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THE JOURNAL OF GENERAL MICROBIOLOGY

THE PREPARATION OF PAPERS

'The more words there are, the more words there are about which doubts may be entertained.'
JEREMY BENTHAM (1748–1832)

'What can be said at all can be said clearly; and whereof one cannot speak thereof one must be silent.'
LUDWIG WITTGENSTEIN: *Tractatus Logico-philosophicus* (tr. C. K. Ogden

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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.

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

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Immunofluorescence Examination of Typical *Staphylococcus aureus* Variants and L-forms for Clumping Factor

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(*Accepted for publication 24 September 1969*)

SUMMARY

Encapsulated coagulase-positive staphylococci which do not clump with fibrinogen possess the clumping factor antigen as well as other cell-wall antigens characteristic of *Staphylococcus aureus*. Variants which were negative for both coagulase and clumping factor not only lacked the clumping factor antigen but also other *S. aureus* surface antigens. L-forms did not remove clumping inhibiting antibody from *S. aureus* antisera. Antisera which were relatively high in clumping-inhibiting antibodies, but low in agglutinins, gave no fluorescence with coagulase-negative, clumping factor-negative variants or L-forms. The clumping factor, probably a cell-wall component of *S. aureus*, is absent in the penicillin-induced, stable L-form.

INTRODUCTION

The characteristic clumping of coagulase-positive staphylococci in the presence of fibrinogen is attributed to a factor (bound coagulase or clumping factor) which is presumed to be a surface component of the cocci. Duthie (1954) reported that an antigen of *Staphylococcus aureus*, which was released into the culture medium upon autolysis, induced production of antibodies which inhibited the clumping reaction. This antigen has not been detected in staphylococcal extracts which precipitate fibrinogen (Rotter & Kelly, 1966; Blackstock, Hyde & Kelly, 1968). Kato & Omori (1959) verified the observation of Duthie (1954) that trypsinized *S. aureus* cocci lose the ability to clump in fibrinogen solution, and they no longer absorb the clumping-inhibiting antibodies from antiserum. It was assumed that the release of clumping factor into the medium of ageing cultures, as well as its loss upon refrigeration and trypsinization, is probably due to a 'peeling off' of cell-surface substance. Gorrill, Klyhn & McNeil (1966) found that clumping factor was either destroyed or removed by sonic vibrations. A remnant of cell-wall material may be present on some or all of the so-called L-forms of *S. aureus* (Pratt, 1966; Hamburger & Carleton, 1966); clumping factor could be a component of this cell-wall remnant. Coagulase and clumping factor are closely associated in the bacterial form of typical *S. aureus* strains, and L-forms of these strains are known to produce coagulase (Mattman, Turnstall & Rossmoore, 1961).

In this immunofluorescence study typical coagulase-positive cocci, clumping factor-negative and L-forms of *Staphylococcus aureus* were tested with antisera of known agglutinating and clumping-inhibiting antibody content.

METHODS

Staphylococcal strains. Six typical *Staphylococcus aureus* strains were used: 2095, 3189 and 2253, strains of known serological type (Cohen & Smith, 1964) supplied by Dr J. O. Cohen; J17, serologically of Cowan's group I (from Dr K. Jensen, who used it in his antigen A studies: personal communication); 604, a wild strain isolated here; the SMITH strain (from Dr G. A. Hunt). Also used were: the coagulase-negative, clumping factor-negative variants of strains J17 and 604 designated J17M and 604M, respectively; strain SV, an encapsulated, mouse-virulent variant of the SMITH strain (Hunt & Moses, 1958); and K6, an encapsulated, mouse-virulent strain which was isolated here from the nasopharynx of a healthy carrier in 1956. Although coagulase-positive, neither strain SV nor strain K6 clump in fibrinogen solutions.

Induction and cultivation of L-forms. Five coagulase-positive, clumping factor-positive strains (2095, 2253, 3189, 604 and J17) were induced to L-form growth. The inducing medium was similar to that used by Marston (1961*a*) and Williams (1963). A concentrated inoculum was prepared by centrifuging a 5 to 8 hr Brain-Heart-Infusion (Difco) broth culture (37°) and resuspending the cocci in one-fifth the original volume of broth. One-half millilitre (0·5 ml.) was plated on Brain-Heart-Infusion (Difco) medium which contained 1 % (w/v) Special Agar-Noble (Difco), 5 % (w/v) sodium chloride, 10 % (v/v) horse serum and 1500 units penicillin G/ml. L-colonies, which developed after (37°) incubation, were subcultured first on the inducing agar medium and thereafter in a similar liquid medium. Once stable L-form growth was obtained, subsequent broth cultures were shaken at low speed on a Burrell wrist action shaker during a 48 hr incubation period at 37°. L-forms were also grown in soft agar medium prepared by adding 0·15 % (w/v) Special Agar-Noble (Difco) to the liquid medium. Modifications of the soft agar medium included addition of 4 % (w/v) human fibrinogen (Merck, Sharp & Dohme, West Point, Pa.) or bovine fibrinogen (Armour Fraction I, Armour Pharmaceutical Co., Kankakee, Ill.) for testing clumping factor activity, and incorporation of 1 % (v/v) rabbit plasma in the fibrinogen-soft agar for demonstrating free coagulase (Alami & Kelly, 1959).

Preparation and use of antisera. Immunization of rabbits with typical *Staphylococcus aureus* strains began with two 0·5 ml. intravenous injections of formalinized (2×10^9 cocci/ml.) vaccine in the first week, and was continued, for a total of 8 weeks, with similar, biweekly injections of cocci which had been heated at 65° for 2 hr. Less than 1 % of the heated cocci were viable.

Samples of each antiserum were absorbed with clumping factor-negative *Staphylococcus aureus* variants and with L-forms. L-forms separated from a 300 ml. broth culture were washed once in 3 % (w/v) NaCl solution and antiserum was added to the sedimented growth. Absorption, with washed typical cocci or L-forms, was allowed to proceed at 37° for 2 hr and at 4° for an additional 24 hr. Antisera against three strains were absorbed with lyophilized supernatant fluid of the broth culture in which the L-form had grown, as well as with lyophilized, uninoculated, L-form broth medium. Antisera were held at −21° until used in agglutination, clumping inhibition and indirect fluorescent-antibody tests. In immunodiffusion experiments antiserum was substituted for horse serum in L-form agar medium.

Indirect fluorescent-antibody tests. Fluorescein isothiocyanate-labelled sheep anti-rabbit gamma globulin (Colorado Serum Company, Denver, Colorado) was applied

to cocci and L-forms which had been reacted with pre-immune, unabsorbed immune or absorbed immune rabbit serum. In a control to rule out nonspecific fluorescence, incubation of organisms with antiserum was omitted from the procedure. Slides were made of staphylococci which had been separated from an 18 hr Brain-Heart-Infusion (Difco) culture (37°), washed once and resuspended in saline. Initially, the method used by Karakawa, Rotta & Krause (1965) in immunofluorescence studies of streptococcal L-forms was applied. However, the most satisfactory preparations of staphylococcal L-forms proved to be smears of growth from broth cultures or of colonies from soft (0·15 %) agar cultures. Slides were examined under a Reichert u.v. microscope using a BG-12 exciter filter and an OG-1 barrier filter.

RESULTS

Examination of L-forms for coagulase and clumping factor. The L-form was considered stable when no bacterial colonies developed on penicillin-free medium. The sedimented growth of broth cultures inoculated with L-colonies contained numerous large bodies similar to those described by Marston (1961*b*), Weibull (1963) and Williams (1963). Occasionally, in broth cultures, the L-form gave rise to small discrete colonies which, on transfer to fresh broth, produced the usual diffuse growth. Typical L-colonies developed from either the diffuse growth or the discrete colonies.

Coagulase-production by L-forms was demonstrated by the plasma-fibrinogen-soft agar method as well as by use of broth cultures in the regular plasma clotting test. Clumping factor activity could not be detected in fibrinogen-soft agar because compact colonies sometimes developed in the fibrinogen-free soft agar base. The viscous nature of the L-form growth, on agar or in broth, made it unsuitable for use in the slide clumping test usually applied to staphylococci.

Table 1. *Homologous agglutination titres before and after absorbing Staphylococcus aureus antisera with variants and L-forms of S. aureus*

S. aureus antiserum (immunizing strain)	Unabsorbed antiserum	Antiserum absorbed with: Cocci (strain) J17M*	604M*	SV†	K6†	L-form of immunizing *S. aureus* strain
J17	1/2560	1/1280	1/2560	—‡	1/10	1/1280
604	1/2560	1/2560	1/1280	—	1/10	1/1280
3189	1/5120	1/2560	1/2560	1/10	1/20	1/2560
2253	1/1280	1/1280	1/1280	—	—	1/320
2095	1/2560	1/2560	1/2560	1/10	1/20	1/1280
S	1/1280	1/640	1/640	—	1/10	1/640

* J17M and 604M are coagulase-negative, clumping factor-negative variants of *S. aureus* strains J17 and 604, respectively.

† SV and K6 are encapsulated, coagulase-positive strains which do not clump in fibrinogen.

‡ No reaction observed in lowest serum (1/10) dilution tested.

Agglutination and clumping inhibition reactions. Prior to immunization, sera of rabbits were negative for clumping-inhibiting antibody and in no instance was the agglutination titre for strains used higher than 1/10. Titres of immune sera ranged

from 1/1280 to 1/5120 for agglutinating antibody and from 1/320 to 1/2560 for clumping-inhibiting antibody.

There was no significant change in either reaction as a result of absorption with coagulase-negative, clumping factor-negative (J17M or 604M) cocci, nor did L-form absorption have any appreciable effect, except in the case of strain 2253. In the three instances tested (strains 604, 3189 and 2095) absorption of antiserum with the concentrated supernatant fluid of L-form broth cultures caused no change in titres. The encapsulated, coagulase-positive, clumping factor-negative (SV or K6) cocci removed both agglutinating and clumping-inhibiting antibodies. However, as a result of absorption with these cocci there was a greater decrease in agglutination titres than in clumping inhibition titres.

Tables 1 and 2 show the agglutination and clumping inhibition titres, respectively, of antisera after repeated absorptions with variant staphylococci and with L-forms of the immunizing strain.

Table 2. *Homologous clumping inhibition titres before and after absorbing Staphylococcus aureus antisera with variants and L-forms of S. aureus*

S. aureus antiserum (immunizing strain)	Unabsorbed antiserum	Antiserum absorbed with: Cocci (strain) J17M*	604M*	SV†	K6†	L-form of immunizing *S. aureus* strain
J17	1/2560	1/1280	1/2560	1/160	1/320	1/1280
604	1/1280	1/1280	1/1280	1/20	1/40	1/640
3189	1/2560	1/2560	1/2560	1/160	1/640	1/1280
2253	1/320	1/320	1/320	1/20	1/40	1/160
2095	1/640	1/640	1/640	1/80	1/160	1/320
S	1/2560	1/1280	1/2560	1/320	1/640	1/1280

* J17M and 604M are coagulase-negative, clumping factor-negative variants of *S. aureus* strains J17 and 604M, respectively.

† SV and K6 are encapsulated, coagulase-positive strains which do not clump in fibrinogen.

Immunodiffusion reactions of L-forms. A zone of precipitate surrounded L-colonies on agar medium containing homologous *Staphylococcus aureus* antiserum. Absorption of the antiserum with cocci of the coagulase-negative (J17M or 640M) variants had no observable effect on this reaction. No precipitation developed when the antiserum was absorbed with typical *S. aureus* or with the encapsulated, coagulase-positive, clumping factor-negative (SV or K6) cocci.

Fluorescent-antibody reactions. Results with strain J17, as presented in Table 3, are typical of those obtained when other *Staphylococcus aureus* strains were tested with homologous antisera.

Pre-immune serum caused slight fluorescence of coagulase-positive strains, regardless of their clumping activity, but no fluorescence of the coagulase-negative variants or L-forms was observed. Fluorescence of coagulase-positive staphyloccoci was eliminated by absorption of pre-immune serum with any coagulase-positive strain, but not with coagulase-negative variants or L-forms.

Each unabsorbed antiserum produced bright fluorescence of all coagulase-positive strains and weak fluorescence of the coagulase-negative variants and L-forms.

Absorption of an antiserum with the homologous staphylococcus eliminated fluorescence of all staphylococci and L-forms tested. Use of coagulase-negative variant cocci as absorbing agents had no effect on the ability of an antiserum to produce fluorescence of typical *Staphylococcus aureus*, although the reaction of the encapsulated, coagulase-positive, clumping factor-negative (SV and K6) cocci was somewhat weakened. The fluorescent reaction of typical *S. aureus* was diminished and that of coagulase-negative variants and L-forms was eliminated by absorption of antisera with cocci of strains SV or K6. Surprisingly, absorption with SV or K6 cocci only slightly decreased the effectiveness of *S. aureus* antisera in producing fluorescence of these encapsulated cells.

Table 3. *Effect of absorption of Staphylococcus aureus strain J17 antiserum with clumping factor-negative variants and L-forms of S. aureus on indirect fluorescent-antibody reactions*

	Degree of fluorescence							
			Antiserum absorbed with					
S. aureus tested (strain)	Pre-immune serum	Unabsorbed immune serum	Cocci (strain) J17	Cocci (strain) J17M	Cocci (strain) 604M	Cocci (strain) SV	Cocci (strain) K6	L-form of immunizing strain (J17)
C+/CF+ *								
J17	+†	+++	−	+++	+++	++	++	+++
604	+	+++	−	+++	+++	++	++	+++
2095	+	+++	−	+++	+++	++	++	+++
2253	+	+++	−	+++	+++	++	++	+++
S	+	+++	−	+++	+++	++	++	+++
C+/CF−								
SV	+	+++	−	++	++	++	++	+++
K6	+	+++	−	++	++	++	++	+++
C−/CF−								
J17M	−	+	−	−	−	−	−	+
604M	−	+	−	−	−	−	−	+
L-form								
J17	−	+	−	+	+	−	−	−

* C, coagulase reaction; CF, clumping factor activity.
† Degrees of fluorescence: +++, Strong; ++, moderate; +, weak; −, negligible or none.

DISCUSSION

Variants of *Staphylococcus aureus* which are negative for both coagulase and clumping factor apparently possess few, if any, surface antigens characteristic of typical coagulase-positive staphylococci. These staphylococci were not agglutinated by antisera prepared against typical *S. aureus*. Absorption of anti-*S. aureus* sera with the cocci of coagulase-negative variants caused no significant decrease in *S. aureus* agglutination or clumping inhibition titres. It has been reported that the agglutinating antibody content of sera from rabbits immunized with coagulase-negative variants is remarkably low (Rotter & Kelly, 1966). Loss of protein A (antigen A), as well as of clumping factor antigen, accompanies coagulase-negative variation, as demonstrated by immunodiffusion studies (Blackstock & Kelly, 1968). Protein A has been recognized as a major agglutinogen of *S. aureus* (Mudd, Yoshida, Li & Lenhart, 1963).

