) through the spore membranes and coats.

If DPA plays a role in heat resistance, step (i) may lead to denaturation of vital biopolymers and death of the spore even though DPA may remain trapped or adsorbed in the spore; only step (ii) would result in measurable loss of DPA into the medium.

In our strain 33A (Fig. 1), DPA may have been permanently or transiently released from its binding sites at 75°, resulting in extensive spore inactivation. However, the permeability of spore membranes and coats may not have been sufficiently affected to allow extensive loss of DPA to occur. At the same temperature the membranes or coats of the heat-sensitive strain VH type E may have been disorganized to such a degree that DPA penetrated them freely and was rapidly lost.

The relationship between a spore's content of DPA and its heat resistance, although widely believed, has never been satisfactorily proved. Furthermore, in addition to DPA, other mechanisms have been postulated, e.g. stabilizing –S–S– cross-linking predominant in spore protein (Vinter, 1961), gel-like spore interior analogous to thiolated gelatins (Black & Gerhardt, 1962), or stabilization by peptidoglycan polymer network of the spore cortex (Murrell *et al.* 1969). The existence of several parallel mechanisms would explain why spores of DPA-less mutants (Halvorson & Swanson, 1969), although much less heat-resistant than those of the wild type, are still more heat-resistant than vegetative cells.

Because of the many possible factors which may play a role in the thermal inactivation of spores and which may differ from strain to strain, one would not expect there to exist a simple relationship between heat resistance and either the amount of DPA or the rate of release of DPA during heating.

This work was supported by U.S. Public Health Service Grant UI 00138 and PHS Career Development Award 5-K3-AI-21,763. We thank Dr H. O. Halvorson, Dr. G. Gould, Dr W. G. Murrell and Dr R. Slepecky for their critical review of the manuscript.

## REFERENCES

BLACK, S. H. & GERHARDT, P. (1962). Permeability of bacterial spores. IV. Water content, uptake, and distribution. *Journal of Bacteriology* **83**, 960–967.

BYRNE, A. F., BURTON, H. T. & KOCH, R. B. (1960). Relation of dipicolinic acid content of anaerobic bacterial endospores to their heat resistance. *Journal of Bacteriology* **80**, 139–140.

CHURCH, B. D. & HALVORSON, H. (1959). Dependence of the heat resistance of bacterial endospores on their dipicolinic acid content. *Nature, London* **183**, 124–125.

COLLIER, R. E. & MURTY, G. G. K. (1957). The correlation of dipicolinic acid synthesis with the sporulation of *Clostridium roseum*. *Bacteriological Proceedings* p. 32.

EL-BISI, H. M., LECHOWICH, R. V., AMAHA, M. & ORDAL, Z. J. (1962). Chemical events during death of bacterial endospores by moist heat. *Journal of Food Science* **27**, 219–231.

FOSTER, J. W. (1959). *Dipicolinic Acid and Bacterial Spores*. Booklet containing text of lecture presented on 22 October 1959 at the University of Maryland, College Park, Maryland.

GRAIKOSKI, J. T. & KEMPE, L. L. (1964). Heat resistance of *Clostridium botulinum* type E spores. *Bacteriological Proceedings* p. 3.

HALVORSON, H. O. (1958). *The Physiology of Bacterial Spores*. Technical University of Norway, Trondheim.

HALVORSON, H. O. & SWANSON, A. (1969). Role of dipicolinic acid in the physiology of bacterial spores. In *Spores*, vol. IV, pp.121–132. Edited by L. L. Campbell. Bethesda: American Society for Microbiology.

HASHIMOTO, T., BLACK, S. H. & GERHARDT, P. (1960). Development of fine structure, thermostability and dipicolinate during sporogenesis in a Bacillus. *Canadian Journal of Microbiology* **6**, 203–213.

JANSSEN, F. W., LUND, A. J. & ANDERSON, L. E. (1958). Colorimetric assay for dipicolinic acid in bacterial spores. *Science, New York* **127**, 26–27.

LECHOWICH, R. V. & ORDAL, Z. J. (1960). The influence of sporulation temperature on the thermal resistance and chemical composition of endospores. *Bacteriological Proceedings*, pp. 44–45.

LEVISON, H. S., HYATT, M. T. & MOORE, F. E. (1961). Dependence of the heat resistance of bacterial spores on the calcium:dipicolinic acid ratio. *Biochemical and Biophysical Research Communications* **5**, 417–421.

LUND, A. (1958). In *Annual Report of the Hormel Institute*, 1957/58.

MURRELL, W. G. (1955). *The Bacterial Endospore*. Monograph published by the University of Sydney, Australia.

MURRELL, W. G. & WARTH, A. D. (1965). Composition and heat resistance of bacterial spores. In *Spores*, vol. III, pp. 1–24. Edited by L. L. Campbell & H. O. Halvorson. Ann Arbor, Michigan: American Society for Microbiology.

MURRELL, W. G., OHYE, D. F. & GORDON, R. A. (1969). Cytological and chemical structure of the spore. In *Spores*, vol. IV, pp. 1–19. Edited by L. L. Campbell. Bethesda: American Society for Microbiology.

PERRY, J. J. & FOSTER, J. W. (1955). Studies on the biosynthesis of dipicolinic acid in spores of *Bacillus cereus* var. *mycoides*. *Journal of Bacteriology* **69**, 337–346.

POWELL, J. F. (1953). Isolation of dipicolinic acid (pyridine-2,6-dicarboxylic acid) from spores of *Bacillus megatherium*. *Biochemical Journal* **54**, 210–211.

RIEMANN, H. (1963). *Germination of Bacterial Spores with Chelators*. Copenhagen: Th. Bang.

ROBERTS, T. A. & INGRAM, M. (1965). The resistance of spores of *Clostridium botulinum* type E to heat and radiation. *Journal of Applied Bacteriology* **28**, 125–138.

TANG, T., RAJAN, K. S. & GRECZ, N. (1968). Mixed chelates of Ca(II)-pyridine-2,6-dicarboxylate with some amino acids related to bacterial spores. *Biophysical Journal* **8**, 1458–1474.

VINTER, V. (1961). The formation of cystine-rich structure in sporulating cells and its possible role in the resistance of spores. In *Spores*, vol. II, pp. 127–141. Edited by H. O. Halvorson. Minneapolis, Minnesota: Burgess Publishing Company.

WALKER, H. W., MATCHES, J. R. & AYRES, J. C. (1961). Chemical composition and heat resistance of some aerobic bacterial spores. *Journal of Bacteriology* **82**, 960–966.

WOOLEY, B. C. & COLLIER, R. E. (1965). Changes in thermoresistance of *Clostridium roseum* as related to the intracellular content of calcium and dipicolinic acid. *Canadian Journal of Microbiology* **11**, 279–285.

WYNNE, E. S., SCHMIEDING, W. R. & DAYE, G. T., JUN. (1955). A simplified medium for counting Clostridium spores. *Food Research* **20**, 9–12.

*Journal of General Microbiology* (1970), **63**, 311–316
*With 3 plates*
*Printed in Great Britain*



# The Endoplasmic Reticulum as the Site of Potassium Tellurite Reduction in Yeasts

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SUMMARY

Potassium tellurite ($K_2TeO_3$) reduction in the obligate aerobe *Rhodotorula mucilaginosa* 49 and the facultative anaerobe *Saccharomyces cerevisiae* 1–434 occurs on localized areas of endoplasmic reticulum. The reduction product is granular and is found in close association with the membranes of specific parts of the endoplasmic reticulum. The site of reduction is the same whether *R. mucilaginosa* 49 is grown on a respiratory substrate (sodium malate) or a fermentable substrate (glucose).

Increasing concentrations of $K_2TeO_3$ increasingly inhibit the growth of *Rhodotorula mucilaginosa* 49. Its rate of oxygen uptake is not altered when grown in the presence of 0·04% $K_2TeO_3$, but in *Saccharomyces cerevisiae* 1–434 it decreases under the same conditions. Growth of *S. cerevisiae* 1–434 is completely inhibited with glycerol as substrate in the presence of $K_2TeO_3$.

## INTRODUCTION

The reduction of potassium tellurite ($K_2TeO_3$) to a black insoluble compound by living tissues is generally regarded as indicating enzymic oxidation reduction processes and, at the ultrastructural level, as indicating the localization of certain enzymes or enzyme systems.

Barnett & Palade (1957) showed that in mammalian heart tissue with succinate as substrate tellurite was reduced mainly in the mitochondria, and this observation was interpreted as providing a basis for a histochemical method for demonstrating the presence of a mitochondrial succinic dehydrogenase.

Different sites for reduction of $K_2TeO_3$ in bacteria have been reported. It is said to occur on the mesosomal membranes in *Bacillus subtilis* and the mesosome is consequently ascribed a respiratory function (van Iterson & Leene, 1964). In most other bacteria the reduction product is not associated with mesosomal membranes but instead occurs either near the cytoplasmic membrane (Tchan & Webber, 1966) or randomly in the cytoplasm (Brieger, 1963). Most workers reporting on reduction of $K_2TeO_3$ in bacteria have been unaware of the mechanism of the reaction, as substrates were not carefully controlled. However, Nermut (1967), using glycine as the substrate, localized glycine oxidase activity in *Proteus vulgaris* when $K_2TeO_3$ was the electron acceptor. Tellurium deposits were found only in the close vicinity of the plasmalemma.

Nickerson (1954) noticed that in *Candida albicans* tellurite was reduced to black

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metallic tellurium and selenite to red metallic selenium. Reduction of both was confined to particulate loci visible in light micrographs.

Nagai (1965) found that colonies of respiratory deficient (petites) and respiratory competent (normal) Saccharomyces species varied in their reduction of tellurite depending on culture conditions. Normal colonies turned black while petite colonies remained nearly white, but addition of ammonium sulphate to the medium weakened and sometimes completely reversed the effect. Nagai considered tellurite reduction only as a means of differentiating between petites and normal Saccharomyces colonies.

The present investigation demonstrates that both *Rhodotorula mucilaginosa* and *Saccharomyces cerevisiae* reduce $K_2TeO_3$. The effects of tellurite on growth and respiration have been studied, and the sites of reduction investigated at the ultrastructural level.

## METHODS

*Organisms.* The strains used were *Rhodotorula mucilaginosa* 49, an obligate aerobe, and *Saccharomyces cerevisiae* 1–434, a facultative anaerobe.

*Media. Rhodotorula mucilaginosa* 49 was grown in a medium containing (g./l.): glucose, 20; or sodium malate, 20; and $(NH_4)_2SO_4$, 20; $KH_2PO_4$, 20; yeast extract, 5; in distilled water. *Saccharomyces cerevisiae* 1–434 was grown in a medium containing (g./l.): glucose 20; or glycerol, 40; Bacto-peptone, 20; yeast extract, 10; in distilled water.

*Growth measurements.* Both organisms were grown up to midlogarithmic growth phase, and 0·5 ml. samples were inoculated into 50 ml. of the appropriate medium in conical flasks, and shaken at 30°. For experiments with $K_2TeO_3$, a 0·4% aqueous solution was membrane-filtered, and added aseptically to give a final concentration of 0·04%. Growth of the population was followed by counting yeast cells in a haemocytometer. Extinction measurements could not be used because the black reduction product obscured the true rate of growth.

*Oxygen uptake measurements.* Oxygen uptake was measured at 30° by means of a Rank Oxygen electrode coupled to a chart recorder.

*Electron Microscopy.* Samples of *Rhodotorula mucilaginosa* 49 were harvested, washed in distilled water and fixed in 1% unbuffered-aqueous potassium permanganate for 20 min at room temperature. *Saccharomyces cerevisiae* 1–434 cells were fixed for 1 h. in the same fixative. After centrifuging and washing, the cells were maintained as a pellet, dehydrated in tertiary butyl alcohol mixtures (Johansen, 1940) and embedded in Araldite. Sections were mounted on formvar-coated grids and examined in a Siemens Elmiskop I or a Zeiss EM 9. Sections were poststained for 30 min. in 50% (v/v) ethyl alcohol saturated with uranyl acetate followed by 2 min. in lead citrate (Reynolds, 1963).

## RESULTS

### *Effect of potassium tellurite on normal growth of yeasts*

In the glucose–salts medium in the presence of $K_2TeO_3$ the growth of *Rhodotorula mucilaginosa* 49 was inhibited. The effects of 0·04 and 0·08% $K_2TeO_3$ on the size of the final stationary population and the mean generation time are presented in Table 1.

In liquid medium with glucose as the fermentable substrate, *Saccharomyces cerevisiae* 1–434 multiplied and reduced $K_2TeO_3$. However, tellurite was finally toxic and the

growth rates were reduced in a way similar to those of *Rhodotorula mucilaginosa* 49. On the non-fermentable substrate glycerol both concentrations of tellurite completely inhibited growth.

*Oxygen uptake of yeasts grown in the presence of $K_2TeO_3$*

As $K_2TeO_3$ profoundly affected growth of both yeasts it was decided to investigate whether or not it caused inhibition of respiration. The oxygen uptake of exponentially growing cultures of *Rhodotorula mucilaginosa* 49 in glucose + salts medium in the presence and absence of tellurite was measured; the $K_2TeO_3$ concentrations were 0·04 and 0·08 %. The same number of centrifuged and washed yeast cells were used for all treatments. The cell suspensions were shaken at 30° during the experiment. Sodium azide, a known inhibitor of respiration, was added to test the sensitivity of the system.

Table 1. *The effect of $K_2TeO_3$ on the size of the stationary population and on the mean generation time of Rhodotorula mucilaginosa 49*

| | Control | 0·04 % $K_2TeO_3$ | 0·08 % $K_2TeO_3$ |
|---|---|---|---|
| Final stationary population as a percentage of control | 100 | 61 | 25 |
| Mean generation time | 1·71 h. | 1·85 h. | 2·85 h. |

Table 2. *Summary of oxygen uptake measurements on Rhodotorula mucilaginosa 49 and Saccharomyces cerevisiae 1–434*

Oxygen uptake rates are expressed as a percentage of the control rate.

| | *Rhodotorula mucilaginosa* 49 | *Saccharomyces cerevisiae* 1–434 | |
|---|---|---|---|
| | Glucose | Glucose | Glycerol |
| Control | 100 | 100 | 100 |
| 0·04 % $K_2TeO_3$ | 100 | 36·8 | No growth |
| 0·08 % $K_2TeO_3$ | 100 | 19·8 | No growth |
| $10^{-3}$ M-$NaN_3$ | 100 | 0·0 | 0·0 |

The oxygen uptake was identical for all four treatments, indicating that $K_2TeO_3$ did not inhibit growth by a direct effect on the respiratory pathways in *Rhodotorula mucilaginosa* 49. $10^{-3}$ M-Azide had no discernible effect on oxygen uptake; this is an unexplained result because azide is a potent inhibitor of mitochondrial electron transfer, inhibiting cytochrome $a_3$.

*Rhodotorula mucilaginosa* 49 grown normally to midlogarithmic growth phase and then exposed to 0·04 and 0·08 % $K_2TeO_3$ also showed no change in oxygen uptake.

Oxygen uptake was also measured for samples of *Saccharomyces cerevisiae* 1–434 grown with glucose as substrate and in the presence of 0·04 and 0·08 % $K_2TeO_3$. Tellurite lowered the rate of oxygen uptake (Table 2). Sodium azide completely inhibited oxygen uptake by normally grown *S. cerevisiae* 1–434 cells.

*Electron microscopy of Rhodotorula mucilaginosa 49*

A normal *Rhodotorula mucilaginosa* 49 cell fixed in potassium permanganate showed well-defined membrane-bounded organelles (Pl. 1, fig. 1). The mitochondria were

numerous with well-developed cristae and the central nucleus was enclosed in a double membrane with pores 80 nm. in diameter. A few small vacuoles bounded by a single unit membrane were usually visible.

The endoplasmic reticulum, a system of double membranes forming tubes and flat plates ramifying through the cytoplasm, was not oriented in any particular way in the yeast. It was frequently seen to be in connexion with the nuclear membrane. Unstained sections of midlogarithmic phase *Rhodotorula mucilaginosa* 49 cells grown with glucose as substrate in the presence of 0·04% $K_2TeO_3$ gave poor contrast. Because poststaining did not obscure the reduction product, all micrographs were of poststained sections.

The electron-opaque reduction product was granular and was deposited only on areas of endoplasmic reticulum near the nucleus (Pl. 1, fig. 2; Pl. 2, fig. 3, 4), areas of endoplasmic reticulum which were absent in control cells. This endoplasmic reticulum was of the tubular type, densely packed and showing much branching. No conclusion can be drawn as to the chemical nature of the reduction product. It was not in the form of crystalline tellurium but consisted of granules in close association with membranes of the endoplasmic reticulum (Pl. 1, fig. 2). In addition to the endoplasmic reticulum involved in tellurite reduction, other unbranched lengths of it were present without associated reduction product (Pl. 2, fig. 3).

When sodium malate, a specific respiratory substrate, replaced glucose in the culture medium, in the presence of 0·04% $K_2TeO_3$ the cells reduced tellurite at the same site as when glucose was substrate (Pl. 2, fig. 5). Dense deposits of reduction product were found on endoplasmic reticulum complexes connected to the nuclear membrane, though the latter had no reduction product associated with it.

*Electron microscopy of Saccharomyces cerevisiae 1–434*

For electron microscopy, *Saccharomyces cerevisiae* 1–434 was grown with 4% (w/v) melibiose as the carbon source. Melibiose, a non-repressing fermentable substrate, gave rise to membranes that preserved well (Marchant & Smith, 1968). The endoplasmic reticulum was less well-defined in *S. cerevisiae* 1–434 than in *Rhodotorula mucilaginosa* 49 when fixed in permanganate, and was mainly oriented around the periphery of the cell inside the plasmalemma (Pl. 3, fig. 6). Another difference was that the mitochondrial cristae were more numerous and prominent in *R. mucilaginosa* 49.

Cells of *Saccharomyces cerevisiae* 1–434 grown with 0·04% $K_2TeO_3$ on melibiose showed two types of endoplasmic reticulum (Pl. 3, fig. 7, 8) similar in distribution to those found in tellurite-grown cells of *Rhodotorula mucilaginosa* 49. The reduction product was less prominent than in *R. mucilaginosa* 49, but was also deposited on localized areas of branched endoplasmic reticulum near the nucleus and in connexion with the nuclear membrane (Pl. 3, fig. 7). The normal endoplasmic reticulum was sometimes difficult to distinguish from the endoplasmic reticulum involved in reduction, as the two types were often not clearly separated, but both could be recognized (Pl. 3, fig. 8).

## DISCUSSION

Studies of enzyme localization are valid only if the electron-dense product does not diffuse away from the site of deposition. The tellurite reduction product, tellurium, is insoluble in water and in organic solvents, but it is soluble in hot sulphuric acid and

Bromine water (Nermut, 1963). Although the reduction product has not been identified as tellurium in yeasts, Barnett & Palade (1957) and van Iterson & Leene (1964) consider that the granular product of tellurite reduction is deposited at the site of reduction and does not diffuse away.

*Rhodotorula mucilaginosa* 49 and *Saccharomyces cerevisiae* 1–434 reduced tellurite on localized branched areas of endoplasmic reticulum. These branched forms were absent in normally grown yeasts. It is postulated that the branching systems of membranes carried an enzyme or enzymes that reduced the tellurite which were not present on the normal lengths of less-branched endoplasmic reticulum.

Because potassium permanganate does not fix ribosomes, it does not permit a distinction between rough and smooth endoplasmic reticulum. Hence it is possible that the two types of endoplasmic reticulum observed in the present study differed in this respect. The above ultrastructural study implies that when a cell is exposed to a high internal concentration of tellurite ions, one type of endoplasmic reticulum proliferates and increases in its content of a particular enzyme or enzymes. This probably occurs with selenite as well, since Nickerson (1954) found that *Candida albicans* reduced tellurite and selenite in particulate loci, and these for tellurite are here demonstrated to be complexes of endoplasmic reticulum.

Barnett & Palade (1957) and Bisaltputra, Brown & Weier (1969) have concluded that dehydrogenases are the primary enzymes involved in tellurite reduction and that respiratory dehydrogenases are located by this reaction. However, respiratory and cytochemical studies on *Rhodotorula mucilaginosa* 49 indicated that tellurite reduction was not mediated by respiratory enzymes. Up to 0·08 % $K_2TeO_3$ had no effect on the rate of oxygen uptake, and when this yeast was grown on a respiratory substrate (sodium malate) in the presence of 0·04 % $K_2TeO_3$ reduction was still confined to localized areas of endoplasmic reticulum, and no reduction product was visible on the mitochondrial membranes.

In *Saccharomyces cerevisiae* 1–434 oxygen uptake is progressively inhibited by increasing concentrations of potassium tellurite. Furthermore, this yeast does not multiply in the presence of glycerol and tellurite, which indicates that respiration is completely inhibited. It appears therefore that the respiratory enzyme system of the facultative anaerobe *S. cerevisiae* 1–434 is sensitive to tellurite whereas respiratory enzymes in the obligate aerobe *Rhodotorula mucilaginosa* 49 are not.

Although the respiratory systems of the two yeasts showed different sensitivities to tellurite, the actual site of reduction was the same for both organisms, namely on a complex of branching endoplasmic reticulum.

This work was supported by a grant to P. S. C. from the Science Research Council.

## REFERENCES

BARNETT, R. J. & PALADE, G. E. (1957). Histochemical demonstration of the sites of activity of dehydrogenase systems with the electron microscope. *Journal of Biophysical and Biochemical Cytology* **3**, 577–588.

BISALTPUTRA, T., BROWN, D. L. & WEIER, T. E. (1969). Possible respiratory sites in a blue-green alga, *Nostoc sphaericum*, as demonstrated by potassium tellurite and tetranitro blue tetrazolium. *Journal of Ultrastructure Research* **27**, 182–197.

BRIEGER, E. M. (1963). In *Structure and Ultrastructure of Micro-organisms*, chap. 19. New York: Academic Press.

VAN ITERSON, W. & LEENE, W. (1964). A cytochemical localization of reductive sites in a Gram-positive bacterium. Tellurite reduction in *Bacillus subtilis*. *Journal of Cell Biology* **20**, 361–376.

JOHANSEN, D. A. (1940). *Plant Microtechnique*. New York: McGraw-Hill.

MARCHANT, R. & SMITH, D. G. (1968). Membranous structures in yeasts. *Biological Reviews* **43**, 459–480.

NAGAI, S. (1965). Differential reduction of tellurite by growing colonies of normal yeast and respiration deficient mutants. *Journal of Bacteriology* **90**, 220–222.

NERMUT, M. V. (1963). Determination of tellurium in the cells of Gram-negative bacteria. *Folia Microbiologica, Praha* **8**, 370.

NERMUT, M. V. (1967). Sites of 'glycine oxidase' activity in *Proteus vulgaris*. *Canadian Journal of Microbiology* **13**, 551–556.

NICKERSON, W. J. (1954). An enzymatic locus participating in cellular division of a yeast. *Journal of General Physiology* **37**, 483–494.

REYNOLDS, E. S. (1963). The use of lead citrate at high pH as an electron-opaque stain in electron microscopy. *Journal of Cell Biology* **17**, 208–212.

TCHAN, Y. T. & WEBBER, A. J. (1966). Fine structure and tellurite reduction in *Azotobacter vinelandii*. *Archiv für Mikrobiologie* **54**, 215–228.

## EXPLANATION OF PLATES

Abbreviations: n, nucleus; m, mitochondrion; v, vacuole; er, endoplasmic reticulum; te, reduction product

### PLATE 1

Fig. 1. Normal cell of *Rhodotorula mucilaginosa* 49 grown to midlogarithmic phase in glucose–salts medium. × 40,500.

Fig. 2. Section of a *R. mucilaginosa* 49 cell, grown in glucose–salts medium in the presence of 0·04 % $K_2TeO_3$. Granular reduction product is deposited on a localized area of endoplasmic reticulum. × 80,000.

### PLATE 2

Potassium tellurite reduction in *Rhodotorula mucilaginosa* 49

Fig. 3. Midlogarithmic phase cell grown in a glucose–salts medium with 0·04 % $K_2TeO_3$. Tellurite reduction occurs on the area of densely packed endoplasmic reticulum near the nucleus ($er_1$). Other lengths of endoplasmic reticulum not involved in reduction are present near the cell membrane ($er_2$). × 56,000.

Fig. 4. Cell grown under the same conditions as described for fig. 3. The reduction product is visible on the localized area of branching endoplasmic reticulum near the nucleus. × 57,600.

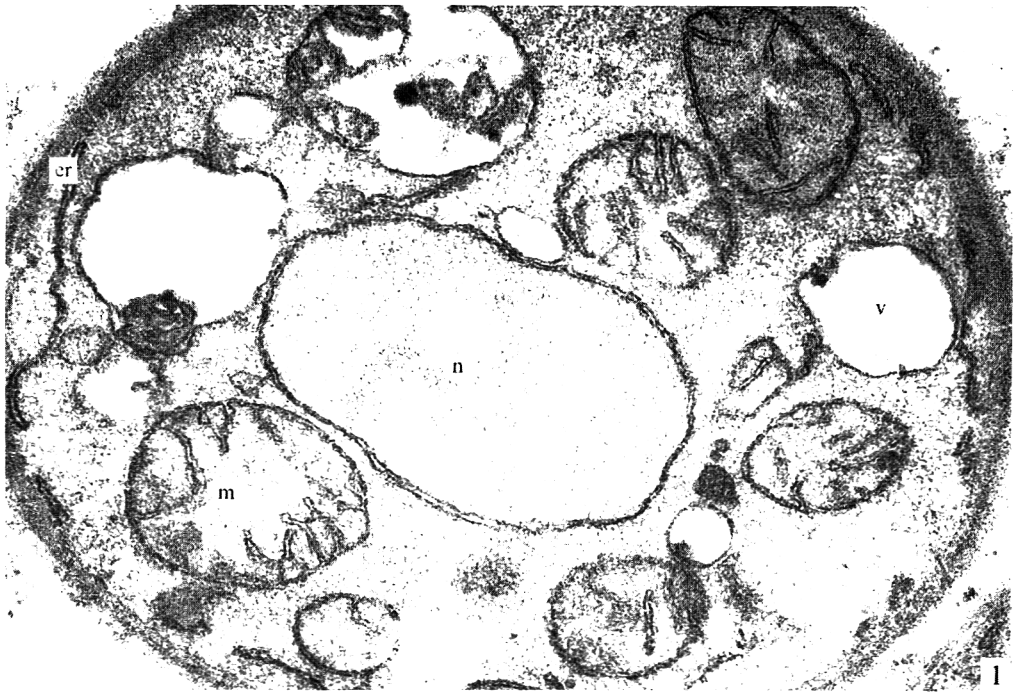
Fig. 5. Section of a cell grown on sodium malate in the presence of 0·04 % $K_2TeO_3$. Reduction product is deposited on the membranes of the endoplasmic reticulum, which is itself connected to the nuclear membrane. × 68,000.

### PLATE 3

Fig. 6. Control cell of *Saccharomyces cerevisiae* 1–434 grown on melibiose. The mitochondrial membranes are poorly defined and the endoplasmic reticulum is oriented mainly around the periphery of the cell. × 41,600.

Fig. 7. Midlogarithmic phase cell of *S. cerevisiae* 1–434 grown on 0·04 % $K_2TeO_3$ and melibiose. Reduction product is deposited on the area of endoplasmic reticulum in connexion with the nuclear membrane ($er_1$). The normal endoplasmic reticulum around the periphery of the cell ($er_2$) is not involved with reduction. × 54,000.

Fig. 8. *S. cerevisiae* 1–434 grown under the same conditions as in fig. 7. The reduction product is restricted to an area of branching endoplasmic reticulum, not present in normal cells ($er_1$); $er_2$, normal endoplasmic reticulum. × 50,000.

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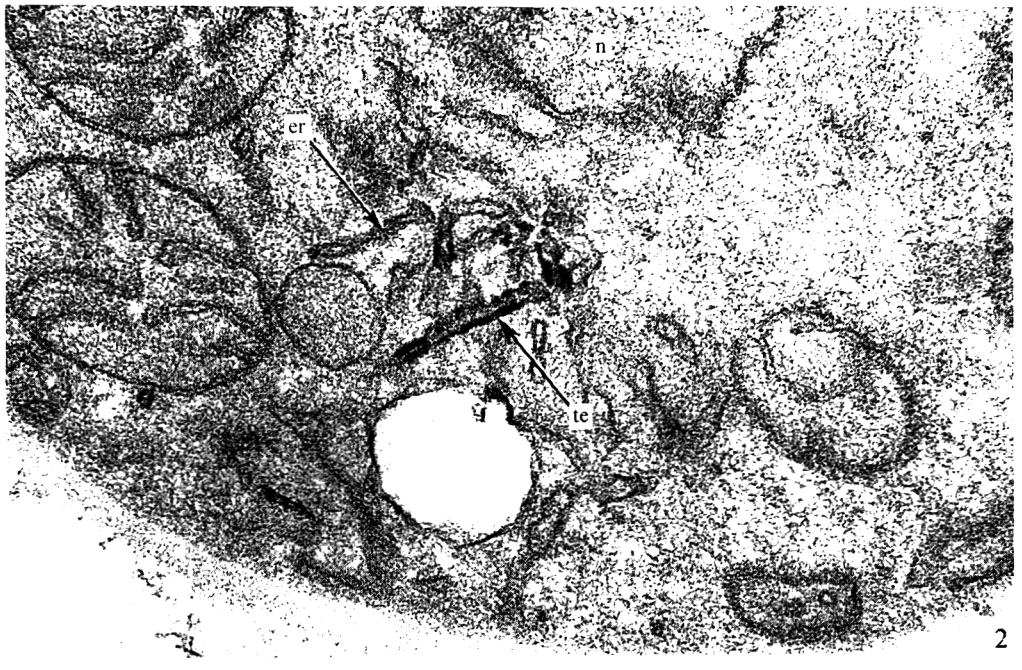


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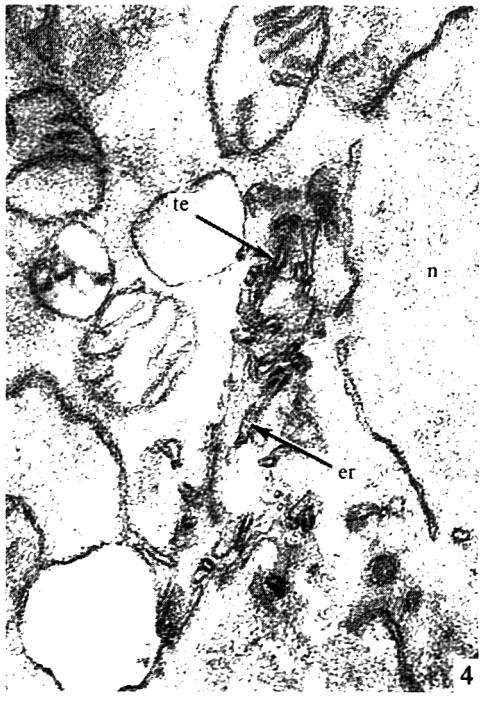


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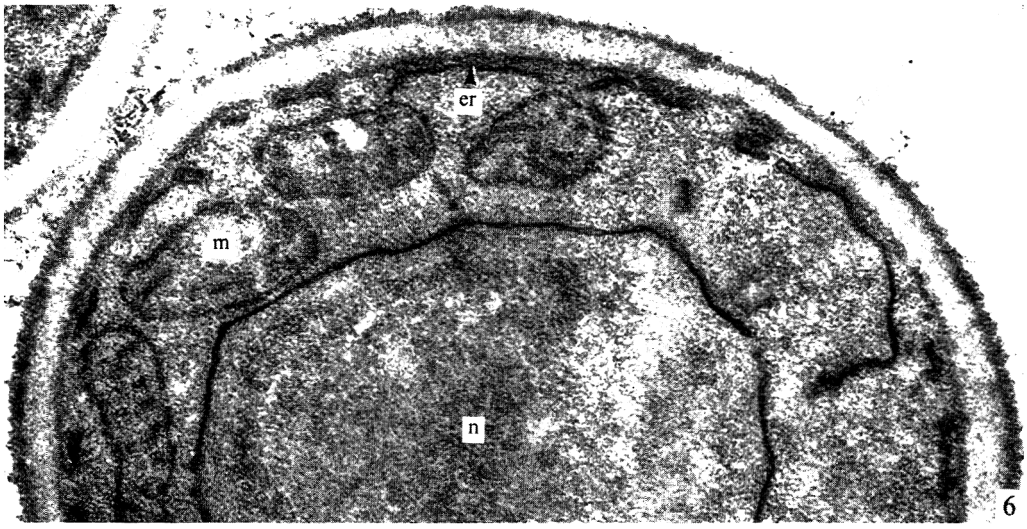


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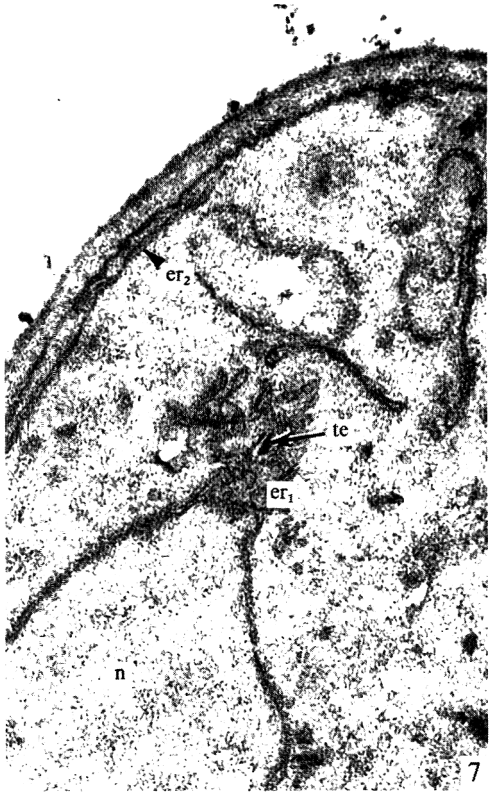


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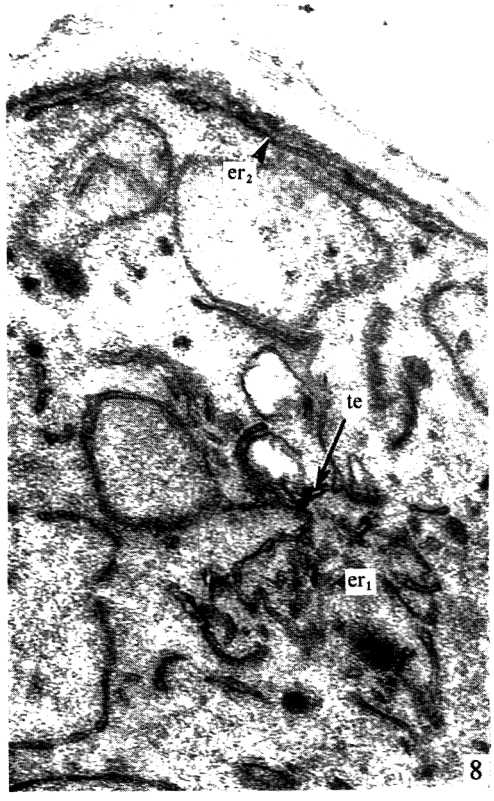


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*Journal of General Microbiology* (1970), **63**, 317–324
*Printed in Great Britain*



# The Use of Amides as Nitrogen Sources by *Aspergillus nidulans*

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(*Accepted for publication 26 August 1970*)

SUMMARY

The utilization of amides as nitrogen sources by *Aspergillus nidulans* has been studied. Formamide is hydrolysed by a specific formamidase enzyme. A second amidase is responsible for the utilization of acetamide, acrylamide, glycolamide and glycineamide. Propionamide, butyramide, valeramide and hexamide are also substrates for this enzyme, but other enzymes may contribute to their use as nitrogen sources. The aromatic amides, benzamide, phenylacetamide and nicotinamide are good nitrogen sources, but are probably not substrates for the acetamidase. Methyl carbamate and malonamide are neither substrates for the acetamidase nor serve as sole sources of nitrogen for *A. nidulans*. β-Hydroxypropionamide, lactamide and fumaramide are very poor nitrogen sources and are probably not substrates for the acetamidase. N-substituted amides are not nitrogen sources.

A large number of amides including non-substrate amides, induce formamidase or acetamidase or both. Acrylamide, although a good substrate for the acetamidase, does not induce its synthesis.

## INTRODUCTION

Amidase enzymes (acylamide-amidohydrolase, EC 3.5.1.4) hydrolyse amides and produce the corresponding carboxylic acid and ammonia. They have been described in rabbit liver extracts (Bray, James, Thorpe & Wasdell, 1950) and various species of mycobacteria (Draper, 1967; Halpern & Grossowicz, 1957; Kimura, 1959; Nagayama, Konno & Oka, 1961). Some fungi can use amides as sources of carbon or nitrogen and possess amidases (Zittle, 1951; Steiner, 1959). In *Pseudomonas aeruginosa* a single amidase enzyme was found to hydrolyse a number of amides. The enzyme was inducible by certain amides and subject to catabolite repression (Kelly & Clarke, 1962). Mutant strains, altered in the regulation of amidase synthesis, have been isolated (Brammar, Clarke & Skinner, 1967).