The encapsulated variants tested (SV and K6) evidently have cell-wall antigens in

common with those of typical *Staphylococcus aureus* strains. Following absorption of *S. aureus* antisera with either SV or K6 cocci, homologous agglutination titres were drastically decreased. Partial removal of clumping-inhibiting antibodies by SV and K6 was an unexpected finding. Although they do not clump in the presence of fibrinogen, these staphylococci apparently have the antigen associated with the clumping reaction. The same conclusion was reached by Blackstock (1968), who found that antisera prepared against such encapsulated staphylococci contained high titres of clumping-inhibiting antibodies. Their failure to clump with fibrinogen could be due to a non-specific masking effect of the capsule. Another possibility is that a factor essential to cell-fibrinogen interaction may be separate from, though closely associated with, the antigen which functions in the clumping inhibition reaction (Kato & Omori, 1959). Recently, it was shown that the capsular polysaccharide of the coagulase-positive, clumping factor-negative staphylococci inhibited the fibrinogen precipitating activity of extracts of *S. aureus* (Blackstock *et al.* 1968). However, inhibition of clumping by this polysaccharide was not demonstrated.

L-forms of typical *Staphylococcus aureus* strains were no more effective in removing agglutinins and clumping-inhibiting antibodies than were coagulase negative, clumping factor-negative variants. A single absorption with the homologous L-form caused no change in titre. A second L-form absorption produced the results shown in Tables 1 and 2, i.e. a slight decrease in both agglutination and clumping-inhibition titres probably due to dilution of the antisera. Upon centrifugation of broth cultures, L-form growth was deposited in a viscous sediment with sufficient water content to account for the dilution.

Immunodiffusion experiments indicated that the L-forms liberated one or more antigens which were shared with the immunizing *Staphylococcus aureus* and with the encapsulated variants, but not with the coagulase-negative staphylococci. Free coagulase could be one such antigen. From results of preliminary studies, it appeared that clumping factor was not released into the medium by the L-form. Concentrated supernatant fluid of L-form broth cultures did not interfere with the clumping-inhibiting antibody activity of antisera.

Certain results of the fluorescent-antibody tests are noteworthy. Although pre-immune rabbit sera contained antibodies which caused slight fluorescence of coagulase-positive staphylococci, these sera failed to react with the coagulase-negative variants or the L-forms. Immunization with *Staphylococcus aureus* induced antibodies which gave weak fluorescence with both coagulase-negative variants and L-forms. Identical antigens have been found in extracts of the membranes of the coccal form and the L-form of *S. aureus* (Pratt, 1966). Absorption of antisera with either encapsulated or nonencapsulated, coagulase-positive staphylococci eliminated the fluorescent reaction of L-forms. Coagulase-negative variants were unable to remove the antibody responsible for fluorescence of L-forms.

Evidence that the L-form does not possess clumping factor can be found in results of the clumping-inhibition and fluorescent-antibody tests. L-forms did not remove clumping-inhibiting antibodies from *Staphylococcus aureus* antiserum. By use of the encapsulated staphylococci (SV and K6) as absorbing antigens, antisera with agglutinin titres of 1/10 or less and clumping inhibition titres in the range of 1/80 to 1/640 were prepared. Such antisera did not cause fluorescence of the L-forms

Application of the fluorescent-antibody technique to L-forms was most successful

when growth was taken from broth or soft agar cultures. Entire colonies, easily separated from the soft agar medium, were particularly suitable for these studies.

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Basidiospore Germination in the Wood-destroying Fungus *Lenzites saepiaria*

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SUMMARY

Basidiospores of *Lenzites saepiaria* are not dormant and resemble vegetative mycelium in physiological properties and in the way they initiate outgrowth on a suitable substrate. Moistened spores respire significantly and autolyse in the absence of growth substrates; glucose, acetate or succinate are oxidized without lag. Optimal germinants, such as malt extract, allow outgrowth of 95 % of the spores in 4 to 5 hr.

Studies with [^{14}C]leucine, [^{3}H]uridine, [^{3}H]thymidine, [^{59}Fe]$FeCl_3$ and [^{32}P]NaH_2PO_4 suggest that RNA and protein are synthesized at low rates which increase immediately after addition of a medium supporting germination; synthesis of DNA and Fe-porphyrins commences after approximately 2·5 hr. During germination and outgrowth, glucose is used primarily for synthesis of cellular material and little is converted to CO_2. Glucose is initially utilized by the hexose monophosphate shunt and the Embden–Meyerhof pathway becomes active as outgrowth progresses. Exogenous organic acids are utilized primarily as sources of energy or membrane synthesis; CO_2 evolution is high and incorporation of these substrates into cellular components is low. Much of the incorporation may involve the fixation of CO_2 produced from substrate oxidation.

INTRODUCTION

The prevalence of spore-forming organisms in nature attests to the survival advantage these structures confer. However, the roles that spores play in the life cycle of organisms may vary. The wood-rotting Basidiomycetes produce an astronomical number of spores. The great number of spores produced compared to the number that reach an environment in which they can grow would suggest that spores as dormant or resistant cells need not play a significant role in the life cycle of these organisms. If such is the case, it is reasonable to expect that 'spore germination' in these organisms is not a unique, irreversible event that transforms a metabolically inactive cell, but a process similar to the resumption of growth by vegetative hyphae. This report is concerned with spore germination in basidiospores of *Lenzites saepiaria* and the intracellular events involved in the transformation of a basidiospore into a growing hypha.

METHODS

Materials. All substrates were obtained commercially and used without further purification. Unless indicated otherwise, all compounds tested were dissolved in water and sterilized by autoclaving after adjusting pH to 5·4 to 5·5 with HCl or

KOH. The DuPont untreated cellulose film was soaked (12 hr) before use in 0·12 N-HCl and washed with distilled water. [^{32}P]sodium phosphate (monobasic), [5-^{3}H]uridine, [methyl-^{3}H]thymidine and [^{59}Fe]ferric chloride were obtained from New England Nuclear Corp., Boston, Mass. All other radioactive materials were obtained from Nuclear Chicago Corp., Des Plaines, Ill.

Spores of *Lenzites saepiaria* were collected from naturally produced fruiting bodies on rotting Southern Yellow Pine (*Pinus* sp.) and from laboratory-grown fruiting structures produced by a stock culture (FR-I). The spores were cast into a stream of dry air and caught on filter paper. Collected spores were stored over activated silica gel or $CaSO_4$ at 4°.

Mycelium of *Lenzites saepiaria* was grown by inoculating aseptically collected spores (10^6 to 10^8/l. medium) into 2 l. of sterile 1 % malt extract. Aeration and agitation were provided by bubbling sterile filtered air (1 l./min.) through the medium. Mycelial pellets were harvested at 36 to 72 hr by filtration on a fritted glass filter, washed with 250 ml. of cold sterile distilled water, remoistened to allow complete swelling of the mass and stored at 4°. Mycelial pellets were starved to deplete endogenous reserves by shaking in sterile distilled water for 24 hr. All experiments were conducted at 30° in the dark except short-term respirometry or radio-respirometry experiments.

Germination experiments. Germination in water suspension was studied as described previously (Walkinshaw & Scheld, 1965) except that aliquots were withdrawn and plated on water agar to eliminate counting difficulties from movement of liquid films. Germination was also studied on 0·3 % purified agar (Difco) containing various substrate combinations. Agar was melted by steaming 30 min. and substrates were added after steaming. The viability of spores in all experiments was ascertained by plating samples on 1·5 % purified agar and incubating 5 hr; during this time, non-viable spores lysed. Dry weights are corrected for the percentage of non-viable spores present.

Respiration measurement. Respirometry procedures were those of Umbreit, Burris & Stauffer (1964); detailed conditions are given in the text. The continuous radio-respirometry procedures developed by Wang *et al.* (1958) were used to measure the evolution of [^{14}C]carbon dioxide from [^{14}C]labelled substrates. The column contained 10 ml. of 2+1 absolute ethanol+monoethanolamine and 200 µl. samples were removed at intervals for determination of [^{14}C]carbon dioxide evolved. Corrections were applied for decrease in column volume resulting from evaporation and sampling.

Substrate incorporation. Spores were germinated with labelled substrates according to conditions given in the text. Periodically samples were removed and washed on membrane filters (B-6 Schleicher and Schuell Co., Keene, N.H.) with cold 5 % trichloroacetic acid (TCA). The filters were placed in vials and dried before addition of scintillation fluid.

Fractionation. Radioactive mycelium or spores (about 0·1 mg.) on a 25 mm. millipore filter (Solvinert, pore size 0·45 µ) in a millipore microfiltration apparatus heated by passing hot water or steam through a jacket on the filter funnel was washed successively with 10 ml. cold water followed by: (1) four 3 ml. volumes of cold 5 % TCA; (2) a 3 ml. rinse with 95 % ethanol followed by four 3 ml. volumes of Bloors solution (ether+ethanol 1+3, v/v), the filter heated to 55° for 3 min. during each wash; (3) three washes with 3 ml. of 5 % TCA held at 90° for 15 min. and a final 3 ml.

rinse without heating; (4) four 3 ml. washes with concentrated formic acid at 90° for 3 min. during each wash; (5) four 1 min. washes with 4 % KOH at 100°. These procedures separate selectively small molecules, lipid, nucleic acids (Schneider, 1945), protein and amorphous wall fractions. Washings from each treatment were combined and 100 μl. samples were counted for radioactivity. Wall material remaining on the filter after the hot KOH treatment was triturated overnight at 4° with 0·2 ml. 26N-H_2SO_4, diluted to 1 ml. with water and 100 μl. taken for radioactivity determination.

Radioactivity measurement. 12 ml. scintillation fluid [4 g. 2,5-diphenyloxazole and 0·1 g. 1,4-bis-2-(5-phenyloxazolyl) benzene/l.] was added to samples dehydrated with 2 ml. absolute ethanol, if necessary, and radioactivity assayed in a Mark I analyser (Nuclear Chicago Corp.).

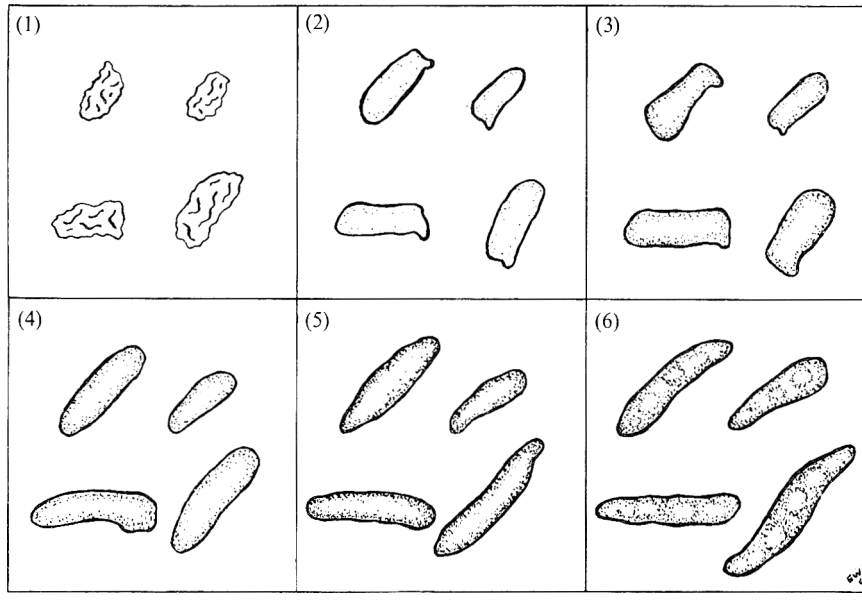


Fig. 1. *Lenzites saepiaria* basidiospores at different stages of swelling and outgrowth. (1) Dry spores at 0 hr; (2) spores after wetting at 0 hr; (3) to (6) spores 1, 2, 3, 4 hr after addition of germinant.

RESULTS

Properties of basidiospores. Damp *Lenzites saepiaria* spores or dry spores exposed to moist atmosphere lost viability within a few hours. Spores in free water lost viability in 1 to 3 days at 30° or after several weeks at 4°. Thirty-six hr mycelium could be incubated at 30° in water for over a month without complete loss of viability.

Visible germination. Germination of the basidiospore appeared as a uniform swelling of the bean-shaped spore with subsequent elongation of one or both ends into a hypha (Fig. 1). At the optimal temperature (30 to 35°) and with malt extract as substrate, 80 to 90 % of the spores exhibited outgrowth after 4 hr; under less optimal conditions, equivalent growth took several days.

Respiratory activity. Powder dry spores had low respiratory activity and instantaneously began to respire rapidly (Fig. 2*a*) in water. Mycelium outgrowing in malt extract (Fig. 2*b*) had a respiration rate similar to spores (Fig. 3*d*) but a significantly

lower oxygen uptake. The Respiratory Quotient (R.Q.) calculated for spores respiring endogenously and germinating in malt extract suggested (Table 1) that the respiration substrates were lipid and carbohydrate respectively. Respiration was sensitive to cell concentration, particularly at a higher temperature (Table 2). Under the conditions the germination rate in a respirometer vessel lagged behind the rate achieved in more dilute suspension.

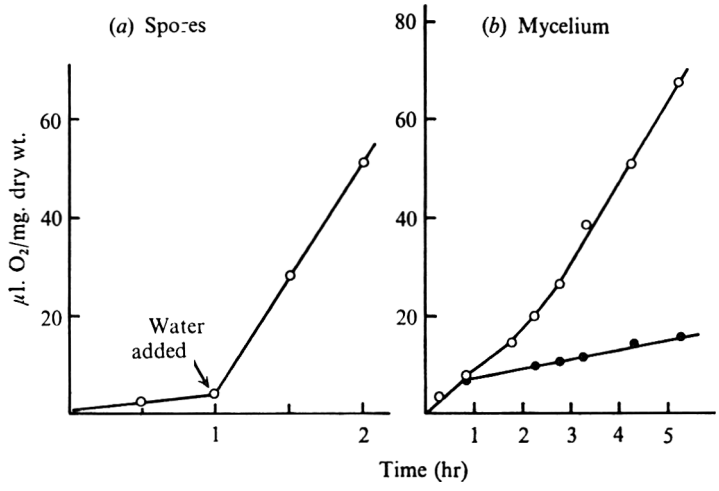


Fig. 2. Respiration by *Lenzites saepiaria* basidiospores before and after moistening (*a*) and by mycelium (*b*). For (*a*) desiccated spores were placed in the main chamber of a respirometer vessel and after 15 min. equilibration at 35° respiration was measured for 1 hr. Water was introduced through the vent plug, and after 15 min. equilibration, tipped into the main chamber. (*b*) Mycelium was grown from spores in 1 % malt extract, harvested and resuspended to the original volume in water. After starvation for 24 hr the mycelium was filtered, placed in respirometer vessels at 3 to 4 mg. dry wt/vessel and incubated at 30°. ●, Endogenous; ○, malt extract.

Table 1. *Respiration of Lenzites saepiaria spores on selected substrates*

One to two mg. dry wt spores were suspended in 5 mM potassium phosphate buffer, pH 5·4, and equilibrated 15 min. before adding substrate to final vol. 2·4 ml. and measuring respiration at 30° for 60 min. Values represent averages of two to three trials of two replicate determinations. Oxygen uptake in µl./mg. dry wt/hr.

Substrate	Concentration mg./flask	Oxygen uptake	R.Q.
Endogenous	—	45	0·62
Glucose	36	61·5	0·75
Succinate (Na)	3·2	66	0·81
Acetate (Na)	1·6	82·5	0·87
Pyruvate (Na)	2·2	72	1·12
Malt Extract	80	81	0·96

Synthetic activity. The synthesis of protein ([^{14}C]leucine), ribonucleic acid (RNA) ([^{3}H]uridine) and phosphate-containing compounds ([^{32}P]NaH_2PO_4) was initiated immediately on wetting spores while synthesis of deoxyribonucleic acid (DNA) ([^{3}H]thymidine) and respiratory pigments ([^{59}Fe]$FeCl_3$) started only after hyphal elongation was evident (Fig. 3). Synthesis by 36 hr mycelium under comparable conditions was similar.

Contribution of exogenous substrates to germination. Carbohydrates in water solution generally supported germination while amino acids and organic acids did not (Tables 3 and 4). Intermediate and short-chain fatty acids and some sugars, including cellobiose, inhibited germination in the presence of maltose.

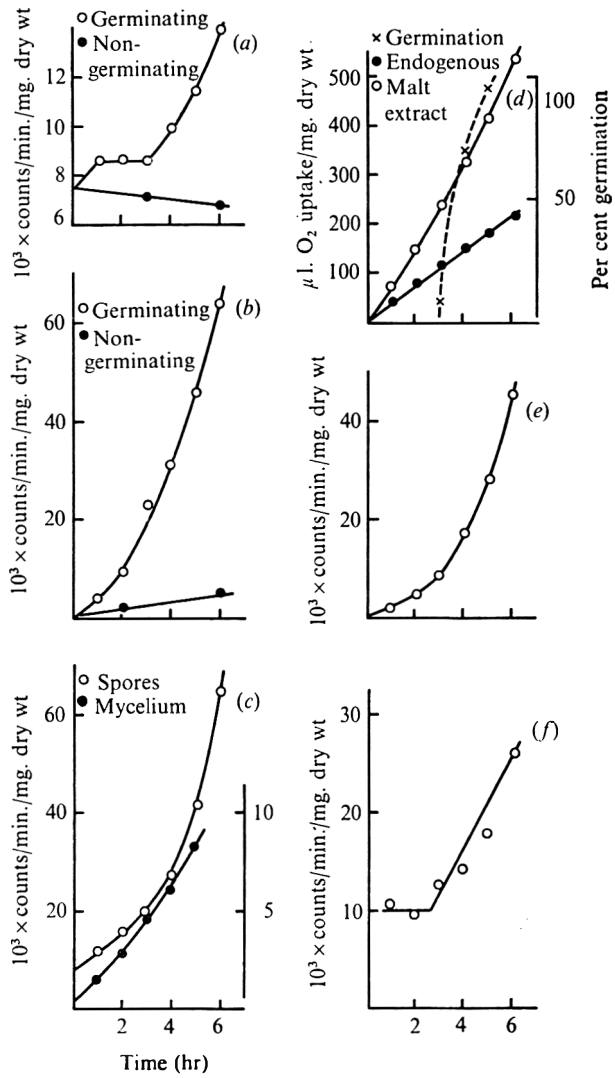


Fig. 3. Studies on the metabolic events occurring during the germination and initial outgrowth of *Lenzites saepiaria* basidiospores. For all experiments with labelled substrates, 0·7 mg. spores were shaken in 250 ml. flasks containing 70 ml. 1 % malt extract and labelled substrate. At intervals 5 ml. samples were removed and washed with water and then cold 5 % TCA on membrane filters. Labelled compounds were: (*a*) 0·05 μc. [^{59}Fe]$FeCl_3$; (*b*) 5 μc. [^{32}P]NaH_2PO_4; (*c*) 0·05 μc. [U-^{14}C]leucine; (*e*) 10 μc. [^{3}H]uridine; (*f*) 10μc. [^{3}H]thymidine. In (*c*) mycelium was grown for 36 hr from spores in 1 % malt extract, harvested, starved by shaking for 24 hr in water and added at 3 mg. dry wt/vessel. For the respirometer experiments in (*d*), 2 mg. spores were suspended in water in a respirometer vessel. After 15 min., 1·2 ml. 8 % malt extract was added from the side arm. Per cent germination was measured in flasks removed at the indicated times.

Purified agar at 1·5 % contained adequate nutrients to support germination. Agar at 0·3 %, melted by a brief steaming, did not support germination; in this, tricarboxylic acid cycle intermediates had a marked effect on the germination rates in contrast to their inactivity in water (Fig. 4). Maltose or glucose added together with stimulatory compounds accelerated germination and subsequent outgrowth further. High levels of CO_2 replaced organic acids when maltose was present.

Table 2. *Respiration of glucose by Lenzites saepiaria spores at various cell densities*

Spores were suspended in 2 ml. 5 mM potassium phosphate buffer, pH 5·4. 200 μmoles glucose added from the side arm after 15 min. equilibration. Oxygen uptake as μl. O_2/mg. dry wt/hr. Values are means of triplicate determinations.

Spores (mg./vessel)	Oxygen uptake	
	At 30°	At 35°
0·85	37	48
1·70	44	59
3·40	47	52
6·80	44	27

Table 3. *Germination of Lenzites saepiaria basidiospores with various carbon sources*

Spores from wild or laboratory-grown fruiting bodies were cast directly on to 1 mm. films of distilled water in glass Petri dishes and incubated at 30°. Carbohydrates at 0·1 M, amino acids (adjusted to pH 5·4 with KOH) at 0·01 M. Mean % germination recorded after 18 hr for four to six separate trials.

Carbon source	% germination
Maltose	95
Fructose	70
Glucose	65
Mannose	60
Cellulose (Cellophane)	60
Starch (soluble)	95
Xylose	55
Sucrose	55
Ribose	45
Galactose	45
Gluconic acid	35
Lactose	25
Glutamic acid	90
Aspartic acid	75

Utilization of uniformly labelled carbon compounds. Seventy-five to 80 % of the [U-^{14}C]glucose metabolized by spores germinated in malt extract was incorporated (Fig. 5). Spores incubated with glucose in buffer (20 μmoles glucose/mg. spores) behaved similarly. Outgrowth experiments employing continuous collection procedures indicated a similar incorporation pattern by spores which had outgrown for 6 hr; there was a reduced ratio in 36 hr mycelium (Fig. 6). Spores germinated in uniformly labelled succinate or acetate incorporated 50 % or more of the labelled substrate metabolized during the first 60 min. of germination. After 60 min. incorporation declined abruptly and the spores began to evolve a major portion of the labelled substrate as CO_2 (Fig. 7). An appreciable lag in CO_2 evolution during the first 60 min. of growth of starved mycelia (Fig. 6) suggested that the same change in ratio of uptake of labelled substrate to CO_2 evolution occurred.

Table 4. *Tests of various compounds for synergy with maltose in the germination of Lenzites saepiaria spores*

The compounds were tested under the conditions given in Table 3 in maltose in water and in maltose in 0·3 % purified agar. Germination was arbitrarily considered significant if 20 % or more were germinated at 18 hr.

Stimulatory compounds	Inhibitory compounds	Compounds having no effect
Lactate	Cellobiose	Mannitol
Pyruvate	Sorbose	Sorbitol
Acetate	Rhamnose	Arabinose
Citrate	Trehalose	Glucosamine HCl
α-Oxo-glutarate	Caprate	*N*-Acetyl-α-D-glucosamine
Succinate	Caprylate	Turanose
Malate	Caproate	Myristate*
Oxalacetate	Valerate	Laurate*
Oleate*	Butyrate	
Stearate*	Propionate	
Palmitate*	Cysteine	
Leucine		
Lysine		
Alanine		
Glutamine		
Phenylalanine		
Tryptophan		
Methionine		
Arginine		

* Tested at 0·001 M.

Table 5. *Fates of [UL-^{14}C]glucose and [UL-^{14}C]acetate added to proliferating mycelium and germinating spores of Lenzites saepiaria*

Mycelium from a spore inoculum was harvested from malt extract culture after 36 hr, washed and incubated in 5 mM potassium phosphate buffer, pH 5·5, with labelled substrate and extracted after 4 hr. Spores germinated in malt extract plus the indicated substrates were harvested and extracted after 6 hr. Recorded as % total activity incorporated in mycelium or germinated spores.

	Mycelium		Spores	
Fraction	[UL-^{14}C]glucose	[UL-^{14}C]acetate	[UL-^{14}C]glucose	[UL-^{14}C]acetate
Cold TCA	3·9	25·2	8·1	34·6
Ethanol-ether	6·0	21·7	18·7	34·5
Hot TCA	33·0	10·7	23·1	9·8
Hot formic	27·0	26·9	21·6	11·3
Hot KOH	10·6	7·3	12·0	2·4
Residue (cell wall)	19·5	8·2	16·4	7·3

In both spores and 36 hr mycelium, a high proportion of the metabolized glucose was incorporated into nucleic acid and wall fractions. A high proportion of the incorporated acetate was found in lipid and protein fractions (Table 5). Some label from glucose was incorporated into the KOH-insoluble wall fraction in less than 1 hr after initiation of germination. During the first hr of incubation, most of the acetate incorporated was in the lipid fraction (Fig. 7). Succinate and propionate were incorporated in the same manner as acetate.

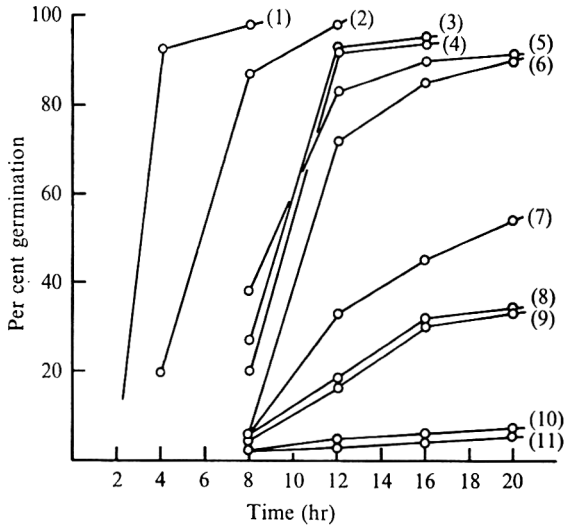


Fig. 4

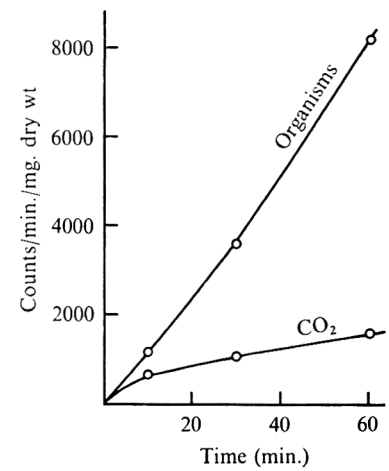


Fig. 5

Fig. 4. Germination of *Lenzites saepiaria* basidiospores with various carbon sources. Agar (0·3 %) was melted by steaming for 30 min. and cooled to 55° before addition of substrates. Carbohydrates were added directly to 1 % (w/v); amino acids and organic acids were added as 0·1 M solutions to 0·01 M. Spores were cast directly on to the agar surface. Test no. 3 was made in a desiccator from which 25 % of the air was withdrawn and replaced with CO_2. Incubation at 30°. Counts were made of 100 spores/plate in 6 replicates. (1) 1 % malt extract; (2) maltose+acetate; (3) maltose+25 % CO_2; (4) glucose+acetate; (5) glutamic acid; (6) Na acetate; (7) Na α-oxo-glutarate; (8) maltose; (9) Na succinate; (10) glucose; (11) glycine.

Fig. 5. Utilization of [^{14}C]glucose by *Lenzites saepiaria* basidiospores germinating in malt extract. Spores germinated at 30° in 4 % malt extract in respirometer vessels with 1 μc. (1 μc/μmole) [U-^{14}C]glucose. [^{14}C]carbon dioxide was trapped in 20 % KOH on filter papers in the centre well. At intervals, germination in selected flasks was stopped by adding 0·2 ml. 2N-H_2SO_4 from the side arm. Papers were removed, damp-dried, soaked in 2 ml. of absolute ethanol and counted. Organisms were washed with cold water (2 × 5 ml.) followed by cold 5 % TCA (2 × 5 ml.) on membrane filters. Filters were dried and counted.

Table 6. *Evolution of* [^{14}C]*carbon dioxide by basidiospores of Lenzites saepiaria incubated with specifically labelled glucose at different stages of outgrowth*

Spores germinated in dilute suspension in a large volume of malt extract broth. Duplicate samples containing about 2 mg. spores were withdrawn at intervals, centrifuged, washed twice and resuspended in 10 ml. of 5 mM phosphate buffer, pH 5·4. After 10 min., 1 μmole specifically labelled glucose (0·1 μc./μmole) was added and the CO_2 evolved was collected continuously.

Hours after initiation of germination	c.p.m. evolved as [^{14}C]carbon dioxide after 2 hr exposure to labelled substrate at 30°	
	[1-^{14}C]glucose	[3,4-^{14}C]glucose
0	10,774	817
1	12,000	1,237
3	18,897	2,746
8	11,872	35,567

Utilization of specifically labelled carbon compounds. In ungerminated spores and during the early stages of outgrowth most of the CO_2 evolved from glucose originated from the C–1 position indicating that the pentose phosphate shunt was the predominant route of glucose metabolism. As outgrowth progressed, there was an increasing proportion that originated from the C–3 and C–4 positions suggesting that glycolytic activity became dominant (Table 6).