The observation that acetamide was a good nitrogen source for *Aspergillus nidulans* led to a study of amide utilization and amidase synthesis. A survey of amides was made to assess the number and substrate specificities of amidase enzymes and mutants altered in their ability to utilize amides were isolated.

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## METHODS

*Strains.* The wild-type strain was *bi1*, a biotin auxotroph, originating from the Department of Genetics, University of Glasgow. The isolation of mutants altered in amidase synthesis has been described elsewhere (Hynes & Pateman, 1970*a*, *b*).

*Chemicals and media.* Analytical grade chemicals were used. Media were as described by Pontecorvo *et al.* (1953) and Cove (1963). Supplements were stored at 4° as concentrated sterile solutions. Solutions of amides (Fluka Co.) were sterilized by Seitz filtration. Growth tests were carried out as described by Cove (1963). Plates were incubated at 35° for 2 to 4 days.

*Growth of mycelium for enzyme assays.* Mycelium was grown in 200 ml. of medium in 1 l. flasks shaken at 25° (Cove, 1966) and harvested by filtration through nylon net, washed with water, blotted dry and frozen.

*Preparation of cell-free extracts.* These were prepared as described by Cove (1966). All extracts were made in 100 mM-tris-HCl buffer, pH 7·2, and centrifuged at about 70,000*g* for 25 to 35 min. in a refrigerated centrifuge. Protein was determined by the Folin method (Lowry, Rosebrough, Farr & Randall, 1951) and was usually 1 to 2 mg./ml. of extract.

*Enzyme assays.* The amidase assay, which was based on the ammonia determination method of Muftic (1964), has been described elsewhere (Hynes, 1970). All substrate amides were used at concentrations of 10 to 20 mM in the reaction mixture.

Enzyme activities are expressed in nmoles of ammonia released/min./mg. protein.

## RESULTS

### *Growth of the wild type on amides as sole nitrogen sources*

Amides (at concentrations of 10 to 50 mM) were added as sole nitrogen sources to glucose-minimal medium. Growth of *Aspergillus nidulans* on the amides was compared with the sparse 'nitrogen-free' growth of the organism on media lacking a nitrogen source. Formamide, acetamide, propionamide, butyramide, valeramide, hexamide, glycineamide, glycolamide, benzamide, phenylacetamide and nicotinamide were good nitrogen sources. Nicotinamide was a nitrogen source, but resulted in abnormal, inhibited growth. β-Hydroxypropionamide, lactamide and fumaramide were only very poor nitrogen sources. Acrylamide, malonamide, methyl-carbamate and N-substituted amides (*N*-methylformamide, *N*-methylacetamide, *N*-ethylformamide, *N*-methylpropionamide, *N*-ethylacetamide, *N*,*N*-dimethylformamide and *N*,*N*-dimethylacetamide) were not nitrogen sources for *A. nidulans*.

*Interaction between amides as nitrogen sources.* Amides which were not nitrogen sources were tested for inhibition of growth when acetamide or formamide was the sole nitrogen source. Malonamide, methyl-carbamate, *N*-methylformamide, *N*-methylacetamide, *N*-ethylformamide, *N*-methylpropionamide and *N*,*N*-dimethylacetamide at concentrations of 10 to 50 mM did not affect growth on acetamide (concentrations of 10 to 50 mM). Similarly, 10 mM methyl-carbamate, malonamide, *N*-ethylformamide, *N*-methylformamide, *N*-methylacetamide and *N*,*N*-dimethylformamide did not affect growth on 10 mM-formamide. Thus the non-utilizable amides did not inhibit either uptake or hydrolysis of acetamide or formamide.

*Description of mutants.* The isolation of mutants altered in amidase synthesis has

been described elsewhere (Hynes & Pateman, 1970). The mutants used to investigate the utilization of amides were: (*a*) *fmdS*$^-$1, unable to grow on formamide as a nitrogen source and lacking amidase activity with formamide as a substrate; (*b*) *amdS*$^-$ mutants, unable to grow on acetamide and lacking amidase activity with acetamide as a substrate; (*c*) *amdR*$^c$ mutants, able to grow on acrylamide and to grow very strongly on acetamide as a nitrogen source. These mutants have high, partly constitutive acetamidase activities.

Table 1. *Growth of wild-type and mutant strains on various amides as sole nitrogen sources added to glucose-minimal medium*

| | Strain | | | | |
|---|---|---|---|---|---|
| Nitrogen source* | Wild type | *amdR*$^c$2 | *amdS*$^-$17 | *fmdS*$^-$1 | *amdS*$^-$17; *fmdS*$^-$1 |
| Formamide | + | + | + | C | ○ |
| Acetamide | + | +++ | ○ | + | ○ |
| Propionamide | + | ++ | ± | + | ± |
| Butyramide | + | ++ | ± | + | ± |
| Valeramide | + | + | ± | + | ± |
| Hexamide | + | ++ | + | + | + |
| Glutamine | + | + | + | . | . |
| Asparagine | + | . | + | . | . |
| Glycolamide | + | ++ | ○ | + | ○ |
| Glycineamide | + | ++ | ○ | + | ○ |
| Benzamide | + | + | + | ± | ± |
| Phenylacetamide | + | + | ± | ± | ± |
| β-Hydroxypropionamide | ± | ± | ± | ± | ± |
| Lactamide | ± | ± | ± | ± | ± |
| Fumaramide | ± | ± | ± | ± | ± |
| Nicotinamide | + | + | + | . | . |
| Urea | + | + | + | + | + |

* Concentration, 10 mM.

Symbols on different media are not equivalent. +++ = Very strong growth; ++ = strong growth; + = growth; ± = poor growth; ○ = 'nitrogen-free' growth.

*Growth properties of mutants on amides.* Table 1 shows the growth of the wild-type and mutant strains on various amides as nitrogen sources. All strains except those containing the *fmdS*$^-$1 mutation grew well on formamide. The *fmdS*$^-$1 mutant grew as well as the wild type on all other amides except benzamide and phenylacetamide. These results indicated that there was an amidase specific for formamide as a substrate, i.e. a formamidase. The effect of the *fmdS*$^-$1 mutation on benzamide and phenylacetamide utilization may have been the result of formamide being a product of their breakdown rather than their being substrates for the formamidase.

The *amdS*$^-$ mutation resulted in inability to grow on acetamide, glycolamide and glycineamide, but did not affect growth on formamide. This indicated that there was an amidase, called an acetamidase, which was solely responsible for the utilization of these amides. *amdR*$^c$ mutants have high acetamidase activities and were found to grow better on these three amides than did the wild type.

Propionamide, butyramide, valeramide and hexamide were clearly substrates for the acetamidase, since growth of the *amdS*$^-$ mutants was significantly less on these amides than that of wild type. In addition, the *amdR*$^c$ mutants grew better than wild type on these amides. There appeared to be other pathways for the utilization of

these amides because *amdS*⁻ and *amdS*⁻ *fmdS*⁻ strains could grow reasonably well on them, and growth on hexamide was particularly strong. The *amdS*⁻ and *amdR*ᶜ mutations also appeared to affect growth on phenylacetamide. However, it cannot be excluded that this was caused by production of acetamide from phenylacetamide.

β-Hydroxypropionamide, lactamide and fumaramide were extremely poor nitrogen sources for all strains and so may not have been substrates for either the formamidase or the acetamidase. Urea, asparagine and glutamine were very good nitrogen sources for *Aspergillus nidulans*, and growth on these was not affected in any of the mutants tested, which indicated that the acetamidase and formamidase enzymes did not contribute significantly to their utilization. The *amdS*⁻ mutation did not affect growth on nicotinamide and so this amide was probably not a substrate for the acetamidase.

*Enzyme assays with different amides as substrates.* The amidase assay used can be applied to all potential substrates capable of releasing ammonia. It was therefore possible to compare the hydrolysis of a variety of amides by crude extracts with that of acetamide.

Table 2. *Amidase activities of the wild type grown on amides as nitrogen sources*

| | Nitrogen source† | | | | | | |
|---|---|---|---|---|---|---|---|
| Substrate* | Urea | Form-amide | Acet-amide | Propion-amide | Butyr-amide | Valer-amide | Hex-amide |
| Formamide | 21 | 107 | 132 | 162 | 173 | 248 | 293 |
| Acetamide | 14 | 114 | 103 | 71 | 76 | 76 | 90 |
| Propionamide | 31 | 106 | 83 | 73 | 105 | 120 | 113 |
| Butyramide | 28 | 95 | 83 | 81 | 113 | 120 | 112 |
| Hexamide | 21 | 79 | 70 | 63 | 90 | 105 | 166 |

* 20 mM in 100 mM pyrophosphate buffer, pH 7·2.
† Added at concentration of 10 mM to glucose minimal medium.

Methyl carbamate and malonamide gave less than 5 % of the activity with acetamide in extracts of wild-type and *amdR*ᶜ strains. Therefore these amides were not nitrogen sources probably because they were not substrates for an amidase enzyme. Acrylamide gave 70 to 80 % of the activity with acetamide as a substrate in a number of extracts of wild-type and *amdR*ᶜ strains. Since *amdR*ᶜ mutants have increased acetamidase levels, this showed that acrylamide was a good substrate for the acetamidase and that other enzymes did not contribute significantly to its hydrolysis. Acrylamide was not a nitrogen source for wild type *Aspergillus nidulans* and, as shown below, this was caused by its lack of inducer activity.

Table 2 shows the activities of extracts of wild-type mycelia that has been grown on a number of amides as nitrogen sources with these same amides used as substrates. Growth on all the amides caused increased amidase activities compared with the activity on urea. The propionamidase, butyramidase and hexamidase activities of mycelia grown on urea were higher than the acetamidase activity, while these activities of mycelia grown on formamide and acetamide were slightly lower than the acetamidase activity. In mycelia grown on the other amides, activities were similar to or higher than the corresponding acetamidase activities. Notably there was a considerably higher hexamidase activity in extracts of mycelia grown on hexamide.

Table 3 shows the activities with different amides as substrates in extracts of mutants

with altered acetamidase activities. The *amdR*$^c$ mutant, which had higher acetamidase activities than the wild type, also showed higher activities with the other amides as substrates, although they were generally much lower than the corresponding acetamidase activities. These results are in agreement with the finding that *amdR*$^c$ mutants grew better than the wild type on these amides as nitrogen sources, indicating that propionamide, butyramide, valeramide and hexamide were substrates for the acetamidase.

Table 3. *Amidase activities of wild-type, amdS*$^-$*17 and amdR*$^c$*6 strains grown on urea plus various amides*

| | Nitrogen source† | | | | | | |
|---|---|---|---|---|---|---|---|
| Substrate* | Urea | Urea + formamide | Urea + acetamide | Urea + propionamide | Urea + butyramide | Urea + valeramide | Urea + hexamide |
| Wild type | | | | | | | |
| Acetamide | 27 | 41 | 55 | 30 | 23 | 29 | 38 |
| Propionamide | 24 | 31 | 55 | 34 | 37 | 53 | 64 |
| Butyramide | 46 | 50 | 96 | 43 | 45 | 51 | 58 |
| Valeramide | 14 | 20 | 48 | 22 | 14 | 29 | 45 |
| Hexamide | 29 | 25 | 43 | 27 | 21 | 37 | 84 |
| *amdS*$^-$17 | | | | | | | |
| Acetamide | 5 | 5 | 4 | 8 | 3 | 6 | 15 |
| Propionamide | 21 | 25 | 38 | 27 | 26 | 36 | 38 |
| Butyramide | 21 | 15 | 18 | 22 | 26 | 23 | 38 |
| Valeramide | 16 | 11 | 21 | 19 | 20 | 13 | 29 |
| Hexamide | 18 | 12 | 27 | 19 | 26 | 23 | 38 |
| *amdR*$^c$6 | | | | | | | |
| Acetamide | 222 | 381 | 223 | 248 | 180 | 228 | 180 |
| Propionamide | 63 | 143 | 111 | 114 | 121 | 154 | 210 |
| Butyramide | 97 | 136 | 76 | 75 | 64 | 89 | 105 |
| Valeramide | 75 | 122 | 46 | 87 | 64 | 75 | 105 |
| Hexamide | 70 | 191 | 60 | 111 | 103 | 132 | 197 |

* 20 mM in 100 mM-pyrophosphate buffer, pH 7·2.
† Urea was added at 1·25 mM and amides at 10 mM to glucose-minimal medium.

The *amdS*$^-$ mutant had very low acetamidase activities (close to the accurate limit of the assay), but significant activities with the other amides as substrates. Therefore hydrolysis of these amides could be accomplished by an enzyme (or enzymes) other than the acetamidase. This agreed with the growth tests which showed that strains containing *amdS*$^-$ would grow on propionamide, butyramide, valeramide and hexamide as nitrogen sources. There was not good evidence for induction of other amidase enzymes by the amides. In the *amdS*$^-$ strain grown on urea plus amides the activities were similar to that of *amdS*$^-$ grown on urea alone. This point requires further investigation.

*Induction of amidase enzymes by amides.* A large number of amides were tested for inducer activity by growing mycelium on urea plus the amide being tested and comparing activities with those of mycelia grown on urea alone. A low concentration of urea (1·25 mM) was used to reduce the effects of repression, which was especially important in the control of the formamidase. Most amides were not very good inducers of amidase enzymes (Table 4). However, *N*-methylacetamide was a good inducer of both activities, while $\beta$-hydroxypropionamide, which was a very poor nitrogen source

and probably not a substrate for the acetamidase, was a very good inducer of the acetamidase but not of the formamidase. Benzamide, which was a very good nitrogen source, induced both enzymes. However, it is not known if this was due to benzamide itself, or to some breakdown product. Another aromatic amide, nicotinamide, was not an inducer.

Table 4. *The induction of the acetamidase and the formamidase in wild type by various amides*

| Inducing amide* | Induction ratio† | |
|---|---|---|
| | Acetamidase | Formamidase |
| Acetamide | 2·5 | 2·1 |
| Fumaramide | 1·4 | 0·8 |
| β-Hydroxypropionamide | 3·7 | 1·0 |
| Lactamide | 1·3 | 0·6 |
| Methyl carbamate | 1·2 | 1·6 |
| Benzamide | 3·6 | 2·0 |
| Nicotinamide | 1·0 | 0·8 |
| *N*-methylacetamide | 2·0 | 1·8 |
| *N*-methylformamide | 1·7 | 1·1 |
| *N*,*N*-dimethylformamide | 1·1 | — |
| *N*-ethylacetamide | 1·0 | — |

* Concentration, 10 mM.

† Induction ratio $= \frac{\text{activity on urea + inducer}}{\text{activity on urea}}$.

Table 5. *Summary of the utilization of amides by the acetamidase and formamidase enzymes*

| Amide | Nitrogen source | Substrate for formamidase | Substrate for acetamidase | Inducer of formamidase | Inducer of acetamidase |
|---|---|---|---|---|---|
| Formamide | + | + | − | + | + |
| Acetamide | + | − | + | + | + |
| Propionamide | + | − | + | + | + |
| Butyramide | + | − | + | + | + |
| Valeramide | + | − | + | + | + |
| Hexamide | + | − | + | + | + |
| Glycolamide | + | − | + | n.d. | + |
| Glycineamide | + | + | − | n.d. | + |
| Benzamide | + | − ? | − ? | + ? | + ? |
| Phenylacetamide | + | − ? | − ? | n.d. | n.d. |
| Nicotinamide | + | n.d. | − | − | − |
| β-Hydroxypropionamide | ± | − | − | − | + |
| Lactamide | ± | − | − | − | ± |
| Fumaramide | ± | − | − | − | ± |
| Acrylamide | − | − | + | n.d. | − |
| Malonamide | − | − | − | n.d. | n.d. |
| Methylcarbamate | − | − | − | + | ± |
| *N*-methylformamide | − | n.d. | n.d. | − | + |
| *N*-methylacetamide | − | n.d. | n.d. | + | + |
| *N*-ethylformamide | − | n.d. | n.d. | n.d. | n.d. |
| *N*-methylpropionamide | − | n.d. | n.d. | n.d. | n.d. |
| *N*-ethylacetamide | − | n.d. | n.d. | n.d. | − |
| *N*,*N*-dimethylformamide | − | n.d. | n.d. | n.d. | − |
| *N*,*N*-dimethylacetamide | − | n.d. | n.d. | n.d. | n.d. |

n.d. = Not determined; + = yes; ± = weak; − = no.

The above results were complicated by repression of the acetamidase by glucose (Hynes, 1970). Therefore induction experiments were carried out in carbon-free medium. Under these conditions acetamide and β-hydoxypropionamide, but not acrylamide, were found to induce the acetamidase. This therefore explained why acrylamide, although a good substrate for the enzyme, was not a nitrogen source for *Aspergillus nidulans*.

*Summary of amide utilization.* Table 5 summarizes amide utilization by *Aspergillus nidulans*. Some of these data are based solely on growth tests, and negative results in particular should be regarded with caution. Uptake of amides may complicate some of the conclusions about the amides which were not nitrogen sources. However, the inducing activity of amides such as methylcarbamate, *N*-methylacetamide and β-hydroxypropionamide suggested that these were taken up to some extent. Preliminary experiments on the uptake of [$^{14}$C]acetamide were carried out with wild-type and *amdS*$^-$ strains. No accumulation of acetamide was found and so *A. nidulans* may lack an uptake system for acetamide.

## DISCUSSION

*Aspergillus nidulans* has been shown to possess an amidase specific for formamide as a substrate and a second amidase responsible for the utilization of acetamide and a number of other amides. Other enzymes contribute to the utilization of longer chain amides and aromatic amides; possibly hydrolysis of longer chain amides occurs as a side reaction of such enzymes as esterases or proteases. Aromatic amides may be broken down without hydrolysis of the amide group. Some fungi can metabolize aromatic compounds (Cain, Bilton & Darrah, 1968).

Asparagine and glutamine are not substrates for the formamidase or the acetamidase There may be a specific asparaginase and a specific glutaminase for the utilization of these amides. *Aspergillus nidulans* possesses a urease enzyme, which is lacking in mutants unable to grow on urea as a nitrogen source. These mutants map at loci distinct from the *amdS* and *fmdS* loci (Scazzocchio & Darlington, 1968).

A large number of amides (including N-substituted amides) are not nitrogen sources for *Aspergillus nidulans*. N-substituted amides may not be substrates for amidase enzymes, since it is known that *A. nidulans* can use amines (which would be the products of hydrolysis of N-substituted amides) as nitrogen sources (Arst & Cove, 1969).

Many amides can act as inducers of the formamidase and acetamidase enzymes. These include both substrate and some non-substrate amides. Induction of the formamidase is not specific, although this enzyme is specific for formamide as a substrate. The inducer specificities of the acetamidase and the formamidase are similar but not exactly the same, e.g. β-hydroxypropionamide is a good inducer for the acetamidase, but not for the formamidase. Acrylamide, although a good substrate for the acetamidase, is not an inducer of its synthesis and is consequently not a nitrogen source. This allows the isolation of regulatory mutants producing the acetamidase constitutively (Hynes & Pateman, 1970*a*, *b*).

The use of amides by *Aspergillus nidulans* contrasts with that by *Pseudomonas aeruginosa* where there appears to be only one amidase which is most active with acetamide and propionamide; formamide is a poorer substrate (Kelly & Clarke, 1962). This enzyme, in addition to having hydrolytic amidase activity, also has acyl group transferase activity with hydroxylamine as an acyl acceptor, and consequent

formation of hydroxamates. Preliminary evidence (M. J. Hynes & P. J. Wright, unpublished) suggests that the *A. niduians* acetamidase also has transferase activity. Variations in the acetamidase activity of extracts of wild-type, *amdS*$^-$ and *amdR*$^c$ strains corresponded with parallel variations in transferase activities. There appears to be a single amidase in *Mycobacterium smegmatis* with activity for formamide and butyramide (Draper, 1967). In this case the amidase activity can be dissociated from transferase activity. In animal tissues, an amidase has been shown to have greatest activity with amides with 5 to 7 carbon atoms (Bray *et al.* 1950).

## REFERENCES


ARST, H. N. & COVE, D. J. (1969). Methylammonium resistance in *Aspergillus nidulans*. *Journal of Bacteriology* **98**, 1284–1293.

BRAMMAR, W. J., CLARKE, P. H. & SKINNER, A. J. (1967). Biochemical and genetical studies with regulator mutants of the *Pseudomonas aeruginosa* 8602 system. *Journal of General Microbiology* **47**, 87–102.

BRAY, H. G., JAMES, S. P., THORPE, W. V. & WASDELL, M. R. (1950). The fate of certain organic acids and amides in the rabbit. II. Further observations on the hydrolysis of amides by tissue extracts. *Biochemical Journal* **47**, 294–299.

CAIN, R. B., BILTON, R. F. & DARRAH, J. A. (1968). The metabolism of aromatic acids by micro-organisms. Metabolic pathways in the fungi. *Biochemical Journal* **108**, 797–828.

COVE, D. J. (1963). *Studies of the Biochemistry and Genetics of Inorganic Nitrogen Metabolism in Aspergillus nidulans*. Ph.D. Thesis, University of Cambridge.

COVE, D. J. (1966). The induction and repression of nitrate reductase in the fungus *Aspergillus nidulans*. *Biochimica et Biophysica Acta* **113**, 51–56.

DRAPER, P. (1967). The aliphatic acylamide amidohydrolase of *Mycobacterium smegmatis*: its inducible nature and relation to acyl transfer to hydroxylamine. *Journal of General Microbiology* **46**, 111–123.

HALPERN, Y. S. & GROSSOWICZ, N. (1957). Hydrolysis of amides from mycobacteria. *Biochemical Journal* **65**, 716–720.

HYNES, M. J. (1970). Induction and repression of amidase enzymes in *Aspergillus nidulans*. *Journal of Bacteriology* **103**, 482–487.

HYNES, M. J. & PATEMAN, J. A. J. (1970*a*). The genetic analysis of regulation of amidase synthesis in *Aspergillus nidulans*. I. Mutants able to utilize acrylamide. *Molecular and General Genetics* **108**, 97–106.

HYNES, M. J. & PATEMAN, J. A. J. (1970*b*). The genetic analysis of regulation of amidase synthesis in *Aspergillus nidulans*. II. Mutants resistant to fluoracetamide. *Molecular and General Genetics* **108**, 107–116.

KELLY, M. & CLARKE, P. H. (1962). An inducible amidase produced by a strain of *Pseudomonas aeruginosa*. *Journal of General Microbiology* **27**, 305–316.

KIMURA, T. (1959). Studies on metabolism of amides in Mycobacteriaceae. III. Amidases and transferases in extracts from Mycobacteriaceae. *Journal of Biochemistry, Tokyo* **46**, 1271–1279.

LOWRY, O. H., ROSEBROUGH, M. J., FARR, A. L. & RANDALL, R. J. (1951). Protein measurement with the Folin phenol reagent. *Journal of Biological Chemistry* **193**, 265–275.

MUFTIC, K. C. (1964). A new phenol–hypochlorite reaction for ammonia. *Nature, London* **201**, 622–623.

NAGAYAMA, H., KONNO, K. & OKA, S. (1961). Formamidase in mycobacteria and its use in differentiating saprophytic mycobacteria from other mycobacteria. *Nature, London* **190**, 1219–1220.

PONTECORVO, G., ROPER, J. A., HEMMONS, L. M., MACDONALD, K. D. & BUFTON, A. W. J. (1953). The genetics of *Aspergillus nidulans*. *Advances in Genetics* **5**, 141–238.

SCAZZOCCHIO, C. & DARLINGTON, A. J. (1968). The induction and repression of the enzymes of purine breakdown in *Aspergillus nidulans*. *Biochimica et Biophysica Acta* **166**, 557–568.

STEINER, M. (1959). The utilization of amino and amide nitrogen by *Endomycopsis vernalis* and other yeasts (with special reference to metabolic reactions at the surface of the cells). *Symposia of the Society for Experimental Biology* **13**, 177–192.

ZITTLE, C. (1951). Hydrolysis of acid amides and amino acid amides. In *The Enzymes*, vol. 1, part 2, p. 922. Edited by J. B. Sumner and K. Myrback. New York: Academic Press.

*Journal of General Microbiology* (1970), **63**, 325–331
*Printed in Great Britain*



# The Influence of Growth-limiting Substrate and Medium NaCl Concentration on the Synthesis of Magnesium-binding Sites in the Walls of *Bacillus subtilis* var. *niger*

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(*Accepted for publication 27 August 1970*)

SUMMARY

A comparative study has been made of the influence of growth environment on the properties of the walls of *Bacillus subtilis* var. *niger* organisms, particularly with respect to ability to bind magnesium ions. The medium NaCl concentration and the nature of the growth-limiting substrate, in chemostat cultures, both affected markedly the subsequent affinity and capacity of the bacterial walls for $Mg^{2+}$ adsorption. These changes correlated with differences in the phosphate content of the walls, which in turn related largely to changes in wall teichoic acid content. Thus it seemed that whenever a constraint to the adsorption of $Mg^{2+}$ was applied (either by growing the organisms in a $Mg^{2+}$-limited environment, or in one containing high concentrations of a competing ion), walls were synthesized that had an increased teichoic acid content and an increased affinity for magnesium ions. These results support the thesis that anionic polymers in the walls of Bacillus organisms (i.e. teichoic acids and teichuronic acids) are involved in cation assimilation.

## INTRODUCTION

We reported previously (Tempest, Dicks & Meers, 1967) that the affinities with which *Bacillus subtilis* and *Aerobacter aerogenes* cell walls bound magnesium ions were different, and that this difference correlated with the ability of the Gram-negative organisms to outgrow the bacilli in $Mg^{2+}$-limited mixed cultures in a chemostat. The above mentioned adsorption experiments were made with organisms grown in $Mg^{2+}$-limited culture only; but since the wall composition of organisms is known to vary with the chemical nature of the environment (Ellwood & Tempest, 1967; Tempest, Dicks & Ellwood, 1968), it was of interest to determine whether the affinity with which organisms bound $Mg^{2+}$ at their surfaces also varied with changes in the nature of the growth limitation. In this connexion, Tempest & Meers (1968) reported that *A. aerogenes* had a lower tolerance for NaCl when its growth was limited by $Mg^{2+}$ availability than when its growth was limited by the availability of any other essential nutrient. This suggested that sodium ions impeded $Mg^{2+}$ assimilation, possibly by competing for adsorption sites on the bacterial surface. Since adsorbed $Mg^{2+}$ probably has an important functional role in bacteria (Gray & Wilkinson, 1965; Asbell & Eagen, 1966; Kushner & Onishi, 1966; Tempest & Strange, 1966), it seemed possible that bacteria might adapt to environments containing low $Mg^{2+}$ concentrations,

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or high $Na^+$ concentrations, either by increasing the number of sites available for $Mg^{2+}$ adsorption or by changing the chemical nature of these sites such that their affinity for $Mg^{2+}$ was increased. These possibilities have been investigated and the results are reported here. A preliminary account of this work has been published (Meers & Tempest, 1969).

## METHODS

*Organism. Bacillus subtilis* var. *niger* (obtained from Fort Detrick, Maryland, U.S.A.; probably the same as ATCC 9372) and *Aerobacter aerogenes* (NCTC 418) were maintained by monthly subculture on tryptic–meat–digest agar slopes containing glucose (0·2%, w/v).

*Growth conditions.* Organisms were grown in 0·5 l. chemostats (Herbert, Phipps & Tempest, 1965) in the simple salts media described by Tempest & Meers (1968) and by Ellwood & Tempest (1969). The chemostat was operated at a dilution rate of 0·3 $h.^{-1}$ and at a temperature of 35°; the pH value was automatically maintained throughout at pH $7·0 \pm 0·05$.

*Experimental procedures.* To determine the capacity and affinity of binding of $Mg^{2+}$ to bacteria, the following procedure was adopted: 2 ml. volumes of culture, each containing about 3 mg. equivalent dry weight of organisms, were centrifuged at 3000 *g* for 5 min. Usually the bacterial pellet was then sufficiently compacted for the supernatant fluid to be removed by decantation; otherwise it was carefully removed using a Pasteur pipette. The bacterial pellet was then dispersed in 4 ml. of 0·85% (w/v) NaCl solution, using a 'Whirlimixer' (Fisons Scientific Apparatus Ltd, Loughborough, Leicestershire), recentrifuged, the supernatant fluid again discarded and the pellet retained. Washing the pellets in saline removed the surface-adsorbed $Mg^{2+}$ that was associated with organisms grown in the presence of an excess of this cation (Strange & Shon, 1964). The pelleted organisms were then suspended in various 2 ml. volumes of 0·85% (w/v) NaCl solution, and 0·017% (w/v) NaCl solution, that contained concentrations of $Mg^{2+}$ between 1 and 256 $\mu$g./ml., and left to equilibrate for 2 to 3 min. at room temperature before being separated by centrifugation. Changing the contact time did not alter the extent of $Mg^{2+}$ adsorption, indicating that equilibrium was established rapidly. Occluded $Mg^{2+}$ was removed by washing the organisms, once, in a small volume (4 to 5 ml.) of water. Finally, samples were 'wet-ashed' (with 1 ml. volumes of 60% (w/v) $HClO_4$), diluted to an appropriate volume with water and analysed for $Mg^{2+}$ by using an E.E.L. Atomic Adsorption Spectrophotometer (model 140). From these data, plus determinations of the dry weight of organisms in the original suspension (Tempest, Hunter & Sykes, 1965), the adsorbed $Mg^{2+}$ (per mg. dry weight of organisms) could be calculated along with the equilibrium extracellular $Mg^{2+}$ concentration.

The above method was also used to determine the capacity and affinity for $Mg^{2+}$ of bacterial wall preparations which had been obtained by the method of Tempest *et al.* (1968). With bacterial wall preparations the starting suspensions contained 1 mg. freeze-dried walls/ml.; each centrifugation step was of 15 min. duration (at 3000 *g*).

Potentiometric titration curves were obtained by the method of Brown (1965) using 50 mg. quantities of freeze-dried bacterial walls suspended in 5 ml. volumes of water, or of solutions of various salts. These suspensions could be repeatedly titrated between values of pH 2·0 and 7·0, giving reproducible results. However, at pH values greater than 8·0 the walls changed irreversibly.

Isolation and examination of wall teichoic acids and teichuronic acids were carried out as described previously (Tempest *et al.* 1968).

## RESULTS

The $Mg^{2+}$ adsorbed by saline-washed suspensions of *Bacillus subtilis* and *Aerobacter aerogenes* organisms (from $Mg^{2+}$-limited and $PO_4^{3-}$-limited cultures) when suspended in 0·017% (w/v) NaCl solutions containing graded concentrations of $MgCl_2$ are shown in Fig. 1 *a*, *b*. This low concentration of NaCl was chosen in order to simulate the salts concentration normally present in the various growth media. It is clear (Fig. 1) that the maximum amount of $Mg^{2+}$ that each sample could adsorb (that is, its $Mg^{2+}$-binding capacity) varied with the growth environment. Thus $Mg^{2+}$-limited *B. subtilis* organisms had a greater capacity for $Mg^{2+}$ adsorption than had the organisms from a $PO_4^{3-}$-limited culture. And similar, though quantitatively less marked, results were obtained with the organisms from *A. aerogenes* chemostat cultures.

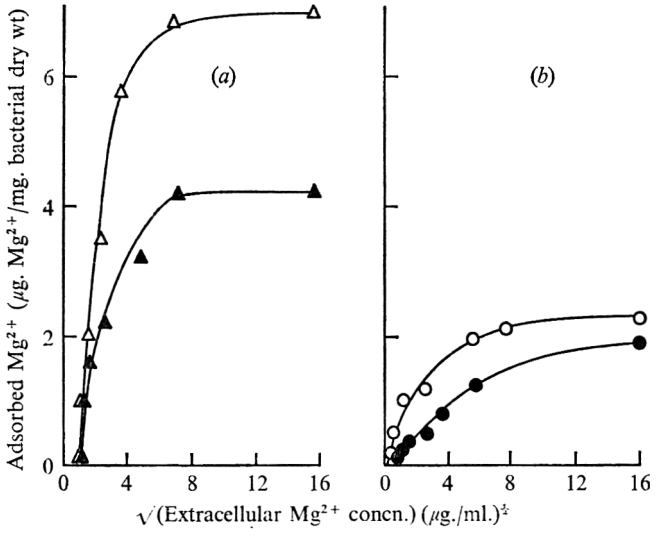


Fig. 1. Magnesium ion-binding capacity of (*a*) *Bacillus subtilis* var. *niger* organisms and (*b*) *Aerobacter aerogenes* organisms that had been grown in chemostat cultures ($D$ = 0·3 $h.^{-1}$, 35°, pH 7·0) in simple salts environments with either $Mg^{2+}$ availability limiting growth (open symbols) or $PO_4^{3-}$ availability limiting growth (blocked symbols). Data were obtained as described in Methods; solutions of $MgCl_2$ each contained 0·017% (w/v) NaCl.

Although *Aerobacter aerogenes* organisms generally had a much lower capacity for $Mg^{2+}$ adsorption than had *Bacillus subtilis* organisms, they nevertheless bound $Mg^{2+}$ more avidly (Tempest *et al.* 1967). Adsorption of $Mg^{2+}$ was competitively inhibited by $Na^+$; therefore, by measuring the $Mg^{2+}$ bound by the variously grown samples of organisms (or isolated bacterial walls) when suspended in solutions of NaCl (0·85%, w/v) containing graded $Mg^{2+}$ concentrations, the specific affinity of each of the cell surfaces for $Mg^{2+}$ could be assessed. When organisms from variously limited *B. subtilis* chemostat cultures were thus compared (Fig. 2) it was evident that $Mg^{2+}$-limited organisms had a much greater affinity for $Mg^{2+}$ than had organisms from other *B. subtilis* cultures. But, in almost every case, addition of 4% (w/v) NaCl to the medium

in which the organisms were growing caused the synthesis of bacteria whose walls had an increased affinity for $Mg^{2+}$ adsorption (Fig. 2). Thus it seemed that whenever a constraint was applied to the uptake of $Mg^{2+}$ (either by limiting growth by the availability of $Mg^{2+}$ or by adding substantial amounts of $Na^+$ to the growth environment), bacterial walls were synthesized that had an improved affinity for $Mg^{2+}$ binding.

It is also evident (Fig. 2) that $PO_4^{3-}$-limited *Bacillus subtilis* organisms had a particularly low affinity for $Mg^{2+}$. Addition of 4% (w/v) NaCl to these $PO_4^{3-}$-limited cultures led to a particularly marked increase in $Mg^{2+}$ binding affinity. Since $Mg^{2+}$-limited and $PO_4^{3-}$-limited *B. subtilis* organisms had walls that were substantially different chemically (Tempest *et al.* 1963), an attempt was made to relate these changes in magnesium-binding affinity to changes in wall macromolecule content and composition.

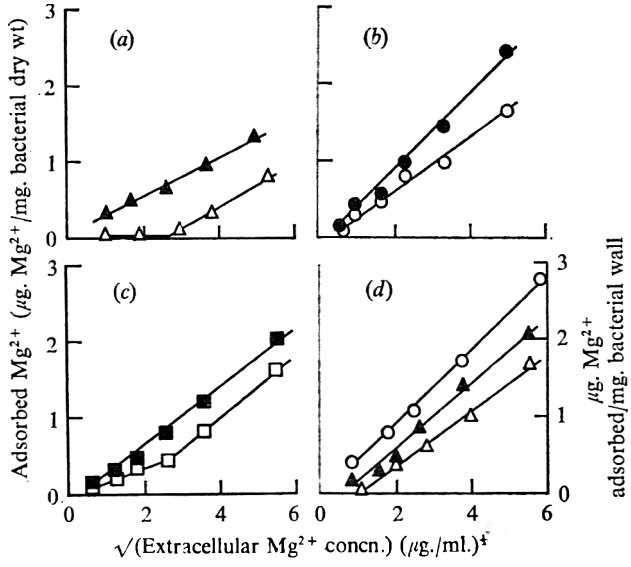


Fig. 2. Magnesium ion-binding affinity of *Bacillus subtilis* var. *niger* organisms, and wall preparations. The open symbols refer to organisms grown in the absence of added NaCl and the closed symbols to those grown in the presence of 4% (w/v) NaCl. Organisms were grown at a dilution rate of 0·3 $h.^{-1}$ (35°, pH 7·0) in (*a*) $PO_4^{3-}$-limited environments, (*b*) $Mg^{2+}$-limited environments, and (*c*) $K^+$-limited environments. Data were obtained as described in Methods; solutions of $MgCl_2$ each contained 0·85% (w/v) NaCl. Similar data were obtained with isolated bacterial wall preparations (*d*); samples were prepared from $Mg^{2+}$-limited organisms (○), $PO_4^{3-}$-limited organisms (△), and from $PO_4^{3-}$-limited organisms grown in the presence of 4% (w/v) NaCl (▲).

In order to investigate further the nature of the anionic $Mg^{2+}$-binding sites, potentiometric titration experiments were undertaken on preparations of isolated *Bacillus subtilis* walls. With walls of $Mg^{2+}$-limited *B. subtilis* organisms, the pH titration curve showed three prominant areas of buffering (Fig. 3*a*); one in the region of pH 2·2 (due to primary phosphate groups), one at pH 3·6 (due to carboxyl groups) and one at pH 5·8 (also possibly due to phosphate) (see Galdiero, 1968; James & Brewer, 1968). In contrast, the walls of $PO_4^{3-}$-limited *B. subtilis* organisms lacked buffering at pH 2·2 and 5·8 but had a greatly increased buffering capacity at pH 3·4 (Fig. 3*b*). These findings were consistent with the presence of much teichoic acid in the walls of $Mg^{2+}$-limited organisms and much teichuronic acid in the walls of $PO_4^{3-}$-limited organisms.