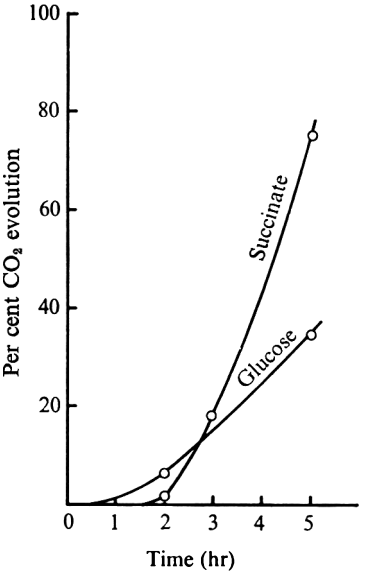


Fig. 6. Utilization of [^{14}C]glucose and succinate by *Lenzites saepiaria* mycelium outgrowing in malt extract. Mycelium was grown from spores in 1 % malt extract, harvested at 36 hr, starved by incubation for 24 hr in water and 4 mg. added to 25 ml. medium in 125 ml. Erlenmeyer flasks fitted with side arms and an inlet tube for scrubbing gas. The medium was 1 % malt extract with 1 μc. [U-^{14}C]glucose or 0·75 μc. [U-^{14}C]succinate. After 10 min. equilibration the mycelium was added from the side arm. [^{14}C]CO_2 evolution was measured continuously (see Methods). Incorporation of label was measured at the end of the experiment by filtering a sample and washing with cold 5 % TCA on a membrane filter and counting. CO_2 evolution as % total carbon utilized.

Table 7. *Evolution of* [^{14}C]*carbon dioxide from specifically labelled propionate by spores of Lenzites saepiaria germinating in malt extract*

After 10 min. equilibration, spores (about 2 mg.) were dumped from the side arm of a 125 ml. flask into the main compartment containing 25 ml. of 1 % malt extract and 0·5 μmoles Na propionate. The flask was fitted with an inlet tube for scrubbing gas which flowed into an absorption column. The absorption column contained initially 10 ml. of 2:1 ethanol-ethanolamine and samples for CO_2 estimation were withdrawn hourly. Incorporation was measured at the end by withdrawing a sample of spores, filtering onto a membrane filter, washing with cold 5 % TCA and counting. CO_2 evolution as % total carbon taken up (CO_2 evolved + propionate incorporated) in 4 hr.

	[^{14}C]carbon dioxide derived from position		
Incubation (hr)	C–1	C–2	C–3
1	2·6	0·1	2·1
2	23·7	6·9	12·6
3	48·4	21·1	24·5
4	82·2	41·0	35·5

The high R.Q. values suggested that CO_2 originated from simple decarboxylation of organic acids. Experiments with several specifically labelled organic acids, including propionic acid (normally considered an inhibitor of fungal growth), indicated that utilization was via normal pathways (Table 7).

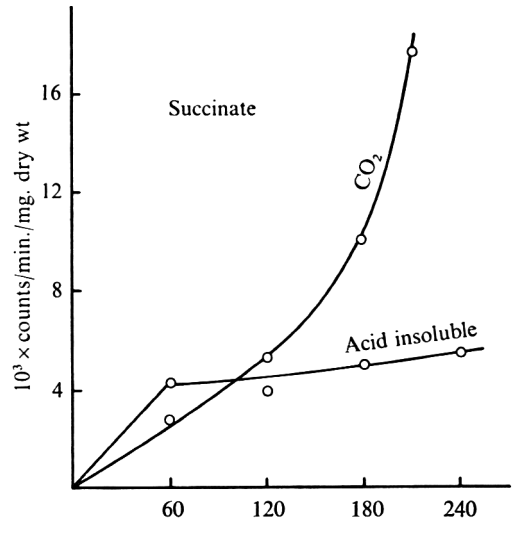
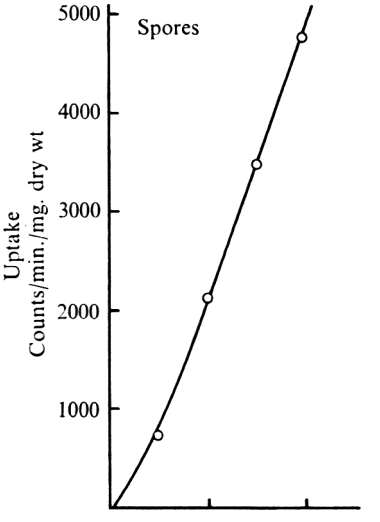


Fig. 7



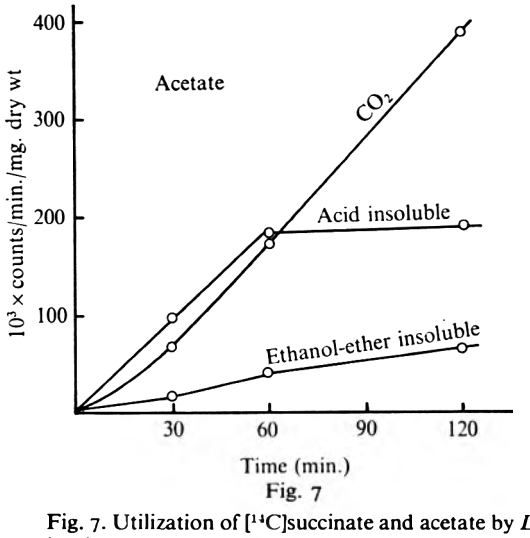


Fig. 8

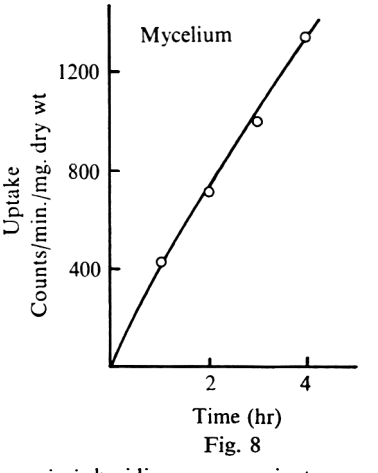


Fig. 7. Utilization of [^{14}C]succinate and acetate by *Lenzites saepiaria* basidiospores germinating in malt extract. Spores germinated at 30° in 4 % malt extract in respirometer vessels with 0·75 μC. (1 μC./μmole) [U-^{14}C]succinate or [U-^{14}C]acetate. [^{14}C]Carbon dioxide was trapped in 20 % KOH on filter papers in the centre wells. At intervals, germination in selected flasks was stopped by adding 0·2 ml. 2N-H_2SO_4 from the side arm. Centre well papers were damp-dried, soaked with 2 ml. absolute ethanol and counted. Parallel samples were filtered on Teflon membrane filters and washed with cold water (2 × 5 ml.) followed by cold 5 % TCA (2 × 5 ml.) or with cold water and TCA followed by hot (50°) ethanol-ether, 2:1 (3 × 3 ml.). Filters were oven dried then counted.

Fig. 8. Incorporation of [^{14}C]glucose by germinating spores and outgrowing mycelium of *Lenzites saepiaria*. Approximately 0·7 mg. spores or 3·0 mg. of 36 hr mycelium were added to 250 ml. flasks containing 70 ml. of 1 % malt extract and 1 μC. [U-^{14}C]glucose and shaken at 30° in a water bath. At intervals 5 ml. samples were washed with cold water and cold 5 % TCA on membrane filters. Filters were oven dried and counted.

Fixation of CO_2 in germination. In all experiments involving CO_2 collection a lag in early CO_2 evolution was observed which implied fixation of the metabolic CO_2. Added [^{14}C]carbon dioxide was fixed in germinating spores; incubation under 25 % CO_2 decreased incorporation of [^{14}C]acetate by 30 % during the first hr of germination.

Utilization of reserves during germination. Lipids were apparently present in basidiospores but no significant amount of carbohydrate reserve. The absence of carbohydrate reserves was reflected in the abrupt glucose incorporation curves exhibited by both spores and mycelium (Fig. 8).

DISCUSSION

The term germination used throughout this report is probably inappropriate when applied to the process whereby *Lenzites saepiaria* basidiospores initiate outgrowth. Germination usually implies a unique and irreversible event in a specialized cell. The basidiospores of this organism appeared relatively unspecialized in spite of their origin and distinct morphology. They exhibited no remarkable degree of resistance to a harsh environment, excepting desiccation, and in all other respects were less able to survive than vegetative hyphal cells.

The physical appearance of germinating basidiospores also suggested an absence of specialization. There was no clearly defined transition point between spore swelling and hyphal elongation, and the original spore cell quickly became indistinguishable from the elongating hypha. Ultrastructurally basidiospores and mycelium are very similar (Hyde & Walkinshaw, 1966). Basidiospores contain a full complement of cell organelles and do not synthesize additional ones until outgrowth occurs. Further, no striking changes in cellular or cell wall organization occurred upon outgrowth.

Exposure of basidiospores to germination (outgrowth) conditions did not result in respiration or synthesis patterns expected of cells which must undergo metamorphosis in order to grow. Respiratory rates were high in ungerminated spores and increased only as the amount of cell material increased. There was no apparent need for synthesis of protein before outgrowth began. Incorporation curves which served as indices of RNA and protein synthesis (Fig. 3) suggest exponential growth curves having their origins at zero time. Moreover, the abrupt glucose incorporation curve suggested that enzyme systems required for the utilization of this substrate were present and fully active.

Qualitatively there was little difference in the patterns of respiration, synthesis of cellular components, or utilization of substrates by outgrowing basidiospores and vegetative mycelium. The few qualitative differences and the generally lower activity of mycelium probably reflected the presence of a substantial number of older cells with a decreased metabolic rate in the mycelium.

Most general studies of spore germination include data on respiration. The impression gained from the present studies was that respiration as measured was useful only as a general indication of relative activity. Any experiment with *Lenzites saepiaria* spores under the crowded conditions of a respirometer vessel was subject to error and should be interpreted with caution. The oxygen uptake for *L. saepiaria* spores was higher than those reported for other fungal spores (Barash, Conway & Howard, 1967; Niederpruem, 1964; Gottlieb & Caltrider, 1963; Tokoro & Yanagita, 1966; Walkinshaw, 1968). It is possible that spores of other fungi respire at a lower rate, but spore respiration has usually been measured in much heavier suspensions than

were found optimal for this organism. Outgrowth in a respirometer vessel lagged behind that in dilute suspension even though the highest oxygen uptake was observed in the denser suspensions (Table 2). Also, there was no difference in rate of outgrowth at 30° and 35° although a significant difference in oxygen uptake was observed. Respiration of various substrates and the rate of outgrowth may not be directly related (Walkinshaw, 1968).

A shift in the pathways of glucose utilization in outgrowing spores has been noted for other fungi (Newburgh & Cheldelin, 1958). While this change may represent a transformation from spore metabolism to mycelial cell metabolism, an alternative explanation is that the pentose phosphate activity remains associated with the hyphal tip, and that glycolytic activity develops in cells or parts of the cell behind the differentiating apex. In support of this, the pentose phosphate activity (as measured by the preponderance of CO_2 originating from the C–1 of glucose) during the first 8 hr remained essentially constant as did the volume of functional hyphal tip. Glycolytic activity increased steadily, paralleling the increase in cell volume. It is reasonable to expect localization of activity at or near the growing tip since this is where the requirement for pentose for nucleotides, as well as reducing power for membrane synthesis, would be greatest. Yanagita & Kogane (1963) in the Aspergilli, demonstrated that there is no forward transport of [^{32}P]-labelled compounds; further, there are large pools of nucleotides in the apex (Nishi, Yanagita & Maruyama, 1968). A similar situation might be expected in *Lenzites saepiaria.*

During the first hour of incubation in a germination medium, most of the label from exogenously added [^{14}C]organic acids was extracted from spores in the lipid fraction. This suggested that lipid synthesis was one of the early events in basidiospores that began outgrowth. Membrane synthesis or changes in configuration would be expected as a preliminary to wall synthesis. The stimulatory effect of organic acids could thus be explained, although the high endogenous respiration and apparently adequate lipid reserve was inconsistent with a requirement for exogenous materials. CO_2 alone could stimulate germination in the same manner as organic acids and an increased CO_2 tension (Fig. 4) displaced the incorporation of [^{14}C]acetate into cells. Obviously metabolic CO_2 was rapidly fixed. Since membrane synthesis occurred concomitantly with the initiation of outgrowth, it was likely that the CO_2 was required for lipid and membrane synthesis as suggested by Choe & Bertani (1968). One of the main roles of exogenous organic acids could be the generation of CO_2.

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Some Properties of the Galactanase Secreted by *Phytophthora infestans* (Mont.) De Bary

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SUMMARY

Studies on the galactanase produced by *Phytophthora infestans* were facilitated by an improved culture medium for enzyme production. The enzyme degraded potato pectin until a proportion of the galactose units were released; short oligosaccharides probably occurred among the products of the reaction. The enzyme rapidly reduced the viscosity of potato and lupin pectin solutions, with a slower concomitant release of reducing groups. In addition it released total carbohydrate more rapidly than galactose from potato cell walls; these results indicate that the enzyme is an endo glycano-hydrolase. It had a limited effect on the cohesion of discs of potato tuber tissue.

INTRODUCTION

Most fungal plant pathogens produce enzymes which degrade polygalacturonate and a smaller number produce enzymes which degrade cellulose (Wood, 1967); microbial polygalacturonate hydrolases and lyases and cellulases are quite well characterized (Bateman & Millar, 1966; Demain & Phaff, 1957; Wood, 1960). On the other hand, glycosidases which degrade araban, galactan and xylan, which are often important components of plant cell walls, have been less widely found and less fully characterized. Arabanases have been reported from various microbial sources (Byrde & Fielding, 1965; Fuchs, Jobsen & Wouts, 1965; McClendon, Somers & Heuberger, 1960) and the arabanase secreted by *Phytophthora palmivora* has been examined in some detail (Akinrefon, 1968). Xylanases are known to be secreted by certain fungal plant pathogens (Hancock, 1967; Sumere, Sumere-de Preter & Ledingham, 1957). Galactanase activity has been attributed to *Botryosphaeria ribis* (McClendon *et al.* 1960) and to *Sclerotinia sclerotiorum* (Hancock, 1967), though never studied in any detail.

However, galactanase activity has been found in culture filtrates of *Phytophthora infestans* (Knee & Friend, 1968). The enzyme liberated galactose from potato pectin and from lupin pectin which contains a β-1,4 linked galactose polymer (Hirst, Jones & Walder, 1947) and it had negligible β-galactosidase activity. The object of this paper is to report more fully on this enzyme. *Phytophthora infestans* produces pectin methyl esterase (Clarke, 1966; Grossmann, 1963) and though Grossmann (1963) reports the presence of endopolygalacturonase, this was not found by Clarke (1966) nor was any evidence of it encountered in the work described below.