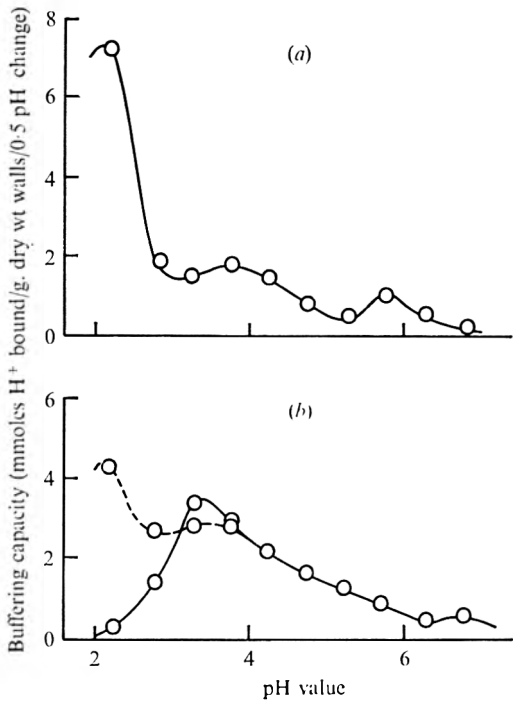


Fig. 3. Buffering capacity of wall preparations from *Bacillus subtilis* var. *niger* organisms grown in (*a*) a $Mg^{2+}$-limited environment and (*b*) a $PO_4^{3-}$-limited environment that contained either no added NaCl (solid line) or 4% (w/v) NaCl (broken line). Suspensions of walls (50 to 100 mg. in 5 ml. water) were titrated with 0·1 N-HCl or 0·1 N-NaOH, using an Agla micrometer syringe (Burroughs Wellcome & Co., London). The wall suspensions were continuously mixed during the course of the titration by sparging with oxygen-free nitrogen gas. In order to derive the data shown above, blank values, obtained by titrating 5 ml. volumes of water, were substracted from the values obtained with the wall suspensions.

Table 1. *The influence of growth environment on the phosphate content of isolated walls of Bacillus subtilis var. niger, and on their capacity and affinity of binding of* $Mg^{2+}$

To measure the $Mg^{2+}$-binding capacity, wall preparations were suspended in 100 mM-$MgCl_2$ solution and then washed repeatedly with deionized water until the bound $Mg^{2+}$ content was constant. To measure the $Mg^{2+}$-binding affinity, wall preparations were suspended in a solution of $MgCl_2$ (0·5 mM) in 0·85% (w/v) NaCl. The walls were separated by centrifugation, washed once with deionized water and the adsorbed $Mg^{2+}$ determined by atomic adsorption spectrophotometry. The values for $Mg^{2+}$-binding affinity (below) are expressed as ratios of $Mg^{2+}$ adsorbed by wall preparation to $Mg^{2+}$ adsorbed by an equivalent weight of $Mg^{2+}$-limited *B. subtilis* walls.

| Growth limitation | Medium NaCl content (g./l.) | Wall phosphorus content (g./100 g. dry walls) | $Mg^{2+}$-binding capacity ($\mu$g./mg. dry wt) | $Mg^{2+}$-binding affinity |
|---|---|---|---|---|
| $Mg^{2-}$ | 0·1 | 5·8 | 15·6 | 1·00 |
| $Mg^{2-}$ | 40·0 | 6·4 | 19·0 | 1·30 |
| $PO_4^{3-}$ | 0·1 | 0·3 | 13·3 | 0·54 |
| $PO_4^{3-}$ | 40·0 | 3·0 | 17·5 | 0·73 |

Addition of 100 mM-$MgCl_2$ to the bacterial wall suspensions caused a marked shift in the titration curves showing that this cation was bound by both the phosphate and carboxyl groups (see Galdiero, Lembo & Tufano, 1968).

Addition of 4% (w/v) NaCl to the $PO_4^{3-}$-limited medium in which *Bacillus subtilis* was grown caused an appreciable change in the wall phosphate content and $Mg^{2+}$-binding capacity (Table 1) and a marked change in the pH titration curve (Fig. 3*b*) and $Mg^{2+}$-binding affinity (Table 1). The most obvious explanation for these observations was that the presence of NaCl in the growth medium induced, in some way, the synthesis of some wall-bound teichoic acid in the $PO_4^{3-}$-limited organisms. When the walls of these organisms were isolated, hydrolysed with 6 N-HCl (3 h., 100°) and analysed chromatographically (Tempest *et al.* 1968), amounts of glucose and glycerol monophosphate, and traces of glycerol diphosphate, were detected. These substances, which are characteristic of a glycerol teichoic acid, were absent from hydrolysed wall preparations of $PO_4^{3-}$-limited organisms grown in the absence of NaCl. Clearly (Table 1), the phosphorus content, the $Mg^{2+}$-binding affinity and the buffering capacity at pH 2·2, of wall preparations each increased with addition of 4% (w/v) NaCl to the growth environment but the three factors did not change proportionately.

## DISCUSSION

The data given in this paper show unequivocally that the walls of bacteria (*Bacillus subtilis* var. *niger* and *Aerobacter aerogenes*) changed markedly in response to changes in the environment in which they were grown. In particular, when the environment was adjusted so that a constraint was applied to the adsorption of $Mg^{2+}$ (that is, by growing the organisms in a medium that contained low concentrations of $Mg^{2+}$ or high concentrations of $Na^+$), walls were synthesized that had an increased affinity for magnesium ions. With cultures of *B. subtilis* var. *niger*, the increased affinity for $Mg^{2+}$ correlated with an increased teichoic acid content of the walls and it is reasonable to conclude, therefore, that teichoic acids are involved in the process of $Mg^{2+}$ assimilation, particularly when this nutrient is not readily accessible. In this context, it has been suggested by Archibald *et al.* (1961) that teichoic acids, being strongly anionic, may be functionally involved in the movement of ions between the organism and its environment; the results reported here accord fully with this hypothesis.

The fact that the walls of $PO_4^{3-}$-limited *Bacillus subtilis* var. *niger* organisms, though lacking teichoic acid, contained an alternative anionic polymer adds further support to the suggestion that these polymers play an essential role in the flux of charged metabolites across the cell wall and plasma membrane. However, the $Mg^{2+}$-binding affinity of teichuronic acid, although not negligible, was clearly much less than of teichoic acid (Table 1) and presumably was functionally inadequate when the growth environment contained much NaCl. Addition of 4% (w/v) NaCl to $PO_4^{3-}$-limited cultures of *B. subtilis* caused the synthesis of walls containing both teichoic acid and teichuronic acid, and these walls had a greatly increased $Mg^{2+}$-binding affinity.

It has been reported (Kushner & Onishi, 1966) that extremely halophilic Gram-negative bacteria require a high concentration of $Mg^{2+}$ for growth. Furthermore, the proteins in their envelopes are, compared with mesophilic cell walls, extremely acidic (Brown, 1963). It would be of interest to know whether the walls of these halophilic organisms have a strong affinity for magnesium ions; if our thesis is correct, this should be so.

The results reported here reinforce our previous conclusion (Tempest & Ellwood, 1969) that the walls of bacteria are highly variable entities which can change structurally (and, presumably, functionally) in such a way as to fit the organism for life in a wide variety of environments.

We are grateful to Dr D. C. Ellwood for many valuable discussions during the course of this work, and to Mr T. H. Dunham for skilled technical assistance.

## REFERENCES

ARCHIBALD, A. R., ARMSTRONG, J. J., BADDILEY, J. & HEY, B. (1961). Teichoic acid and the structure of bacterial cell walls. *Nature, London* **191**, 570–572.

ASBELL, M. A. & EAGON, R. G. (1966). Role of multivalent cations in the organization, structure and assembly of the cell wall of *Pseudomonas aeruginosa*. *Journal of Bacteriology* **92**, 380–387.

BROWN, A. D. (1963). The peripheral structures of Gram-negative bacteria. *Biochimica et Biophysica Acta* **75**, 425–435.

BROWN, A. D. (1965). Hydrogen ion titrations of intact and dissolved lipoprotein membranes. *Journal of Molecular Biology* **12**, 491–508.

ELLWOOD, D. C. (1970). The wall content and composition of *Bacillus subtilis* var. *niger*, grown in a chemostat. *Biochemical Journal* **118**, 367–373.

ELLWOOD, D. C. & TEMPEST, D. W. (1967). Teichoic acid or teichuronic acid in the walls of *Bacillus subtilis* var. *niger*, grown in a chemostat. *Biochemical Journal* **104**, 69P.

ELLWOOD, D. C. & TEMPEST, D. W. (1969). Control of teichoic acid and teichuronic acid biosyntheses in chemostat cultures of *Bacillus subtilis* var. *niger*. *Biochemical Journal* **111**, 1–5.

GALDIERO, F. (1968). Hydrogen ion binding of bacterial cell wall. *Experientia* **24**, 352–353.

GALDIERO, F., LEMBO, M. & TUFANO, M. A (1968). Affinity of various cations for *Staphylococcus aureus* cell wall. *Experientia* **24**, 34–36.

GRAY, G. W. & WILKINSON, S. G. (1965). The effect of ethylenediaminetetra-acetic acid on the cell walls of some Gram-negative bacteria. *Journal of General Microbiology* **39**, 385–399.

HERBERT, D., PHIPPS, P. J. & TEMPEST, D. W. (1965). The chemostat: design and instrumentation. *Laboratory Practice* **14**, 1150–1161.

JAMES, A. M. & BREWER, J. E. (1968). Non-protein components of the cell surface of *Staphylococcus aureus*. *Biochemical Journal* **107**, 817–821.

KUSHNER, D. J. & ONISHI, H. (1966). Contribution of protein and lipid components to the salt response of envelopes of an extremely halophilic bacterium. *Journal of Bacteriology* **91**, 653–660.

MEERS, J. L. & TEMPEST, D. W. (1969). The influence of environment on the synthesis of magnesium-binding sites on *Bacillus subtilis* var. *niger* cell walls. *Journal of General Microbiology* **55**, x.

STRANGE, R. E. & SHON, M. (1964). Effects of thermal stress on viability and ribonucleic acid of *Aerobacter aerogenes* in aqueous suspension. *Journal of General Microbiology* **34**, 99–114.

TEMPEST, D. W. & ELLWOOD, D. C. (1969). The influence of growth condition on the composition of some cell wall components of *Aerobacter aerogenes*. *Biotechnology and Bioengineering*, **11**, 775–783.

TEMPEST, D. W. & MEERS, J. L. (1968). The influence of NaCl concentration of the medium on the potassium content of *Aerobacter aerogenes* and on the interrelationships between potassium, magnesium and ribonucleic acid in the growing bacteria. *Journal of General Microbiology* **54**, 319–325.

TEMPEST, D. W. & STRANGE, R. E. (1966). Variation in the content and distribution of magnesium, and its influence on survival, in *Aerobacter aerogenes* grown in a chemostat. *Journal of General Microbiology* **44**, 273–279.

TEMPEST, D. W., DICKS, J. W. & ELLWOOD, D. C. (1968). Influence of growth condition on the concentration of potassium in *Bacillus subtilis* var. *niger* and its possible relationship to cellular ribonucleic acid, teichoic acid and teichuronic acid. *Biochemical Journal* **106**, 237–243.

TEMPEST, D. W., DICKS, J. W. & MEERS, J. L. (1967). Magnesium-limited growth of *Bacillus subtilis* in pure and mixed cultures in a chemostat. *Journal of General Microbiology* **49**, 139–147.

TEMPEST, D. W., HUNTER, J. R. & SYKES, J. (1965). Magnesium-limited growth of *Aerobacter aerogenes* in a chemostat. *Journal of General Microbiology* **39**, 355–366.

*Journal of General Microbiology* (1970), **63**, 333–345
*Printed in Great Britain*



# Molar Growth Yields and Fermentation Balances of *Lactobacillus casei* L 3 in Batch Cultures and in Continuous Cultures

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(*Accepted for publication 28 August 1970*)

SUMMARY

Fermentation balances determined for different substrates in batch and continuous cultures of *Lactobacillus casei* revealed two pathways of pyruvate conversion by this organism, a reduction to lactate and the phosphoroclastic cleavage. Pyruvate formed anaerobically from mannitol and citrate was split by the phosphoroclastic enzyme. Lactate was the main fermentation product formed during aerobic growth on mannitol and anaerobic and aerobic growth on glucose. In glucose-limited continuous cultures pyruvate conversion was dependent on the dilution rate. At low dilution rates glucose was fermented exclusively to acetate, ethanol and formate. At high rates only small amounts of acetate, ethanol and formate were formed and lactate production was maximal. Lactate dehydrogenase of *L. casei* had an absolute requirement for fructose-1,6-diphosphate and manganous ions. The specific activity of lactate dehydrogenase did not differ significantly at different dilution rates. It was concluded that the intracellular level of fructose-1,6-diphosphate controlled the pathway of pyruvate conversion. In batch cultures $Y_{ATP}$ values were between 18·2 and 20·9. No evidence for oxidative phosphorylation was found. In continuous cultures $Y_{ATP}$ values varied from 18·7 at low dilution rates to 23·5 at high dilution rates. From the dependence of $Y_{ATP}$ on the dilution rate, a maintenance coefficient of $1{\cdot}52 \times 10^{-3}$ was calculated. The $Y_{ATP}$ value corrected for energy of maintenance was 24·3. The possibility that the molar growth yields were erroneously high because of assimilation of growth substrate into intracellular polysaccharides, or because of energy yield from components of the medium other than the added energy source, was excluded.

## INTRODUCTION

In a previous paper (de Vries & Stouthamer, 1968) we reported two pathways for pyruvate conversion in bifidobacteria: a reduction of pyruvate to L-lactate and the phosphoroclastic split. The amount of pyruvate converted via either pathway depended on the growth substrate. Indications were found that more lactate and less acetate and formate were formed from growth substrates metabolized rapidly than from substrates which permitted only slow growth. Exact data were not given because bifidobacteria were difficult to culture. In the present report, investigations are extended to a strain of *Lactobacillus casei*. Previously, van den Hamer (1960) found that suspensions of this organism fermented glucose almost completely to lactate, whereas appreciable amounts of volatile acids were formed from ribose. Preliminary experi-

ments with ribose and pyruvate indicated that the phosphoroclastic split of pyruvate operates in this organism. The purpose of the present study was to examine the relation between growth rate and pattern of fermentation products in a strain of *L. casei* and to determine the factor which regulates the pathway of pyruvate conversion. Molar growth yields and $Y_{ATP}$ values (g. dry weight of bacteria formed/mole ATP) were also measured.

From the investigations of Bauchop & Elsden (1960) and others it was concluded that $Y_{ATP}$ was a constant for different micro-organisms (reviewed by Forrest, 1969; Stouthamer, 1969). The generally accepted value of $Y_{ATP}$ was 10·5. However, some recent work questions the concept of a constant $Y_{ATP}$ value for different micro-organisms. Hobson & Summers (1967) found that molar growth yields of some rumen bacteria varied with growth rate. From optimum growth yields and known fermentation reactions they calculated a $Y_{ATP}$ value of about 20 for *Bacteroides amylophilus* and *Selenomonas ruminantium*, growing on maltose and glucose respectively, and 15 for lipolytic bacterium 5S growing on fructose. Moustafa & Collins (1968) found $Y_{ATP}$ values of 17 (range 15·3 to 20) for certain Streptococcus strains. They observed a rapid autolysis of the cells after exhaustion of the energy source from the medium and were careful to determine growth yields at the moment of maximum growth. Smalley, Jahrling & VanDemark (1968) measured anaerobic and aerobic molar growth yields of *Streptococcus faecalis* and found evidence for oxidative phosphorylation in this bacterium. However, growth yield measurements of two strains of *Lactobacillus casei* indicated that in these strains electron transport to oxygen was not coupled to any additional energy-yielding system (Brown & VanDemark, 1968).

## METHODS

*Organism and growth conditions.* All experiments were made with *Lactobacillus casei* L3. This strain was used by van den Hamer (1960) and was obtained from K. C. Winkler (Laboratory of Microbiology, State University, Utrecht, The Netherlands). *L. casei* was maintained as stab cultures in tomato-agar (Oxoid) supplemented with 2% (w/v) glucose. The basal medium used for the growth experiments had the following composition (per l. water): peptone (Oxoid), 10 g.; beef extract (Oxoid), 3 g.; yeast extract (Oxoid), 5 g.; Tween 80, 1 ml.; $K_2HPO_4$, 3·5 g.; $KH_2PO_4$, 1·5 g.; $MgSO_4.7H_2O$, 0·1 g.; $MnSO_4.4H_2O$, 0·025 g.; pH 6·8. Glucose, mannitol and sodium citrate were added as filter-sterilized solutions. Cultures were grown at 37°.

*Growth experiments in batch cultures.* Most growth yield determinations were carried out in the 2 l. vessel of the Microferm laboratory fermentor (New Brunswick Scientific Co., New Brunswick, N.J., U.S.A.). A 1000 ml. volume of the basal medium supplemented with growth-limiting concentrations of glucose, mannitol or citrate (7·5 or 10 mM) was inoculated with 50 ml. of an overnight culture grown on the same substrate. Anaerobic conditions were obtained by passing a stream of pure nitrogen through the culture (agitation at 100 rev./min.). For aerobic cultures moderate aeration was used (agitation at 600 rev./min.). Growth of fermentor cultures was followed by periodically measuring the extinction at 660 nm. in a Unicam SP 600 spectrophotometer, with 1 cm. cuvettes. When the maximum extinction was reached, two samples (100 ml.) were taken for the determination of the growth yield. Growth yields were measured by filtration on membrane filters of constant weight as described

previously (de Vries & Stouthamer, 1968; Stouthamer, 1969). A few growth yield determinations were made with cultures grown in 250 ml. bottles with different growth-limiting concentrations of glucose (0 to 10 mM). Both for fermentor cultures and bottle cultures corrections were made for the dry weight of the medium (about 0·01 mg./ml.).

*Growth experiments in continuous cultures.* Continuous culture studies were made in the Microferm laboratory fermentor mentioned above. In this apparatus a peristaltic-action pump (model T6*S*; Sigmamotor, Inc., Middleport, N.Y., U.S.A.) transferred nutrient from the nutrient reservoir to the growing vessel. It was adjusted manually to deliver the desired volume of the nutrient at a continuous rate. A second pump transferred the culture from the growing vessel to a holding vessel, and operated intermittently through the electronic level-controller. The growth vessel had a volume of 2 l. The working volume was usually about 1000 ml. The dilution rate varied from 0·12 to 0·66 $h.^{-1}$. Continuous cultures were started as batch cultures. Medium, 800 ml., containing 5 mM-glucose was inoculated with 200 ml. of an overnight culture grown in the same medium. After growth had ceased, a regulated flow of fresh medium to the culture was started. The culture was run for at least six doubling times to allow establishment of a steady state. Then, duplicate samples (100 ml.) were taken for the determination of the dry weight of bacteria. Supernatant fluids were used for the determination of pH value, fermentation products and residual glucose.

*Determination of glucose and fermentation products.* Glucose, L-lactate, and ethanol were determined enzymically by means of a Biochemica Test Combination (C. F. Boehringer und Soehne, GmbH, Mannheim, Germany). Total lactate was measured according to Barker & Summerson (1941). Acetate was determined by the enzymic method of Rose, Grunberg-Manago, Korey & Ochoa (1954), which is based on the colorimetric determination of acetyl phosphate according to Lipmann & Tuttle (1945). Pyruvate was determined according to Friedemann & Haugen (1943). Acetoin was determined according to Westerfeld (1952). In all determinations, the medium was used as a blank and medium was added to standard solutions. Formate was measured with formate–nitrate reductase from *Proteus mirabilis* S503. This bacterium was grown in closed bottles (1 l.) completely filled with Oxoid MRVP medium supplemented with 69 mg. sodium azide/l. Under these growth conditions formate–nitrate reductase was induced and the hydrogenlyase system was repressed (de Groot & Stouthamer, 1970). After incubation overnight at 32°, bacteria were harvested by centrifugation and washed at least three times with 0·067 M-potassium phosphate buffer (pH 6·8). The sediment was suspended in 15 ml. of this buffer. The bacteria were then disrupted in a MSE Mullard ultrasonic disintegrator (60 W at 20 kc./sec.). The crude bacterial-free broken preparation was used to determine formate by a modification of the method described by Itagaki & Suzuki (1964). The reaction was done in a Thunberg tube. The main tube contained, in 1·7 ml., 90 $\mu$mole potassium phosphate (pH 6·0), 25 $\mu$mole potassium nitrate, 0·25 mg. methylene blue, and 0·3 ml. extract of *P. mirabilis*. Samples, blanks and standard solutions (0·5 and 1 $\mu$mole) made to 0·3 ml. with distilled water, were placed in the side arm. Anaerobic conditions were obtained by alternately evacuating and filling with nitrogen five times. The tube was then inverted to mix the contents. After incubation at 30° for 90 min. 0·1 ml. of the incubation mixture was used to determine nitrite by a modification (Van't Riet, Stouthamer & Planta, 1968) of the method described by Nicholas & Nason (1957). By the use of internal standards and media supplemented with ethanol, it was ascertained

that ethanol and other fermentation products could not act as hydrogen donors in the reduction of nitrate. The medium itself gave a slow reaction for which a correction was applied.

*Determination of total polysaccharide and intracellular (soluble) polysaccharide.* Bacteria from 600 ml. of medium from a batch or continuous culture were harvested by centrifugation and washed once with 0·85% (w/v) NaCl. The sediment was suspended in 15 ml. of the NaCl solution. The dry weight of bacteria was determined by filtration of two 2 ml. portions of this suspension. Total polysaccharide was determined in whole cells with anthrone reagent (Trevelyan & Harrison, 1952) using glucose as a standard. Intracellular carbohydrate was measured by the anthrone method after the polysaccharides were isolated from the organisms as described by Zevenhuizen (1966).

*Determination of lactate dehydrogenase.* Bacteria harvested from 400 ml. of batch or continuous culture were washed twice with 0·067 M-sodium potassium phosphate buffer (pH 6·8) and resuspended in 10 ml. of this buffer. Bacteria-free extracts were prepared with a MSE Mullard ultrasonic disintegrator (60 W, 20 kc./sec). The determination of lactate dehydrogenase was performed at 25° in quartz cuvettes (1 cm. light path) with an Unicam SP 820 constant-wavelength scanner. The complete assay mixture (2·5 ml.) contained: histidine buffer of different pH values, 0·08 M; pyruvate, 4 mM; NADH, 0·3 mM; fructose-1,6-disphosphate, 1·2 mM; $MnCl_2.4H_2O$, 4 mM; extract, about 0·004 mg. protein/ml. assay mixture. This assay could be performed with such a small protein concentration that no interference by NADH oxidase was obtained. The extinction was followed at 340 nm. The specific activity of lactate dehydrogenase was calculated by using the extinction coefficient of NADH ($6·2 \times 10^6$ $cm.^2$/mole). Protein was measured with the Folin reagent (Lowry, Rosebrough, Farr & Randall, 1951) with bovine serum albumin as a standard.

The production of L-lactate from pyruvate and NADH by extracts of *Lactobacillus casei* was determined at pH 6·5 in the presence of manganous ions and at pH 6·0 in the absence of manganous ions. The reaction mixture was as described above except that more NADH (2 mM) and more extract (0·5 mg. protein/ml. reaction mixture) were added. The reaction was done in Thunberg cuvettes under nitrogen to eliminate NADH oxidation by oxygen. A control without added NADH was run in parallel. After NADH added had been completely oxidized (as shown by measuring the extinction at 340 nm.), protein was precipitated by adding 0·1 ml. 3 N-$H_2SO_4$. In the supernatant fluid, L-lactate was measured enzymically as described above. The exact amount of NADH added was measured at 340 nm. in an appropriate dilution in buffer.

*Enzymes and chemicals.* Acetate kinase, NADH, ATP and fructose-1,6-diphosphate were obtained from C. F. Boehringer und Soehne GmbH, Mannheim, Germany; formate and citrate from E. Merck, Darmstadt, Germany; pyruvate, acetoin, glucose and mannitol from B.D.H. Chemicals Ltd, Poole, Dorset. All reagents were of Analar grade.

## RESULTS

### *Growth of Lactobacillus casei in batch cultures*

Growth of *Lactobacillus casei* with limiting concentrations of energy source was logarithmical up to the growth maximum. After growth had ceased, the extinction of the

cultures remained constant for at least 6 h. This indicated that lysis did not occur. The relation between total growth of *L. casei* and the concentration of glucose in the medium was linear up to a concentration of 10 mM-glucose under both anaerobic and aerobic conditions. Higher glucose concentrations were not tested. Only slight growth was obtained in the basal medium without an added energy source.

The results obtained for *Lactobacillus casei* growing in batch cultures with limiting concentrations of glucose, mannitol or citrate are summarized in Table 1. Under both anaerobic and aerobic conditions 80% of the glucose metabolized was recovered as lactate. About 90% of the lactate formed was L-lactate. Traces of acetate, ethanol, formate, acetoin and pyruvate accounted for about 5% of the glucose carbon. Apparently a few other products were formed from glucose.

Under aerobic conditions mannitol was primarily converted to lactate, whereas under anaerobic conditions, besides lactate, large amounts of acetate, ethyl alcohol and formate were formed (Table 1). Assuming that $x$ mole of lactate is formed/mole mannitol, the theoretical fermentation balance for anaerobic growth on mannitol should be:

$$\text{mannitol} \rightarrow x \text{ lactate} + (\tfrac{1}{2} - \tfrac{1}{2}x) \text{ acetate} + (1\tfrac{1}{2} - \tfrac{1}{2}x) \text{ ethanol} + (2 - x) \text{ formate}.$$

According to this equation the maximal lactate production amounts to only 1 mole/mole mannitol, as under anaerobic conditions, besides pyruvate, acetyl phosphate is required as hydrogen acceptor. Lactate formed (Table 1) was much less than 1 mole/mole mannitol. The fermentation balance (Table 1) fitted the theoretical balance well. There was a long lag before anaerobic growth on mannitol started. Under aerobic conditions no lag was observed. During the first steps in the degradation of mannitol, NADH is formed. Presumably, aerobic growth on mannitol was facilitated by oxidation of NADH by oxygen. Under aerobic conditions operation of the phosphoroclastic enzyme did not seem to occur.

The fermentation balance for citrate (Table 1) shows that citrate was fermented by citratase, oxaloacetate decarboxylase and the phosphoroclastic enzyme. The C-recovery of the fermentation balance was very good. Just as was found for anaerobic growth on mannitol, a long lag was observed before anaerobic growth on citrate started.

For calculating $Y_{ATP}$ it was assumed that 2 moles ATP were formed in the conversion of 1 mole glucose or mannitol to pyruvate, and that 1 mole ATP was formed/mole acetate arising from pyruvate. From Table 1 it can be seen that the $Y_{ATP}$ values were very high (range 18·2 to 20·9). $Y_{ATP}$ values for anaerobic growth on mannitol and citrate were somewhat lower than the other values. The aerobic molar growth yield for mannitol showed that electron transport from NADH to oxygen was not coupled with oxidative phosphorylation.

### *Growth of Lactobacillus casei in continuous cultures*

The results of growth experiments in glucose-limited continuous cultures of *Lactobacillus casei* are summarized in Table 2. At dilution rates below 0·25 $h.^{-1}$ no lactate was formed. Instead, pyruvate arising from glucose was split to acetyl phosphate and formate by the phosphoroclastic enzyme. Acetyl phosphate formed was partly converted to acetate with concomitant formation of ATP, and was partly reduced to ethanol. At dilution rates above 0·25 $h.^{-1}$ the amounts of acetate, ethanol and formate

Table 1. *Summary of results from growth experiments with Lactobacillus casei in batch cultures*

| Substrate | Growth condition | Specific growth rate ($h.^{-1}$) | Products formed (mole/mole of substrate) | | | | C-recovery (%) | ATP formation (mole/mole of substrate) | Molar growth yield (g./mole) | $Y_{ATP}$ (g. mole) |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | Lactate | Acetate | Ethanol | Formate | | | | |
| Glucose | Anaerobic | 0·60 | 1·6 | 0·05 | 0 | 0·1 | 83 | 2·05 | 42·9* | 20·9 |
| Glucose | Aerobic | 0·51 | 1·6 | 0·06 | 0 | 0 | 82 | 2·06 | 41·9* | 20·3 |
| Mannitol† | Anaerobic‡ | 0·31 | 0·4 | 0·22 | 1·29 | 1·6 | 97 | 2·22 | 40·5 | 18·2 |
| Mannitol† | Aerobic | 0·33 | 1·6 | 0·10 | 0 | 0 | 83 | 2·10 | 41·7 | 19·9 |
| Citrate† | Anaerobic‡ | 0·32 | 0·05 | 1·96 | 0·05 | 0·70 | 81§ | 0·96 | 18·2 | 19·0 |

* Calculated from the relation between total growth of *L. casei* and the concentration of glucose.
† The data for citrate and mannitol represent the mean of values found in two or three independent cultures (7·5 mM-mannitol; 10 mM-sodium citrate). Differences between individual values were small.
‡ In these cases a long lag time was observed.
§ The theoretical C-recovery (without accounting for $CO_2$ formed by decarboxylation of oxaloacetate) is 83·3 %.

Table 2. *Fermentation balances, molar growth yields and $Y_{ATP}$ values for Lactobacillus casei growing in continuous cultures with a limiting concentration of glucose**

| Dilution rate ($h.^{-1}$) | Products formed (mole/mole glucose) | | | | C-recovery (%) | ATP formation (mole/mole glucose) | Molar growth yield (g./mole) | $Y_{ATP}$ (g./mole) |
|---|---|---|---|---|---|---|---|---|
| | Lactate | Acetate | Ethanol | Formate | | | | |
| 0·125† | 0 | 0·99 | 0·99 | 1·70 | 94 | 2·99 | 55·8 | 18·7 |
| 0·140 | 0 | 1·06 | — | 1·56 | — | 3·06 | 58·7 | 19·2 |
| 0·159†‡ | 0·20 | 0·88 | 0·87 | 1·86 | 99 | 2·88 | 56·4 | 19·6 |
| 0·168 | 0·05 | 1·05 | 0·94 | 1·76 | 98 | 3·05 | 62·0 | 20·3 |
| 0·244 | 0·02 | 0·98 | 0·92 | 1·63 | 92 | 2·98 | 63·2 | 21·2 |
| 0·290 | 0·22 | 0·90 | — | 1·58 | — | 2·90 | 61·5 | 21·2 |
| 0·390 | 0·7 | 0·68 | 0·60 | 1·14 | 97 | 2·68 | 59·4 | 22·2 |
| 0·400 | 0·7 | 0·68 | — | 0·88 | — | 2·68 | 60·4 | 22·5 |
| 0·500 | 1·5 | 0·20 | 0·13 | 0·50 | 94 | 2·20 | 48·8 | 22·2 |
| 0·503 | 1·5 | 0·13 | — | — | — | 2·13 | 47·4 | 22·3 |
| 0·600 | — | 0·24 | — | 0·28 | — | 2·24 | 52·6 | 23·5 |

* For all cultures the glucose concentration was 5 mM.
† The medium used for these cultures was supplemented with histidine, tryptophan, arginine and aspartate (all concentrations 5 mM)
‡ In this culture the pH was 5·6.

decreased with increasing dilution rates, and lactate production was observed. At high dilution rates (0·50 to 0·60 h.$^{-1}$) only small amounts of acetate, ethanol and formate were formed, and lactate production was maximal. C-recoveries were from 92 to 99%. No glucose was detected in the effluent culture up to a dilution rate of 0·60 h.$^{-1}$. At the dilution rate 0·66 h.$^{-1}$ the culture slowly washed out and glucose was detected in the culture. At this dilution rate the glucose fermented was almost completely converted to lactate. At most dilution rates the pH of the culture was 6·3. In an experiment (dilution rate 0·159 h.$^{-1}$) in which the culture had a lower pH value, formation of a small amount of lactate was detected. Probably the fermentation pattern is somewhat influenced by pH value.

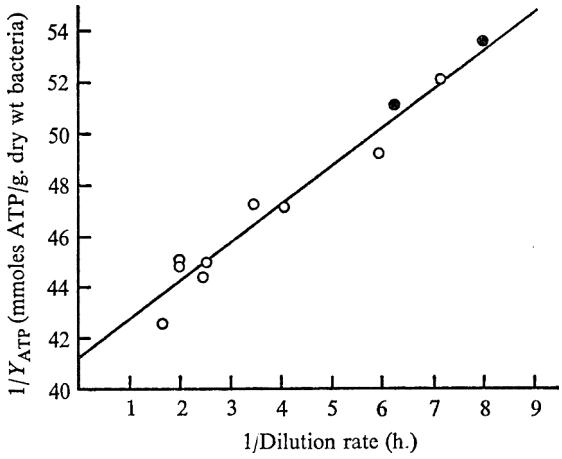


Fig. 1. Plot of $1/Y_{ATP}$ against 1/dilution rate for *Lactobacillus casei*. Each point is the mean of two determinations in one culture. ○, Points found for the basal medium (5 mM-glucose); ●, these points were found for a medium to which arginine, histidine, tryptophan and aspartate (all concentrations 5 mM) were added in addition to glucose.

Just as was found in batch cultures, $Y_{ATP}$ values in continuous cultures were high. The $Y_{ATP}$ value for glucose (g. dry weight of bacteria/mole of ATP) varied from 18·7 at low dilution rates to 23·5 at high dilution rates. To test the possibility that the organism was capable of utilizing for energy some compounds in the medium besides glucose, continuous cultures (dilution rates 0·125 and 0·159 h.$^{-1}$) were run with the basal medium supplemented with glucose, arginine, histidine, aspartate and tryptophan (all 5 mM). From Table 2 it can be seen that neither the amounts and type of products formed nor the growth yield indicated any fermentation and energy yield from the amino acids added. Therefore, the values found in this enriched medium were included in the calculation of the maintenance coefficient and $Y_{ATP}$ (max.) (Fig. 1).

### *Calculation of maintenance energy*

Pirt (1965) showed that for a continuous culture in which growth was limited by the energy source the molar growth yield ($Y_G$), corrected for energy of maintenance, could be calculated from the relationship

$$1/Y = m_S/\mu + 1/Y_G,$$

where $m_S$ was the substrate requirement for energy of maintenance/unit amount organism/unit time, $\mu$ was the specific growth rate and $Y$ was the apparent molar

growth yield. In the steady state $\mu$ was identical with the dilution rate $D$. This expression is valid only when the amount of energy derived from the growth substrate is constant at all dilution rates. In our experiments the amount of ATP formed/mole glucose was dependent on the dilution rate. In this case, the following relation holds

$$1/Y_{ATP} = m_e/\mu + 1/Y_{ATP} \text{ (max.)},$$

where $Y_{ATP}$ (max.) is the growth yield/mole of ATP corrected for energy of maintenance, $m_e$ is the amount of ATP required for maintenance (per unit amount of organism per unit time). $Y_{ATP}$ is the apparent growth yield/mole of ATP. A plot of $1/Y_{ATP}$ versus $1/D$ results in a function whose intercept at the ordinate is the reciprocal of $Y_{ATP}$ (max.) and whose slope is $m_e$.

In Fig. 1 the reciprocal of $Y_{ATP}$ values found for *Lactobacillus casei* are plotted against the reciprocal of the dilution rate. By linear regression analysis the best fit line was calculated. From the slope of this line a maintenance coefficient of $1{\cdot}52 \times 10^{-3}$ (moles ATP used/g. dry weight bacteria/h.) was calculated. From the intercept on the ordinate a $Y_{ATP}$ (max.) value of 24·3 (g. dry weight bacteria/mole ATP) was calculated.

### *Polysaccharide content of Lactobacillus casei in continuous cultures and batch cultures*

Assimilation of glucose into intracellular polysaccharides might have been the reason for the high molar growth yields found. However, intracellular (soluble) polysaccharides were not detected in *Lactobacillus casei* after growth in batch cultures with a limiting concentration of glucose. Both in batch cultures and in continuous cultures growing at different dilution rates, the total polysaccharide content of the bacteria was about 11%. This value is quite normal for the percentage of cell-wall polysaccharides in bacteria (Gunsalus & Shuster, 1961).

### *Demonstration of lactate dehydrogenase*

Initially, difficulties were met in the demonstration of lactate dehydrogenase in extracts of *Lactobacillus casei*. Previously it had been found that lactate dehydrogenase of bifidobacteria and *Streptococcus bovis* had absolute requirements for fructose-1,6-diphosphate (Wolin, 1964; de Vries, Gerbrandy & Stouthamer, 1967). However, addition of fructose-1,6-diphosphate to the reaction mixture for the determination of lactate dehydrogenase of *L. casei* did not result in the demonstration of this enzyme. A highly active lactate dehydrogenase was detected when, besides fructose-1,6-diphosphate, manganous chloride was added to the reaction mixture (Fig. 2). From this it is evident that both manganous chloride and fructose-1,6-diphosphate were required for the activity and that the oxidation of NADH was indeed caused by reaction with pyruvate. The concentration of fructose-1,6-diphosphate required to yield 50% of maximal activity of lactate dehydrogenase was 0·3 mM. This concentration was about six times higher than that required to activate the lactate dehydrogenase of the bifidobacteria (de Vries, Gerbrandy & Stouthamer, 1967). Fructose-1,6-diphosphate could not be replaced by other intermediates of the glycolytic system, or of glycogen synthesis, or by ATP. Manganous chloride could not be replaced by magnesium chloride. The demonstration of lactate dehydrogenase was done at pH 6·5 (Fig. 2). When the pH value of the reaction mixture was decreased the specific activity of lactate dehydrogenase increased and the requirement for manganous ions gradually disappeared (Fig. 3). In fact, at values below pH 6·1, manganous ions had a weakly

inhibitory effect (Fig. 3). Both at pH 6·5 in the presence of manganous ions and at pH 6·0 in the absence of manganous chloride, a stoichiometric amount of L-lactate was formed as compared with the amount of NADH oxidized.

The specific activities of lactate dehydrogenase in *Lactobacillus casei* harvested from batch and continuous cultures are shown in Table 3. Despite the fact that no lactate

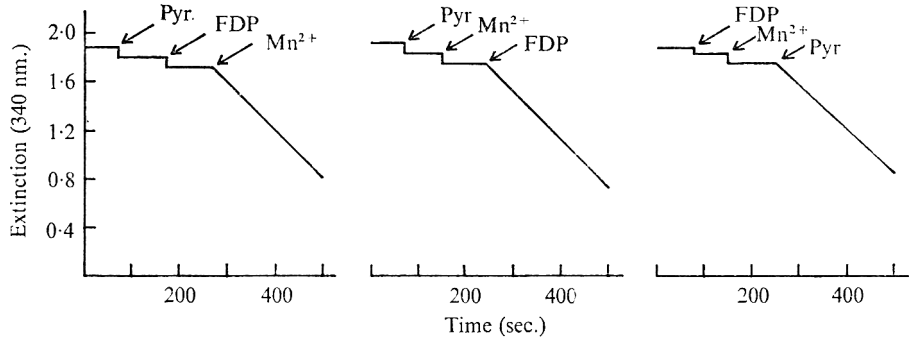


Fig. 2. Requirement of lactate dehydrogenase of *Lactobacillus casei* for fructose-1,6-diphosphate and manganous ions. The complete reaction mixture contained histidine buffer (pH 6·5), pyruvate, NADH, fructose-1,6-diphosphate and manganous chloride (see text). Pyruvate (Pyr), fructose-1,6-diphosphate (FDP) and manganous chloride ($Mn^{2+}$) were added at the points indicated by arrows.

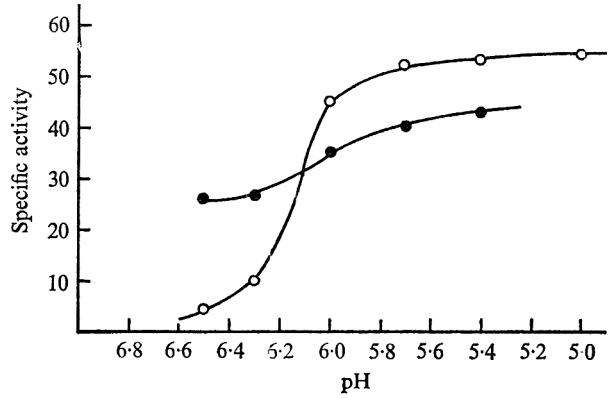


Fig. 3. Influence of pH and manganous ions on the specific activity of lactate dehydrogenase. ○, No manganous ions added; ●, manganous ions added. Specific activity is expressed as μmole NADH oxidized/mg. protein/min. Determinations were made with a crude cell-free extract.

Table 3. *Specific activity of lactate dehydrogenase of Lactobacillus casei growing in continuous culture and in batch culture*

| Dilution rate ($h.^{-1}$) | Specific activity* |
|---|---|
| 0·12 | 21·4 |
| 0·40 | 18·3 |
| Batch culture | 25·8 |

The reaction mixture contained histidine buffer (pH 6·5), pyruvate, NADH, fructose-1,6-diphosphate, manganous chloride and cell-free extract; for details see text.

* Expressed as μmole NADH oxidized/mg. protein/min.

was formed from glucose at low dilution rates, the specific activity of lactate dehydrogenase was not appreciably different from that at high dilution rate. In batch cultures the specific activity of lactate dehydrogenase was somewhat higher than in continuous cultures.

## DISCUSSION

Continuous culture studies showed that the fermentation balance of glucose for *Lactobacillus casei* was dependent on the growth rate. At high growth rates glucose was fermented predominantly to lactate, at low growth rates the phosphoroclastic enzyme functioned in the breakdown of glucose; acetate, ethanol and formate were the fermentation products. It is interesting that under conditions of poor glucose supply the organism adapted itself to a pathway which yielded more ATP/mole of glucose than when glucose was in excess and which gave, consequently, a higher growth yield/mole of glucose than was achieved when glucose was in good supply. An increase in the proportion of volatile fatty acids with decreasing growth rate has been observed by other investigators. Hobson & Summers (1967) reported an increase in the proportion of volatile fatty acids (acetic acid and propionic acid) with decreasing growth rates for a lipolytic bacterium growing on glycerol. For *Bacteroides amylophilus* the amount of volatile acids (acetic acid, formic acid) formed from maltose, varied with growth rate, showing a peak at dilution rate 0·29 $h.^{-1}$ (Hobson & Summers, 1967). The results of Rosenberger & Elsden (1960) and Hempfling, Mainzer & VanDemark (1969) showed that at slow growth rates the amounts of volatile fatty acids formed from glucose by *Streptococcus faecalis* were higher than at faster growth rates.

It would be interesting to know the regulation mechanism which determines the choice between the two pathways of pyruvate conversion in *Lactobacillus casei*. Our attempts to show the phosphoroclastic enzyme in extracts of *L. casei* were unsuccessful (unpublished results). Lactate dehydrogenase turned out to have an absolute requirement for fructose-1,6-diphosphate and manganous ions. The demonstration of large amounts of lactate dehydrogenase in extracts of *L. casei* grown at low dilution rates rules out the possibility that the absence of lactate formation observed at low dilution rates is a consequence of repression of lactate dehydrogenase synthesis. The absolute requirement of lactate dehydrogenase for fructose-1,6-diphosphate suggests that the intracellular concentration of this compound controls the pathway of pyruvate conversion. It seems plausible that with a fast supply of growth substrate, the intracellular level of fructose-1,6-diphosphate is higher than with a slow substrate supply. With a slow substrate supply the intracellular level of fructose-1,6-diphosphate might be too low to activate lactate dehydrogenase, and another pathway of pyruvate conversion, the phosphoroclastic split, is used.

For growth on mannitol a second hydrogen acceptor must be present in addition to pyruvate. Under anaerobic conditions acetyl phosphate functions as a hydrogen acceptor. Therefore, for anaerobic growth on mannitol the phosphoroclastic enzyme is required. Under aerobic conditions no ethanol and formate were formed from mannitol. Thus the phosphoroclastic enzyme does not function under aerobic conditions. In a number of micro-organisms it has been shown that the phosphoroclastic enzyme is sensitive to oxygen (Lindmark, Paolella & Wood, 1969). During aerobic growth of *Lactobacillus casei* on mannitol oxygen serves as hydrogen acceptor. In accordance with the results of Brown & VanDemark (1968) no evidence for oxidative

phosphorylation was found. However, the presence of NADH oxidase is physiologically useful for the micro-organism since it facilitates aerobic growth on mannitol. The high lactate yield during aerobic growth on glucose and mannitol demonstrates that *in vivo* the lactate dehydrogenase activity is much higher than the NADH oxidase activity.

In the present investigation high $Y_{ATP}$ values were found for *Lactobacillus casei* (18·2 to 20·9 in batch cultures with different substrates; 18·7 to 23·5 in glucose-limited continuous cultures). The possibility that the organism was capable of utilizing for energy some compounds in the medium other than the added energy source could be ruled out for two reasons. First, the curve relating growth yield and substrate concentration in batch cultures was a straight line passing through the origin. Secondly, growth yield and amounts of fermentation products in glucose-limited continuous cultures were not influenced by addition of arginine, tryptophan, histidine and aspartate; compounds which are known to act as energy source for some bacteria. The high C-recoveries, especially in the fermentation of citrate and mannitol and in glucose-limited continuous cultures, eliminate the possibility that *L. casei* could gain ATP from unknown side reactions. Accumulation of intracellular polysaccharides, which is known to increase growth yields, was not observed. Therefore, it must be concluded that the high molar growth yields and $Y_{ATP}$ values found for *L. casei* are indeed real. The $Y_{ATP}$ values found are much higher than the value of 10·5 found by Bauchop & Elsden (1960) and several other investigators for different micro-organisms. The $Y_{ATP}$ values found are similar to those reported by Hobson & Summers (1967) for some rumen bacteria and are somewhat higher than those found by Moustafa & Collins (1968) for certain Streptococcus strains.

In most cases, higher $Y_{ATP}$ values are noted in complex media rather than in defined or partially defined media. Brown & VanDemark (1968), who used a simpler medium than we did, found a lower $Y_{ATP}$ value (10·8) for another strain of *Lactobacillus casei*. The most striking influence of the composition of the medium on $Y_{ATP}$ has been found in *Zymomonas mobilis*. Senez & Belaïch (1965) found that the molar growth yield of this organism on glucose in minimal medium is only 4·11 g. dry weight of bacteria/mole of glucose, whereas in complex medium it is 8·61 g./mole. The high growth yields in complex medium can be explained by the presence of all monomers needed for polymer synthesis in the medium. The lower growth yields in simpler medium might be explained by the assumption that large amounts of energy are expended to synthesize monomers from other constituents of the medium. However, the authors feel that another explanation may be more important. The large difference between the growth yields of *Z. mobilis* in complex and chemically defined medium (Senez & Belaïch, 1965) can be explained by the assumption that the rate at which one or more essential compounds are synthesized in defined medium is rate-limiting, which would cause uncoupling of growth and energy production (Stouthamer, 1969). More published examples of uncoupling between growth and energy production by nutrient limitation have been quoted by Stouthamer (1969). Therefore, we suggest that the difference between growth yields in complex and defined media may be mainly due to this reason.

The maintenance coefficient of *Lactobacillus casei* calculated from the plot of the reciprocal of $Y_{ATP}$ versus the reciprocal of the dilution rate, was $1{\cdot}52 \times 10^{-3}$ mole ATP/g. dry weight bacteria/h. This value was much lower than the values calculated by

Pirt (1965) for *Aerobacter aerogenes*, *A. cloacae* and lipolytic bacterium 5S. Von Meyenburg (1969) found a low maintenance coefficient, $1{\cdot}57 \times 10^{-3}$ mole ATP/g. dry weight of organisms/h., for *Saccharomyces cerevisiae*. It is questionable whether the low maintenance coefficient found for *L. casei* is the sole reason for the high $Y_{ATP}$ values found for this micro-organism. A more probable reason for the high $Y_{ATP}$ values could be a very effective coupling between catabolism and anabolism, and the absence of adenosine triphosphatase.

## REFERENCES


BARKER, S. B. & SUMMERSON, W. H. (1941). The colorimetric determination of lactic acid in biological material. *Journal of Biological Chemistry* **138**, 535–554.

BAUCHOP, T. & ELSDEN, S. R. (1960). The growth of micro-organisms in relation to their energy supply. *Journal of General Microbiology* **23**, 457–469.

BROWN, J. P. & VANDEMARK, P. J. (1968). Respiration of *Lactobacillus casei*. *Canadian Journal of Microbiology* **14**, 829–835.

FORREST, W. W. (1969). Energetic aspects of microbial growth. *Symposia of the Society for General Microbiology* **19**, 65–86.

FRIEDEMANN, T. E. & HAUGEN, G. E. (1943). Pyruvic acid. II. The determination of keto acids in blood and urine. *Journal of Biological Chemistry* **147**, 415–442.

DE GROOT, G. N. & STOUTHAMER, A. H. (1970). Regulation of reductase formation in *Proteus mirabilis*. II. Influence of growth with azide and of haem deficiency on nitrate reductase formation. *Biochimica et Biophysica Acta* **208**, 414–427.

GUNSALUS, I. C. & SHUSTER, C. W. (1961). Energy-yielding metabolism in bacteria. In *The Bacteria*, vol. 2, *Metabolism*, pp. 1–58. Edited by I. C. Gunsalus and R. Y. Stanier. New York and London: Academic Press.

VAN DEN HAMER, C. J. A. (1960). *De koolhydraat-stofwisseling van melkzuurbacteriën*. Thesis, University of Utrecht, The Netherlands.

HEMPFLING, W. P., MAINZER, S. E. & VANDEMARK, P. J. (1969). Invariance of *Y*(Adenosine triphosphate) of *Streptococcus faecalis* 10 cl during anaerobic continuous culture. *Bacteriological Proceedings*, p. 172.

HOBSON, P. N. & SUMMERS, R. (1967). The continuous culture of anaerobic bacteria. *Journal of General Microbiology* **47**, 53–65.

ITAGAKI, E. & SUZUKI, S. (1964). Microdetermination of formic acid in periodate oxidation mixtures. *Journal of Biochemistry, Tokyo* **56**, 77–80.

LIPMANN, F. & TUTTLE, L. C. (1945). A specific micromethod for the determination of acyl-phosphates. *Journal of Biological Chemistry* **159**, 21–28.

LINDMARK, D. G., PAOLELLA, P. & WOOD, N. P. (1969). The pyruvate formate-lyase system of *Streptococcus faecalis*. *Journal of Biological Chemistry* **244**, 3605–3612.

LOWRY, O. H., ROSEBROUGH, N. J., FARR, A. L. & RANDALL, R. J. (1951). Protein measurement with the Folin phenol reagent. *Journal of Biological Chemistry* **193**, 265–275.

VON MEYENBURG, H. K. (1969). Energetics of the budding cycle of *Saccharomyces cerevisiae* during glucose-limited aerobic growth. *Archiv für Mikrobiologie* **66**, 289–303.

MOUSTAFA, H. M. & COLLINS, E. B. (1968). Molar growth yields of certain lactic acid bacteria as influenced by autolysis. *Journal of Bacteriology* **96**, 117–125.

NICHOLAS, D. J. D. & NASON, A. (1957). Determination of nitrate and nitrite. In *Methods of Enzymology*, vol. 3, pp. 981–984. Edited by S. P. Colowick and N. O. Kaplan. New York and London: Academic Press.

PIRT, S. J. (1965). The maintenance energy of bacteria in growing cultures. *Proceedings of the Royal Society of London* B, **163**, 224–231.

ROSE, I. A., GRUNBERG-MANAGO, M., KOREY, S. R. & OCHOA, S. (1954). Enzymatic phosphorylation of acetate. *Journal of Biological Chemistry* **211**, 737–756.

ROSENBERGER, R. F. & ELSDEN, S. R. (1960). The yields of *Streptococcus faecalis* grown in continuous culture. *Journal of General Microbiology* **22**, 726–739.

SENEZ, J. C. & BELAÏCH, J. P. (1965). Etude microcalorimétrique de la croissance bactérienne et du contrôle de l'activité métabolique par le phosphate. In *Régulation chez les Micro-organismes*, pp. 357–363. Paris: Editions du Centre National de la Recherche Scientifique.

SMALLEY, A. J., JAERLING, P. & VANDEMARK, P. J. (1968). Molar growth yields as evidence for oxidative phosphorylation in *Streptococcus faecalis* strain 10 cl. *Journal of Bacteriology* **96**, 1595–1600.

STOUTHAMER, A. H. (1969). Determination and significance of molar growth yields. In *Methods in Microbiology*, vol. 1, pp. 629–663. Edited by J. R. Norris and D. W. Ribbons. New York and London: Academic Press.

TREVELYAN, W. E. & HARRISON, J. S. (1952). Studies on yeast metabolism. I. Fractionating and microdetermination of cell carbohydrates. *Biochemical Journal* **50**, 298–303.

VAN'T RIET, J., STOUTHAMER, A. H. & PLANTA, R. J. (1968). Regulation of nitrate assimilation and nitrate respiration in *Aerobacter aerogenes*. *Journal of Bacteriology* **96**, 1455–1464.

DE VRIES, W., GERBRANDY, SJ. J. & STOUTHAMER, A. H. (1967). Carbohydrate metabolism in *Bifidobacterium bifidum*. *Biochimica et Biophysica Acta* **136**, 415–425.

DE VRIES, W. & STOUTHAMER, A. H. (1968). Fermentation of glucose, lactose, galactose, mannitol and xylose by bifidobacteria. *Journal of Bacteriology* **96**, 472–478.

WESTERFELD, W. W. (1952). Colorimetric determination of acetoin plus diacetyl. In *Analytical Methods for Bacterial Fermentations*, p. 39. Edited by A. C. Neish. Saskatoon, Saskatchewan: National Research Council of Canada.

WOLIN, M. J. (1964). Fructose-1,6-diphosphate requirement of streptococcal lactic dehydrogenases. *Science, New York* **146**, 775–777.

ZEVENHUIZEN, L. P. T. M. (1966). *Intracellular Polysaccharide of Arthrobacter*. Thesis, University of Amsterdam, The Netherlands.

*Journal of General Microbiology* (1970), **63**, 347–356
*Printed in Great Britain*



# Nuclear Size in Plasmodia of the True Slime Mould *Didymium nigripes*

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(*Accepted for publication 1 September 1970*)

SUMMARY

Nuclei of different diameters, containing different numbers of chromosomes, are found in all macroscopic plasmodia of the true slime mould *Didymium nigripes*. The relative proportions of small and large nuclei vary in different plasmodial clones. Over extended periods of time C6 wild-type clones maintain a constant proportion of small nuclei whereas Y2–1, a yellow mutant, does not. Nuclear fusion (syngamy) does not occur prior to plasmodium formation in this isolate; neither is it delayed until the plasmodium has formed. The rate of formation of large nuclei in plasmodia was calculated. When plasmodia with differing nuclear size ratios were fused, the observed shifts in the nuclear size ratio suggested the possibility that parasexual processes could exist in the isolate under study.

## INTRODUCTION

During the course of its life cycle, the true slime mould *Didymium nigripes*, passes through several morphologically distinct phases. Uninucleate spores germinate to form uninucleate amoebae. After several days of amoeboid growth and division, plasmodia form and continue to grow until they attain macroscopic size. When starved, plasmodia differentiate into fruiting bodies; within these fruiting bodies cytoplasmic cleavage occurs and uninucleate spores are formed, thus completing the life cycle. In many true slime moulds, plasmodia are initiated when two amoebae fuse; thus spores and amoebae are haploid, and plasmodia are diploid. The isolate of *D. nigripes* under study differs from a typical slime mould in that plasmodia form without prior amoebal or nuclear fusion: the plasmodia and amoebae both possess the same ploidy level (S. Kerr, 1968*a*, *b*).

Phase contrast microscope observations of young plasmodia, and of smear preparations of pieces of older plasmodia which have grown to macroscopic size, have shown that mitotic division within a single plasmodium is an approximately synchronous process. Accordingly, it is logical to assume that all nuclei in a given plasmodium are in the same stage of the cell cycle at a given time; this has been demonstrated in a closely related slime mould, *Physarum polycephalum* (Nygaard, Guttes & Rusch, 1960). Despite synchrony of the cell cycle, young plasmodia occasionally contain nuclei of different diameters. By the time a plasmodium has grown to macroscopic size, nuclei of two size classes are invariably present, and occasional nuclei of extraordinary size are also encountered.

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Chromosome counts in the strains of *Didymium nigripes* under investigation have made it possible to identify the approximate chromosome number of each of these size classes of nuclei (S. Kerr, 1968*a*, *b*). In effect, a plasmodium of one of these strains possesses a population of nuclei with approximately 32 to 33 chromosomes (small nuclei), nuclei with 64 to 66 chromosomes (large nuclei), and nuclei with over 100 chromosomes. The same studies showed that the proportion of small nuclei in populations of young plasmodia (with 2 to 16 nuclei) was not significantly different from the proportion of small nuclei in the populations of amoebae from which those plasmodia were derived. Thus no obvious change in ploidy level of nuclei occurs at the time of plasmodium formation.

The investigations reported here represent an attempt to determine whether a constant ratio of small to large nuclei is maintained by a plasmodium after its formation, or whether the nuclear fusion which is expected to occur during the slime mould life cycle might occur progressively during the time when a plasmodium has achieved macroscopic size. An attempt was made to characterize the infrequent changes in nuclear size ratio which occur, and to look for information as to whether or not the plasmodium possesses some means of regulating the ploidy level of its nuclear population.

## METHODS

*Organisms.* Plasmodia were maintained on GPY/5 agar (glucose, peptone, yeast extract) spread with *Aerobacter aerogenes* (N. Kerr, 1961) and serially subcultured to fresh media at 2 day intervals. Each plasmodial stock was clonally derived from the progeny of a single amoeba. Two strains of a single isolate of *Didymium nigripes* were studied: C6 wild type (N. Kerr, 1965) and Y2–1, a yellow mutant isolated on actidione agar (N. Kerr & Waxlax, 1968*b*).

*Determination of nuclear size.* Nuclear size was determined using a Zeiss GFL phase contrast microscope with a 63X neofluor objective and 12·5X oculars. Smear preparations were made by placing small pieces of plasmodium on a slide in a drop of glycerine containing aceto-orcein, adding a coverslip, and disrupting the plasmodium by pressing gently on the coverslip. Nuclear size in young plasmodia was determined by observing living plasmodia growing on thin agar which had been transferred to a slide, covered with a coverslip, and sealed.

When nuclear size ratios in macroscopic plasmodia were determined, the size of 500 nuclei was determined and the number of small nuclei per 500 was recorded. No attempt was made to assign ploidy levels to extraordinarily large nuclei. Since within a plasmodium the mixing of contents occurs very rapidly (N. Kerr & Waxlax, 1968*a*), the nuclear population of small pieces of the plasmodium should be representative of the whole. Five hundred was chosen as the sample size because it was found that, with this size sample, counts from different portions of the same plasmodium duplicated each other.

*Fusion of plasmodia.* Plasmodia were fused by placing equal-sized pieces of two different plasmodia growing on agar adjacent to each other and next to a shelf of agar of the same thickness (see N. Kerr, 1965). During a period of 3 to 4 h. such plasmodia migrate to the shelf of agar, come in contact with each other, and fuse.

*Preparation of microplasmodia.* Microplasmodia (small pieces of plasmodium) were prepared by scraping a plasmodium from the plate of agar, suspending it in 0·01 M

pH 6·5 phosphate buffer (SS), homogenizing the suspension with a Vortex Junior mixer for 30 sec. and then allowing the suspension to stand for 30 min. During this time the larger fragments of plasmodium settle to the bottom, and small pieces (many containing single nuclei) can be removed from the top of the suspension and plated on agar with *Aerobacter aerogenes*. After 2 to 3 days such microplasmodia grow to macroscopic size and can be isolated and subcultured in the same manner as other plasmodia.

## RESULTS

### *Changes in nuclear size composition of macroscopic plasmodia*

The proportion of small nuclei present in a plasmodial nuclear population was determined at weekly intervals in ten clonally derived plasmodia (plasmodia derived originally from a single amoeba) from each of two strains—the C6 wild type and the Y2–1 mutant. Representative data from three clones of each strain are shown in Table 1.

Table 1. *Number of small nuclei observed in clones of plasmodia of Didymium nigripes strains C6 and Y2–1*

In each observation, total number of nuclei counted was 500. The C6 plasmodia were examined for two months and the mutant plasmodia (Y2–1) for 100 days. The statistical significance of the observed changes was determined by using a 2 × 2 contingency table. Statistically significant changes ($P = 0{\cdot}01$) are denoted by an asterisk.

| | Age in days after spore germination | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | 11–12 | 17–18 | 27–29 | 35–36 | 41–43 | 48–49 | 57–58 | 63 |
| Plasmodial clone | | | | | | | | |
| C6–1 | 369 | 369 | 388 | 390 | 397 | 395 | 398 | — |
| C6–7 | 361 | 372 | *435 | 414 | 410 | 416 | 417 | 417 |
| C6–10 | 431 | 438 | *384 | 404 | *468 | 466 | 461 | 466 |
| Y2–1A | 463 | 451 | *387 | *307 | *351 | *401 | 404 | 435 |
| Y2–1B | 123 | *404 | *136 | *350 | *395 | 399 | 370 | 397 |
| Y2–1F | 418 | *292 | *196 | *137 | *353 | *303 | *368 | *225 |

If no change in ploidy level were occurring in the plasmodium, one would expect to find relatively little change in the proportion of small nuclei over extended periods of time. In this respect, C6 plasmodia showed very few changes of statistical significance: four of the ten plasmodia showed no changes at all and three more underwent only one change. One plasmodium behaved differently and demonstrated changes of nuclear size ratio at almost every observation. In general, C6 plasmodia did not undergo significant changes of ploidy level after 4 weeks of age. In contrast, most of the yellow clones exhibited changes in the nuclear size ratio at almost every observation. A summary of the changes in nuclear size in all 20 clones is included in Table 2.

There was no trend in the direction of significant changes, i.e. to more large nuclei or to more small nuclei. Of 13 changes detected in C6, eight resulted in more large nuclei and five in more small nuclei. Of 60 changes in Y2–1 clones, 32 led to more large nuclei and 28 led to more small nuclei. Within a given plasmodium, both continuation of a trend, e.g. to even more large nuclei, and alteration of direction of change, were noted on successive observations.

In order to determine whether the observed long-term stability of nuclear size ratio is a property of the C6 strain or was simply due to the fact that the previously selected clones possessed a majority of small nuclei, a second series of C6 clones was isolated which showed three types of nuclear size ratio, i.e. mainly large, mainly small, and an appreciable proportion of both large and small (designated as mixed). In general, C6 clones, regardless of initial nuclear size composition, showed less tendency to undergo changes in nuclear size ratio than did Y2–1 clones (Table 3).

Table 2. *Number of plasmodia showing changes in nuclear size ratios*

Table records number of plasmodia showing change in nuclear size ratio/total number of plasmodia examined ($P = 0{\cdot}01$).

| | Age of plasmodia (weeks) | | | | | | |
|---|---|---|---|---|---|---|---|
| Strain | 3 | 4 | 5 | 6 | 7 | 8 | 9 |
| C6 | 1/9 | 5/10 | 2/7 | 3/7 | 0/9 | 1/9 | 1/7 |
| Y2–1 | 7/10 | 7/10 | 7/10 | 7/10 | 5/10 | 4/10 | 6/10 |

Table 3. *C6 plasmodia showing changes in nuclear size ratios*

Table records number of plasmodia showing changes in nuclear size ratio/total number of plasmodia examined ($P = 0{\cdot}01$).

| | Age of plasmodia (weeks) | | | | | |
|---|---|---|---|---|---|---|
| Strain | 1 | 2 | 3 | 4 | 5 | 6 |
| Mainly small nuclei | 1/3 | 1/2 | 2/3 | 1/3 | 0/1 | 0/1 |
| Mainly large nuclei | 2/3 | 3/3 | 2/3 | 0/3 | 0/3 | 0/3 |
| Mixed | 1/4 | 4/4 | 1/4 | 0/4 | 0/3 | 0/3 |
| Total | 4/10 | 8/9 | 5/10 | 1/10 | 0/7 | 0/7 |

*Origin of large nuclei in the nuclear population*

A previous study (S. Kerr, 1968*a*, *b*) has shown that in populations of amoebae and in populations of young plasmodia derived from these amoeboid populations the proportion of small nuclei did not differ significantly. However, macroscopic plasmodia often possessed more large nuclei than the populations of amoebae from which they were derived. Occasional fortunate observations made with the phase contrast microscope revealed two methods by which large nuclei are formed: (i) fusion of two nuclei during interphase and (ii) failure of mitotic daughter nuclei to separate after telophase, with subsequent fusion. Large nuclei formed by both methods remained large throughout subsequent divisions, yielding large daughter nuclei.

The rate of formation of large nuclei in young plasmodia (4 to 16 nuclei) was calculated in populations of cells 12 to 30 h. after they had been plated on agar following incubation procedures which give the largest numbers of plasmodia (N. Kerr, 1961). Cells were plated at densities such that secondary fusion of several plasmodia could not occur and account for the mixture of nuclear sizes. Two media were used: GPY/5, which gives a nuclear generation time of 4 to 6 h., and a phosphate-buffered minimal media (SS; S. Kerr, 1967) which gives a nuclear generation time of 6 to 8 h. Of 1125 plasmodia observed, 84 plasmodia contained both large and small nuclei.

The rate of formation of large nuclei was calculated in the following manner. Nuclear generations were defined such that generation$_0$ was from plasmodial dif-

ferentiation until division to the two-nucleate stage, generation$_1$ was the two-nucleate stage until the second division, generation$_2$ was the four-nucleate stage until the third division, etc. The simplest hypothesis explaining the generation of origin of large nuclei was accepted for each mixed plasmodium observed. For example, if one large and six small nuclei were present, only one hypothesis could explain this change of size as one event—namely that two small nuclei fused during generation$_3$. Fusion could have occurred either between interphase nuclei or as a failure of mitosis but the generation of change would remain the same. Using this system, size change which occurred during the amoeboid phase or during generation$_1$ would not be detected, for in these cases all resulting nuclei would be of uniform size.

On the medium which resulted in the longer generation time (SS), the observed rate of change per generation was higher (Table 4). In seven cases mixed plasmodia were observed which could be explained only by assuming that a large nucleus had split into two smaller ones. These data are not included in Table 4.

Table 4. *Rate of formation of large nuclei in young plasmodia*

| Growth medium | Generation | Number of plasmodia observed | Number of plasmodia with mixed nuclear size | Number of nuclear generations elapsed | Rate of change per nuclear generation |
|---|---|---|---|---|---|
| GPY/5 | 2 | 293 | 14 | 2240 | $6{\cdot}3 \times 10^{-3}$ |
| GPY/5 | 3 | 185 | 6 | 2136 | $2{\cdot}8 \times 10^{-3}$ |
| GPY/5 | 4 | 82 | 5 | 1312 | $3{\cdot}8 \times 10^{-3}$ |
| Total | 2–4 | 560 | 25 | 5688 | $4{\cdot}4 \times 10^{-3}$ |
| SS | 2 | 304 | 25 | 2224 | $11{\cdot}2 \times 10^{-3}$ |
| SS | 3 | 161 | 19 | 2016 | $9{\cdot}4 \times 10^{-3}$ |
| SS | 4 | 91 | 8 | 1456 | $5{\cdot}5 \times 10^{-3}$ |
| Total | 2–4 | 558 | 52 | 5696 | $9{\cdot}1 \times 10^{-3}$ |

The second column shows the generation in which the large nuclei were estimated to have been formed first.

*Control of nuclear size ratio*

Several approaches were used in an attempt to determine whether a control mechanism exists in the plasmodium so that the nuclear size ratio is maintained at a fairly constant level.

*Behaviour of microplasmodia.* In a preliminary study, ten microplasmodia were isolated from each of three parental clones and their nuclear size ratios were compared to the ratios of their respective parents. Derivatives of one parental clone showed no significant difference in nuclear size ratios from the parent. Four and five of the isolated microplasmodia of the other clones differed from the parents after a 2 day period of time.

A second series of 12 microplasmodia were selected which differed from the nuclear size ratio of the parental clones. The ratios in these microplasmodia were followed for 15 to 18 days to see if they would show any tendency to revert to the parental nuclear size ratio. Three of these microplasmodial lines underwent no change of ratio; six underwent a change of ratio but did not achieve a ratio like that of the parent; and three reverted to a ratio like that of their parents. Thus there seems to be no pronounced tendency for a given plasmodial stock to regulate back to a specific nuclear size ratio.

*Fusions of plasmodia with unlike ratios.* Further attempts were made to determine whether or not maintenance of ploidy level and changes in ploidy level might be under some type of cytoplasmic control. Microplasmodia of C6 were isolated which showed three distinctive types of nuclear ratios: mainly small, mainly large, and an approximately equal number of small and large nuclei (mixed). These microplasmodia were grown to macroscopic size and then pieces of approximately equal size were fused with other pieces containing the same and different nuclear ratios in all possible combinations. Nuclear size ratios of the plasmodia derived from the fusion were determined 1 to 3 days later. Similar fusions were performed with clonally derived plasmodia. No difference in behaviour was found between plasmodia of these two origins.

Table 5. *Fusions between plasmodia of different nuclear size ratios*

| Type of fusion | Number of fusions | Number of changes in nuclear size ratio | Direction of change |
|---|---|---|---|
| (*a*) Controls (two pieces of the same plasmodium) | | | |
| Mainly large | 8 | 0 | — |
| Mainly small | 8 | 1 | More small |
| Mixed | 10 | 9 | More small |
| (*b*) Fusions between clones | | | |
| Large + large | 5 | 0 | — |
| Small + small | 5 | 3 | More small |
| Mixed + mixed | 7 | 4 | More small |
| Large + small | 12 | 12 | More small |
| Large + mixed | 13 | 13 | More small |
| Small + mixed | 16 | 13 | 11 More small<br>2 More large |

Where there was a significant change in ratio ($P = 0{\cdot}01$) the direction of the change in nuclear size is shown in the last column.

The nuclear size ratios resulting from 84 such fusions were compared to a mean value derived from the parental stocks by means of a $2 \times 2$ contingency table (Table 5). The behaviour of plasmodia derived from such fusions was extremely consistent. Ploidy level changes generally occurred whenever one of the parental plasmodia contained an appreciable proportion of small nuclei (i.e. plasmodia characterized as either small or mixed) unless a given clone was being fused to a piece of itself. Of 55 changes in nuclear size ratio, all but two resulted in more small nuclei. In these two cases the 'small' parent for the fusions was the same clone.

*Fusions of C6 and Y2–1 plasmodia.* Clonally derived plasmodia of the C6 strain were fused with clonally derived plasmodia of the Y2–1 strain. Such 'heteroplasmodia' were examined weekly for 5 weeks in an attempt to determine whether they exhibited behavioural characteristics of either of the parental strains. These heteroplasmodia were compared both to their parents and to compiled data from other clones (Table 6).

In this case, 'heteroplasmodia' showed behavioural traits somewhat intermediate to the two parental types. The reduction in the number of ploidy level changes after a month of growth was reminiscent of the behaviour of the C6 whereas the percentage and magnitude of the changes observed before this time was more like the behaviour observed in the yellow mutant clones.

The C6+Y2–1 heteroplasmodia were characterized by loss of pigmentation a few hours after formation. After the 5 weeks of observation the composition of the plasmodia was checked by two methods for the continued presence of both parental types. Microplasmodia were isolated and incubated until colour could be determined. The heteroplasmodia were also allowed to fruit, the resulting spores were plated so they would give rise to clones, and then the colour of the resulting amoeboid clones was scored. With the exception of one plasmodium which had become yellow during the 5 weeks (and which yielded only yellow progeny), both parental types were recovered from all the heteroplasmodia.

Table 6. *C6+Y2–1 heteroplasmodia ploidy level changes*

Table records number of plasmodia showing changes in nuclear size ratio/total number of plasmodia examined ($P = 0{\cdot}01$).

| | Age of plasmodia (weeks) | | | | |
|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 |
| Type of plasmodia | | | | | |
| All C6 | 4/10 | 8/9 | 6/19 | 6/20 | 2/14 |
| All Y2–1 | 1/2 | 10/12 | 10/12 | 8/12 | 8/12 |
| C6 parents | 1/3 | 0/3 | 0/3 | 0/3 | 0/3 |
| Y2–1 parents | 1/2 | 2/2 | 2/2 | 1/2 | 1/2 |
| Fusion plasmodia; equal parental contribution | 4/6 | 4/6 | 4/6 | 4/6 | 0/6 |
| Fusion plasmodia; 1/3 Y2–1; 2/3 C6 | 1/2 | 2/2 | — | — | — |
| Fusion plasmodia; 2/3 Y2–1; 1/3 C6 | 1/2 | 1/2 | 2/2 | 1/2 | 0/2 |
| Total of fusions | 6/10 | 7/10 | 6/8 | 5/8 | 0/8 |

## DISCUSSION

### *Mixture of nuclear sizes in plasmodia*

The mixture of nuclear sizes as reported here for *Didymium nigripes* is not a new observation for true slime moulds. Ross (1966), Koevenig & Jackson (1966) and Guttes & Guttes (1969) have recently reported several sizes of nuclei which represent different ploidy levels in the plasmodium of *Physarum polycephalum*. In addition, Ross reported several ploidy levels in amoebae of *Badhamia curtisii*. Therrien (1966) used microspectrophotometry to measure the DNA content of individual nuclei of a single plasmodium of the isolate of *D. nigripes* used in the current investigation and found that these nuclei may have vastly differing amounts of DNA, even though all the nuclei in a single plasmodium should be in the same phase of the cell cycle at a given time. Counts of the number of chromosomes present also show that nuclei of several ploidy levels are present (S. Kerr, 1968*a*, *b*). The present study reported only the proportion of nuclei of a certain size class which were present. Very rarely, nuclei of smaller diameter were encountered: routinely the 'large' nuclei varied greatly in nuclear diameter.