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METHODS

In general the methods for culture of *Phytophthora infestans*, enzyme and substrate preparation, and enzyme assay were as described previously (Knee & Friend, 1968). In the previous work low activities had been a hindrance and it was found that the addition of casein hydrolysate (Difco Ltd.) at the rate of 2 g./l. to frozen bean medium resulted in greatly enhanced activity in the culture filtrates. Although a supplement of potato or lupin pectin gave a smaller enhancement it gave no advantage when added with casein hydrolysate. It was convenient to remove reducing sugars from the filtrates and further concentrate activity by ammonium sulphate precipitation, resuspension and dialysis; approximately 70 % of total activity was recovered after these procedures. For routine assay of these more active preparations reducing sugar was estimated in duplicate 0·5 ml. samples taken at time intervals from a reaction mixture comprising 0·2 ml. enzyme, 2·0 ml. potato pectin solution (5 mg./ml.), 1·0 ml. citrate-phosphate buffer (pH 4·0, 0·062 *M* with respect to citrate) and 1·8 ml. water.

RESULTS

pH activity relationship

Using the assay procedure of Knee & Friend (1968), the activity of *Phytophthora infestans* galactanase was studied in relation to pH values between 2·5 and 7·0 (Fig. 1). Below pH 4·0 the reaction slowed markedly with time so that while pH 3·5 gave the optimal value at 30 min, at 60 and 90 min. the optimum shifted to between pH 4·0 and 5·0. When the 30 min. reaction period was preceded by a 60 min. incubation of the enzyme in buffer without substrate, the measured enzyme activity was reduced more at pH 3·5 than at pH 4·5.

Extent of degradation of substrate

Acid hydrolysis of potato pectin liberates galactose, estimated chromatographically (Wilson, 1959) as 3·2 μmoles/mg. After 24 hr digestion of potato pectin with galactanase the reducing sugar liberated was 2·4 μmoles/mg., estimated in terms of galactose equivalents. However, paper chromatography (Fischer & Dörfel, 1955) of this digest, followed by estimation of the galactose, indicated that 1·5 μmoles/mg. were present. Other spots, which gave a weak reaction with aniline hydrogen phthalate reagent, were visible on this chromatogram; they did not correspond in position to arabinose or galacturonic acid (the other monosaccharide residues present in potato pectin) and may have been galactose-containing oligosaccharides.

It was thought possible that a more complete liberation of galactose might occur at low substrate concentrations. Thus initial activity and galactose produced in 24 hr were estimated using a range of potato pectin concentrations, but the same enzyme concentration throughout.

From the results shown in Fig. 2 it can be seen that after a rapid liberation of reducing sugar, the extent of which increased with substrate concentration, a slower reaction set in so that after 24 hr a constant proportion of the substrate was converted to reducing sugar. The reaction followed a similar course with lupin pectin as substrate.

Viscosity change during action of galactanase

Potato and lupin pectin do not form highly viscous solutions. High concentrations (20 mg./ml. digest) of the pectins were necessary to follow changes in viscosity during the action of galactanase. Viscosity measurements were based on the time taken for the meniscus of a solution at 20° to pass between two graduations on a

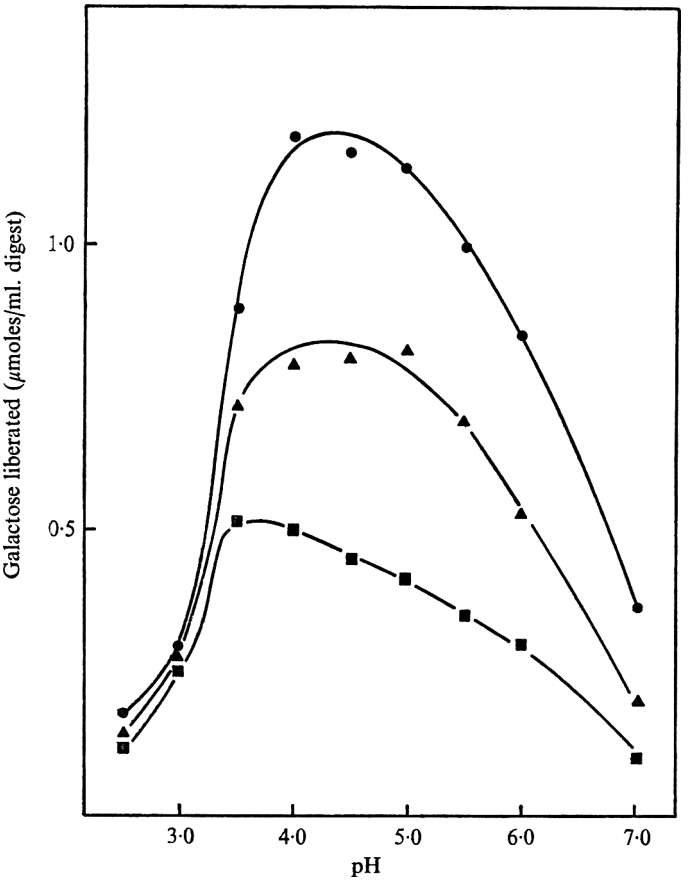

Fig. 1. pH/activity relationship of *Phytophthora infestans* galactanase. —●—●—, Galactose released after 90 min. incubation; —▲—▲—, galactose released after 60 min. incubation; —■—■—, galactose released after 30 min. incubation.

vertically held pipette. As can be seen from Fig. 3, the enzyme rapidly reduced the viscosity of lupin pectin until about 2 hr after the start of the experiment when a limiting value was approached. On the other hand reducing sugar was liberated at a more even rate over 4 hr, which was the duration of the experiment. From the experiments previously described it was to be expected that 48 μmoles/mg. reducing sugar would be formed if this reaction had gone to completion; at 2 hr, when viscosity had almost reached its lowest value, less than 10 μmoles had been released.

The viscosity of a reaction mixture including boiled enzyme did not change throughout the course of the experiment and essentially similar results were obtained when potato pectin was used as a substrate.

Potato tuber cell walls as a substrate for galactanase

Potato and lupin pectin are degradation products of polymers present in intact cell walls. In assessing the function of galactanase in the pathogenic interaction of *Phytophthora infestans* and potato tissue it is important to know how the native polymers would be attacked. Thus potato cell walls were prepared with the minimum of destructive pretreatment as follows.

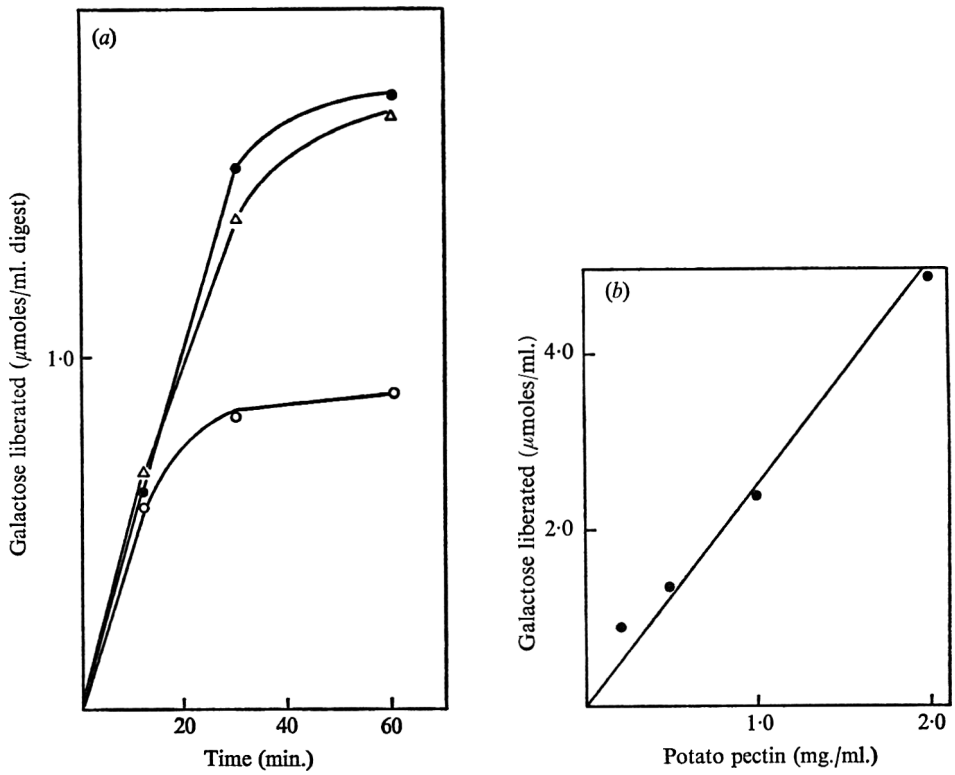

Fig. 2. Degradation of potato and lupin pectins by *Phytophthora infestans* galactanase. (*a*) Initial reaction rates. ●—●, Potato pectin substrate (1·0 mg./ml.); ○—○, potato pectin substrate (0·5 mg./ml.); △—△, lupin pectin substrate (1·0 mg./ml.). (*b*) Extent of degradation of potato pectin after 24 hr incubation with the enzyme; the effect of substrate concentration.

Peeled King Edward potatoes were chopped and 100 g. disintegrated in a blender in 200 ml. of acetone, chilled to −20°. The slurry was filtered through cheese cloth and the residue washed with a further 200 ml. chilled acetone, followed by 200 ml. chilled 70% ethanol and 1000 ml. iced water. The remaining 'paste' was frozen at −20° and ground to a powder in a chilled pestle and mortar; this powder was resuspended in 0·05 M-phosphate buffer (pH 7·5), filtered on muslin, washed with more buffer, followed by water and finally frozen at −40°. This procedure gave a cell wall preparation relatively free from starch and cytoplasmic contamination, as seen under the microscope, and it did not darken in colour on being allowed to stand in air.

A quantity of the frozen cell wall paste was suspended in deionized water to give approximately 12 mg. cell wall/ml. suspension, equivalent to 5 mg./ml. potato pectin.

Samples (2·0 ml.) of this wall suspension were transferred to flasks to which other additions were as for routine assay of the enzyme. Some of the flasks included fresh enzyme while in others the enzyme had been held at 100° for 10 min.; the flasks were incubated at 25° with constant agitation for definite periods of time after which the contents were filtered on sintered glass (porosity 3) and carbohydrate in the filtrates estimated by three methods.

Negligible quantities of carbohydrate appeared in the filtrates from incubations

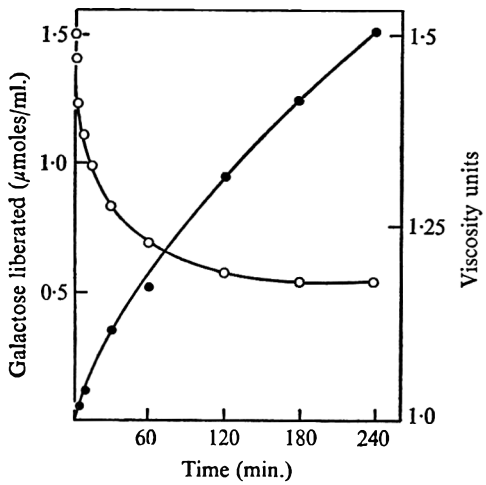

Fig. 3

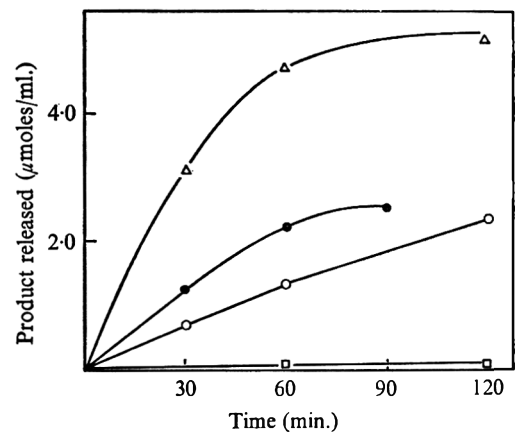

Fig. 4

Fig. 3. Viscosity change and reducing sugar liberated by action of *Phytophthora infestans* galactanase on lupin pectin. O—O, Change of viscosity relative to water; ●—●, reducing sugar liberated.

Fig. 4. Action of a *Phytophthora infestans* galactanase preparation on potato tuber cell walls and on potato pectin. △—△, Total carbohydrate released from wall; O—O, reducing sugar released from wall; □—□, uronide released from wall; ●—●, reducing sugar released from potato pectin.

with boiled enzyme. In filtrates from incubations with fresh enzyme total carbohydrate, estimated by the procedure of Devor (1950), increased rapidly to near a limiting value at two hours (Fig. 4). In these estimations, using randomly presulphonated α-naphthol reagent, the absorption spectra of the products in the range 400 nm. to 600 nm. were typical of the sugar residues present (M. Knee & J. Friend, unpublished results). In the filtrates from galactanase digests of the cell walls the relative absorbances at 480 nm., 555 nm. and 575 nm. were almost exactly as predicted from a pure galactose sample.

Reducing sugar was liberated from the wall preparation at a little more than half the rate from a potato pectin substrate, and accounted for an increasing proportion of the total carbohydrate released (Fig. 4).

Uronide material in the filtrates was estimated by the procedure of McCready & McComb (1952) and corrected for neutral sugar present. As can be seen from Fig. 4, very little was liberated in the course of the experiment.

Action of galactanase on potato tuber discs

Discs of King Edward potato tuber (1·0 cm. diameter, 0·2 mm. thick) were floated in batches of twenty on citrate-phosphate buffer (pH 4·0) in Petri dishes. A galactanase preparation was added to some of the dishes, to others was added a solution of commercial Pectinase (Sigma Chemicals Ltd.) of comparable glycosidase activity, and a control dish was included where water was added in place of enzyme.

The discs in various solutions were tested at various times for cohesion by attempting to draw them apart with mounted needles and by lifting them from the solution with a pair of forceps. Where galactanase was present the discs remained firm for several hours; 24 hr after the start of the experiment there was a marked loss of cohesion, assessed with mounted needles, by comparison with control discs. At this stage the Pectinase-treated discs were completely lacking in cohesion.

DISCUSSION

In a previous paper (Knee & Friend, 1968) it was reported that galactanase activity was detected in culture filtrates when *Phytophthora infestans* was grown on media containing lupin or potato pectin. It now appears that these supplements are not necessary for enzyme production; on the other hand the possibility that galactanase is an adaptive enzyme cannot be excluded as the basic culture medium may have included substances responsible for enzyme induction.