In the light of both chromosome counts and Therrien's calculation of DNA content, it seems expedient at the present time not to assign small nuclei to a haploid class, but simply to regard nuclei of various sizes as several different multiples of an, as yet, undetermined haploid number of chromosomes.

*Nuclear size fluctuations*

Observations dealing with maintenance of nuclear size ratios or change in these ratios over a period of time have not been reported previously for the true slime moulds. The calculated rate of formation of large nuclei in populations of young plasmodia is great enough to account for all of the changes in nuclear size ratio which have been observed. After examining the data collected in this study, it is rather difficult to construct a model for control of nuclear size ratios. If formation of large nuclei continued to occur at the initial rate, plasmodia would soon be composed solely of large nuclei. Although indirect evidence for formation of small nuclei from large nuclei was obtained, this rate was much lower and could not account for the maintenance of a constant ratio of small to large nuclei. During sporulation large-scale destruction of nuclei has been observed: some of the nuclei became enclosed in vacuoles and were gradually destroyed. Nuclear destruction of this type is rarely observed in a growing C6 plasmodium, however, and so it seems unreasonable to assume that a plasmodium maintains a fairly constant nuclear size ratio by large scale destruction of its large nuclei.

Yellow plasmodia underwent massive fluctuations in size classes and at the same time differed from C6 in other ways. Yellow plasmodia were less hardy and underwent an ageing process which was characterized by loss of the ability to sporulate, loss of the ability to fuse with other plasmodia and, finally, loss of the ability to grow (N. Kerr & Waxlax, 1968*b*). Smear preparations of yellow plasmodia undergoing division showed that most extremely large and occasional large nuclei failed to divide at the same time as the small nuclei were dividing. Yellow plasmodia contained degenerating nuclei in vacuoles and also possessed a much greater proportion of polyploid nuclei, and on occasions a majority of the nuclei have seemed to be of extraordinary diameter. However, there was no correlation between the onset of ageing symptoms, accumulation of polyploid nuclei, and changes in the nuclear size ratio.

*Is nuclear size controlled*?

One hypothesis which must be examined is that a plasmodium may be able to control the proportions of nuclei in its various size classes and thus maintain what seems to be an optimum distribution of sizes. In this case one would expect a clonally derived plasmodium not only to maintain a fairly constant distribution of sizes in its nuclear population, but also for tiny pieces of this plasmodium to achieve the same ratio. In cases where microplasmodia were isolated and nuclear size ratios studied, there was no indication that maintenance of a special ratio was occurring.

An alternate hypothesis might be that a fairly constant nuclear size ratio is maintained simply by divison of all existing nuclei and that changes in ratio are precipitated only by sampling errors during subculture. Although this hypothesis might explain the behaviour of microplasmodia, several types of observations suggest that this hypothesis in untenable. Rapid mixing of all cytoplasmic components within a plasmodium has been reported within the isolate studied using both stained and radioactively labelled plasmodia (N. Kerr & Waxlax, 1968*a*). Samples 1 mm.$^2$ contain several thousand nuclei and nuclear ratios from various portions of the same plasmodium in samples of this size are virtually identical. Subculture blocks 1 cm.$^2$ would then be

expected to contain representative distribution of the nuclear sizes present. In most cases a constant ratio was maintained by this subculture technique.

If the nuclear ratio remains constant through a passive means, then it becomes difficult to explain several observations: (*a*) the relatively constant rate of formation of large nuclei in young plasmodia, (*b*) the results from fusions between pieces of plasmodia with different size ratios, in which the resulting size ratio was unlike the mean derived from the two parental ratios and yet all nuclei present seemed to divide synchronously, and (*c*) the rather extensive alterations in nuclear size ratio in yellow plasmodia.

The fact that ploidy level changes do occur is particularly interesting since critical cytological and genetic evidence of standard sexual processes are lacking in this organism. Perhaps the behaviour observed can be considered analogous to parasexual processes described by Pontecorvo (1958) in the fungus *Aspergillus nidulans*. The sequence of events described by Pontecorvo involved rare fusion of haploid nuclei to form a diploid nucleus and subsequent segregation of recombinant nuclei with eventual haploidization. The occasional fusion of nuclei in *Didymium nigripes* and variability of chromosome number (S. Kerr, 1968*a*, *b*) and DNA content (Therrien, 1966) could be cited as evidence for diploidization followed by progressive haploidization. A search for parasexual processes by genetic analysis has not yet been attempted in *D. nigripes*: at the same time the cytological observations reported here suggest that parasexuality should not be excluded as a possible genetic mechanism in the true slime moulds.

I wish to thank Dr Norman Kerr for the use of his laboratory and for helpful discussion on the preparation of this paper. Parts of this work were supported by two grants to Dr Norman Kerr—the Biomedical Research Fund of the Graduate School, University of Minnesota, and N.I.H. Institute of Allergy and Infectious Disease grant AI-05521.

## REFERENCES

GUTTES, E. & GUTTES, S. (1969). Replication of nucleolus-associated DNA during $G_2$ Phase in *Physarum polycephalum*. *Journal of Cell Biology* **43**, 229–236.

KERR, N. (1961). A study of plasmodium formation by the true slime mould *Didymium nigripes*. *Experimental Cell Research* **23**, 603–611.

KERR, N. (1965). Disappearance of a genetic marker from a cytoplasmic hybrid plasmodium of a true slime mould. *Science, New York* **147**, 1586–1588.

KERR, N. & WAXLAX, J. (1968*a*). The fusion of plasmodia of a true slime mould grown on various nutrients and the mixing of fused plasmodia. *Transactions of the American Microscopical Society* **87**, 197–200.

KERR, N. & WAXLAX, J. (1968*b*). A yellow variant of the Eumycetozoan *Didymium nigripes* which exhibits ageing. *Journal of Experimental Zoology* **168**, 351–361.

KERR, S. (1967). A comparative study of mitosis in amoebae and plasmodia of the true slime mould *Didymium nigripes*. *Journal of Protozoology* **14**, 439–445.

KERR, S. (1968*a*). *Cytological observations on plasmodial differentiation in the true slime mould Didymium nigripes*. PhD. Dissertation, University of Minnesota.

KERR, S. (1968*b*). Ploidy level in the true slime mould *Didymium nigripes*. *Journal of General Microbiology* **53**, 9–15.

KOEVENIG, J. L. & JACKSON, R. C. (1966). Plasmodial mitosis and polyploidy in the Myxomycete *Physarum polycephalum*. *Mycologia* **53** 662–667.

NYGAARD, O. F., GUTTES, S. & RUSCH, H. P. (1960). Nucleic acid metabolism in a slime mould with synchronous mitosis. *Biochimica et Biophysica Acta* **38**, 298–306.

PONTECORVO, G. (1958). *Trends in Genetic Analysis*, pp. 119–124. New York: Columbia University Press.

ROSS, I. K. (1966). Chromosome numbers in pure and gross cultures of Myxomycetes. *American Journal of Botany* **53**, 712–718.

THERRIEN, C. D. (1966). Microspectrophotometric measurement of nuclear deoxyribonucleic acid content in two myxomycetes. *Canadian Journal of Botany* **44**, 1667–1675.

*Journal of General Microbiology* (1970), **63**, 357–366
*Printed in Great Britain*



# An Extension of the Theory of the Chemostat with Feedback of Organisms. Its Experimental Realization with a Yeast Culture

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(*Accepted for publication 4 September 1970*)

SUMMARY

Homogeneous continuous-flow culture with some mechanical means for concentrating the biomass in the fermenter is termed a chemostat with feedback. The theory of the system has been reformulated and extended to include concentration of the biomass outside or inside the culture vessel by means such as centrifugation, filtration or gravity sedimentation. An important feature of the general system is the provision of two effluents, one of concentrated biomass and the other of diluted biomass. Experimentally, feedback was realized by filtration of one effluent stream. By this means, steady states were obtained over a wide range of flow rates; the values of biomass and growth-limiting substrate were in good agreement with the theory. The maximum biomass output rate of the chemostat was increased fourfold.

## INTRODUCTION

The chemostat with feedback was first represented by Herbert (1961) as shown in Fig. 1*a*. The continuous culture was depicted with a single effluent stream provided with a device, such as a centrifuge, to concentrate the organisms and return part of the concentrate to the culture. Increase in the organism concentration in the fermenter enables the process rates to be increased above those possible in the simple chemostat (Herbert, Elsworth & Telling, 1956). This method of increasing microbial process rates is required where the concentration of substrate is fixed and cannot be increased as, for example, in effluent disposal or where the substrate solubility, or toxicity of products limits the possible substrate concentration.

The initial formulation of the theory by Herbert (1961) has been followed in subsequent treatments (for example: Fencl, 1966; Pirt, 1969). Hitherto there have been no reports of experiments deliberately designed to test the theory and to explore the extent to which it can be realized in practice. Indeed, it has seemed too difficult to achieve on the normal laboratory scale because of the lack of a simple device to control the concentration of the biomass and feed a known fraction of it back into the culture, especially under aseptic conditions. Now the re-examination of the system reported here has lead to an extension of the theory and to the development of a simple experimental means of realizing the system.

## THEORY

The chemostat with feedback, as originally represented (Herbert, 1961) with a single effluent stream, is shown in Fig. 1*a*. In practice it is difficult to conceive of any controllable device for concentrating just the amount of biomass required for the feedback. In general, both concentrated and dilute biomass streams would be generated as shown in Fig. 1*b* and a part of the concentrated stream would be fed back.

Two other systems of 'feed back' are represented in Fig. 1. In the system shown in Fig. 1*c* a filter is used to remove from the culture a dilute suspension of organisms; in the extreme case this may be a cell-free fluid. Culture containing concentrated suspension of organisms is taken directly from the culture vessel through a constant-level device. A fourth system of feedback is depicted in Fig. 1*d*. This system consists of a culture in which the organisms growing at the bottom of the vessel are continuously enriched with biomass sedimenting from the upper zones. Thus effluent drawn from the top of the culture forms the dilute stream of organisms. The concentrated stream is pumped out of the lower part of the culture.

### *Notation*

$V$ = volume of growing culture in the fermenter (l.)
$F$ = rate of flow of medium into the culture (l./h.)
$D = F/V$ = dilution rate (h.$^{-1}$)
$s_r$ = concentration of growth-limiting substrate in fresh medium (g./l.)
$s$ = concentration of growth-limiting substrate in culture (g./l.)
$x$ = biomass (organism) concentration (g./l.)
$c$ = fraction of total effluent stream which contains concentrated biomass
$Y$ = growth yield (g. biomass/g. growth-limiting substrate utilized)
$\mu$ = specific growth rate (h.$^{-1}$)
$\mu_m$ = maximum specific growth rate (h.$^{-1}$)
$t$ = time (h.)
$a$, $g$, $k$ are dimensionless coefficients
$K_s$ = saturation constant (g./l.) for the growth-limiting substrate, in the Monod relation, $\mu = \mu_m s/(K_s+s)$.

A bar over a symbol (for example, $\bar{x}$) indicates that it is a steady-state value.

### *Chemostat with feedback*

The system represented in Fig. 1*b* will be considered first. The net rate of medium flow through the fermenter is $F$. A fraction $a$ of the total flow out of the fermenter ($F_s$) is fed back; thus $F_s = F + aF_s$, whence $F_s = F/(1-a)$. It is supposed that sedimentation of organisms concentrates the biomass by the factor $g$ so that the concentration of biomass in the feedback line is $gx$ where $g > 1$. Only a part of the biomass concentrate is fed back, the remainder forms the concentrated biomass effluent with flow rate $cF$. The dilute biomass effluent has the concentration $kx$ where $k < 1$. The subsequent analysis is made with the simplifying assumption that the time spent by the culture in the feedback line is negligible.

The rate of biomass production can be obtained from the organism balance for the fermenter, which is

$$\text{increase in biomass} = \text{growth} - \text{output} + \text{feedback}.$$

In the infinitely small time interval $dt$ the balance will be

$$V dx = \mu x V\, dt - F_e x\, dt + aF_e gx\, dt. \tag{1}$$

On dividing through by $dt$, substituting for $F_e$ and putting $D = F/V$ we obtain

$$\frac{dx}{dt} = \left\{\mu - \frac{(1-ag)}{1-a} D\right\} x. \tag{2}$$

Let $\beta = (1-ag)/(1-a)$ then

$$\frac{dx}{dt} = (\mu - \beta D)x. \tag{3}$$

In the steady state $dx/dt = 0$ and $\bar{\mu} = \beta D$. It should be noted that $\beta$ must be a positive fraction since $ag < 1$ (or the biomass fed back would not be $< 100\%$, which is essential for steady states) and $ag > a$.

The balance for the growth-limiting substrate in the fermenter is given by

Increase in substrate = input − substrate used for growth − output + feedback, that is,

$$V ds = Fs_r\, dt - (\mu x/Y) V dt - F_e s\, dt + aF_e s\, dt. \tag{4}$$

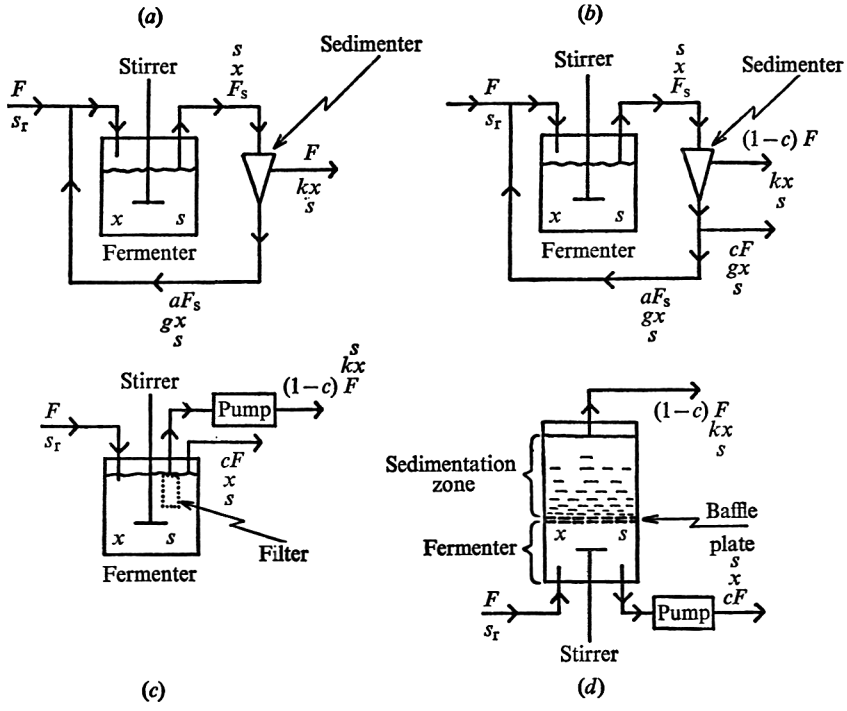


Fig. 1. The chemostat with feedback of biomass. The symbols represent the concentrations of biomass ($x$), growth-limiting substrate ($s$) and flow rate ($F$) at various points. (*a*) Single effluent system with sedimenter which concentrates the biomass by the factor $g$ in the feedback stream. (*b*) Dual effluent system with concentrated biomass effluent (flow rate, $cF$) and diluted biomass (flow rate, $(1-c)F$). (*c*) Filter feedback system. The diluted biomass effluent is extracted through the filter. (*d*) Fermenter with sedimentation zone. The baffle plate would serve to separate the homogeneous fermenter zone from the sedimentation zone. Concentrated biomass is removed at the base of the fermenter and diluted biomass from the top of the sedimenter.

On dividing by $V\,dt$ we obtain

$$\frac{ds}{dt} = D(s_r - s) - \frac{\mu x}{Y}. \tag{5}$$

To solve for the steady-state values of $x$ and $s$, which are obtained when $dx/dt = ds/dt = 0$, we substitute for the variable $\mu$ by means of the Monod relation, $\mu = \mu_m s/(K_s + s)$. Hence

$$\bar{s} = \frac{\beta DK_s}{\mu_m - \beta D} \quad \text{and} \quad \bar{x} = \frac{Y}{\beta}(s_r - \bar{s}). \tag{6}$$

At the critical dilution rate ($D_c$) the organisms grow at the maximum rate which the medium will allow. This value of the dilution rate is obtained by putting $\mu = \beta D_c$ and $s = s_r$ in the Monod relation, that is,

$$D_c = \frac{\mu_m}{\beta}\frac{s_r}{K_s + s_r}, \tag{7}$$

and when $K_s \ll s_r$, $D_c \approx \mu_m/\beta$.

The concentration of biomass in the dilute effluent ($kx$) can be found from the biomass balance

biomass leaving fermenter = biomass in concentrated effluent
+ biomass in diluted effluent + biomass in feedback,

that is,

$$F_4 x = cFgx + (1-c)Fkx + aF_8 gx. \tag{8}$$

On simplifying and rearranging we obtain

$$k = \frac{\beta - cg}{1-c}. \tag{9}$$

The single effluent system shown in Fig. 1*a* can be regarded as a special case of the dual effluent system in which $c = 0$. Hence the equations for $\bar{x}$ and $\bar{s}$ are unaffected but $k = \beta$.

*Chemostat with filter feedback*

The filter feedback system is depicted in Fig. 1*c*. Just as in the preceding system there are two effluent streams, one containing concentrated biomass and the other diluted biomass. The biomass balance for the fermenter is

increase in biomass = growth − output in concentrated stream
− output in diluted stream,

that is, for the infinitely small time interval $dt$

$$V\,dx = \mu x V\,dt - cFx\,dt - (1-c)Fkx\,dt. \tag{10}$$

On substituting $D = F/V$ and rearranging we obtain

$$\frac{dx}{dt} = \left[\mu - \left\{c + (1-c)k\right\}D\right]x. \tag{11}$$

Let $\gamma = c + (1-c)k$, then the value of $\mu$ in the steady state, when $dx/dt = 0$, is $\bar{\mu} = \gamma D$.

The balance for growth-limiting substrate is

increase in substrate = input − substrate used for growth
− substrate in both effluents,

that is,

$$V\,\mathrm{d}s = Fs_r\,\mathrm{d}t - (\mu x/Y)V\,\mathrm{d}t - Fs\,\mathrm{d}t, \tag{12}$$

hence

$$\frac{\mathrm{d}s}{\mathrm{d}t} = D(s_r - s) - \frac{\mu x}{Y}. \tag{13}$$

Putting $\mathrm{d}x/\mathrm{d}t = 0$ and $\mathrm{d}s/\mathrm{d}t = 0$ in equations (11) and (13) and substituting for $\mu$ we obtain for the steady state

$$\bar{s} = \frac{\gamma DK_s}{\mu_m - \gamma D} \quad \text{and} \quad \bar{x} = \frac{Y}{\gamma}(s_r - \bar{s}). \tag{14}$$

The critical dilution rate is given by substituting $\mu = \gamma D_c$ and $s = s_r$ in the Monod relation; hence $D_c \approx \mu_m/\gamma$ when $s_r \gg K_s$.

An important special case of the chemostat with filter feedback occurs when cell-free medium is extracted through the filter. Then $k = 0$ and $\gamma = c$, and the steady-state values of $x$ and $s$ become

$$\bar{s} = \frac{cDK_s}{\mu_m - cD}$$

and

$$\bar{x} = \frac{Y}{c}(s_r - \bar{s}). \tag{15}$$

The fourth system of feedback depicted in Fig. 1*d* theoretically is identical with the filter feedback system. This involves the simplifying assumptions that all the growth occurs in the lower zone (to which nutrient is added) and that the organisms are unaffected by their stay in the sedimentation zone.

In the three cases of feedback considered the expressions giving the steady-state concentrations of organisms and growth-limiting substrate are formally the same, the only difference being in the factor $\beta$, $\gamma$ or $c$, the reciprocal of which expresses the increase in the biomass concentration over that which would be obtained in the chemostat without feedback. Because of its fundamental significance, the factor $1/\beta$, $1/\gamma$ or $1/c$ is termed the 'feedback factor'. The theory has been tested by use of the filter feedback system.

## METHODS

*Apparatus.* The continuous-flow cultures were made in the CECA (Gallenkamp, Christopher Street, London, E.C.2) type chemostat. The culture vessel was based on the 152 × 305 mm. Pyrex pipeline as used by Elsworth, Meakin, Pirt & Capell (1956). The culture volume was in the range 1·5 to 2·0 l.

*Culture filtration.* The filter feedback system was achieved by insertion of Whatman glass fibre filter tubes (Reeve Angel Ltd, 14 New Bridge Street, London, E.C.4) in the culture vessel. The filters excluded particles > 2 $\mu$m. diameter. For filtration with a yeast concentration up to 1·5 g. dry wt./l., three filters of type A 1 (60 × 12 mm., total filter area 78 cm.$^2$) were used; for filtration rates with yeast cell densities up to 3·5 g. dry wt./l. four filters of type B 2 (60 × 26 mm.; total filter area 180 cm.$^2$) were inserted

in the fermenter. One end of each filter was closed with a rubber bung; the other end was connected by means of a polypropylene adaptor (Portex Ltd, Hythe, Kent) to a stainless steel tube and the joints sealed with Araldite. The stainless steel tube was passed through a bung in one of the ports on the lid of the fermenter. The steel tube was then connected by silicone tubing to one of the peristaltic pumps on the apparatus which provided the suction for filtration. The filtration rate (up to 2·5 l./h.) was monitored by one of the burettes provided for medium flow rate measurement.

*Culture conditions.* The culture temperature was controlled at 30°. During the steady states the pH value was maintained at $5{\cdot}5 \pm 0{\cdot}1$ by the buffer in the medium. Antifoam (0·1 ml. polypropylene glycol 2000) was added every 2 h. Normally, vortex aeration by free agitation was used in the vessel. However, because the presence of the filters partially interfered with the vortex formation, it was decided to use a relatively low stirrer speed (900 rev./min.) with air sparged through the culture at 600 ml./min. The sparger consisted of a steel tube of bore 2 mm. dipping to the bottom of the culture vessel.

*Medium.* The nutrient medium had the following composition (g./l.): glucose, 1·5; $Na_2HPO_4.2H_2O$, 1·75; $KH_2PO_4$, 13·5; $MgSO_4.7H_2O$, 0·25; $CaCl_2.2H_2O$, 0·05; $NH_4Cl$, 2·0; $FeSO_4.7H_2O$, $3 \times 10^{-6}$; $KMnO_4$, $3 \times 10^{-7}$; $ZnSO_4.7H_2O$, $4{\cdot}2 \times 10^{-6}$; $CoNO_3.6H_2O$, $5 \times 10^{-7}$; KI, $1{\cdot}0 \times 10^{-6}$; m-inositol, $1{\cdot}25 \times 10^{-3}$; thiamine, $2{\cdot}5 \times 10^{-4}$; nicotinic acid, $2{\cdot}5 \times 10^{-4}$; riboflavin, $2{\cdot}5 \times 10^{-4}$; calcium pantothenate, $2{\cdot}5 \times 10^{-4}$; *p*-aminobenzoic acid, $1{\cdot}25 \times 10^{-4}$; pyridoxin, $2{\cdot}5 \times 10^{-4}$; *d*-biotin, $6{\cdot}25 \times 10^{-6}$.

The medium was prepared in four parts: (1) glucose solution, (2) phosphates, (3) ammonium chloride and magnesium sulphate, (4) the calcium chloride, vitamins and other minerals. Solutions (1), (2) and (3) were sterilized by autoclaving at 121°; solution (4) was sterilized by Seitz filtration. To make 20 l. of medium, 10 l. solution (1), 6 l. solution (2), 3·945 l. solution (3) and 0·055 l. solution (4) were mixed aseptically.

*Inoculum.* A loopful of the organism (*Saccharomyces cerevisiae*, National Collection of Yeast Cultures, strain 229) was transferred to 5 ml. of the nutrient medium and incubated at 30° until turbid. This culture was used to inoculate 200 ml. nutrient medium in a 2 l. flask. After overnight incubation on an orbital shaker at 30° the culture was used to inoculate the CECA fermenter.

*Analytical methods.* The turbidity of the organism suspension was read on an EEL colorimeter with a green filter. The turbidity was related to the mass of the water-washed organism dried at 105°. Residual glucose in the culture was determined with glucose oxidase after rapid removal of organisms by membrane filtration.

## RESULTS

The experiments were designed to test the theoretical equations relating the steady-state concentrations of biomass and growth-limiting substrate to the dilution rate and specific growth rate. Consequently it was necessary to vary the dilution rate, the feed-back factor ($1/c$) and determine the steady-state concentrations of yeast and of glucose (the growth-limiting substrate). A yeast rather than a bacterial species was used as the test organism because filtration of the culture was thereby greatly facilitated. Also, because of the low pH value, the culture was unlikely to support growth of any bacterial contaminant. This 'resistance' was useful because, during cultivation, filters were changed by removing the top plate of the fermenter. During the latter stages of

the work there was a low degree of bacterial contamination (< 3 bacteria/100 yeast cells). This low degree of bacterial contamination contributed a negligible amount to the biomass and, it was shown, had no effect on the biomass:turbidity ratio.

The filtered effluent stream was virtually cell-free; the ratio of the number of yeasts in the filtrate to the number in the culture being about $10^{-4}$. The useful life of the filters varied greatly with the filtration rate and the yeast concentration. When the filters started to block during steady-state operation, the filtration rate was increased by periodic reversal of the flow through the filter so as to pump back about 15 ml. of filtrate. This could conveniently be done since the flow inducer on the CECA was provided with a switch to reverse the direction of flow. The complete procedure of back-flushing consisted of pumping 15 ml. of filtrate into the burette used for flow-rate measurement, then pumping this filtrate back through the filters. The time for this operation was from 30 to 60 sec. The frequency of back-flushing increased with the filtration rate and yeast concentration. The frequency was less than once every 10 min. except at the highest dilution rate (about 1·5 $h.^{-1}$) when filters were back-flushed at 4 min. intervals. During this procedure the medium supply was stopped. This necessitated a correction to the medium flow rate, which was taken to be the mean rate given by, pumping rate $x$ (sum of 'on' periods/sum of 'on' and 'off' periods).

*Determination of critical dilution rate ($D_c$)*

The critical dilution rate was determined from the rate of wash-out of the culture when the dilution rate exceeded $D_c$. We then have, when $s \gg K_s$, $\mu \approx \mu_m$ and $\mu_m$ is given by

$$\ln x = (\mu_m - cD)t + \ln x_0, \tag{16}$$

where $x_0$ = biomass when $t = 0$. Thus $\mu_m$ can be calculated from the slope of a plot of $\ln x$ against $t$ and $D_c$ was given by $\mu_m/c$. In order to facilitate the filtration and maintain $c$ constant at the high values of $D$ used for wash-out (1·24 $h.^{-1}$ with $c = 0{\cdot}47$ and 1·83 $h.^{-1}$ with $c = 0{\cdot}25$), the yeast concentration was first reduced, by wash-out, to about a quarter of the maximum value. Then excellent straight-line relations were found between $\ln x$ and $t$ over a period of 1·5 h. or more. The value for $\mu_m$ found was $0{\cdot}42 \pm 0{\cdot}01$ $h.^{-1}$, with $c = 0$, 0·47 and 0·25. The value of $\mu_m$ found in batch culture was virtually identical (0·41 $h.^{-1}$). Thus the critical dilution rates were equal to the theoretical values.

*Steady states.* The criterion for the steady state was that three turbidity measurements taken at 30 min. intervals agreed within 1·5 % and showed no upward or downward trend. The steady-state values of the yeast and glucose concentrations are shown in Fig. 2 and 3. The glucose concentrations with the lower dilution rates and higher feedback factor were too low to be measured (< 5 μg./ml.). The value of the saturation ($K_s$) was found to be 0·025 g./l. from a plot of the reciprocals of $\bar{\mu}$ and $\bar{s}$ (Fig. 4).

The graphs show good agreement between the experimental and theoretical values of $\bar{x}$ and $\bar{s}$. In the case of the chemostat without feedback, at the high dilution rates $\bar{x}$ rose above the theoretical value. This deviation can be attributed to some growth of the yeast on the instrument probes in the culture vessel, producing an 'apparatus effect' (Herbert, *et al.* 1956). With $c = 0{\cdot}25$, near to the critical dilution rate the biomass deviated below the theoretical value. This deviation could be due to error in the

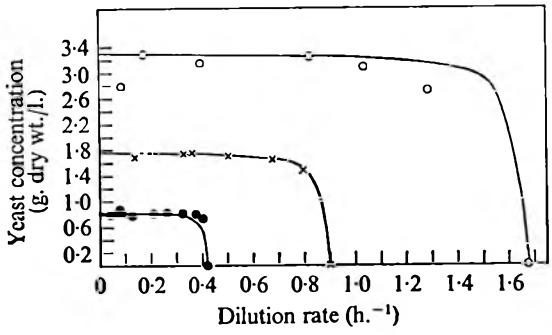


Fig. 2

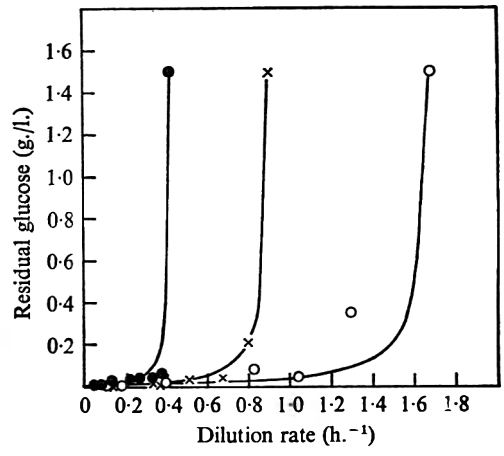


Fig. 3

Fig. 2. Yeast concentration as function of dilution rate: ●, without feedback; ×, with feedback ($1/c$ = 2·14); ○, with feedback ($1/c$ = 4·0). The lines drawn are the theoretical curves with $Y$ = 0·55 g. yeast dry wt./g. glucose, $K_s$ = 0·025 g./l., $s_r$ = 1·50 g./l., $\mu_m$ = 0·42 h.$^{-1}$.

Fig. 3. Residual glucose concentrations in steady states at different dilution rates: ●, without feedback; ×, with feedback ($1/c$ = 2·14); ○, with feedback ($1/c$ = 4·0).

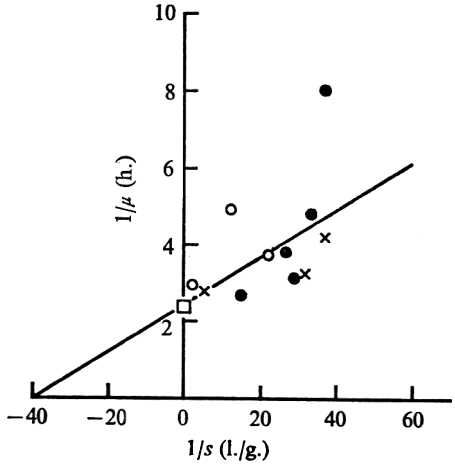


Fig. 4

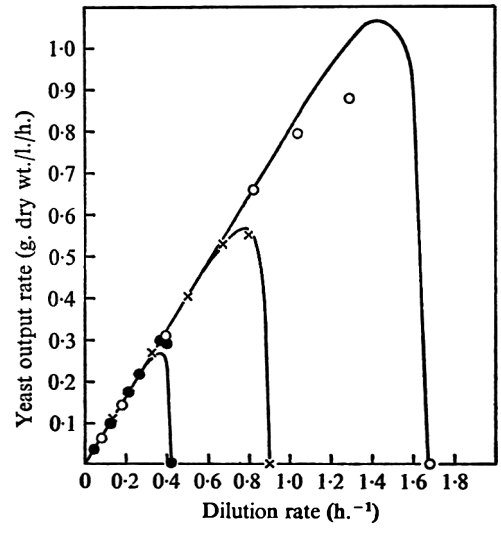


Fig. 5

Fig. 4. Plot of reciprocal of growth-limiting substrate concentration ($1/s$) against the reciprocal of the specific growth rate ($1/\mu$): ●, no feedback; ×, with feedback ($1/c$ = 2·14); ○, with feedback ($1/c$ = 4·0); □, $\mu_m$ from unsteady state.

Fig. 5. Output rate of biomass ($D\bar{x}$) in steady states at different dilution rates: ●, without feedback; ×, with feedback ($1/c$ = 2·14); ○, with feedback ($1/c$ = 4·0). The lines drawn are the theoretical curves with $Y$ = 0·55 g. yeast dry wt./l., $K_s$ = 0·025 g./l., $s_r$ = 1·50 g./l., $\mu_m$ = 0·42 h.$^{-1}$.

flow rate introduced by the frequent back-flushing of the filters necessary at such a high flow rate, combined with high yeast concentration. At this point the filtration technique was stretched to its limit. The deviation in $\bar{x}$ at the lowest dilution rates can be attributed to maintenance energy effects (Pirt, 1965) which were not allowed for.

The rates of biomass output are plotted in Fig. 5. They show how dramatically feedback can increase the output rate of a chemostat.

## DISCUSSION

The theory of the chemostat with feedback formulated here unifies various systems of continuous culture aimed at increasing the concentration of biomass in a culture with a fixed substrate concentration. This new analysis, in contrast to the original formulation (Herbert, 1961), shows that in general the system requires two effluent streams, one of concentrated, and the other of diluted biomass. The results show that filtration of part of the effluent stream provides a simple means of realizing feedback on the laboratory scale. Sortland & Wilke (1969) devised a 'filtration fermenter' from which cell-free medium was extracted through a rotating membrane filter whilst concentrated biomass was removed in a second stream. However, the theory of this system was not related to that of the chemostat with feedback and the experimental data dealt with only transient states and did not indicate the range of steady states possible.

The 'tower fermenter' (Royston, 1966), which is used in the beer fermentation, is a system which achieves feedback by sedimentation in the fermenter but the fermenter has only one outlet provided at the top of the fermenter from which a diluted biomass stream emerges. In such a culture the biomass concentration will continually increase until the culture becomes solid and therefore only unsteady states can be obtained. With this type of fermenter it seems to be the practice to run off periodically some of the dense culture at the base. If the tower fermenter were provided with an outlet for the concentrated biomass at the base, it would represent a feedback system of the type shown in Fig. 1*d*. The theory is, of course, oversimplified in that it assumes the organisms would not change in the sedimentation zone. Clearly, if they remained long in the sedimentation zone the organisms would go into a resting state or stationary phase of growth which would take some time to reverse when the organisms re-entered the fermenter zone.

The laboratory realization of the chemostat with feedback makes possible many studies on the acceleration of microbial processes such as brewing, effluent disposal, utilization of wastes such as whey and mineral leaching, where normally the substrate concentration is fixed and consequently the organism concentration is low without feedback. Feedback also provides a means of increasing output rate where an inhibitory product is formed at a concentration ($P$) proportional to the substrate concentration, that is, $P = bs_r$ where $b$ is a constant. Such a process could be oxidation of ammonia to nitrite by Nitrosomonas. The method is also one sought after in animal tissue cell culture (for example, Himmelfarb, Thayer & Martin, 1969) where either nutrient solubility, in particular, cystine, or an unknown growth-limiting factor limits maximum cell concentration.

The filtration technique needs to be developed. The filters used in this study are probably much more limited in their applicability to bacterial cultures. New types of filter such as those of sintered steel (Pall (U.K.) Ltd, Walton Road, Farlington, Portsmouth, Hants) may provide the desired improvement in performance.

## REFERENCES

ELSWORTH, R., MEAKIN, L. R. P., PIRT, S. J. & CAPELL, G. H. (1956). A two litre scale continuous culture apparatus for micro-organisms. *Journal of Applied Bacteriology* **19**, 264–278.

FENCL, Z. (1966). A theoretical analysis of continuous culture systems. In *Theoretical and Methodological Basis of Continuous Culture of Micro-organisms*. Edited by I. Malek & Z. Fencl. New York: Academic Press.

HERBERT, D. (1961). A theoretical analysis of continuous culture systems. In *Continuous Culture*, monograph no. 12, pp. 21–53. London: Society of Chemistry and Industry.

HERBERT, D., ELSWORTH, R. & TELLING, R. C. (1956). The continuous culture of bacteria: a theoretical and experimental study. *Journal of General Microbiology* **14**, 601–622.

HIMMELFARB, P., THAYER, P. S. & MARTIN, M. E. (1969). Spin filter culture: the propagation of mammalian cells in suspension. *Science, New York* **164**, 555–557.

PIRT, S. J. (1965). The maintenance energy of bacteria in growing cultures. *Proceedings of the Royal Society* B **163**, 224–231.

PIRT, S. J. (1969). Microbial growth and product formation. *Symposia of the Society for General Microbiology* **19**, 199–221.

ROYSTON, M. G. (1966). Tower fermentation of beer. *Process Biochemistry* **1**, 215–221.

SORTLAND, L. D. & WILKE, C. R. (1969). Growth of *Streptococcus faecalis* in dense culture. *Biotechnology and Bioengineering* **11**, 805–841.

*Journal of General Microbiology* (1970), **63**, 367–370
*Printed in Great Britain*



# SHORT COMMUNICATIONS

## Effect of Glutaraldehyde on Protoplasts of *Bacillus megaterium*

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(*Accepted for publication 7 August 1970*)

Glutaraldehyde is a 5-carbon dialdehyde with a wide antimicrobial spectrum; its effectiveness is markedly increased by buffering, usually with sodium bicarbonate, between pH 7·5 and 8·5. Its properties as an antimicrobial agent and chemosterilizer have been reviewed by Rubbo, Gardner & Webb (1967), and it is used extensively as a fixative in electron microscopy. We suggested (Munton & Russell, 1970) that glutaraldehyde acted on the wall of *Escherichia coli*, although other sites of action were also possible. We report here the effects of the dialdehyde on the stabilization of wall-less forms (protoplasts) with particular emphasis on their ability to withstand osmotic shock.

### METHODS

*Organism and method of culture. Bacillus megaterium* NCTC 6005 was grown for 18 h. at 37° in 100 ml. of nutrient broth (Oxoid) in a shaking incubator operating at 70 oscillations/min. The culture was centrifuged, and the resulting pellet washed twice with sterile glass-distilled water and finally resuspended in sterile water to give the required bacterial density.

*Chemicals.* Aqueous solutions of glutaraldehyde, with or without added sodium bicarbonate (0·3%, w/v), were prepared from a 25% solution (Kodak Ltd, Kirby, Liverpool). Other chemicals were of analytical reagent grade. Lysozyme was purchased from British Drug Houses Ltd, London.

*Measurement of turbidity changes.* Optical density (O.D.) was measured with the Unicam SP 600 spectrophotometer using 1 cm. cells. The reference cell contained distilled water.

*Protoplast formation.* Protoplasts of *Bacillus megaterium* were formed by suspending the bacteria (0·6 mg. dry wt/ml.) in a medium containing sucrose (0·5 M), lysozyme (200 $\mu$g./ml.) and phosphate buffer (0·013 M, pH 7·2) at 0°. Formation of protoplasts was complete after 30 to 60 min. Magnesium sulphate, $MgSO_4.7H_2O$, was added to give a final concentration of $5 \times 10^{-3}$ M. The protoplasts were deposited by centrifugation at 500 ***g*** for 30 min. and resuspended in the same stabilized buffer (phosphate + sucrose + $Mg^{2+}$) to the required O.D. value.

*Stability of glutaraldehyde-treated protoplasts.* This was investigated in two ways: (*a*) 1·5 ml. of protoplast suspension was added to 8·5 ml. volumes of aqueous glutaraldehyde solutions, water, or stabilized buffer; (*b*) protoplast suspensions (9 ml.) were

pretreated with 1 ml. glutaraldehyde for 5, 30 or 60 min., after which 1·5 ml. was added to 8·5 ml. of water, and O.D. measured at intervals. Untreated protoplasts were similarly diluted in stabilized buffer as control. Results are expressed as % of the O.D. of the control in buffer at 0 min. Samples were also examined under a phase-contrast microscope (×40c).

## RESULTS

*Effect of glutaraldehyde on O.D. of protoplast suspensions.* Low glutaraldehyde concentrations slightly reduced the O.D. of protoplast suspensions, whereas high concentrations give a significant increase. The reduction in O.D. was not associated with any decrease in protoplast numbers. Optical density readings at 450 and 660 nm. showed no significant difference and yellow colour production (Munton & Russell, 1970) is thus not involved in O.D. changes. The effect of glutaraldehyde was unchanged by the addition of sodium bicarbonate (0·3 %, w/v), although bicarbonate alone reduced the O.D. of protoplast suspensions.

Table 1. *Lysis of protoplasts of Bacillus megaterium in aqueous or alkaline glutaraldehyde (method (a))*

Readings are expressed as % increases (+) or decreases (−) of O.D. at 450 nm. of control in stabilizing buffer at 0 min.

| | Time (min.) | | | | | |
|---|---|---|---|---|---|---|
| | 0 | 5 | 30 | 60 | 90 | 120 |
| Stabilized buffer | 0 | 0 | 0 | 0 | −2·1 | −2·4 |
| Water | 0 | −56·8 | −56·8 | −56·8 | −57·0 | −57·0 |
| Protoplast suspension diluted with glutaraldehyde (% w/v) | | | | | | |
| 0·01 | 0 | −49·6 | −48·3 | −48·3 | −47·7 | −47·2 |
| 0·1 | 0 | −45·4 | −43·8 | −43·5 | −43·5 | −42·8 |
| 0·2 | 0 | −38·3 | −35·7 | −35·2 | −35·7 | −35·7 |
| 0·6 | 0 | +11·1 | +4·5 | −1·3 | −12·4 | −19·8 |
| 1·0 | 0 | +21·7 | +16·8 | +7·2 | −0·7 | −0·9 |
| Protoplast suspension diluted with 0·3 % (w/v) sodium bicarbonate and glutaraldehyde (% w/v) | | | | | | |
| 0·0* | 0 | −57·0 | −57·0 | −57·0 | −56·5 | −56·5 |
| 0·01 | 0 | −56·9 | −54·8 | −52·0 | −49·8 | −49·5 |
| 0·1 | 0 | −52·0 | −50·1 | −49·5 | −49·5 | −49·5 |
| 0·2 | 0 | −52·0 | −52·0 | −49·5 | −49·5 | −49·5 |
| 0·6 | 0 | −49·7 | −48·4 | −48·0 | −48·0 | −48·0 |
| 1·0 | 0 | −47·0 | −47·0 | −46·5 | −46·5 | −46·5 |

* Protoplasts in 0·3 % (w/v) sodium bicarbonate only.

*Stability of glutaraldehyde-treated protoplasts.* More protoplast stabilization occurred in aqueous than in alkaline glutaraldehyde (Table 1, method (*a*)) and was complete at high aldehyde concentrations. The O.D. with high concentrations of aqueous glutaraldehyde was higher than that of the control, because of the increase in protoplast density, as outlined above. The subsequent reduction in O.D. (Table 1, with 0·6 % and 1·0 % aqueous glutaraldehyde) results from aggregation of the stabilized protoplasts in acid medium, as observed microscopically.

Alkaline glutaraldehyde (method (*a*)) slightly stabilized protoplasts only at high concentrations, e.g. 0·6 and 1·0% (Table 1). Negligible lysis of protoplasts occurred when they were diluted with stabilizing buffer (Table 1).

Pretreatment with aqueous glutaraldehyde before dilution in water (method (*b*)) stabilized the protoplast suspensions; 30 min. treatment with an aldehyde concentration as low as 0·01 % (w/v) conferred some stabilization of protoplasts. The O.D. of suspensions of untreated protoplasts diluted in water fell by 65 %, whereas that of suspensions treated with 0·01 % (w/v) glutaraldehyde for 30 and 60 min. fell by 40 % and 20 % respectively. Protoplasts treated with 0·01 % (w/v) glutaraldehyde were observed to swell in water. This was reflected in a two-stage decrease in O.D. When pretreated with 0·2 % (w/v) glutaraldehyde there was no subsequent swelling of protoplasts in water and no lysis.

## DISCUSSION

Lysis of protoplasts in media of low osmotic pressure is due to water uptake and subsequent bursting of the membrane, although mechanical factors may also be involved (Weibull, 1958; Edebo, 1961; Razin & Argaman, 1963; Marquis, 1967; Corner & Marquis, 1969). Marquis (1965) and Marquis & Corner (1967) have concluded that the protoplast membrane is perforated with small aqueous channels through which lipophobic molecules could diffuse. Protoplasts are stabilized by formaldehyde, osmium tetroxide (Fitz-James, 1958), acid pH (Edebo, 1961) and ethanol or ethanol–acetic acid fixation which permanently immobilizes protoplasts (Fitz-James, 1958).

Glutaraldehyde-treated protoplasts do not lyse in water, possibly because aqueous glutaraldehyde is acid (pH 3·4 to 5) and will precipitate protoplasmic constituents. However, even at pH 8 (with bicarbonate) glutaraldehyde stabilizes protoplasts to some extent, suggesting an inherent stabilization (fixation) property of the molecule.

Slight swelling of protoplasts has been observed at low aldehyde concentrations and short contact times. In such conditions, the membrane seems partially stabilized, allowing some water to enter, and in some cases burst the protoplast or produce a swollen form. High concentrations of alkaline glutaraldehyde do not allow this swelling, water access is prevented probably by blocking off the aqueous channels, and the membrane is strengthened to resist lysis.

## REFERENCES

CORNER, T. R. & MARQUIS, R. E. (1969). Why do protoplasts burst in hypotonic solutions? *Biochimica et Biophysica Acta* **183**, 544–558.

EDEBO, L. (1961). Lysis of bacteria. 3. On the stability of protoplasts and spheroplasts in different pH ranges. *Acta Pathologica Microbiologica Scandinavia* **53**, 121–128.

FITZ-JAMES, P. C. (1958). Studies on the morphology and nucleic acid content of protoplasts of *Bacillus megaterium*. *Journal of Bacteriology* **75**, 369–382.

MARQUIS, R. E. (1965). Osmotic stability of bacterial protoplasts related to molecular size of stabilizing solutes. *Biochemical and Biophysical Research Communications* **20**, 580–585.

MARQUIS, R. E. (1967). Osmotic sensitivity of bacterial protoplasts and the response of their limiting membrane to stretching. *Archives of Biochemistry and Biophysics* **118**, 323–331.

MARQUIS, R. E. & CORNER, T. R. (1967). Permeability changes associated with osmotic swelling of bacterial protoplasts. *Journal of Bacteriology* **93**, 1177–1178.

MUNTON, T. J. & RUSSELL, A. D. (1970). Aspects of the action of glutaraldehdye on *Escherichia coli* *Journal of Applied Bacteriology* **33**, 410–419.

RAZIN, S. & ARGAMAN, M. (1963). Lysis of Mycoplasma, bacterial protoplasts, spheroplasts and L-forms by various agents. *Journal of General Microbiology* **30**, 155–172.

RUBBO, S. D., GARDNER, J. F. & WEBB, R. L. (1967). Biocidal activities of glutaraldehyde and related compounds. *Journal of Applied Bacteriology* **30**, 78–87.

WEIBULL, C. (1958). Bacterial protoplasts. *Annual Review of Microbiology* **12**, 1–26.

*Journal of General Microbiology* (1970), **63**, 371–374
*Printed in Great Britain*



# Alternative Carbon Assimilation Pathways in Methane-utilizing Bacteria

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(*Accepted for publication 31 August 1970*)

An examination of three species of methane-utilizing bacteria has shown (Lawrence, Kemp & Quayle, 1970) that the bacteria use one of two pathways of carbon assimilation, the serine pathway (Heptinstall & Quayle, 1970) or the ribose phosphate cycle of formaldehyde fixation (Kemp & Quayle, 1967). The isolation of many new types of methane-utilizing bacteria by Whittenbury, Phillips & Wilkinson (1970) has made possible an extension of these studies, and this paper reports the results of an examination of eight of the new isolates. The examination is based on the distribution of two key enzymes, each of which appears to be specifically involved in one of the assimilation pathways, namely hydroxypyruvate reductase (serine pathway) and hexose phosphate synthetase (ribose phosphate cycle).

## METHODS

*Growth of the organisms.* The following organisms were kindly provided by Dr R. Whittenbury, Department of General Microbiology, University of Edinburgh (the symbols in parentheses denote the particular strain): '*Methylosinus sporium*' (5), '*Methylosinus trichosporium*' (OB3b), '*Methylocystis parvus*' (OB BP), '*Methylomonas agile*' (Y1), '*Methylomonas rosaceus*' (BG2), '*Methylomonas methanica*' (25), '*Methylococcus minimus*' (TMC) and '*Methylobacter capsulatus*' (1521). The bacteria were grown in liquid salts medium (Foster & Davis, 1966) under an atmosphere of methane–air (1:1) in conical flasks shaken at 30°. '*Methylobacter capsulatus*' would grow in liquid medium only if the starter culture was supplemented with an aqueous extract of agar. The extract was prepared from Difco agar by the method of Dworkin & Foster (1956) and 0·5 ml. (containing 22·5 mg. dry wt) was added to 10 ml. of the starter culture. *Pseudomonas methanica* (Dworkin & Foster, 1956), *Methylococcus capsulatus* (Foster & Davis, 1966) and *Methanomonas methanooxidans* (Stocks & McCleskey, 1964) were grown up in a fermentor on methane as carbon source using the growth technique described by Lawrence *et al.* (1970). The bacterial paste harvested from the fermentor was freeze-dried and stored at $-15°$ until used.

*Preparation of bacteria-free extracts.* Bacteria, 1 g. (wet wt) or 0·1 g. (dry wt), were suspended in 5 ml. of 20 mM-sodium phosphate buffer, pH 7·0, supplemented with 5 mM-magnesium chloride, sonicated until maximum breakage had occurred (< 8 min.) in an MSE sonicator (type 100 W) and the resulting suspensions centrifuged at 90,000 *g* for 30 min. The supernatant fraction was poured off and the pellet resuspended in 5 ml. of the same buffer used to suspend the bacteria.

*Enzyme assays.* The activities of hexose phosphate synthetase and hydroxypyruvate reductase in the supernatant and pellet fractions were measured by the procedures of Lawrence *et al.* (1970). The specific activity of hydroxypyruvate reductase is expressed as μmoles of NADH oxidized/h./mg. protein. The specific activity of hexose phosphate synthetase is expressed as μmoles of [$^{14}$C]formaldehyde fixed/h./mg. protein. In the assays for hexose phosphate synthetase, control assays were performed by incubating [$^{14}$C]formaldehyde with extract in the absence of ribose 5-phosphate. The control values were subtracted from the test values obtained with ribose 5-phosphate present in the assay mixture.

*Protein determinations.* Protein was determined by the method of Folin and Ciocalteu as described by Layne (1957), with bovine serum albumin as standard.

## RESULTS AND DISCUSSION

The enzyme distribution in the eight new isolates and in *Pseudomonas methanica* (Dworkin & Foster), *Methylococcus capsulatus* (Foster & Davis) and *Methanomonas methanooxidans* (Stocks & McCleskey) is shown in Table 1. It can be seen that the clear division of organisms into those which contain hydroxypyruvate reductase and those which contain hexose phosphate synthetase, initially seen in the case of the

Table 1. *Specific activities of hydroxypyruvate reductase and hexose phosphate synthetase in extracts of methane-grown bacteria*

| Organism | Test of membrane system | Hydroxypyruvate reductase activity (μmoles/mg. protein/h.) | | Hexose phosphate synthetase activity (μmoles HCHO fixed/mg. protein/h.) | |
|---|---|---|---|---|---|
| | | Supernatant | Pellet | Supernatant | Pellet |
| '*Methylosinus sporium*' (5) | II | 80 | n.d. | n.d. | n.d. |
| '*Methylosinus trichosporium*' (OB3b) | II | 46·3 | n.d. | n.d. | n.d. |
| '*Methylocystis parvus*' (OB BP) | II | 91 | n.d. | n.d. | n.d. |
| *Methanomonas methanooxidans* (Stocks & McCleskey) | II | 72·3 | n.d. | 1·11 | 0·54* |
| '*Methylomonas agile*' (Y1) | I | n.d. | n.d. | 18·7 | 8·70 |
| '*Methylomonas rosaceus*' (BG2) | I | n.d. | n.d. | 108·8 | 15·5 |
| '*Methylomonas methanica*' (25) | I | n.d. | n.d. | 78·1 | 31·4 |
| '*Methylobacter capsulatus*' (1521) | I | n.d. | n.d. | 35·7 | 18·2 |
| '*Methylococcus minimus*' (TMC) | I | n.d. | n.d. | 132·2 | 17·8 |
| *Pseudomonas methanica* (Dworkin & Foster) | I | 0·62 | n.d. | 54·8 | 10·6* |
| *Methylococcus capsulatus* (Foster & Davis) | I | n.d. | n.d. | 18·2 | 22·5* |

n.d. = None detectable.

* The pellet fraction was washed thrice with 20 mM-phosphate buffer, pH 7·0, + 5 mM-$MgCl_2$ before assay.

last-named three organisms by Lawrence *et al.* (1970), precisely holds over the wider range that have now been tested. Whittenbury *et al.* (1970) have tentatively classified the methane-utilizers on the basis of morphology, fine structure and type of resting stage into two vibrioid groups, '*Methylosinus*' and '*Methylocystis*', and three rod/coccoid groups, '*Methylomonas*', '*Methylobacter*' and '*Methylococcus*'. On this new

classification, *Mtn. methanooxidans* (Stocks & McCleskey) appears to be a strain of '*Methylosinus*' (R. Whittenbury, personal communication), *Ps. methanica* (Dworkin & Foster) is a strain of '*Methylomonas methanica*', and *Mtc. capsulatus* (Foster & Davis) remains similarly named. The 11 organisms in Table 1 which have now been screened, although they only constitute a small sample of the total number of strains isolated, are nevertheless representative of each of the different groups proposed by Whittenbury *et al.* (1970). It thus seems likely that the dichotomy of assimilation pathway observed in this sample of organisms will be a general property amongst methane-utilizing bacteria.

All the methane-utilizing bacteria which have been examined so far possess complex internal membrane systems (Proctor, Norris & Ribbons, 1969; Whittenbury, 1969; Davies & Whittenbury, 1970; Whittenbury *et al.* 1970) which have been divided into two basic arrangements (Davies & Whittenbury, 1970): a series of bundles of disc-shaped vesicles distributed throughout the organism (type I) and a system of paired membranes running throughout the organism or aggregated at the periphery (type II).

It is most interesting to note that the subdivision of the organisms into two classes based on assimilation pathway corresponds exactly with their subdivision based on membrane system. Thus an organism using the serine pathway possesses a type II membrane system, whereas an organism using the ribose phosphate cycle possesses a type I membrane system. It therefore appears likely that the methane-utilizing bacteria, far from being a closely similar group of organisms as might be anticipated from their striking nutritional similarity of being able to grow only on methane or methanol, may in fact be divided into at least two major groups possessing fundamental differences in their mode of carbon assimilation and system of internal membranes. These differences are wide enough to suggest that the two groups of organisms may have evolved quite separately as methane utilizers. If this were so, it raises the question as to why both groups have convergently evolved as obligate methylotrophs.

We acknowledge generous financial support from the Agricultural Division, Imperial Chemical Industries Ltd, Billingham, Co. Durham, and the Science Research Council for a Studentship held by A. J. L.

## REFERENCES

DAVIES, S. L. & WHITTENBURY, R. (1970). Fine structure of methane-utilizing bacteria. *Journal of General Microbiology* **61**, 227–232.

DWORKIN, M. & FOSTER, J. W. (1956). Studies on *Pseudomonas methanica* (Söhngen) nov. comb. *Journal of Bacteriology* **72**, 646–659.

FOSTER, J. W. & DAVIS, R. H. (1966). A methane-dependent coccus, with notes on classification and nomenclature of obligate, methane-utilizing bacteria. *Journal of Bacteriology* **91**, 1924–1931.

HEPTINSTALL, J. & QUAYLE, J. R. (1970). Pathways leading to and from serine during growth of *Pseudomonas* AM 1 on $C_1$ compounds or succinate. *Biochemical Journal* **117**, 563–572.

KEMP, M. B. & QUAYLE, J. R. (1967). Uptake of [$^{14}$C]formaldehyde and [$^{14}$C]formate by methane-grown *Pseudomonas methanica* and determination of the hexose labelling pattern after brief incubation with [$^{14}$C]methanol. *Biochemical Journal* **102**, 94–102.

LAWRENCE, A. J., KEMP, M. B. & QUAYLE, J. R. (1970). Synthesis of cell constituents by methane-grown *Methylococcus capsulatus* and *Methanomonas methanooxidans*. *Biochemical Journal* **116**, 631–639.

LAYNE, E. (1957). In *Methods in Enzymology*, vol. 3, p. 447. Edited by S. P. Colowick & N. O. Kaplan. New York: Academic Press.

PROCTOR, H. M., NORRIS, J. R. & RIBBONS, D. W. (1969). Fine structure of methane-utilizing bacteria. *Journal of Applied Bacteriology* **32**, 118–121.

STOCKS, P. K. & MCCLESKEY, C. S. (1964). Morphology and physiology of *Methanomonas methanooxidans*. *Journal of Bacteriology* **88**, 1071–1077.

WHITTENBURY, R. (1969). Microbial utilization of methane. *Process Biochemistry* **4**, 51–56.

WHITTENBURY, R., PHILLIPS, K. C. & WILKINSON, J. F. (1970). Enrichment, isolation and some properties of methane-utilizing bacteria. *Journal of General Microbiology* **61**, 205–218.

*Journal of General Microbiology* (1970), **63**, 375–377
*Printed in Great Britain*



# Surface Charges of Two Types of *Bacillus megaterium* Spores Differing in Their Response to *n*-Butane

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(*Accepted for publication 8 September 1970*)

Rode & Foster (1965) showed that the germination of spores of *Bacillus megaterium* is sometimes affected by hydrocarbons such as *n*-butane: one type of spore (AL), which responds germinatively to L-alanine and inosine, is inhibited by *n*-butane; a second type of spore (GN), which responds to glucose and $KNO_3$, is not inhibited, but even accelerated a little by the same compound. The study of the biological activity of these kinds of compounds in respect of bacterial spores is useful in elucidating the mechanism of germination. In this communication, the effect of *n*-butane on the superficial charges of the spores was investigated; differences in electron microscopical morphology have already been shown by Rode (1968): the alanine type spores have a veined surface with a superficial beaded ultrastructure pattern, the glucose type spores have the well-known prominent equatorial ridge and polar knob appearance.

## METHODS

*Bacillus megaterium* ATCC 19213 (designated AL strain) and QM B1551 (designated GN strain) were used. Spores were produced on an agar medium according to the methods described by Rode & Foster (1960). *n*-Butane (98 mole % purity) was obtained from Tokyo Kasei Co.

## RESULTS AND DISCUSSION

*n*-Butane was bubbled continuously into 5 ml. water suspensions of the two types of spores (O.D. = 0·5, about $10^8$/ml.) in a 20 ml. beaker, for the time tested, through a tapering glass tube at 2 to 3 bubbles/sec., and at every min. the pH value of the suspensions was determined by a Hitachi model M5 pH-meter. The test was at room temperature, and no special precautions were taken to prevent loss of gas from the suspensions during measurement. Fig. 1*a* shows that both spore suspensions usually started at about pH 6·2. The pH increased rapidly to a maximum value; the rate of increase depended on the speed of gassing. After that, the pH values did not vary, unless the gas bubbling stopped, when they rose slightly. Bubbling *n*-butane into a water control caused no changes in pH. The pH value of the AL spore suspension was more alkaline than that of GN spores. Flushing with air for a few seconds to remove the gas resulted in prompt falls in the pH values of both spore suspensions to the initial levels. A second bubbling of *n*-butane again caused a rise of the pH values of both spore suspensions, showing that the change in pH values of the suspensions caused by *n*-butane was reversible. Some kind of change, therefore, in superficial charge located on some special parts of the spores might have been induced by *n*-butane through a structural change of surrounding water. The spore coats which enclose the central

core of dormant spores consist primarily of macromolecules such as proteins or polysaccharides. The changes of physiochemical characteristics of macromolecules by non-polar compounds have recently received the attention of many investigators. Wetlaufer & Lovrien (1964) found that action of hydrocarbons in an aqueous solution resulted in changes in viscosity, pH value, optical rotation and ultraviolet absorption spectra of proteins such as bovine serum albumin or $\beta$-lactoglobulin.

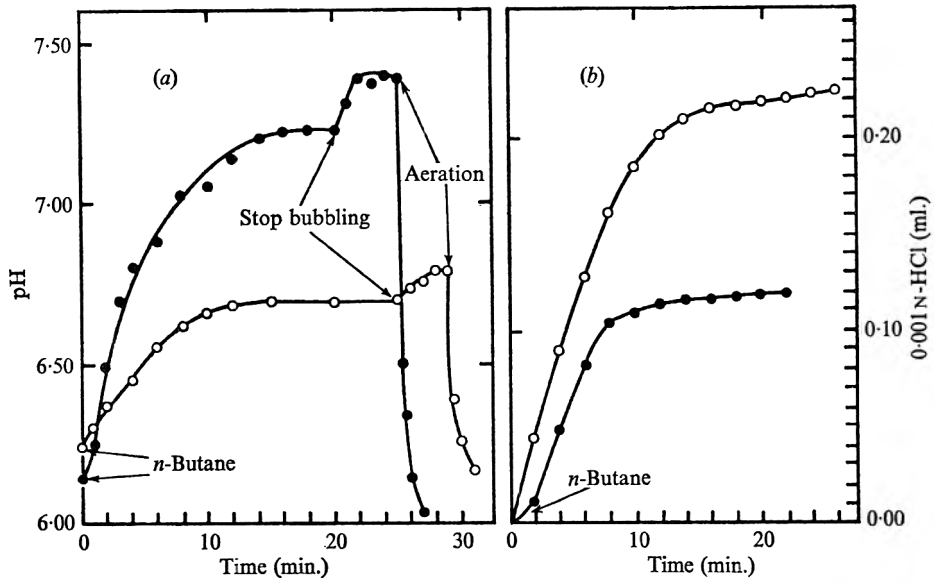


Fig. 1. Change in the pH values (*a*) and the amounts of titratable HCl (*b*) of spore suspensions induced by continuous bubbling of *n*-butane. Spores of *Bacillus megaterium* ATCC 19213 (—●—) and of QM B 1551 (—○—) were suspended in water at a concentration of O.D. = 0·5 (Hitachi electric colorimeter, model EPO-B, red filter).

The change in the concentration of intrinsic hydrogen ions of *n*-butane-treated spore suspensions were examined by means of a Radiometer model TTT 1c pH-stat. Five ml. of spore suspension or control spore-free water were introduced into the pH-stat cell covered with a fitting Teflon disc, which was bored to receive the electrodes, magnetic stirring bar, and the tube for flushing gas and air. While being bubbled with *n*-butane at room temperature, the sample solutions were titrated automatically with 0·001 N-HCl ($f$ = 1·004) to bring the pH values back to the initial pH 6·2. As shown in Fig. 1*b*, the amounts of HCl solution needed for the back titration increased with time and finally reached constant values for the respective spore suspensions. However, while the $\Delta$pH was smaller, the $\Delta H^+$ (about 0·22 ml.) was larger in the case of GN spores, and the reverse was the case with AL spores ($\Delta H^+$, about 0·12 ml.), which suggests that the buffer action of the former was stronger than that of the latter, and suggests further that there existed more ionizable residues on the surface of the former.

The next feature examined was whether such a buffer action (presence of ionizable groups on the surface) of spores was of a substantive nature or one induced by *n*-butane. After the pH values of the suspensions were adjusted to about 3 with a few drops of dilute HCl, a pH-titration curve of 10 ml. spore suspensions (O.D. = 0·9) was constructed

by means of the pH-stat using 0·05N-NaOH ($f = 0·997$). A large quantity of alkali was consumed, due to negative charges such as $COO^-$, between pH 3 and 4·5 in both cases. Both titration values increased gradually in parallel with the pH values. The titration values of GN spores, however, were always higher than those of AL spores; for example, at pH 7, GN spores = 0·035 ml., AL spores = 0·010 ml., when the initial value of both suspensions was pH 4·5. No noticeable inflexion point in the curve showing the pKa value was observed in either case (i.e. if there were an inflexion point, less than $1·8 \times 10^{-4}$N-NaOH would have been needed to detect it). These results suggest that most parts of the surface of both types of spores are occupied by non-polar regions. When *n*-butane is introduced into the spore suspensions, microconfigurations of spores are easily altered, possibly through the structural changes of water covering the surfaces; consequently the pK values of Brönstedt's acid are increased. These reversible changes of $H^+$ equilibrium might have been induced by configurational changes unfolding, stabilization or other changes in macromolecular fractions probably existing in some special sites of the spore surfaces. GN Spores have more ionizable groups as compared with AL spores and the buffer action against the change of $H^+$ equilibrium (ex. $RCOO^- + H^+ \rightarrow RCOOH$, $RNH_2 + H^+ \rightarrow RNH_3^+$) caused by an interaction with *n*-butane is stronger, resulting in the smaller amount of change of net $H^+$.

The evidence presented here agrees with the view of Rode (1968) that GN spores may have a greater number of available binding sites for $H^+$ than AL spores. Furthermore, the differences in the charges of two types of spores seem to have some connexion with the fact that at germination each responds quite differently to *n*-butane.

The authors are indebted to Dr K. Hayashi of Kyushu University for his helpful advice on this work.

REFERENCES

RODE, L. J. & FOSTER, J. W. (1960). Mechanical germination of bacterial spores. *Proceedings of the National Academy of Sciences of the United States of America* **46**, 118–128.

RODE, L. J. & FOSTER, J. W. (1965). Gaseous hydrocarbons and the germination of bacterial spores. *Proceedings of the National Academy of Sciences of the United States of America* **53**, 31–38.

RODE, L. J. (1968). Correlation between spore structure and spore properties in *Bacillus megaterium*. *Journal of Bacteriology* **95**, 1979–1986.

WETLAUFER, D. B. & LOVRIEN, R. (1964). Induction of reversible structural changes in proteins by nonpolar substances. *Journal of Biological Chemistry* **239**, 596–603.

*Journal of General Microbiology* (1970), **63**, 379–382
*Printed in Great Britain*



# Taxonomically Significant Group Antigens in Rhizobium

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(*Accepted for publication 24 September 1970*)

Earlier studies of the antigenic determinants of Rhizobium were almost entirely concerned with agglutinogens and revealed considerable strain specificity, the recognition of which was enhanced by a distinction between flagellar and somatic antigens (Bushnell & Sarles, 1939; Vincent, 1941, 1942; Kleczkowski & Thornton, 1944; Purchase, Vincent & Ward, 1951; Means, Johnson & Date, 1964). In some cases this specificity depended on complex patterns of shared antigens, in others on complete non-cross reactivity between strains that belonged to the same species. Agglutination could not therefore be depended on for the recognition of species or groups of species of rhizobia. On the other hand, the technique has proved valuable for the definition of serotypes and the labelling of strains used for experimental purposes.

Some attention has recently been given to the demonstration of soluble antigens by the gel-diffusion technique, using either the bacteria or isolated fractions as source of antigen (Dudman, 1964; Humphrey & Vincent, 1965; 1969*a*, *b*; Scheffler & Louw, 1967; Failly & Blachère, 1968; Škrdtela, 1969). This technique can be used for the recognition of serotypes within a species, but this may be complicated if additional antigens are released by mechanical breakage of the bacteria, or, in a less-controlled fashion, as a result of leakage from bacteria in calcium-deficient cultures (Humphrey & Vincent, 1965), or in old cultures, or from bacteria that may have been damaged by lyophilization or freezing and thawing.

The occurrence of what appeared to be internal antigens suggested the possibility that they might reveal a group reactivity not seen in the more specific antigens associated with the bacterial surface. Evidence for this was referred to briefly in our earlier report (Humphrey & Vincent, 1965) and is supported by the more comprehensive tests reported in the present paper.

Antisera were developed in rabbits against two strains of *Rhizobium trifolii* (SU 329 (TA 1) and SU 297/31) and a strain of *R. meliloti* (U 45) grown in a defined liquid medium (Vincent, 1962). The suspensions were injected at first intramuscularly with Freund's complete adjuvant and later intravenously without adjuvant, following the detailed procedure already described (Humphrey & Vincent, 1965). Such antisera had an agglutination titre of 6400 and when diffused against washed homologous bacteria, broken in a Mickle disintegrator, revealed several groups of lines which have been labelled *a*, *b* and *c* in order from the antigen well towards the antiserum well. The *a* lines of *R. trifolii* have been identified as being due to specific agglutinating antigen (Humphrey & Vincent, 1969*b*), the remainder, not found when young intact rhizobia are used in the antigen well, can be attributed to 'internal' antigens. *R. meliloti* seems to provide a similar array of specific agglutinating and non-specific internal antigens.

Antisera differ in the clarity and further splitting of the *b* and *c* lines. An antiserum

to a 21 day culture of *Rhizobium trifolii* SU329 permitted clear recognition of two lines within *b* and *c* ($b_1$, $b_2$, $c_1$, $c_2$) whereas other antisera prepared against 3 day cultures of the same strain or *R. trifolii* SU297/31 did not permit this degree of further resolution although the *b* and *c* lines were quite clear. Antiserum to a 3 day culture of *R. meliloti* U45 showed besides strain specific *a* positioned lines, an array of seven lines in the *b* and *c* positions. The development of antibodies to internal antigens was not dependent on breakage of the bacteria before injection provided the whole bacteria were mixed with adjuvant and the intramuscular route was used. Differences between individual rabbits seemed to be more important in determining the degree of resolution within the major lines. A series of intravenous injections alone, whilst producing a high agglutinin titre, did not appear to be as satisfactory for the production of antibody to internal antigens.

Twenty-nine strains of fast-growing rhizobia (*Rhizobium trifolii*, ten; *R. leguminosarum*, seven; *R. phaseoli*, one; *R. meliloti*, nine, and one each of a fast *Lotus* and *Leucaena* culture), nine slow growers (*R. lupini*, three; *R. japonicum*, one; cowpea rhizobia, four, and a slow Lotus rhizobium) and four Agrobacteria (*Agrobacterium radiobacter*, two; *A. tumefaciens*, two), were used in the present investigation. A small collection of other non-rhizobia was also included (representative Escherichia, Aerobacter, Salmonella, Pseudomonas, Flavobacterium and Bacillus). Rhizobia and the agrobacteria used for the demonstration of internal antigens were grown as shaken cultures in yeast extract liquid medium (Fred, Baldwin & McCoy, 1932) at 26° for 3 days (fast growers) or 8 to 10 days (slow growers). Identical results were obtained when the organisms were cultured in the defined medium, or with the yeast extract contained within the dialysis sac. Other bacteria were harvested from the usual meat infusion agar.

In tests of this kind, the amount of bacterial suspension used as testing antigen is important for the detection and resolution of lines. Too high a concentration can be as misleading as too low a concentration. For example, the *c* lines appeared as a diffuse band at 20 mg./ml. (dry wt) but at 5 mg./ml. were resolved into two clear lines with some antisera or one clear line with others. No single concentration of antigen suspension gave clear resolution of all lines. Comparisons were made, using 0·1 ml./well, of two concentrations (5 and 20 mg./ml.) with patterns that permitted direct comparisons with the homologous reaction.

All except one (an ineffective u.v.-induced mutant of *Rhizobium meliloti*) of the fast-growing rhizobia and the agrobacteria revealed clear internal antigen lines with *R. trifolii* antisera. The patterns revealed using the more highly resolving antiserum to strain SU329 are shown in Table 1. Similar tests with antiserum to *R. meliloti* U45 confirmed and complemented the evidence obtained with *R. trifolii* antisera. All of the fast growers gave clear internal lines. Lines shared with the homologous condition were greatest among the *R. meliloti*, less with three of the four agrobacteria and the mutant *R. meliloti*, Sa 10M, and least with *R. trifolii*, *R. leguminosarum*, *R. phaseoli*, the faster lotus strain and the other agrobacterium. All the slow-growing rhizobia were again negative.

The results are interesting both because they provide a means for quick recognition of fast-growing rhizobia and because of the support they offer other taxonomic evidence showing close relationship between *Rhizobium trifolii*, *R. leguminosarum* and *R. phaseoli*, some, but less close, relationship between these and *R. meliloti*, an evident

relationship between the latter and agrobacteria, and the distinctiveness of the slow-growing rhizobia (Graham, 1964; Heberlein, de Ley & Tijtgat, 1967; t'Mannetje, 1967; Kern, 1968; Moffett & Colwell, 1968; Wu, Gregory & Hauser, 1968; Yao & Vincent, 1969). Common antigens have also been reported amongst strains of *R. japonicum* not sharing an agglutinogen or the specific gel-diffusion line that would occupy the *a* position (Škrdtela, 1969). The same phenomenon appears to be involved.

On the other hand, our results contain a warning as to the confusion that could arise in strain identification by gel-diffusion technique if the specificity of the surface antigens were to be confused by the undetected release of internal 'group' antigens in old or mishandled cultures.

Table 1. *Patterns of internal antigens observed with Rhizobium trifolii (SU329) antiserum*

The fast-growing strains are divided into two groups (*a*, *b*) using established characteristics of these species and the internal antigen lines found in the present work.

| | Line identification | | | |
|---|---|---|---|---|
| Fast-growing strains | $b_1$ | $b_2$ | $c_1$ | $c_2$ |
| (*a*) *R. trifolii* (8)* | | | | |
| *R. leguminosarum* (6) | + | + | + | + |
| *R. phaseoli* (1) | | | | |
| *R. trifolii* (2) | + | + | − | + |
| *R. leguminosarum* (1) | − | + | + | + |
| (*b*) *R. meliloti* (6) | + | − | + | − |
| *A. tumefaciens* (1) | | | | |
| *A. radiobacter* (1) | + | − | − | + |
| Leucaena rhizobium (1) | | | | |
| *A. tumefaciens* (1) | − | + | − | − |
| *A. radiobacter* (1) | | | | |
| *R. meliloti* (2) | + | − | − | − |
| Lotus rhizobium (SU 343)† | | | | |
| *R. meliloti* (1)‡ | − | − | − | − |
| Slow-growing strains | | | | |
| *R. lupini* (3) | | | | |
| *R. japonicum* (1) | − | − | − | − |
| Cowpea rhizobia (4) | | | | |
| Lotus rhizobium (1)§ | | | | |
| Other bacteria: non-rhizobia | − | − | − | − |

* Number of strains showing pattern given in parentheses.

† Fast-growing lotus strain.

‡ Ultraviolet-induced ineffective mutant (Sa 10 M) of Sa 10 (Failly & Blachère, 1968). The parent strain gave $b_1$ and $c_1$ lines with antiserum to *R. trifolii* SU 329.