It was suggested previously that the simplest explanation of the data on the pH/activity relationship is that the enzyme is progressively inactivated at low pH values. Some experimental support for this suggestion was obtained by pre-incubating the enzyme with buffer at various low pH values for 1 hr before addition of substrate. The pre-incubated samples showed less activity than untreated controls: 60 % activity was retained at pH 3·5 and 70 % at pH 4·5. If this explanation is correct then the results discussed below are subject to the proviso that activity has been studied at a pH value somewhat above the optimum for activity.

In the reaction of galactanase with potato pectin at pH 4·0 a rapid liberation of reducing sugar occurred initially and continued for a period of time dependent upon the substrate concentration. Subsequently there was a much slower release of reducing groups until after 24 hr a constant proportion of the original substrate had been degraded. Quantitative paper chromatography showed that free galactose comprised less than two thirds of the reducing sugar at 24 hr. The presence of probable oligosaccharides, which could not be estimated, on the chromatogram, may have accounted for the difference and A. L. J. Cole (personal communication) has confirmed the likely presence of the dimer, trimer and tetramer of galactose by another chromatographic method. It is characteristic of endoglycosidases that short oligosaccharides such as the dimer and trimer are not attacked or are attacked slowly (Phaff, 1966; Schwimmer, 1950; Whelan & Roberts, 1953), and the relative progress of the curves for loss of viscosity and liberation of reducing sugar from potato and lupin pectin are suggestive of an endoglycosidase, as is the reaction of galactanase with a tuber cell wall preparation. The excess of total carbohydrate over reducing sugar liberated in the latter experiment probably reflects the polymeric state of the material released; once in solution this material would be further degraded by the enzyme. Thus two

reactions probably occur, attack on the substrate in the wall leading to its dissolution and further degradation in solution.

Little or no uronide-containing material was liberated from the potato tuber cell wall preparation. This suggests that galactan in the walls is either independent of polyuronide or is attached to it only as side chains. Work on pectin composition has emphasised the importance of polyuronide as a structural component and the negative influence, if any, of galactan and araban side chains on the cohesive properties of this polymer (Gould, Rees, Richardson & Steele, 1965; Stoddart, Barrett & Northcote, 1967).

However, galactan forms a large proportion of potato tuber cell walls (Friend & Knee, 1969), and galactanase had a marked physical effect on a potato pectin substrate and a lesser effect on the cohesion of potato discs. Thus the enzyme might aid the penetration of tuber tissue during pathogenesis by *Phytophthora infestans*. Though activity was present in infected potato tuber discs degradation of galactan in this material was limited (Friend & Knee, 1969).

The limitations of the work stem from various sources, particularly the fact that both enzyme and substrates must be regarded as impure. Galactan as such probably does not exist in nature (Barrett & Northcote, 1965) though it is possible to prepare relatively pure galactose polymers, for instance from lupin pectin (Hirst *et al.* 1947). In view of the degraded nature of such a preparation it would be necessary to determine the average degree of polymerization of the polymer molecules, or preferably to derive fractions of uniform molecular weight by gel filtration, which could be tested separately as substrates. Analogous work has been carried out on α-amylase from various sources (Bird & Hopkins, 1954; Whelan, Bailey & Roberts, 1953; Schwimmer, 1950; Whelan & Roberts, 1953) and on an endopolygalacturonase (Phaff, 1966).

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Effect of Starvation on the Viability and Cellular Constituents of *Zymomonas anaerobia* and *Zymomonas mobilis*

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SUMMARY

Zymomonas anaerobia and *Z. mobilis*, grown on glucose + peptone + yeast-extract medium, degraded no endocellular carbohydrate, DNA and protein even in prolonged starvation. No significant qualitative changes in protein content during starvation were detected by disc gel electrophoresis of crude extracts. Both organisms had a high content of RNA (22 % w/w) which was degraded on starvation. In *Z. anaerobia* RNA decreased linearly to 5 % of the dry weight in 125 hr. With *Z. mobilis*, half the RNA was degraded in the first 24 hr of starvation after which time the decline was much slower. $MgCl_2$ (33 mM) prevented RNA breakdown. During growth, the intracellular ATP concentration increased from 0·5 to 1·0 μg./mg. dry wt, but began to decrease exponentially in the last generation before growth ceased because of glucose exhaustion. Intracellular ATP content correlated with viability determined by slide culture. The addition of 33 mM-$MgCl_2$ to the starvation medium did not affect ATP content, but increased viability. On prolonged starvation (up to 7 days), populations whose viability had fallen to 3 % possessed unimpaired ability to produce ATP from glucose; only after even longer starvation periods was this ability impaired.

INTRODUCTION

During recent years considerable attention has been focused on the nature of the endogenous metabolism of micro-organisms (for reviews, see Dawes & Ribbons, 1962, 1964) although this work has been confined mainly to aerobic and facultatively anaerobic organisms. By investigating the changes in chemical composition which occur during starvation an insight can be afforded into the substrates utilized in endogenous metabolism and their relation to the viability of the organisms. Anaerobic bacteria, which obtain their energy by substrate-level phosphorylation reactions which are well charted, ought to be particularly amenable to investigation, especially in relation to the problem of 'maintenance energy' (Dawes & Ribbons, 1964). Forrest & Walker (1965) studied the endogenous metabolism of the homofermentative organism *Streptococcus faecalis* which degrades glucose to lactate via the Embden–Meyerhof pathway, and Thomas (1968) has investigated *Streptococcus lactis*. Accordingly, for the present work, *Zymomonas anaerobia* (McGill, Dawes & Ribbons, 1965; McGill, 1966) and *Z. mobilis* (Stern, Wang & Gilmour, 1960; Dawes, Ribbons & Large, 1966) were chosen; they ferment glucose to ethanol and carbon dioxide by the Entner–Doudoroff (1952) pathway. Characteristic of this pathway is an energy yield of 1 mole ATP/mole glucose fermented, i.e. half that obtained by the Embden–Meyerhof pathway (Elsden & Peel, 1958).

Most of our work was done with *Z. anaerobia*, but where the results with *Z. mobilis* differed, they have been reported. A preliminary account of the earlier part of this work has appeared (Large & Dawes, 1966).

METHODS

Maintenance and growth of organisms. Zymomonas anaerobia (NCIB 8227) was maintained as a stab culture in agar and also in liquid medium. The basal liquid medium contained (g./l.): D-glucose, 20; Difco Bactopeptone, 10; Difco yeast extract, 10. When required, it was solidified with 15 g. New Zealand agar substitute/l. (in the case of the slide cultures 10 g. Oxoid Agar No. 1/l. was used). Cultures were incubated at 30°. *Zymomonas mobilis* (NCIB 8938) was maintained in a similar manner. This organism grew with a doubling time of about 2 hr, compared with 3·5 hr for *Z. anaerobia*.

Growth of larger quantities of either organism was achieved by transfer of an inoculum from the stock culture to 20 ml. sterile liquid medium. When this culture was gassing vigorously (after about 12 hr) it was used to inoculate one or more litres of medium in round flat-bottomed flasks or aspirators filled to the neck. *Zymomonas anaerobia* attained stationary phase in 20 hr at 30°, while *Z. mobilis* took 12 to 15 hr.

For starvation experiments organisms were harvested aseptically in closed, sterile centrifuge bottles and the cultures were examined for contamination by phase-contrast microscopic examination of a wet smear.

Measurement of bacterial concentration. This was performed turbidimetrically with a Unicam SP 600 spectrophotometer at 570 nm. Extinctions were related to population density by a dry wt calibration curve.

Preparation of washed suspensions for starvation experiments. After the appropriate period of growth organisms were harvested aseptically and washed in sterile phosphate buffer (below), pH 6·8, and suspended in a suitable volume of the same buffer in a sterile measuring cylinder. In earlier experiments the suspension was then distributed into sterile 50 ml. conical flasks, which were flushed with N_2, sealed with sterile silicone rubber stoppers and shaken at 30°. In later experiments the apparatus described by Dawes & Holms (1958) was used in which nitrogen was bubbled through the suspension at 30° via a sintered glass aerator. This apparatus bore a tap at the base through which samples could be removed aseptically, and had a small side arm into which substrate could be introduced aseptically at the start of the experiment, and subsequently tipped into the suspension without admitting air to the flask.

Preparation of extracts and disc electrophoresis. Zymomonas anaerobia organisms (about 70 mg. dry wt) were suspended in 2·5 ml. ice-cold 67 mM-phosphate buffer (pH 6·8), disrupted for 1·5 min. in an M.S.E. 60 W ultrasonic disintegrator and centrifuged for 10 min. at 25,000*g* to remove whole cells and debris. Samples of the supernatant fluid containing 100 μg. protein were used for polyacrylamide disc electrophoresis as described by Davis (1964).

Determination of ATP. The ATP content of organisms was measured by the firefly luciferase technique based on the method of Forrest & Walker (1965). Cole, Wimpenny & Hughes (1967) pointed out that for accurate assay of intracellular ATP there should be as little lag as possible between harvesting of the organisms and extraction of the ATP; that, ideally, samples ought not to be harvested by centrifugation, and the samples should not be chilled. In the present work the method developed by Cole *et al.* (1967), in which growing bacteria were pipetted into $HClO_4$, did not extract

the ATP from *Z. anaerobia* satisfactorily, so they were centrifuged before extracting the ATP. This was done within 5 min., the organisms being centrifuged for 2 min. at 20,000*g* at room temperature in a Servall Superspeed SS-1 centrifuge. ATP was extracted from the unwashed packed organisms by using 0·6 N-H_2SO_4 as described by Forrest & Walker (1965) except that 20 mM-Na_3PO_4 was used to neutralize the acid instead of KOH.

The apparatus used was a high-gain photomultiplier tube (E.M.I. type 6097S) with a Nuclear Enterprises type N.E. 5353 high voltage supply (Damoglou & Dawes, 1968). The current output of the tube was measured after conversion to voltage on a Yellow Springs Model 80 laboratory recorder (Yellow Springs Instrument Co. Inc., Yellow Springs, Ohio, U.S.A.). The firefly lantern extract and the estimation method were as described by Forrest & Walker (1965).

Viability determination by anaerobic slide culture. The slide culture method of Postgate, Crumpton & Hunter (1961) was modified slightly for anaerobic bacteria. Since *Zymomonas anaerobia*, long thought to grow only anaerobically (Shimwell, 1937, 1950) was found during this work to tolerate oxygen and to grow slowly under aerobic conditions, rigid precautions to ensure anaerobiosis during harvesting and preparation of the slide culture were not necessary. Starved suspensions were diluted 1/10 with sterile 67 mM-phosphate buffer (pH 6·8) and one drop applied to the agar on the slide. After drying and removal of excess moisture, the cultures were sealed with sterile buffer under circular coverslips, placed in Petri dishes and incubated in an anaerobic jar filled with oxygen-free N_2 at 22°, high viability samples for 48 hr, samples of low viability for 72 hr, and the numbers of divided and undivided bacteria (single or paired) were counted under a Watson Microsystem 70 phase-contrast microscope and the % viability calculated. No further increase in the % viability was noted when the incubation times were prolonged further.

Extraction of amino acid pool. Bacteria (5 mg. dry wt) were centrifuged down and suspended in 5 ml. water. After adding one drop of 2 N-H_2SO_4, the suspension was heated in a water bath at 100° for 10 min. with a glass bulb condenser on the tube. The cell debris was centrifuged down, and the supernatant fluid analysed for amino acids.

Chemical determinations. Ethanol was determined enzymically (Kaplan & Ciotti, 1957) by using liver alcohol dehydrogenase 340–L2 (Sigma–London Chemical Co.). Standards prepared from ethanol standardized by specific gravity determination were estimated simultaneously. Glucose in suspensions of starved bacteria was determined by the method of Nelson (1944), but for bacterial growth media the glucose oxidase+horseradish peroxidase method (Huggett & Nixon, 1957) with the Boehringer blood sugar test combination TC–M–II was used, since constituents of the peptone produced false positive reactions with the Nelson reagent. Pyruvate was estimated by the direct method of Friedemann & Haugen (1943), amino acids by the method of Yemm & Cocking (1955) and carbohydrate by the anthrone method of Trevelyan & Harrison (1952) as modified by Binnie, Dawes & Holms (1960). Ammonia was determined by the indophenol method of Chaney & Marbach (1962), polyphosphate as described by Harold (1960) and poly-β-hydroxybutyrate by the method of Law & Slepecky (1961), using the isolation procedure of Williamson & Wilkinson (1958). Cell protein was determined by the method of Lowry, Rosebrough, Farr & Randall (1951) after suspensions had been boiled for 6 min. in 2·5 N-NaOH.

Bovine serum albumin was used as standard. Cold-acid-soluble nucleotides were estimated by extraction of 5 mg. dry wt bacteria in ice-cold 0·7 N-$HClO_4$ for 10 min. at 0°. After centrifugation at 25,000*g*, the residue was washed three times with 0·7 N-$HClO_4$, the supernatant fluids and washings combined and the extinction read at 260 nm. The residue was hydrolysed with 0·3 N-KOH for 90 min. at 37°. The RNA content was determined on the basis of the extinction at 260 nm., as described by Fleck & Munro (1962), with yeast RNA hydrolysed by the same procedure as a standard. DNA was estimated on the cold-$HClO_4$-washed residue by the indole method of Ceriotti (1952).

Materials and chemicals. Analytical reagent grade chemicals were used where available. Firefly lanterns and ATP were obtained from Sigma–London Chemical Co., (London, S.W.6) and NAD from the Boehringer Corporation (London) Ltd. (London, W.5).

Buffer. KH_2PO_4 (0·067 M) was adjusted to pH 6·8 with 5 N-NaOH.

RESULTS

Changes in cellular constituents during growth and starvation

Carbohydrate content. Zymomonas anaerobia contained about 4 % of its dry wt as carbohydrate, which did not change on starvation up to 118 hr in N_2. *Z. mobilis* contained about 5 % (w/w) carbohydrate, which decreased to about 3 % on starvation up to 118 hr under N_2.

Poly-β-hydroxybutyrate and polyphosphate. These polymers were absent from both organisms.

DNA content. Zymomonas anaerobia contained about 2·7 % (w/w) DNA, and this remained stable on starvation to at least 138 hr.

Protein content. The protein content of *Zymomonas anaerobia* was examined at various stages of growth and during starvation of the harvested and washed bacteria under N_2. During growth the protein content remained essentially constant at 65 to 69 % of the dry wt but decreased to 54 % late in the stationary phase. During starvation of washed suspensions the protein content did not change significantly during starvation up to 150 hr; this result was not altered when the cells were either shaken or left stationary. The protein content was not affected when the glucose concentration in the growth medium was varied from 1 to 5 % (w/v).