§ Slow-growing lotus strain.

Finally, it should be noted that the results we have reported were obtained with preparations from washed deposited bacteria. We see no reason for believing that the group antigenic reactions we have reported depend on extracellular gum, which, in our experience with *Rhizobium trifolii*, appears to be inactive at concentrations far in excess of what would occur in the suspensions of washed bacteria we have used.

This work has been supported by the Australian Research Grants Committee, the Wheat Industry Research Council, and the Rural Credits Division of the Reserve Bank of Australia. The skilled assistance of Mrs B. Netting and Mrs S. Wake is gratefully acknowledged.

REFERENCES

BUSHNELL, U. A. & SARLES, W. B. (1939). Investigations upon the antigenic relationships of the root nodule bacteria of the soybean, cowpea and lupine cross inoculation groups. *Journal of Bacteriology* **38**, 401–410.

DUDMAN, W. F. (1964). Immune diffusion analysis of the extracellular soluble antigens of two strains of *Rhizobium meliloti*. *Journal of Bacteriology* **88**, 782–793.

FAILLY, C. & BLACHÈRE, H. (1968). Identité immunochimique des certains polysaccharides extracellulaires chez diverses espèces de Rhizobium. *Canadian Journal of Microbiology* **14**, 247–251.

FRED, E. B., BALDWIN, I. L. & MCCOY, E. (1932). *Root-nodule Bacteria and Leguminous Plants*. University of Wisconsin Studies in Science no. 5, Madison.

GRAHAM, P. H. (1964). The application of computer techniques to the taxonomy of the root-nodule bacteria of legumes. *Journal of General Microbiology* **35**, 511–517.

HEBERLEIN, G. T., DE LEY, J. & TIJTGAT, R. (1967). Deoxyribonucleic homology and taxonomy of Agrobacterium, Rhizobium and Chromobacterium. *Journal of Bacteriology* **94**, 116–124.

HUMPHREY, B. A. & VINCENT, J. M. (1965). The effect of calcium nutrition on the production of diffusible antigens of *Rhizobium trifolii*. *Journal of General Microbiology* **41**, 109–118.

HUMPHREY, R. A. & VINCENT, J. M. (1969*a*). Correlation between surface charge density of whole cells and electrophoretic movement of isolated somatic antigen of *Rhizobium trifolii*. *Journal of Bacteriology* **98**, 845–846.

HUMPHREY, B. A. & VINCENT, J. M. (1969*b*). The somatic antigens of two strains of *Rhizobium trifolii*. *Journal of General Microbiology* **59**, 411–425.

KERN, H. (1968). Beitrage zur Taxonomie der Rhizobiaceae. *Archiv für Mikrobiologie* **63**, 278–291.

KLECZKOWSKI, A. & THORNTON, H. G. (1944). A serological study of root-nodule bacteria from pea and clover inoculation groups. *Journal of Bacteriology* **48**, 661–672.

T'MANNETJE, L. (1967). A re-examination of the taxonomy of the genus *Rhizobium* and related genera using a numerical analysis. *Antonie van Leeuwenhoek* **33**, 477–491.

MEANS, U. M., JOHNSON, H. W. & DATE, R. A. (1964). Quick serological method of classifying strains of *Rhizobium japonicum* in nodules. *Journal of Bacteriology* **87**, 547–553.

MOFFETT, M. L. & COLWELL, R. R. (1968). Adansonian analysis of the Rhizobiaceae. *Journal of General Microbiology* **51**, 245–266.

PURCHASE, H. F., VINCENT, J. M. & WARD, L. M. (1951). Serological studies of the root-nodule bacteria. IV. Further analysis of isolates from Trifolium and Medicago. *Proceedings of the Linnean Society of New South Wales* **76**, 1–6.

SCHEFFLER, J. G. & LOUW, H. A. (1967). The serological characteristics of the clover rhizobia in the soils of the Stellenbosch district. *South African Journal of Agricultural Science* **10**, 161–174.

ŠKRDTELA, V. (1969). Serological analysis of eleven strains of *Rhizobium japonicum*. *Antonie van Leeuwenhoek* **35**, 77–83.

VINCENT, J. M. (1941). Serological studies of the root-nodule bacteria. I. Strains of *Rhizobium meliloti*. *Proceedings of the Linnean Society of New South Wales* **66**, 145–154.

VINCENT, J. M. (1942). Serological studies of the root-nodule bacteria. II. Strains of *Rhizobium trifolii*. *Proceedings of the Linnean Society of New South Wales* **67**, 82–86.

VINCENT, J. M. (1962). Influence of calcium and magnesium on the growth of Rhizobium. *Journal of General Microbiology* **28**, 653–663.

WU, L., GREGORY, K. F. & HAUSER, M. M. (1968). DNA homologies in Rhizobium species. *Bacteriological Proceedings* **68**, 19–20.

YAO, P. Y. & VINCENT, J. M. (1969). Host specificity in the root hair 'curling factor' of *Rhizobium* spp. *Australian Journal of Biological Sciences* **22**, 413–423.

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# THE SOCIETY FOR GENERAL MICROBIOLOGY

*The Society for General Microbiology held its fifty-ninth General Meeting at Oxford on Thursday and Friday, 24 and 25 September 1970. The following communications were made.*

## SYMPOSIUM: FUNCTION AND METABOLISM OF NUCLEIC ACIDS

**Effects of Purine Nucleotide Concentrations on RNA Synthesis in *Escherichia coli*.** By K. BURTON (*Department of Biochemistry, The University, Newcastle upon Tyne, NE1 7RU*)

On transfer to purine-free medium, purine auxotrophs cease net synthesis of nucleic acids before there are appreciable changes in the amounts of intracellular ATP (Gallant, J. & Harada, B. (1969), *Journal of Biological Chemistry* **244**, 3125; Thomas, G. A., Varney, N. F. & Burton, K., submitted to *Biochemical Journal*). Although a sensitive allosteric control of ribonucleotide reductase could conceivably stop DNA synthesis, it is not immediately clear why RNA polymerase, with its low $K_m$ (*c.* 15 $\mu$M), does not exhaust the ATP pool.

*PurB*$^-$ (i.e., adenylosuccinase-less) strains convert hypoxanthine, xanthine or guanosine to GTP but not to ATP and then rapidly synthesize RNA at the expense of intracellular ATP, ADP and AMP until ATP falls to approximately one-third of its original level; NAD is not depleted (Jeffries, G. A. & Burton, K. (1969), *Biochemical Journal* **114**, 42P). In fact, RNA synthesis appears to be more dependent upon changes in the amount of GTP than upon the level of ATP and in any case there is considerably less GTP. This helps to explain the behaviour in purine-free media since there is a more rapid fall of GTP than of ATP and since adenine nucleotides are converted to guanine nucleotides only when adenine nucleotides are in excess (Magasanik, B. & Karibian, D. (1960), *Journal of Biological Chemistry* **235**, 450). Because bacterial membranes bind ATP and GTP (Weissbach, H., Redfield, B. & Kaback, H. R. (1969), *Archives of Biochemistry and Biophysics* **135**, 66), the small changes in total GTP may reflect much larger changes in the proportion of free GTP available to RNA polymerase.

*E. coli* RNA is known to be initiated with pppA– or pppG– at the 5′-ends; pyrimidine starts have not been identified (Jorgensen, S. E., Buch, L. B. & Nierlich, D. P. (1969), *Science, New York* **164**, 1067). ATP and GTP have tenfold lower affinities for the initiation site of RNA polymerase than for the polymerization site (Anthony, D. D., Wu, C. W. & Goldthwait, D. A. (1969), *Biochemistry, Easton* **8**, 246; Wu, C. W. & Goldthwait, D. A. (1969), *Biochemistry, Easton* **8**, 4450, 4458) suggesting that purine deficiency would act by stopping initiation more than chain growth. However, this need not be so if the allosteric controls of nucleotide interconversion ensure that only one of ATP or GTP is limiting RNA synthesis. For example, if GTP were limiting we could expect chains beginning with pppA– to be initiated but to grow slowly because of the lack of GTP. In fact, experiments with rifampicin support the latter argument in showing that chain growth is inhibited at least as much as chain initiation is inhibited.

DNA/RNA hybridization experiments indicate that purine starvation increases the ratio of RNA polymerase molecules bound to the cistrons for mRNA to the number bound to rRNA and tRNA cistrons. While a specific mechanism could conceivably suppress the synthesis of stable RNA, non-specific reduction of chain growth with a smaller effect upon chain starts will automatically increase the ratio of mRNA synthesis to stable RNA synthesis

because the polymerase molecules are so closely packed upon the rRNA and tRNA cistrons (Mueller, K. & Bremer, J. (1968), *Journal of Molecular Biology* **38**, 329). On adding either adenine or hypoxanthine to a purine-starved *purB*$^-$ strain, the synthesis of stable RNA is restored to normal within 4 min. at 25°.

Amino acid starvation of RC$^{str}$ bacteria has a similar effect in selectively inhibiting the synthesis of stable RNA (Midgley, J. E. M. & Gray, W. J. H., in preparation); for earlier work see Eldin, G., Stent, G. S., Baker, R. F. & Yanofsky, C. ((1968), *Journal of Molecular Biology* **37**, 25). Gallant, J. & Harada, B. ((1969), *Journal of Biological Chemistry* **244**, 3125) have suggested that RNA synthesis is inhibited because the amino acid starvation causes a deficiency of ATP and especially of GTP. However, there are important differences between amino acid starvation and purine starvation indicating different mechanisms for the control of RNA synthesis. In particular, amino acid starvation acts primarily upon the initiation of stable RNA and, when amino acids are restored, the synthesis of stable RNA is regained much more slowly than it is when purines are restored after purine starvation.

**Suppressor Transfer RNAs and Their Use in the Study of tRNA Structure and Function.** By J. D. SMITH (*Medical Research Council, Laboratory of Molecular Biology, Cambridge*)

Mutations resulting in chain terminating or missense codons within a messenger RNA can be corrected during translation in strains of *Escherichia coli* carrying certain suppressor genes. Some suppressor strains arise by mutations which alter the coding properties of specific transfer RNAs. The altered transfer RNA can translate the chain terminating or missense codon as an acceptable amino acid.

In one instance sequence analysis has shown the suppressor mutation to result in a base-change in the anticodon of a tRNA, so changing its codon recognition. This is the $SU_{III}$ amber suppressor which allows the translation of UAG as tyrosine. It is a mutation in one of the tyrosine tRNA genes of *E. coli* changing the anticodon from G$^+$UA (G$^+$ is a modified guanylic acid residue) to CUA (Goodman, H. M., Abelson, J., Landy, A., Brenner, S. & Smith, J. D. (1968), *Nature, London* **217**, 1019; Goodman, H. M., Abelson, J. N., Landy, A. Zadrazil, S. & Smith, J. D. (1970), *European Journal of Biochemistry* **13**, 461). This type of suppressor mutation requires the existence of more than one gene specifying tRNAs with similar coding properties. The evidence for the origin of other suppressors in *E. coli* will be reviewed.

Mutants of the $SU_{III}$ tRNA gene have been used in a study of the relation between nucleotide tRNA structure and function (Abelson, J. N., Gefter, M. L., Barnett, L., Landy, A., Russell, R. L. & Smith, J. D. (1970), *Journal of Molecular Biology* **47**, 15). The approach is to isolate mutants of $SU_{III}$ which have partially or completely defective suppressor activity. Many of these specify tRNAs with single base substitutions and the properties of some of these will be described. From many of these mutants second site revertants specifying second base changes in the tRNA have been obtained. The tRNAs specified by the revertants give information on the structural and functional interaction of different bases in the tRNA sequence.

**The Structure of the Ribosome.** By I. O. WALKER (*Department of Biochemistry, University of Oxford*)

The bacterial ribosome may be reversibly dissociated into two subunits unequal in size and molecular weight. Each subunit is a complex of one molecule of ribonucleic acid and several protein molecules. In addition each ribosome has spermidine and about 2500 magnesium ions bound to it. The structural role of the great majority of the magnesium ions appears to be non-specific and they act simply to neutralize the phosphate groups on the RNA. Removal of these ions causes the ribosome or its subunits to unfold without dissociation of the proteins from the RNA. A comparison with RNA of the spectral and hydrodynamic properties of native ribosomes and subunits unfolded in EDTA or urea reveals the importance of protein interactions in maintaining the quaternary structure of the ribosome.

The chemical and physical structure of ribosomal proteins and ribosomal RNA and the nature of the protein-RNA interactions will be discussed.

The enzymic degradation of native and unfolded subunits with an endonuclease, specific for single-stranded nucleic acid, yields information about the folding of RNA within the native subunit.

Finally, the nature of the interaction between the ribosomal subunits and between the ribosome and transfer RNA will be discussed.

**The Synthesis of Ribosomes in Bacteria.** By G. TURNOCK (*Department of Biochemistry, University of Leicester*)

Analysis of the mechanism of synthesis of ribosomes in bacteria has been approached in four principal ways: (i) by kinetic analysis of the incorporation of specific radioactive precursors, (ii) by inference with the normally close interrelationship between the synthesis of RNA and protein to give cells with elevated concentrations of ribosomal precursors, (iii) by isolation of mutants with defects in the assembly process, and (iv) by study of the reassembly of ribosomes *in vitro* from their constituent RNA and proteins.

Kinetic studies of the incorporation of radioactive nucleic acid precursors into ribosomes in *E. coli* during exponential growth (McCarthy, B. J., Britten, R. J. & Roberts, R. B. (1962), *Biophysical Journal* **2**, 57; Mangiarotti, G., Apirion, D., Schlessinger, D. & Silengo, L. (1968), *Biochemistry* **7**, 456) have provided evidence for the following pathways of synthesis for the 30 *s* and 50 *s* ribosomal subunits.

(*a*) Growing RNA $\longrightarrow$ 26 *s* $\longrightarrow$ 30 *s*
0·17% 3·5% 29·5%

(*b*) Growing RNA $\longrightarrow$ 32 *s* $\longrightarrow$ 43 *s* $\longrightarrow$ 50 *s*
0·33% 0·4% 7·0% 59·0%

The figure under each class of particle refers to the amount of RNA it contains as a percentage of the total ribosomal RNA of the cell. The first detectable precursors, with sedimentation coefficients of about 26 *s* and 32 *s*, contain 16 *s* RNA and 23 *s* RNA respectively. The presence of discrete holdup points in the assembly process may reflect the lack of a particular protein or a requirement for a relatively rare conformational change in a precursor before it can participate in the next reaction.

Treatment of *E. coli* with low concentrations of chloramphenicol, insufficient to give complete inhibition of protein synthesis, leads to accumulation of particles believed to be identical with the 32 *s* and 43 *s* precursors to the 50 *s* subunit (Osawa, S., Otaka, E., Itoh, T. & Fukui, T. (1969), *Journal of Molecular Biology* **40**, 321). Both particles contain 23 *s* RNA that is undermethylated with respect to that found in a 50 *s* subunit whilst 5 *s* RNA is not found in either of them, suggesting that it is incorporated in the last stage in the pathway. The 32 *s* particle was found to possess three and the 43 *s* particle nine of the 19 proteins detectable in the 50 *s* ribosomal subunit.

Relatively few mutants with defects in the assembly of ribosomes have so far been obtained. One with a high concentration of particles similar to the immediate precursor to the 50 *s* subunit has been described (MacDonald, R. E., Turnock, G. & Forchhammer, J. (1967), *Proceedings of the National Academy of Sciences, U.S.A.* **57**, 141). The particles are not the same as the 43 *s* particles because they contain 5 *s* RNA and appear to have a full complement of proteins, suggesting that the mutation has created a new holdup point in the pathway of synthesis (Mehdi, Q. & Wild, D. G., unpublished results). Recently it has been found that a significant number of cold-sensitive mutants of *E. coli* appear to have conditional blocks in ribosome synthesis at a variety of points (Guthrie, C., Nashimoto, H. & Nomura, M. (1969), *Proceedings of the National Academy of Sciences, U.S.A.* **63**, 384). These strains should help to increase our knowledge of ribosome genetics and of the properties of the precursors.

An important contribution to our understanding of ribosome synthesis has come from the demonstration that 30 *s* subunits may be reformed *in vitro* from 16 *s* RNA and the purified proteins (Traub, P. & Nomura, M. (1969), *Journal of Molecular Biology* **40**, 391).

The reassociation does not depend on addition of the proteins to the reaction mixture in a particular order and requires only a reasonable ionic strength and a temperature of 40°. The latter seems to be essential for a conformational change involving the RNA and a group of proteins which will associate with the RNA at 0°.

**The Fate of Nucleic Acids During Mixed Infections with Bacteriophages.** By MARY R. LUNT (*Microbiology Unit, Department of Biochemistry, University of Oxford*)

When bacteria infected by a T-even phage strain are superinfected with further T-even phage, genetic markers of the secondary phage appear infrequently among the progeny virus particles. This phenomenon is known as temporal exclusion (Dulbecco, R. (1952), *Journal of Bacteriology*, **63**, 209). When the secondary phage are labelled with $^{32}P$ their DNA is largely converted to acid-soluble fragments (Graham, A. F. (1953), *Annales de l'Institut Pasteur, Paris* **84**, 90). This superinfection breakdown only occurs when the host bacteria contain normal levels of the enzyme endonuclease-I. Secondary infection of host strains deficient in endonuclease-I is not accompanied by solubilization of the superinfecting phages' DNA, although temporal exclusion occurs normally (Fielding, P. E. & Lunt, M. R. (1970), *Journal of General Virology* **6**, 333). It is suggested that in these instances temporal exclusion is primarily caused by virus-induced changes at the cell surface which prevent complete injection of superinfecting phage DNA, the solubilization of which is a secondary process largely caused by the host endonuclease-I.

Bacteria infected with T 5 phage exclude secondarily infecting T 5 but without breakdown of the superinfecting DNA, even in bacterial hosts which have normal levels of endonuclease-I. It is possible that impaired release of the secondary T 5 phages' DNA may be the cause of its exclusion and protection from host or other nucleases.

When bacteria deficient in endonuclease-I are infected with either T 4 or T 5 phages and then superinfected with $^{32}P$-labelled $\lambda$ phage, a fraction (10 to 25 %) of the $\lambda$ phage DNA is converted to acid-soluble fragments. Extensive (40 to 50 %) breakdown of superinfecting T 2 or T 4 phage DNA is brought about by endonuclease-I-deficient bacteria which have been infected with T 5 phage. It seems likely that this breakdown may be initiated by a T 5-induced enzyme, for example the deoxyribonuclease which can convert viral DNA molecules to high molecular weight fragments, and which is detectable early in T 5 infection when host DNA breakdown begins (Fielding, P. E. & Lunt, M. R. (1969), *Federation of European Biochemical Societies Letters* **5**, 214).

During simultaneous mixed infections with T 2 and T 4 phages, genetic markers of T 2 are excluded from the progeny, some regions of the T 2 genome being excluded more strongly than others (Russell, R. L. (1966), *Thesis, California Institute of Technology*; de Groot, B. (1967), *Genetics Research* **10**, 13). Recent experiments show that in such infections there is a correlation between the extent of genetic exclusion and the amounts of certain enzymes induced by the excluded T 2 genome. In endonuclease-I-deficient hosts, mixed infections using a $^{32}P$-labelled input strain show that while some breakdown (10 to 14 %) of the T 2 DNA occurs, no breakdown of T 4 DNA is observed (Mahmood, N. & Lunt, M. R., unpublished observations). These results suggest that the partial exclusion of T 2 by T 4 could result from T 4 induced nuclease attack on selected regions of the T 2 genome, thus reducing their availability for genetic transcription and for transmission to progeny phage.

It is suggested that the processes by which a particular phage genetic system dominates another may partly depend on specific recognition of the DNA molecules involved, and which results in nuclease attack directed against one of the viral genomes. Similar processes may also lead to degradation of host cell DNA during infections by virulent phages such as T 4 or T 5. The phenomena resemble some of those associated with incompatibility between different bacterial plasmids, and those concerned in DNA modification and restriction typical of different bacterial strains (Arber, W. (1969), *Annual Review of Biochemistry*, **38**, 467).

**Studies on the Structure of the Genome of Phage Qβ.** By M. A. BILLETER, J. E. DAHLBERG H. M. GOODMAN, J. HINDLEY* and C. WEISSMANN (*Universities of Zürich, Geneva and Bristol*)

The genome of phage Qβ consists of a single RNA strand comprising about 3500 nucleotides. Its three or four cistrons code for the viral coat protein the viral RNA polymerase and for one, or possibly both, of the minor proteins found in the viral particle.

The nucleotide sequence of Qβ RNA is being systematically determined by the following approach. Qβ specific RNA polymerase is used to synthesize segments of viral RNA in synchronized short-time reactions, using α-$^{32}$P-labelled nucleoside triphosphates of high specific activity. By an appropriate choice of incubation conditions it is possible to obtain segments of essentially any desired average length; radioactivity may be introduced at various times during synthesis using either one or any combination of several labelled nucleoside triphosphates. Sequence analysis is performed using the methodology by Sanger and his colleagues and is greatly facilitated by the use of nearest neighbour data and information obtained by sequential labelling. The synthetic reaction directed by minus strands gives rise to 5′ terminal sections of the plus strand; that directed by plus strands yields 5′ terminal segments of the minus strand.

The nucleotide sequence of the first 175 nucleotides of the 5′ terminal region of the plus strand has been established; a further adjoining section of about 150 nucleotides is now being elucidated. The analysis of a stretch of about 16 nucleotides comprising the 5′ terminus of the minus strand is also being completed and will allow the deduction of the complementary 3′ terminal sequence of the plus strand. The main conclusions to be drawn from the sequence established so far are the following: (*a*) the 5′ terminal region begins with a very stable hydrogen-bonded loop; (*b*) no initiation triplet occurs before position 62; (*c*) the sequence required for the initiation of synthesis of the coat protein (PuUGGCN) is not found within the first 300 nucleotides from the 5′ end; (*d*) at least 62 nucleotides at the 5′ and 25 nucleotides at the 3′ terminus are in all probability not used to code for protein and may be reserved for interactions with the viral polymerase (or possibly with other components, such as the viral structural proteins).

In order to localize the coat protein cistron, advantage was taken of the finding that *E. coli* ribosomes bound to intact phage RNA in the presence of formylmet-tRNA$_F$ specifically protect the initiation site of the coat protein cistron against RNase (Argetsinger-Steitz (1969), *Nature, London* **224**, 957; Hindley & Staples (1969), *Nature, London* **224**, 964). A collection of full-length Qβ plus strands was labelled from the 5′ end for various lengths along the molecule. Ribosomes were bound to these RNA preparations and the RNA segment corresponding to the binding site was isolated. It was found that only those molecules which had been labelled to about the 1100th nucleotide or beyond yielded a labelled RNA segment which by nucleotide sequence analysis was identified as the beginning of the coat protein cistron. It is concluded that the coat protein cistron is located in the middle third of the genome and is preceded by another cistron.

Supported by grants from the Schweizerische National fonds, Jane Coffin Childs Fund, British Research Council.

* Paper presented by J. Hindley.

**Nucleic Acids of Animal Viruses.** By J. H. SUBAK-SHARPE (*Institute of Virology, University of Glasgow*)

All animal viruses store their genetic information in nucleic acid but, while any particular type of virus particle always only contains one sort of nucleic acid, different viruses differ in the nucleic acid they contain. Thus characteristically some have double-stranded DNA, others single-stranded DNA, still others double-stranded RNA and a fourth class single-stranded RNA. On this criterion alone the animal viruses can be placed into four classes, but this is of rather limited usefulness. The double-stranded DNA class, for example,

embraces groups of viruses which vary enormously in size, complexity and morphology from the very small and relatively simple icosahedral papovaviruses to the very large and highly complex brick-shaped poxviruses.

The G+C content of the nucleic acid of viruses also varies characteristically between very wide limits as for example between vaccinia (35 %) and pseudorabies (74 %). Although G+C content gives additional information which helps to characterize a nucleic acid, this information suffers from the severe limitation that it gives no clue to the sequential arrangement of the different bases in the nucleic acid.

A considerably more informative characterization of nucleic acid can be attained by the use of doublet analysis. This not only gives the G+C content but also yields the frequency distribution of the 16 possible two-base sequences (doublets) in the nucleic acid. But in addition one can extract from this analysis information concerning the highly characteristic 'general design' (pattern of deviations from random expectation) of the nucleic acid and this is *not* dependent on the G+C content. A characterization of nucleic acid which is independent of the G+C content offers the advantage of a measurement which allows a valid comparison to be made with any other nucleic acid. Thus the general designs of the nucleic acids of different viruses can be compared with each other and with their hosts.

In Glasgow doublet analysis has been carried out on some 20 animal viruses and this had led to the highly intriguing recognition that the general designs fall into three groups: two major and one minor. The first group comprises all *small* viruses (N.A. mol.wt. $\leqslant 5 \times 10^6$) irrespective of whether their genetic information is stored in double-stranded DNA (4), single-stranded DNA (3) or single-stranded RNA (2); their general designs show considerable resemblance to the highly characteristic general design of vertebrate DNA (host cells). In the second group are all *large* double-stranded DNA viruses (mol.wt. $\geqslant 70 \times 10^6$) examined (4) —they have general designs which show no resemblance to the hosts DNA. Moreover, they show relatively little similarity with one another. The minor group comprises the intermediate sized (mol.wt. 19 to $23 \times 10^6$) adenoviruses (6) which show some discernible resemblance to the general design of host DNA and thus occupy a somewhat ambiguous position. All show very considerable similarities to one another in doublet pattern.

As the general design is the characteristic pattern of deviation from random expectation it represents the end-product of the nucleic acids total history of responses to selection pressures. It follows that closely corresponding general designs are likely to have been generated by response to very similar or identical selection pressures. In other words, such general designs suggest evolutionary relationships. The implications of these findings to the problem of the origin of viruses and viral evolution will be discussed.

**The Expression of Genetic Information by Somatic Cell Nuclei.** By H. HARRIS (*Sir William Dunn School of Pathology, University of Oxford*)

Evidence derived by the use of cell fusion will be presented in support of the view that the expression of genetic information by somatic cell nuclei requires the activity of a functional nucleolus. The experiments indicate that the RNA which carries the specifications for the synthesis of proteins and any structural RNA components in the ribosomal complex pass from the nucleus to the cytoplasm together.

## ORIGINAL PAPERS

**Rate of DNA Replication in Thymine-requiring Strains of *Escherichia coli*.** By ARIEH ZARITSKY (*Department of Genetics, University of Leicester*)

In thymineless mutants of *Escherichia coli* the DNA replication velocity can be varied over a 2·5 to 3·0-fold range simply by altering the concentration of thymine in the growth medium (Pritchard, R. H. & Zaritsky, A. (1970), *Nature, London* **226**, 126). The change in velocity does not significantly alter the growth rate, thus demonstrating that the two are not coupled.

The concentration of thymine yielding a given velocity is higher in *E. coli* K 12 *thy⁻tlr⁻* than in *E. coli* 15 T⁻.

The maximum velocity attainable by progressively increasing the concentration of thymine in the growth medium appears to be less than that in *thy⁺* strains (Cooper, S. & Helmstetter, C. E. (1968), *Journal of Molecular Biology* **31**, 519) by more than 20%. Addition of deoxyguanosine increases the replication velocity above that in the respective thymine concentration alone, hence decreasing the minimum concentration needed for normal growth.

Suggestions that bacteria contain a control system adjusting their DNA/mass ratio to a constant value for any given growth rate are made unlikely by our data, which demonstrate that this ratio varies with the external thymine concentration.

**The Thymidine Triphosphate Pool in Wild-type and Thymine-requiring Strains of *Escherichia coli* K 12 and 15.** By I. R. BEACHAM (*Department of Genetics, University of Leicester*)

The thymidine triphosphate (dTTP) pool has been measured in wild-type and low-thymine requiring (*thy⁻tlr⁻*) strains of *E. coli* K 12 and 15, using thin-layer chromatography on poly (ethyleneimine)-impregnated cellulose (Neuhard, Randerath & Randerath (1965), *Analytical Biochemistry* **13**, 211).

Using thymine as a precursor of thymidylate in *thy⁻tlr⁻* mutants, it is found that the size of the dTTP pool increases with increasing concentrations of external thymine. The maximum pool was less than that in *thy⁺* strains. The size of the pool in *E. coli* K 12 (*thy⁻tlr⁻*) was smaller than in 15 T⁻ (555-7) at all thymine concentrations. At low concentrations of thymine the addition of deoxyguanosine (GdR) raised the pool size above the value with thymine alone, as expected if GdR serves as a source of deoxyribose-1-phosphate (see Breitman & Bradford (1967), *Biochimica et Biophysica Acta* **138**, 217). In *E. coli* K 12 the maximum value with thymine alone was not increased by the addition of GdR.

When thymidine was used as a precursor of thymidylate in thymidine phosphorylase negative (*tpp⁻*) derivatives of the *thy⁻* strains, the pool sizes were also less than that in the *thy⁺* strains. In *E. coli* K 12 the pool size decreased with increasing concentrations of thymidine until at 20 $\mu$M thymidine a constant value was reached which was equivalent to the maximum value for thymine alone.

It is concluded that other factors, as well as a limitation of deoxyribose-1-phosphate, limit the size of the dTTP pool in *thy⁻* mutants of *E. coli*.

At concentrations of thymine normally used by workers using *thy⁻* mutants, the velocity of replication is much less than in *thy⁺* strains and the size of the dTTP pool is reasonably correlated with the velocity of replication (Pritchard & Zaritsky (1970), *Nature, London* **226**, 126).

**Induction of the Cytoplasmic Petite Mutation in *Saccharomyces cerevisiae* by Antibacterial Antibiotics.** By D. H. WILLIAMSON, N. G. MAROUDAS and D. WILKIE (*National Institute for Medical Research, Mill Hill, and Botany Department, University College, London*)

When applied to yeast, the antibacterial antibiotics erythromycin and chloramphenicol appear selectively to block the synthesis of proteins in mitochondria, cytoplasmic protein synthesis being relatively unaffected. The basis of this selectivity is uncertain but may reside in different sensitivities of the mitochondrial and cytoplasmic ribosomes. In any event, in the presence of these drugs mitochondria are defective, respiration is prevented, and energy for growth can only be obtained by glycolytic degradation of a fermentable substrate such as glucose. Hitherto this drug-induced repression of the respiratory system was thought always to be reversible, with the implication that no loss of the genetic potential for respiration was involved. However, we have now observed that in certain circumstances exposure of sensitive strains to high concentrations of erythromycin or chloramphenicol can generate cytoplasmic petite mutants in high yields. These mutants result from irreversible loss of the cytoplasmic genetic determinant known as the 'rho factor' which may, in a general sense, be correlated with the presence of normal mitochondrial DNA molecules. The occurrence

of this mutation in the circumstances under consideration cannot be explained by selection of pre-existing spontaneous mutants, since the conversion of the entire population into mutants occurs abruptly, and only after many generations of growth in the presence of the drug, during which period no mutants appear. In one diploid strain, measurements of changes in mitochondrial DNA content throughout the induction process revealed that the synthesis of these molecules continued for some time in the presence of erythromycin, but that the ultimate appearance of the petite mutants was accompanied by an apparently complete loss of mitochondrial DNA. This and other data lead to the tentative conclusion that the induction involves selective inhibition of synthesis of a mitochondrial product which is essential for normal replication of mitochondrial DNA and is present in considerable excess in aerobically grown cells.

**Properties of Male Derivatives of *Escherichia coli* Mutants Defective in Initiation of Chromosome Replication.** By H. G. NANDADASA (*Department of Genetics, University of Leicester*)

On the assumption that replication of plasmids is under negative cytoplasmic control, temperature-sensitive mutants of an F *lac* particle having uncontrolled replication were looked for. It was anticipated that such mutants would be lethal to the host at 42° but give a high frequency of *lac*⁻ revertants due to loss of the F particle. Although unstable temperature-sensitivities were found, none of them were of this type. In one class, on the contrary, the reversion frequency was *enhanced* by the presence of the F particle. These mutants proved to have a chromosomal lesion similar to those classified by others as defective in initiation of chromosome replication.

Known initiation-defective mutants (CRT 83 and CRT 46) also show an enhanced reversion frequency when infected by F and some F prime particles.

On the basis of one model (Pritchard, Barth & Collins (1969), *Symposia of the Society for General Microbiology*, no. XIX, *Microbial Growth*, pp. 263–297), for the control of replication of plasmids it would be predicted that insertion of a plasmid into a host chromosome carrying a lesion preventing initiation of a cycle of chromosome replication might suppress that lesion. Evidence will be presented that the enhanced reversion frequency of such mutants carrying F is due, at least in part, to such suppression by insertion.

**The Timing of Episomal F Replication in *Escherichia coli* at Different Growth Rates.** By JOHN COLLINS (*Department of Genetics, University of Leicester*)

We do not know the extent to which episomes are dependent on the chromosome for the control of their replication (Pritchard, R. H., Barth, P. T. & Collins, J (1969), *Symposia of the Society for General Microbiology*, no. XIX, *Microbial Growth*, pp. 263–297), in particular, the timing of the initiation of their replication. The problem was approached by directly assaying the amount of *F lac* DNA as a percentage of total DNA by DNA/DNA hybridization. The results obtained with cultures with doubling times from 90 min. to 20 min. show that the F *lac* particle is replicating later in the cell cycle than the chromosome origin at the faster growth rates. Thus although the chromosome seems to initiate at a constant cell volume (Pritchard, R. H., Barth, P. T. & Collins, J. (1969), *Symposia of the Society for General Microbiology*, no. XIX, *Microbial Growth*, pp. 263–297, 1969; Donachie, W. D. (1968), *Nature, London* **219**, 1077–1079) at different growth rates, this does not apply to the episome, and a different control mechanism is proposed.

**A Mutant in the Initiation of DNA Synthesis in *Salmonella typhimurium*.** By B. G. SPRATT and R. J. ROWBURY (*Department of Botany and Microbiology, University College, London*)

The initiation of DNA synthesis has been suggested to be of major importance in the control of cell growth (Maaløe, O. & Kjeldgaard, N. O. (1966), *Control of Macromolecular Synthesis*, W. A. Benjamin, Inc.). Temperature-sensitive mutants of DNA synthesis that

show the properties of initiator mutants have been isolated in *Escherichia coli* and *Bacillus subtilis* (Hirota, Y., Ryter, A. & Jacob, F. (1968), *Cold Spring Harbor Symposia on Quantitative Biology* **33**, 677; Gross, J. D., Karamata, D. & Hempstead, P. G. *ibid.* p. 307). We wish to report a temperature-sensitive mutant of *Salmonella typhimurium* apparently defective in the initiation of DNA synthesis at 38°.

The mutant (11G), when transferred from 25° to 38°, continues to synthesize RNA and protein for several hours resulting in the production of filaments and death. DNA synthesis continues for about 40 min., resulting in an increase in DNA of 55% in Casamino acids minimal medium, the amount of DNA expected if rounds of replication are completed at 38° but no new rounds initiated. Changes in supply of DNA precursors and the stability of the DNA are not the cause of the lesion which is irreversible and not corrected by increasing the osmotic pressure of the medium. Phage P 22 can develop in 11G at 38°. Increasing the temperature from 38° to 42° does not decrease the amount of DNA synthesized by 11G, and the addition of chloramphenicol at 38° has no effect on the increment. The amount of DNA synthesized plus chloramphenicol at 25° or 38° in various media closely corresponds to the amount synthesized by 11G at 38° in the same media. This suggests that chloramphenicol and the temperature-sensitive lesion are having similar effects. Chloramphenicol is known to stop the initiation of DNA synthesis without affecting rounds of replication already in progress.

**Production of DNA-less Cells in a Mutant of *Salmonella typhimurium*.** By B. G. SPRATT and R. J. ROWBURY (*Department of Botany and Microbiology, University College, London*)

Inhibition of DNA synthesis by mutation or by chemical means usually results in cessation of cell division (Helmstetter, C. E. & Pierucci, O. (1968), *Journal of Bacteriology* **95**, 1627; Clark, D. J. (1968), *Journal of Bacteriology* **96**, 1214). In *Escherichia coli* mutants have been isolated that continue cell division in the absence of DNA synthesis resulting in the production of cells lacking DNA (Hirota, Y., Jacob, F., Buttin, G. & Nakai, T. (1968), *Journal of Molecular Biology* **35**, 175; Inouye, M. (1969), *Journal of Bacteriology* **99**, 842).

A temperature-sensitive mutant of *Salmonella typhimurium* reported in the above abstract seems to be unable to initiate new rounds of DNA replication at 38°. After a shift to 38° cells divide apparently normally once or twice depending on the growth medium. These divisions probably result in the distribution of the completed chromosomes one to each daughter cell. The cells then elongate to form filaments which after about 1 h. recommence cell division at the ends of the cells. Either end of a filament can produce these small cells, and each end can produce more than one small cell. The small cells are about the same size as normal cells and can be purified on a sucrose gradient. Phage P 22 is unable to develop in these small cells and the cells synthesize no protein, RNA or DNA and contain little and probably no DNA. Addition of nalidixic acid at the time of a shift from 25° to 38° drastically decreases the amount of cell division and completely stops the production of DNA-less cells, although addition of the inhibitor after a period of growth at 38° has much less effect on cell division and allows the production of small cells. We consider the early divisions and the production of DNA-less cells to be the result of the termination of rounds of DNA replication in the absence of new initiations in this mutant at 38°.