The effect of starvation on the qualitative pattern of individual proteins in the soluble fraction of cell extracts was examined by disc electrophoresis on polyacrylamide gel. In extracts of unstarved organisms about 14 different protein bands were distinguished, of which five were especially prominent. After 159 hr starvation, 13 bands were still evident and the same five bands were still prominent. There did not seem to be any major or significant changes in individual protein bands in samples taken during this period.

Amino acids and ammonia. The concentration of ammonia and amino acids in the hot-water-extractable pool of *Zymomonas anaerobia* did not change in starvation periods up to 24 hr. Variable amounts of ammonia (from 0·1 to 0·5 μmole/mg. dry wt) and amino acids (0·02 to 0·2 μmole/mg. dry wt) were released into the medium during these periods; in some experiments as much as 1 μmole ammonia/mg. dry weight was noted.

RNA and cold-acid-soluble nucleotide pool. Zymomonas anaerobia has a high content of RNA (17 to 25 %, average 22 %, of the dry wt of freshly harvested late exponential phase organisms). On prolonged starvation, the RNA content decreased drastically to as low as 3 % after 138 hr (Fig. 1 A). During this period considerable amounts of material having an absorption maximum at 260 nm. were released into the medium, and when expressed as RNA this accounted for all the RNA degraded, since the cold-acid-soluble nucleotide pool remained essentially constant. The degradation of RNA began immediately and continued in a linear fashion during starvation. The degradation was suppressed when the organisms were starved in the presence of 33 mM-$MgCl_2$ (Fig. 1 A).

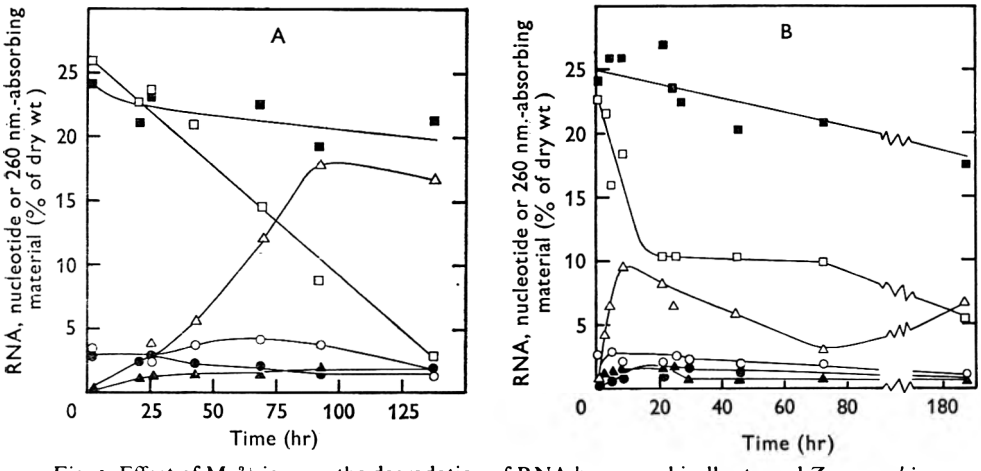

Fig. 1. Effect of Mg^{2+} ions on the degradation of RNA by anaerobically starved *Z. anaerobia* (A) and *Z. mobilis* (B). Bacteria from late exponential phase (10 mg. dry wt) were harvested aseptically and suspended in 10 ml. sterile 67 mM phosphate buffer (pH 6·8) in 50 ml. conical flasks under N_2, with and without 33 mM-$MgCl_2$. Flasks were removed at intervals, bacteria centrifuged down and extinction of the supernatant fluid at 260 nm. measured. Bacteria were fractionated into cold 0·7 N-$HClO_4$-soluble nucleotide pool and RNA, and these were estimated as described in Methods. Results are expressed as % of dry wt bacteria, with yeast RNA as standard. RNA content: ■, in presence of $MgCl_2$; □, in absence of $MgCl_2$. Cold acid-soluble nucleotide pool: ●, in presence of $MgCl_2$; ○, in absence of $MgCl_2$. 260 nm.-absorbing material: ▲, in presence of $MgCl_2$; △, in absence of $MgCl_2$.

The RNA content of *Zymomonas mobilis* in late exponential phase (average 22 % of dry wt) was very similar to that of *Z. anaerobia*, but marked differences in the rate of RNA degradation were observed for *Z. mobilis*. After anaerobic starvation for 24 hr the RNA content had decreased from 22 % to 11 %; this degradation was prevented by 33 mM-$MgCl_2$ (Fig. 1 B). Degradation of RNA was accompanied by a release of 260 nm.-absorbing material into the medium, while the cold-acid-soluble nucleotide pool remained constant. This initial rapid rate of RNA breakdown then declined and for the next 150 hr was about the same as the rate of breakdown in the presence of $MgCl_2$. The initial rapid release of 260 nm.-absorbing material in the first 24 hr appeared to be followed by a slow re-utilization during the succeeding 50 hr, an effect never observed with *Z. anaerobia*. $MgCl_2$ suppressed this release of nucleotides.

Intracellular level of ATP during growth. During growth, the intracellular ATP

content increased as shown in Fig. 2, until the last generation of growth was reached, when a rapid and exponential decrease in ATP began and continued after the glucose in the medium had been exhausted. This decrease was consistently observed in several experiments. In some cases it could be correlated with a decrease in growth rate indicating the approach to the stationary phase, but in other experiments exponential growth was still occurring when the decrease in ATP began.

Effect of addition of substrates on endogenous ATP levels. Within 2 hr of addition of a pulse of glucose (final concentration 2·5 mM) to a suspension of *Zymomonas anaerobia* which had been starved for 25 hr under oxygen-free N_2, the glucose had

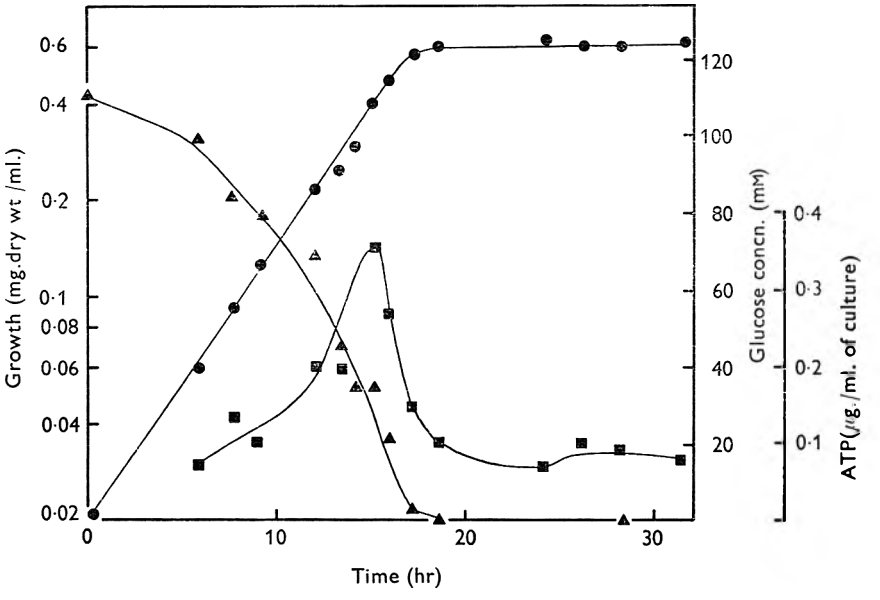

Fig. 2. Intracellular ATP during growth and short-term starvation in growth medium. *Zymomonas anaerobia* was grown anaerobically at 20° and samples were removed aseptically at intervals for measurement of growth (●) and glucose concentration (▲) and extraction of ATP. ■, ATP content (μg./ml. culture).

entirely disappeared from the supernatant fluid (Fig. 3). The concentration of ATP within the cell reflected closely the pattern of glucose utilization. There was a bi-phasic formation of ethanol, the inflexion point (2·1 μmoles) corresponding to 1 mole ethanol/mole glucose added. There then followed a second phase of ethanol formation reaching a maximum with 4·5/2·2 = 2 moles ethanol formed/mole glucose added. The subsequent loss of ethanol from the supernatant fluid was most probably due to volatilization. In a similar experiment in which glucose was replaced by pyruvate there was no detectable ATP formation, thus confirming that decarboxylation of pyruvate was not an energy-yielding reaction. To test whether the organism can obtain energy from ethanol, the effect of a pulse of ethanol on the ATP levels of a 24 hr-starved suspension of *Z. anaerobia* was determined under both aerobic and anaerobic conditions (Fig. 4). The results suggest that even under anaerobic conditions the organism formed small amounts of ATP from ethanol.

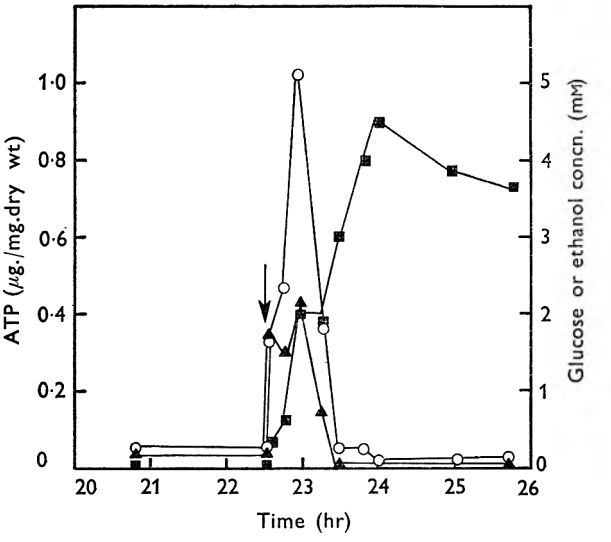

Fig. 3. Effect of anaerobic addition of glucose on ATP levels in starved *Zymomonas anaerobia*. Bacteria (265 mg. dry wt) from late exponential phase were harvested aseptically and suspended in 250 ml. sterile 67 mM-phosphate buffer (pH 6·8) and oxygen-free N_2 bubbled through the suspension. After starvation for 22 hr 625 μmole glucose were added anaerobically (at point shown by arrow) and samples removed at intervals for assay of ATP (○) μg. ATP/mg. dry wt of organisms. The culture supernatant fluids were analysed for glucose (▲) and ethanol (■).

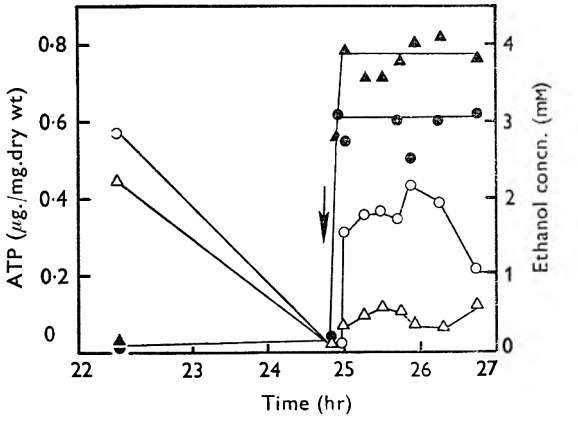

Fig. 4. Effect of addition of ethanol, both aerobically and anaerobically, on ATP levels in starved *Zymomonas anaeroòia*. Conditions were as for Fig. 3, except that two flasks were set up, one bubbled with air, the other with oxygen-free N_2. After 24-hr starvation (at the point shown by an arrow) 1 m-mole ethanol was added to each flask (without introduction of air), and samples removed at intervals. Organisms were harvested by centrifugation and ATP extracted from them. The supernatant fluids were analysed for ethanol concentration: ●, aerobic; ▲, anaerobic. ATP pool (μg. ATP/mg. dry wt); ○, aerobic; △, anaerobic.

Effect of starvation on ability of Zymomonas anaerobia to produce ATP from glucose

The ability to metabolize glucose, and to form ATP from it, remained unimpaired after 143 hr starvation (Fig. 5). In another experiment the starvation was extended to 15 days, after which time the ability to metabolize glucose was impaired, and only low amounts of ATP were formed (maximum, 0·6 μg./mg. dry wt). Bacteria starved for 15 days took 30 hr to metabolize 1 m-mole of glucose as compared with 1 to 2 hr after starvation for 143 hr.

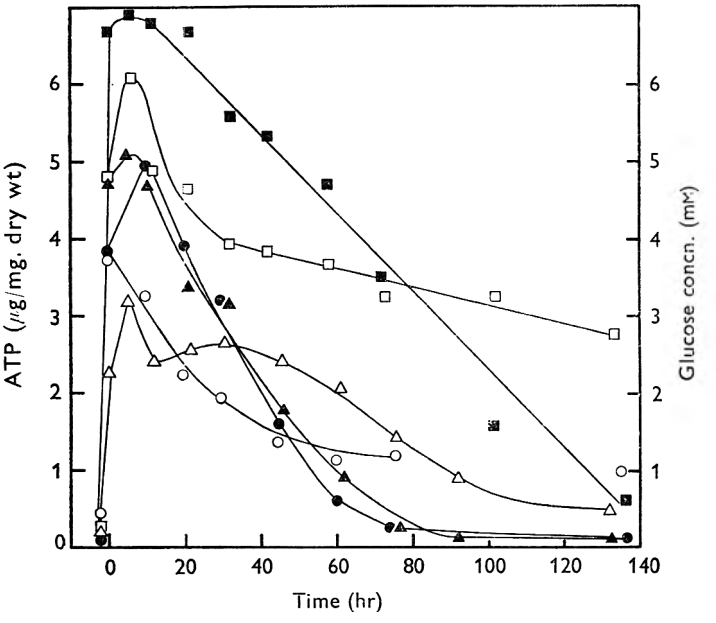

Fig. 5. Effect of extended starvation on the ability of *Zymomonas anaerobia* to produce ATP from glucose anaerobically. Conditions were as for Fig. 3, except that three flasks were set up, each containing 0·4 mg. dry wt organism/ml. and each starved for different periods. After starvation, 1 m-mole glucose was added anaerobically, and samples removed at intervals. Organisms were collected by centrifugation and ATP extracted. The supernatant fluids were analysed for glucose. Solid symbols represent glucose, open symbols ATP content (μg./mg. dry wt), for 23 hr (circles); 47 hr (triangles) and 143 hr (squares) starvation.