**Synthesis of Double-stranded and Single-stranded RNA by Cell-free Extracts of Barley Leaves Infected with Bromegrass Mosaic Virus.** By J. SEMAL and J. KUMMERT (*Faculté des Sciences agronomiques, Gembloux, Belgium*)

Cell-free extracts of barley leaves infected with bromegrass mosaic virus (BMV) displayed virus-specific RNA polymerase activity when incubated with radioactive UTP in the presence of actinomycin D and EDTA (Semal, J. & Kummert, J. (1970), *Journal of General Virology* **7**, 173; Semal, J. (1970), *Annales de Phytopathologie*, in the Press). The RNA product of a short pulse of labelled UTP was essentially resistant to RNAse in high salt, while the product of longer incubations with the radioactive precursor was partially RNAse-sensitive.

Pulse-chase experiments indicated that the early double-stranded product was the precursor of the single-stranded RNA; displacement of the single-stranded product was dependent upon the presence of all four ribonucleotides.

RNA with sedimentation properties of BMV-RNA's were not identified as a major product in the system described, possibly because of the action of endogenous nucleases associated with the leaf fraction. Thermal denaturation of the 14 s double-stranded product of the RNA polymerase reaction generated polydisperse single-stranded RNA, part of which sedimented in the region of the large component of BMV-RNA. The results will be discussed in comparison with those obtained with picornaviruses. Reannealing of the heat-denatured RNA product indicated that the radioactivity was associated with the 'plus' strand of BMV-RNA.

**Pyrimidine Sequences in the Complementary Strand of the Replicative Form of Bacteriophage φX 174 DNA.** By G. K. DARBY (*Department of Virology, University of Birmingham*), L. B. DUMAS (*Department of Biological Sciences, Northwestern University, Evanston, Illinois, U.S.A.*) and R. L. SINSHEIMER (*Division of Biology, California Institute of Technology, Pasadena, California, U.S.A.*)

The complementary strand of $\phi$X RF DNA specifically labelled in the pyrimidines has been synthesized *in vitro* using *E. coli* DNA polymerase and a $\phi$X viral DNA template. The DNA was degraded by the method of Burton, K. & Petersen, G. B. (1960, *Biochem. J.* **75**, 17) to pyrimidine tracts which were dephosphorylated and then separated into isoplithic groups by chromatography on DEAE-Sephadex at pH 8. The elution pattern obtained was compared with that obtained from the pyrimidine tracts in the viral DNA. As the chain lengths of these tracts had been determined earlier by Hall, J. B. & Sinsheimer, R. L. (1963, *Journal of Molecular Biology* **6**, 115) it was possible to deduce the chain lengths of the majority of the complementary strand tracts.

Isopliths were further fractionated on the basis of their compositions by column chromatography on DEAE-Sephadex at pH 3 and on Dowex 50 ($H^+$). The base compositions of the components were assigned by a simple mapping technique, the validity of which was cross-checked by direct analysis of the compositions of a number of components.

The complete catalogue of pyrimidine tracts in the complementary strand of $\phi$X RF DNA was compiled.

**Partial Purification and Properties of a Fungal Growth Stimulant in Anthers which Predisposes Wheat to Attack by *Fusarium graminearum*.** By R. N. STRANGE and H. SMITH (*Department of Microbiology, University of Birmingham*)

Wheat anthers contain a fungal growth stimulant which appears to be important in the establishment of head infection by *Fusarium graminearum* (Strange, R. N. & Smith, H. (1969, *Journal of General Microbiology* **59**, ii). The system used to assay the fungal stimulant *in vitro* did not contain amino acids but paper chromatography demonstrated their presence in the anther extracts. Amino acids, therefore, were examined for growth-stimulating activity. However, none of the 17 amino acids in anther extracts revealed by quantitative amino acid analysis (Technicon Analyzer) nor a mixture of them in the proportions present in anthers were active in the assay.

The stimulant diffused through Visking tubing and paper electrophoresis of the diffusate showed that stimulant activity was divided between neutral and basic components. The components were separated by chromatography of the aqueous diffusate on carboxymethylcellulose adjusted to pH 5 with ammonia. Only the basic component was adsorbed and this was eluted with ammonium acetate. After removal of the buffer by freeze-drying the basic factor was further purified by precipitation of contaminants from ethanol solution with ether (equal volume) and a few drops of hydrochloric acid. All the activity remained in the supernatant and on a dry weight basis this represented an 80-fold purification of the factor over that found in the crude diffusate.

**Growth of Two Rumen Bacteria in Mixed Culture.** By P. N. HOBSON and C. S. STEWART (*Rowett Research Institute, Bucksburn, Aberdeen, AB2 9SB*)

*Selenomonas ruminantium* and *Lactobacillus* species are both constituents of the rumen flora. *Selenomonas* occurs regularly, but lactobacilli usually only occur in high numbers under 'acidosis'-type conditions, when they may dominate the flora. Under these conditions carbohydrate is usually in excess, and growth is similar to a batch culture. Growth of these organisms, in pure and mixed batch cultures, was investigated.

Pure cultures were grown at pH 6·7 and 38° in an anaerobic tryptone yeast medium with varying concentrations of glucose. Maximum growth rate for both organisms was at about 0·8 % glucose, when doubling times were approximately 90 min. (*Selenomonas*) and 150 min. (*Lactobacillus*).

Mixed cultures were grown in the same medium containing 0·9 % glucose inoculated with approximately equal numbers of lactobacilli and selenomonads to give about $2 \times 10^6$ bacteria per ml. medium.

In mixed cultures with initial pH 6·7, differential counts suggested that during the exponential phase the growth of each bacterium was similar to the growth of the same organism in pure culture. As the pH fell the growth rate of *Selenomonas* decreased and at pH 4·4 further growth was inhibited, but the *Lactobacillus* continued to make slow growth.

Varying the initial pH of mixed cultures between 6·9 and 6·2 did not affect growth in the early exponential phase, but a pH inhibitory to the selenomonads developed more rapidly in the cultures of lower initial pH and in the later stages lactobacilli predominated. After 18½ h. from initial pH 6·9 there were $6{\cdot}3 \times 10^8$ selenomonads/ml. and $7{\cdot}7 \times 10^8$ lactobacilli, and after the same time from initial pH 6·2 there were $2{\cdot}6 \times 10^8$ selenomonads and $7{\cdot}8 \times 10^8$ lactobacilli.

The media were highly reduced and no change of $E_h$ occurred during growth. Fermentation patterns were similar in all cultures. It is concluded that predominance of lactobacilli in the rumen is connected principally with changes in pH.

**Excystment of *Hartmannella castellanii*.** By M. P. STRATFORD and A. J. GRIFFITHS (*Department of Microbiology, University College, Cardiff*)

The excystment of cysts of the soil amoeba *Hartmannella castellanii* under axenic conditions is most readily induced by resuspending the cysts in 4 % (w/v) Mycological Peptone; other media and bacterial extracts are less effective or do not induce excystment. In general the cysts exhibit the same nutrient requirements and pH and temperature optima for excystment as for growth. There is some variability in the time required for excystment, but the process is usually completed within 3 to 5 days, and is relatively synchronous within each culture.

The cysts maintain their viability after exposure to low temperatures and extreme water loss. Temperatures in excess of 60° render the cysts non-viable.

Excystment of *Hartmannella* apparently consists of at least three stages: the initiation phase, the pre-emergence phase, and emergence proper (outgrowth) when amoebae appear in the culture. The initiation phase is characterized by an apparent lack of directly observable events, whereas the pre-emergent cysts show increases in dry weight and total acid phosphatase activity, and also a slight increase in $Q_{O_2}$. These observations, together with the lower levels of free acid phosphatase measured in pre-emergent cysts, may indicate renewed endocytotic activity in the 24 h. or so immediately preceding outgrowth. This is confirmed by the appearance of vacuoles in the cyst at this stage. As the changes associated with pre-emergence always occur during the 24 h. or so preceding outgrowth, it is likely that the variability in the time required for excystment is a result of variability in the length of the initiation phase.

After outgrowth structurally perfect but empty cysts remain, it is likely therefore that outgrowth occurs through a pore in the cyst wall.

**A Fermentor Culture System for Sporulation Studies on *Aspergillus niger*.** By J. G. ANDERSON and J. E. SMITH (*Department of Applied Microbiology, University of Strathclyde*)

In fungal sporulation studies the development of a system for the induction of sporulation of *Aspergillus niger* in shake flask culture (Galbraith, J. C. & Smith, J. E. (1969), *Journal of General Microbiology* **59**, 31) is a considerable improvement over static growth conditions. Adaptation of this system for fermentor culture involved changing from pellet growth in shake flasks to a more dispersed growth in the fermentor and it was necessary to initiate some degree of mycelial clumping in the fermentor for maximum conidiophore development.

Nitrogen limitation in a simple glucose, ammonium sulphate and inorganic salts medium gave the maximum induction of conidiation. The first morphological evidence of induction was the production of conidiophore initials from swollen cells, many of which existed before nitrogen exhaustion. Continued uptake of glucose occurred during conidiophore elongation while concurrently there was abundant production of carotenoid pigments. In this medium no vesicles, phialides or spores were produced from the conidiophores. The final stage of the reproductive structure was induced by replacement of the culture into a medium containing a nitrogen source and a tricarboxylic acid cycle intermediate.

A major problem in this system was the induction of sporulation by factors other than nitrogen exhaustion which resulted in a loss of synchrony. This sporulation originated from inoculum cells, either from germinated conidia with a conidial inoculum or from swollen cells with a mycelial inoculum and occurred before nutrient exhaustion. This has been eliminated by a reduction in the oxygen supplied to the culture to a level which although not affecting growth suppresses this early sporulation. Further reduction of aeration rate results in growth limitation, ethanol production, inhibition of nitrogen exhaustion induced sporulation and inhibition of carotenoid production.

Results with this system suggest that sporulation in *A. niger* can be subdivided into at least four stages: (i) production of swollen pre-conidiophore cells, (ii) production of conidiophore initial, (iii) growth of conidiophore, and (iv) production of vesicles, phialides and spores.

**The Cultivation of 'Ovals' from the Ovine Rumen.** By C. G. ORPIN and G. S. COLEMAN (*Department of Biochemistry, Agricultural Research Council Institute of Animal Physiology, Babraham, Cambridge*)

The purpose of this communication is to describe, for the first time, the *in vitro* cultivation of 'Ovals' isolated from the ovine rumen. The 'Ovals' are large ($8 \times 6$ to $20 \times 12$ $\mu$), oval, motile, Gram-negative bacteria. Quin (Quin, J. I. (1943), *Onderspoort Journal of Veterinary Science and Animal Industry* **18**, 91) described an oval organism with similar characteristics from the rumen of sheep; other workers give its dimensions as $6 \times 4$ $\mu$ (Wicken, A. J. & Howard, B. H. (1967), *Journal of General Microbiology* **47**, 207). Quin (1943) and Eadie (Eadie, J. M. (1962), *Journal of General Microbiology* **29**, 563) found larger Ovals in the rumen of cattle and sheep respectively, but give few details.

Rumen fluid from a Clun Forest wether was inoculated into the medium of Coleman (Coleman, G. S. (1969), *Journal of General Microbiology* **57**, 81). After several months, 'Ovals' appeared in cultures incubated at 38–40° under $CO_2$, the supernatant being replaced daily. The omission of grass and starch from the medium freed the cultures of protozoa, leaving a mixed bacterial population. It was found that the daily addition of glucose was essential, but that the medium need only be changed weekly. When higher cell densities were achieved, maintenance was limited to daily feeding with glucose and weekly division of each culture into two, making each up to 10 ml. with fresh medium. Under these conditions, the maximum population attained was $2 \times 10^7$ ml$^{-1}$. The 'Ovals' would not grow in medium gassed with 95 % $N_2$ + 5 % $CO_2$, nitrogen or air; the cysteine could be replaced by 0·02 % sodium thioglycollate or mercaptoethanol, or 0·01 % sodium dithionite or ascorbic acid. Glucose could be replaced by fructose or galactose, and, less efficiently, by sucrose or lactose. Cells incubated with these carbohydrates stained brown with iodine. Replacement

of autoclaved rumen fluid (ARF) with centrifuged ARF, centrifugally fractionated ARF or autoclaved rumen bacteria resulted in decreased growth. The 'Ovals' were sensitive to ampicillin, polymixin B, methicillin, chloromycetin and terramycin at 200 $\mu$g ml.$^{-1}$. No growth occurred in solid media.

**Ultrastructural Changes during Sporulation in *Clostridium pasteurianum*.** By B. M. MACKEY and J. G. MORRIS (*Department of Biochemistry, School of Biological Sciences, University of Leicester*)

Much less is known of the biochemical changes accompanying sporulation in clostridia than has been discovered for the very similar process in bacilli. To some extent this is due to the fact that complex sporulation media must in general be employed for the clostridia.

We have obtained comparatively rapid sporulation of *Clostridium pasteurianum* ATCC 6013, during anaerobic, batch cultivation in a simple, defined medium containing only salts, calcium carbonate, glucose, L-cysteine and ammonium ions, and by electron microscopy we have followed the course of the associated ultrastructural changes. Features of special interest include: (*a*) the formation of spore coat prior to the deposition of spore cortex, (*b*) the multilayered nature of both spore coat and exosporium, (*c*) the possible presence of a central, basal pore in the exosporium.

Examination of the culture by light microscopy showed that onset of sporulation was heralded by a distinctive swelling of the organisms at the end of their exponential growth. During continued incubation at 35°, phase-white spores appeared within 6 to 7 h. and became heat-resistant after a further 5 to 6 h. At this time, approximately 80% of the organisms contained refractile spores. Terminal lysis with liberation of mature spores enclosed within their exosporia, was a protracted process usually completed only after several weeks of storage. Direct electron microscopic assay of the proportion of the population that had at any one time attained a distinctive developmental stage, revealed that from septation to engulfment of the forespore took 1·9 h., spore coat material appeared 2·9 h. after engulfment, and spore cortex deposition commenced 2·6 h. later than spore coat production. It seems that the various developmental stages were attained sufficiently synchronously to allow of meaningful correlative biochemical studies.

We are grateful to Mrs Winifred Ego who kindly supplied us with the strain of *Cl. pasteurianum* employed in these studies.

**Proline Biosynthesis: Regulation by a Positive Control Mechanism?** By D. GAUDIE and H. TRISTRAM (*Department of Botany and Microbiology, University College, London*)

Spontaneous mutations in *Salmonella typhimurium* resulting in resistance to 3,4-dehydro-DL-proline, a toxic analogue of proline, have been located close to, or within *proB*, a locus involved in the early steps of proline biosynthesis. Such mutants, designated *proG*$^r$, produce derepressed levels of $\Delta'$-pyrroline-5-carboxylic acid reductase when grown in minimal medium and synthesis of this enzyme cannot be repressed by proline. Close proximity to *proB* allows several possible alternative suggestions for the function of *proG*. That *proG* is an operator locus can be readily dismissed, since the heterozygote of *proG1054* for *proA* and *proB* is *cis*-recessive. That *proG* determines a regulatory subunit of an enzyme sensitive to feedback inhibition can also be eliminated since a mutation in the locus determining the regulatory subunit of such an enzyme would not account for the derepressed and non-repressible synthesis of $\Delta'$-pyrroline-5-carboxylic acid reductase.

The possibility that *proG* determines a repressor with a positive or negative effect is dependent on whether *proG* is located within or outside *proB*. Heterozygotes of genotype *proG*$^+$ *proB*$^-$ *proA*$^+$/*proG*$^r$ *proB*$^+$ *proA*$^+$ and *proG*$^+$ *proB*$^+$ *proA*$^-$/*proG*$^r$ *proB*$^+$ *proA*$^+$ display different phenotypes, an observation consistent with a location of *proG* within *proB*. If the *proB* gene product is the repressor of a negatively controlled system, point mutations in this locus would only give rise to *pro*$^-$ phenotype if the mutation exerts a polar effect on *proA*. Further, abortive transductants of *proB* with *proA* would be phenotypically *pro*$^-$. However,

in such crosses *pro*$^+$ abortive transductants have been found (Miyake, T. & Demerec, M. (1960), *Genetics* **45**, 755). These observations, including the derepressed and non-repressible synthesis of $\Delta'$-pyrroline-5-carboxylic acid reductase, can be explained by assuming that *proG* is located within *proB* and the *proB* gene product functions as an activator of synthesis of the proline biosynthetic enzymes.

**Properties of a Pyridine-degrading Organism.** By D. A. STAFFORD and A. G. CALLELY (*Department of Microbiology, University College, Cardiff*)

This work arose from studies on coke-oven effluents, which as well as containing many phenolic compounds, whose microbial degradation has been well studied, also contain a number of pyridine compounds. Little information is available on the degradation of pyridine itself, though studies have been made on certain hydroxylated derivatives (Houghton, C., Watson, G. K. & Cain, R. B. (1969), *Biochem. J.* **114**, 75P), and on nicotinic acid derivatives (Hughes, D. E. (1955), *Journal of General Microbiology* **60**, 303; Behrman, E. J. & Stanier, R. Y. (1958), *Journal of Biological Chemistry* **228**, 947). Nicotinic acid degradation in a *Pseudomonas* sp. (Ensign, J. C. & Rittenberg, S. C. (1964), *Journal of Biological Chemistry* **239**, 2285), proceeded via 6-hydroxy-nicotinic acid, the 2,5-dihydroxy and 2,3,6-trihydroxy pyridines, maleamic and maleic acids.

Using conventional enrichment techniques, an oxidase positive Gram-negative rod (most likely a pseudomonad) was isolated from an activated sludge plant treating pyridine compounds. It grew well aerobically in liquid medium containing pyridine (10 mM) as a sole source of carbon, nitrogen and energy, ammonia being produced during growth and pyridine disappearing (estimated by u.v. at $\lambda$ 255 nm.). Good growth occurred with nicotinic acid, 6-hydroxy nicotinic acid and 4-methyl pyridine, but not with the 2- and 3-methyl pyridines or the mono-hydroxypyridines; it grew with maleic acid after a lag of 2 to 3 days. Pyridine oxidation is inhibited by arsenite, pyruvate appearing in the supernatant. Pyridine grown cells do not oxidize any of the mono-hydroxypyridines, nor the 2,3- and 2,6-dihydroxy pyridines, nor maleamic acid; maleic acid is oxidized after a lag period. The inability to oxidize the latter two compounds readily may be due to permeability difficulties, because spectrophotometric studies indicate that maleamic acid can be converted to fumaric acid via maleic acid with cell-free extracts of pyridine grown cells. This activity has not been demonstrated in extracts of the organism grown with succinate.

**The Effect of Oxygen and Nitrate on Respiratory Systems in *Micrococcus denitrificans*.** By LYNDA M. SAPSHEAD and J. W. T. WIMPENNY (*Department of Microbiology, University College, Cardiff*)

Growth in the presence or absence of oxygen or nitrate can alter the composition of microbial electron transport pathways (Wimpenny, J. W. T. (1969), *Symposia of the Society for General Microbiology* **19**, 161). The total amounts of cytochrome *b* and *c* are higher anaerobically than aerobacilly, in *Micrococcus denitrificans* (Scholes, P. & Smith, L. (1968), *Biochimica et Biophysica Acta* **153**, 363). We have investigated the effect of oxygen and nitrate on cytochromes and respiratory enzymes, and their distribution in this organism in greater detail.

Cells were grown on a synthetic medium, containing 0·4% (w/v) glucose and either 0·13M-$NaNO_3$ or c·03M-$NH_4Cl$. Growth was exponential under anaerobic and aerobic conditions and linear under oxygen limitation.

Harvested cells were washed and crushed using the Hughes's press. The DNase-treated crush was fractionated by centrifugation into a washed 22,000*g* pellet—CW/M, a 306,000*g* pellet—65P, and a supernatant-soluble fraction. Cytochromes were determined spectrophotometrically from oxidized versus reduced difference spectra.

Excess oxygen depresses the total amount of cytochromes *b* and *c* in all fractions of nitrate or ammonium grown cells. Cytochrome *b* was never detected in the soluble fraction. The total amount of cytochromes *b* and *c* were not significantly different in the CW/M or 65P fractions. The presence of nitrate *and* the absence of oxygen resulted in a high level

of cytochrome *o* in the CW/M and 65P fractions, suggesting a role for it in nitrate reduction. In anaerobic and oxygen-limited cells, even when nitrate was absent, the cytochrome $a+a_3$ complex was repressed and cytochromes *c* and *o* appeared in the soluble fraction.

The location and specific activities of various respiratory enzymes were measured: isocitrate dehydrogenase and malic dehydrogenase were predominantly soluble. NADH oxidase, succinic cytochrome *c* reductase, cytochrome oxidase, NADH cytochrome *c* reductase and succinic dehydrogenase activities were located in the CW/M and 65P fractions. Neither oxygen concentration nor nitrogen source had any clear effect on enzyme activities or distribution.

**Oxygen-induced Proton Pulses in Various Species of Bacteria.** By JULIAN W. T. WIMPENNY (*Microbiology Department, University College, Cardiff*)

Mitchell (Mitchell, P. & Moyle, J. (1967), *Biochemical Journal* **105**, 1147) has shown that proton extrusion is coupled to respiration in mitochondria. There has been little systematic work with bacteria in this field.

Various micro-organisms were grown on appropriate media, harvested, washed and resuspended in 120 mM-KCl:2 mM glycyl glycine pH 7·0. pH and oxygen tension changes were followed in a closed stirred vessel at 30°. The outputs from a sensitive pH meter and the Clark oxygen electrode were recorded simultaneously. Pulses of oxygen-saturated KCl/glycyl-glycine or anaerobic 50 mM-HCl or KOH were injected from an Eppendorf micropipette. The resulting traces were analysed as described by Mitchell.

All organisms examined showed a small alkaline change when first pulsed with oxygen. In some experiments after several oxygen pulses acidic changes were observed in *Micrococcus denitrificans* and *Staphylococcus albus*. In these organisms and in *Bacillus subtilis* and *B. megaterium* proton pulses induced by oxygen appeared after pretreatment with valinomycin and carbonyl-cyanide-chlorphenylhydrazone or with gramicidin. $H^+$:O stoichimetry lay between 6 and 8 for *M. denitrificans* and for both species of *Bacillus* and was about 4 under normal conditions for *S. albus*.

The conversion of an alkaline pulse to an acid pulse has been followed during a series of oxygen titrations with *M. denitrificans*. A small proton pulse is at first superimposed on the alkaline shift. This gradually increases in size and is finally the dominant effect.

No proton pulses have ever been observed in *Escherichia coli* K 12, *Klebsiella aerogenes*, *Pseudomonas fluorescens*, *Mycobacterium tuberculosis* BCG, *M. phlei*, *Streptococcus lactis* or, as might be expected, intact cells of *Saccharomyces carlsbergensis*.

It seems that under normal conditions proton pulses do not occur in response to oxygen in bacteria, but that they may be detected in some gram positive species under artificial conditions. It is likely from these observations that proton extrusion and uptake are quite tightly coupled.

**The Nature of the Ribonucleic Acid Synthesized *in vitro* by Sendai Virus-induced Ribonucleic Acid-dependent Ribonucleic Acid Polymerase.** By JANET E. HUTCHINSON, B. W. J. MAHY and R. D. BARRY (*Department of Pathology, University of Cambridge*)

The properties of an RNA-dependent RNA polymerase induced in cells infected with Sendai (parainfluenza 1) virus have been reported (Mahy, B. W. J., Hutchinson, J. E. & Barry, R. D. (1970), *Journal of Virology* **5**, 663). The product of enzyme reaction *in vitro* consisted of a complex mixture of RNA species including 34 *s*, 24 *s* and 18 *s* components, 98 % of which could be hybridized with RNA extracted from purified Sendai virus particles. An improved assay system has now been developed in which the effects of endogenous nucleases are minimized and incorporation of nucleoside triphosphates into RNA is increased. RNA synthesized by the enzyme under these conditions contained an additional 57 *s* component, similar to RNA which can be extracted from purified virions (Barry, R. D. & Bukrinskaya, A. G. (1968), *Journal of General Virology* **2**, 71). A ribonuclease-resistant component was also detected in the enzyme product: this sedimented at 20 to 25 *s*, and may

correspond to the 20 to 30 *s* replicative form of Sendai virus RNA recently isolated from Sendai virus-infected cells (Zhdanov, V. M. & Bukrinskaya, A. G. (1970), *Archiv für die gesamte Virusforschung* **29**, 241). The role of this enzyme in the replication of Sendai virus will be discussed.

**Studies on the Nature of Influenza Virus Ribonucleic Acid.** By P. A. BROMLEY and R. D. BARRY (*Department of Pathology, University of Cambridge*)

$^{32}$P-labelled influenza (fowl plague) virus RNA has been separated into seven components by acrylamide plate gel electrophoresis using autoradiography to locate the RNA fractions. Five RNA species are of relatively high molecular weight while the remaining two species are distinct and of low molecular weight. Two high-molecular-weight fractions have so far been recovered intact from acrylamide gels by homogenization of the gel and fractional precipitation of the RNA, and these RNA fractions have been further characterized by sucrose density-gradient centrifugation. Total influenza RNA has been digested with ribonuclease $T_1$ and the digest products separated by a two-dimensional procedure involving high-voltage ionophoresis on cellulose acetate (pH 3·5 urea) followed by thin-layer homochromatography in the second dimension (Brownlee, G. G. & Sanger, F. (1969), *European Journal of Biochemistry* **11**, 395). This procedure allows finger-printing of digests of influenza RNA giving excellent resolution of relatively long base sequences with a terminal guanine residue which may prove definitive in an analysis of RNA of various strains of influenza virus or of RNA fractions within a single strain.

The ribonuclease-resistant, double-stranded RNA that accumulates in cells infected with influenza virus (Pons, M. (1967), *Virology* **31**, 523) has been isolated and shown to be heterogeneous. It can be separated into two components by poly-L-lysine kieselguhr chromatography (Bromley, P. A. & Barry, R. D. (1970), *Analytical Biochemistry*, **36**, 278). One component sediments as a homogeneous peak (7 *s*) on sucrose-gradient analysis. The other component sediments as a heterogeneous band with a peak at 7 *s* and shoulders at 12 *s* and 18 *s*. Further analysis of influenza-induced double-stranded RNA and of its relation to viral RNA will be presented.

**Anti-interferon Factors in the Digestive Tract.** By Q. R. MIRANDA and T. S. L. BESWICK (*Department of Bacteriology and Virology, University of Manchester*)

Field trials with the live polio virus vaccine as reported by Sabin and others have shown that the vaccine does not always produce antibody even in individuals without evidence of pre-existing immunity. This is particularly true of children living under conditions of poor community hygiene in tropical and subtropical regions (Sabin, A. B. (1959), *Live Polio Virus Vaccines*, pp. 14–33, Pan American Sanitary Bureau; Montefiore, D., Collard, P., Jamieson, M. F. & Jolly, H. (1963), *British Medical Journal* i, 1569).

Such children usually show high carriage rates for enteroviruses and their resistance to the vaccine has been generally ascribed to interference by these enteroviruses (Sabin, A. B. (1959), *loc. cit.*). In view of the known sensitivity of interferon to trypsin (Isaacs, A. (1963), *Advances in Virus Research* **10**, 1–35), it seemed of interest to determine whether interferon could exist in significant amounts in the bowel contents of man and animals. We have failed to find detectable levels of interferon in bowel contents but have confirmed their ability to destroy interferon. We have compared the capacity to destroy interferon, the cell toxicity and the enzymic activity of bowel contents from different levels of the intestinal tract and from different species of animal.

Our preliminary results indicate that destruction of interferon is not due to bacterial enzymes and is probably independent of trypsin and chymotrypsin.

**The Accumulation of Ribosome Precursors during Inhibition by Cobalt Chloride.** By M. R. BLUNDELL and D. G. WILD (*Microbiology Unit, University of Oxford*)

When *Escherichia coli* MRE600 is incubated with a concentration of cobalt chloride sufficient to slow but not to stop growth, protein synthesis is inhibited more than the synthesis of RNA. Inhibited cells accumulate three particles that contain ribosomal RNA but are not ribosomes; the properties of these particles are consistent with their being ribosome precursors (Blundell & Wild (1969), *Biochemical Journal* **115**, 213).

More recent work supports this view. The RNA made during inhibition by cobalt chloride was labelled with radioactive uracil, then cells were collected at intervals during incubation in cobalt-free medium. Sucrose gradient analysis showed that the radioactivity of the particles was incorporated into completed ribosomes. This transfer also occurred when the cobalt-free medium contained a concentration of rifampicin sufficient to prevent RNA synthesis; thus the RNA of the particles can be incorporated directly into ribosomes. Chromatography on carboxymethyl-cellulose has shown that the proteins of the three particles are ribosomal proteins.

The particle which contains 16 *s* RNA, and is therefore a putative precursor of 30 *s* ribosomes, sediments as a single peak in sucrose gradients. However, when the RNA and protein made during inhibition by cobalt chloride were labelled with radioactive uracil and lysine respectively, the protein content, relative to RNA, varied in different fractions of this peak. Moreover, the results of carboxymethyl-cellulose chromatography indicate that the more slowly sedimenting species in this region of a gradient contain relatively fewer ribosomal proteins. These results imply that, if the particles accumulated during inhibition by cobalt chloride are 'natural' ribosome precursors, the synthesis of 30 *s* ribosomes may normally involve the participation of more than one precursor.

**Altered Ribosomes after Inhibition of *Escherichia coli* by Rifampicin.** By M. R. BLUNDELL and D. G. WILD (*Microbiology Unit, University of Oxford*)

Rifampicin rapidly halts RNA synthesis in bacteria by inactivating RNA polymerase. During prolonged treatment of *Escherichia coli* MRE600 with this antibiotic, the sedimentation properties of the ribosomes show marked changes. At intervals after addition of rifampicin to exponentially growing cultures, cells were collected and lysed and their contents were analysed by centrifuging through sucrose gradients containing 10 mM-$Mg^{2+}$. A series of conversions was observed. The polysomes decayed rapidly to 70 *s* ribosomes; these then broke down to give two particles which sedimented more slowly than ribosomal subunits, at approximately 45 *s* and 25 *s*. The 45 *s* particle was later converted, 1 to 2 h. after the addition of the rifampicin, to a component whose sedimentation coefficient was approximately 35 *s*. The ribosomal RNA of these particles was intact; the 45 *s* and 35 *s* particles contained 23 *s* RNA and the 25 *s* particle, 16 *s* RNA. The particles could be degraded by ribonuclease in conditions in which normal subunits were apparently unaffected. Both unfolding of the ribosomes and the loss of some protein may be involved in the conversions. No changes in the sedimentation properties of ribosomes were detected when rifampicin was added to extracts from exponentially growing cells.

The particles found after inhibition by rifampicin *in vivo* resemble those present after complete starvation of *E. coli* (Maruyama, Ono & Mizuno (1970), *Biochimica et Biophysica Acta* **199**, 176). We therefore suggest that the action of rifampicin is indirect and may reflect gross metabolic changes after inhibition of RNA synthesis. The altered ribosomes are likely to be inactive in protein synthesis; moreover, ribosomes may be inactivated before changes in their sedimentation properties can be detected. Experiments which measure protein synthesis in intact cells after the addition of rifampicin should be interpreted with care.

**Synthesis of Ribosomes in a Mutant of *Escherichia coli*.** By Q. MEHDI and D. G. WILD (*Microbiology Unit, University of Oxford*)

15–28 is a mutant of *Escherichia coli* 15 that grows more slowly than the parent strain but whose cells have a higher content of RNA. Much of this excess RNA is in '48 *s* particles' which, although containing 23 *s* ribosomal RNA, can be distinguished from 50 *s* ribosomes. In cell-free systems, the 70 *s* ribosomes of 15–28 are less efficient in protein synthesis than those of the parent organism (MacDonald, Turnock & Forchhammer (1967), *Proceedings of the National Academy of Sciences, U.S.A.* **57**, 141).

We have confirmed the suggestion of the authors above that 48 *s* particles are ribosome precursors. However, these particles differ from the immediate precursor to 50 *s* ribosomes that has been studied in normal organisms growing exponentially or in the presence of a low concentration of chloramphenicol (Osawa, Otaka, Itoh & Fukui (1969), *Journal of Molecular Biology* **40**, 321). The final stage in the assembly of 50 *s* ribosomes is thought to involve limited methylation of the 23 *s* RNA of a precursor together with the addition of 5 *s* RNA and some few ribosomal proteins. 48 *s* particles have the same complement of 5 *s* RNA and ribosomal protein as 50 *s* ribosomes, although their RNA is undermethylated. The particles also differ in their sedimentation properties and ribonuclease sensitivity from the precursors to 50 *s* ribosomes that accumulate during inhibition of *E. coli* MRE600 by cobalt chloride (Blundell & Wild (1969), *Biochemical Journal* **115**, 213). The maturation of 50 *s* ribosomes in 15–28 may therefore be atypical. Melting profiles of 48 *s* particles and 50 *s* ribosomes from 15–28 are similar but both differ from those of 50 *s* ribosomes from the parent organism. A biological consequence of this alteration could be the reduced activity in protein synthesis of ribosomes from the mutant.

# Index of Authors

*Small roman numerals refer to pages in the* Proceedings of the Society for General Microbiology

# Index of Subjects

*Small roman numerals refer to pages in the* Proceedings of the Society for General Microbiology

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TOMUS XVII FASCICULUS 1

INDEX



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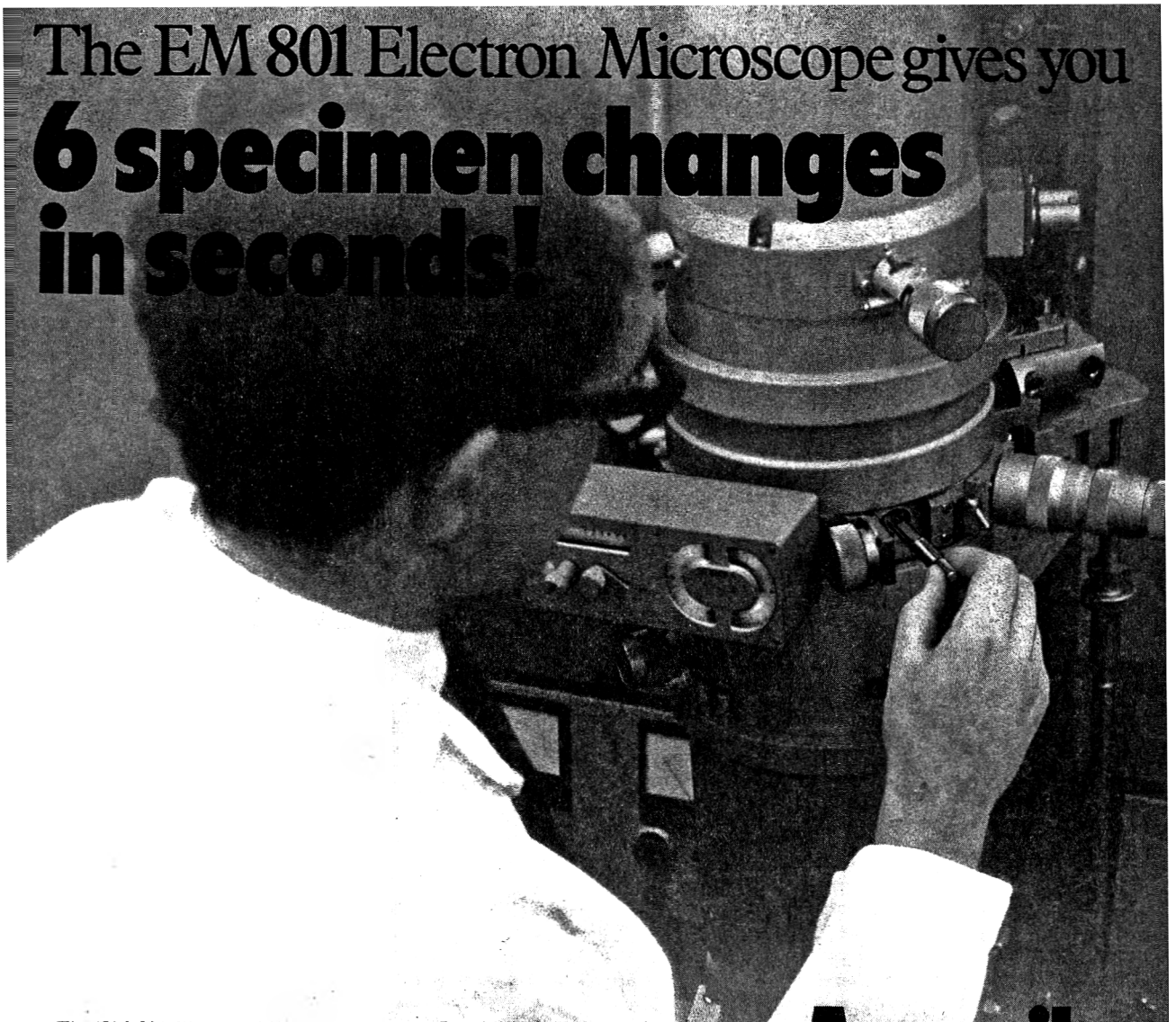

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# The Journal of General Microbiology

Volume 63, Part 3, November 1970



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