Relationship between viability of Zymomonas anaerobia and intracellular concentrations of RNA and ATP

Viability decreased exponentially as soon as starvation began, as might be expected since *Zymomonas anaerobia* contains no energy reserves to protect against starvation. The decrease in viability was accompanied by an exponential decrease in intracellular ATP which was most marked during the initial 6 hr. During longer periods of starvation Mg^{2+}, which prevented degradation of RNA (Fig. 1), seemed to protect the populations. After 175 hr starvation in the presence of 33 mM-$MgCl_2$, the population was 22 % viable as compared with only 3 % in the absence of $MgCl_2$ (Fig. 6). Mg^{2+} did not have a significant effect on the ATP content of the organisms.

An experiment was made to study the effect on ATP amounts of adding glucose to a culture of low viability. Viability was followed as a function of time; after 180 hr, when the viability had decreased to less than 3 %, glucose was added anaerobically (final concentration, 5 mM). The rates of disappearance of glucose and formation and disappearance of ATP were very similar to those shown in Fig. 5. It is apparent that the ability to produce ATP in response to glucose is not lost on prolonged starvation and is, therefore, not related to viability as measured by slide culture.

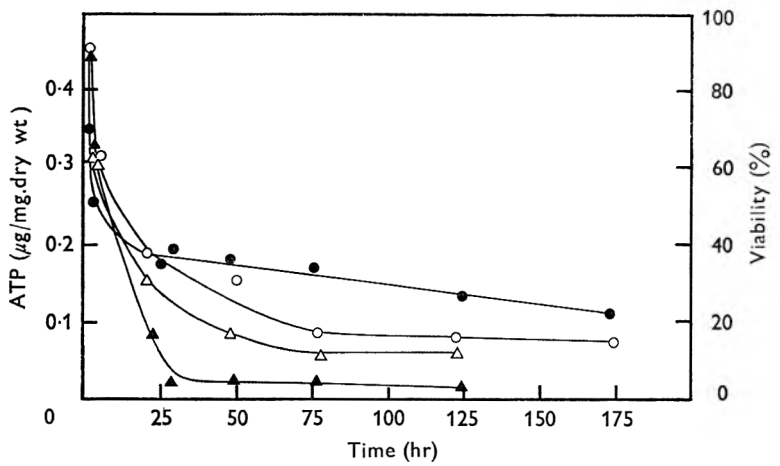

Fig. 6. Effect of prolonged starvation on intracellular ATP levels and viability of *Zymomonas anaerobia* with and without Mg^{2+}. Conditions were as for Fig. 3. △, Intracellular ATP concentration and ▲, viability in the absence of $MgCl_2$; ○, ATP concentration and ●, viability in the presence of 33 mM-$MgCl_2$.

DISCUSSION

A study of the intracellular constituents of *Zymomonas anaerobia* and *Z. mobilis* which are degraded on starvation suggests that, in both organisms, the only constituent which is significantly broken down is RNA. Breakdown of RNA seems to be a common (though by no means universal) response of bacteria to starvation. Thus *Aerobacter aerogenes* (Strange, Dark & Ness, 1961), *Pseudomonas aeruginosa* (Gronlund & Campbell, 1965), *Escherichia coli* (Dawes & Ribbons, 1965), *Sarcina lutea* (Burleigh & Dawes, 1967) and *Streptococcus lactis* (Thomas, 1968) all degrade RNA during starvation. Anaerobic organisms would thus not seem to differ from aerobes in this respect. In all cases, the breakdown of RNA is prevented by Mg^{2+}, which is well known as a ribosome-stabilizing agent (Bowen, Dagley & Sykes, 1959; Wade, 1961). In the case of *P. aeruginosa*, Gronlund & Campbell (1965) have shown that the ribosomes are degraded. Fig. 6 shows that Mg^{2+} ions exert a considerable protective effect against loss of viability in *Z. anaerobia*. In this respect, the organism resembles *A. aerogenes* (Strange & Shon, 1964; Strange & Hunter, 1967) and *S. lactis* (Thomas & Batt, 1968) rather than *E. coli* (Dawes & Ribbons, 1965) or *S. lutea* (Burleigh & Dawes, 1967), where survival is not prolonged by Mg^{2+} ions. Ethanol, unlike pyruvate, leads to the formation of small amounts of ATP, even under anaerobic conditions. This observation suggests that the metabolism of *Z. anaerobia* may be somewhat

more complex than has hitherto been envisaged. The higher level of ATP formed in aerated suspensions is in keeping with the ability of washed cell suspensions of anaerobically grown *Z. anaerobia* to oxidize ethanol without a lag (P. J. Large, unpublished observation). This may indicate that ethanol can be metabolized further by *Z. anaerobia* and that this metabolism may be energy-yielding. Belaïch & Senez (1965) found that *Z. mobilis* would oxidize ethanol, but suggested that the process was not energy-yielding.

While the *ability to produce* ATP is ultimately impaired by starvation, the actual intracellular amounts of ATP seem to be correlated with viability of the cells (Fig. 6). Although several workers (e.g. Cole *et al.* 1967 with *Escherichia coli*) have studied the effects of starvation on ATP levels, the only work of this kind that we are aware of in which viabilities were measured is that of Strange, Wade & Dark (1963). In contrast to the present observations, these workers were unable to detect any correlation between intracellular ATP content of *Aerobacter aerogenes* and viability, the ATP amounts reflecting largely the oxygen tension and solute concentration. A higher ATP content in *E. coli* starved aerobically was also observed by Cole *et al.* (1967). We have been unable to discover any correlation between viability and ability to produce ATP from glucose in *Zymomonas anaerobia*.

The marked decrease in intracellular ATP in the last generation before growth on glucose ceased was observed in several experiments. This phenomenon does not seem to have been explicitly reported by other workers. Forrest & Walker (1965), in *Streptococcus faecalis* growing on glucose, only observed a decline in ATP amounts when the stationary phase had been reached. Cole *et al.* (1967) measured the ATP pool in *Escherichia coli* under various conditions. When the organisms were grown anaerobically an apparent decline in ATP content did occur; in defined medium containing glucose the amount of ATP decreased abruptly in the stationary phase, while in glucose + yeast-extract medium a slow decrease in ATP content occurred in the last half-generation before growth ceased. We suggest that the decrease in ATP content in *Zymomonas anaerobia* during growth on glucose (which is here behaving as energy source) is an indication that the organisms are becoming energy limited. In cultural conditions where growth is limited by some other constituent of the medium the ATP pool might be expected to behave differently.

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Development and Organization of the Aerial Mycelium in *Streptomyces coelicolor*

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SUMMARY

The anatomy of individual colonies of *Streptomyces coelicolor* was studied at various developmental stages *in situ* by means of surface-impressions and thin sections. Young colonies consisted of substrate mycelium composed of a loose network of hyphae with uniform appearance. The uppermost cells produced the very closely packed hyphae of the aerial mycelium. Subsequently the surface layer of the aerial mycelium began to sporulate but became overgrown by young hyphae which formed a new sporulating zone above the first. The process was repeated several times. Many of the early produced spores germinated immediately. When the climax of sporulation was reached the aerial mycelium showed two trends of development: one towards spore formation (in the surface layer) and one towards lysis of non-sporulating hyphae (below the sporulating zone). Sporulation was initiated by coiling of hyphal tips which were then divided by cross walls into chains of spore-sized compartments. The basal non-sporulating parts of the hyphae disintegrated later. The most conspicuous cytological change during lysis was the appearance of large dense granules.

INTRODUCTION

It is characteristic of Streptomycetes growing on solid media to produce two sorts of mycelium: substrate and aerial (Waksman, 1959). A germinating spore forms substrate hyphae which branch frequently and grow radially, developing into a young colony. Subsequently the aerial mycelium is produced. In a study by phase-contrast microscopy Hopwood (1960) confirmed the observations of Erikson (1949) that the aerial hyphae of *Streptomyces coelicolor* originated by simple branching from substrate hyphae and not necessarily from fusion cells as claimed by Klieneberger-Nobel (1947) for *Streptomyces gardneri*, *Streptomyces albosporeus*, *Streptomyces chromogenes* and *Streptomyces madurae*. Nevertheless the results of genetic studies (Hopwood, 1967) suggested that fusion of substrate hyphae, or some kind of conjugation between them, occasionally occurs.

The fine structure of the aerial mycelium in various Streptomycetes as seen in thin sections has been studied by several authors (Hagedorn, 1960; Glauert & Hopwood, 1959, 1960, 1961; Hopwood & Glauert, 1960; Rancourt & Lechevalier, 1964; Bradley & Ritzi, 1968). However in these studies mycelium was unsystematically scraped from colony-surfaces, centrifuged and then prepared for electron microscopy, or, if whole colonies were sectioned, no attempt was made to obtain information on the development and spatial organization of individual colonies. The present

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report describes a study of the changes in fine structure of the aerial and the adjacent parts of the substrate mycelium of *Streptomyces coelicolor in situ* during the development of the colony. Fine structure of the sporulation process is described in an accompanying paper (Wildermuth & Hopwood, 1970).

METHODS

Stock cultures of wild-type *Streptomyces coelicolor* A 3 (2) (*Streptomyces violaceoruber* according to Kutzner & Waksman, 1958) and an adenine/histidine requiring mutant (strain 504, *hisE6*, of the Hopwood stock culture collection) were maintained at 30° on a complex agar medium–'complete medium' (Hopwood, 1967). The auxotrophic mutant, which grows slowly on minimal medium, produced tiny compact colonies (0·4 to 1 mm. in diameter at 7 days) when grown on unsupplemented, chemically defined 'minimal medium' (Hopwood, 1967). These were more suitable for anatomical studies of whole colonies than were the much larger ones of the wild type. Single colonies of both strains at various stages of growth on 'minimal medium' were cut out of the agar and prefixed for 2 hr in ice cold 2·5 % (v/v) glutaraldehyde in cacodylate buffer at pH 7·2 (Glauert & Thornley, 1966; Audrey M. Glauert, personal communication). The vessel in which the prefixation was performed was partially evacuated by means of a water pump to expel the air trapped between the hydrophobic aerial hyphae. Colonies were postfixed at room temperature overnight at atmospheric pressure in 1 % (w/v) osmium tetroxide in veronal-acetate buffer at pH 6·1 (Ryter & Kellenberger, 1958), washed for 2 hr in Ryter–Kellenberger buffer containing 0·5 % (w/v) uranyl acetate, dehydrated in a graded ethyl alcohol series and embedded in Araldite (Glauert & Glauert, 1958). Thin sections were cut on an LKB Ultratome III with glass knives and mounted on carbon- or formvar-coated copper grids. Specimens were examined in a Siemens Elmiskop Ia, operating at 60 or 80 kV at instrumental magnifications between 4,000 and 20,000.

Negatively stained specimens were prepared as follows. The surface of a colony was gently touched with a carbon-coated grid and the impression negatively stained with 1 % (w/v) aqueous potassium phosphotungstate (pH 7) or 2 % (w/v) ammonium molybdate (pH 7) (Brenner & Horne, 1959; Horne, 1965). Unstained impressions were prepared in the same way on cover slips for viewing in the phase-contrast microscope.

RESULTS

Stages in the development of the aerial mycelium. Although environmental variation was minimized the development and final size of the individual colonies varied considerably. In the course of this work it was found that both features depended largely on the depth of the medium and on the population density of the colonies. However unknown factors also influenced the growth of individual colonies considerably, so that it was difficult to estimate the physiological age of a colony; both absolute age and colony diameter proved unsuitable. The most reliable indicator of physiological age was the colour of the aerial mycelium. In a systematic study it was found that colonies of the same colour (but not necessarily of the same age) exhibited rather uniform features in impressions and in sections. However a colony sometimes did not change colour simultaneously over its whole surface. Moreover in

uniformly coloured colonies the thickness of the aerial mycelium varied considerably, the colony often appearing very flat with a diffuse edge. In order to exclude any factor which might affect the uniformity of the results unnecessarily, only compact colonies with a sharp edge and simultaneous change of colour were selected for this study. After such a selection the sections of colonies of the same colour appeared entirely uniform.

It was necessary to distinguish between intact and disintegrating cells. The cytological changes characteristic of cellular disintegration (lysis) are illustrated in Pl. 4, fig. 31–33 in comparison with a healthy cell (Pl. 4, fig. 30). Initially the nuclear fibrils condensed to form a loose network (Pl. 4, fig. 31 and 33, *N*). The cytoplasm was gradually replaced to a great extent by vacuole-like spaces (*S*). Later the membranes, including those of the mesosomes, broke into fragments and often rounded off. Finally the cells consisted only of the wall and some membrane fragments (Glauert & Hopwood, 1960). The dense granules (Pl. 4, fig. 32), whose size varied from about 0·05 to 0·5 μm., also appeared to be symptomatic of the beginning of lysis, since they occurred almost exclusively in cells which showed all the signs of disintegration mentioned above.

Substrate mycelium before the appearance of the aerial mycelium (Pl. 1, fig. 1–4). This mycelium consisted of a loose network of branched and interconnected hyphae. The cell contents and wall appeared homogeneous. The white areas seen, for example, in Pl. 1, fig. 1 are sectioning artifacts and not vacuoles. A few hyphae showed signs of disintegration (Pl. 1, fig. 2). Dense bodies, which are considered symptomatic of ageing hyphae, were only occasionally observed. The population of hyphae was densest near the agar surface (Pl. 1, fig. 1), but, apart from a relatively thin top layer, it was sparse compared with the aerial mycelium.

White aerial mycelium shortly after its appearance (Pl. 1, fig. 5–8). Impressions and sections revealed only closely packed hyphae with homogeneous cell contents and walls. Neither stages of sporulation nor disintegrating cells were seen. In contrast to the substrate hyphae, aerial hyphae often contained vacuoles (Pl. 1, fig. 5, arrows) which at higher magnification were easily distinguished from sectioning artifacts. The population of hyphae was fairly dense and similar at all levels. Dense bodies were as rare as in the substrate mycelium. The boundary between the medium and the air was recognized by the many particles trapped within the agar (Pl. 1, fig. 8, arrows); by definition this borderline indicated the transition from substrate to aerial mycelium.

White aerial mycelium, about one day after its appearance. Impressions revealed spores, singly or in chains, some of them germinating (Pl. 3, fig. 29), and a few aerial hyphae. However sections lacked spores and stages of sporulation; the loose spores were presumably washed off during fixation. Otherwise the aerial mycelium appeared the same as in younger colonies.

Pale grey aerial mycelium (Pl. 1, fig. 9–12). Impressions consisted almost entirely of spore chains and single spores; germinating spores were absent and hyphae were only rarely found.

In sections the aerial mycelium appeared very heterogeneous. Mature spores, mixed with some young hyphae, formed a top layer. Presumably most top spores which were not trapped between hyphae were washed off in the fixative. Owing to the hardness of their walls many spores folded during sectioning. Below the top layer was a deep zone of aerial hyphae and spores. In contrast to the top spores many

were germinating (Pl. 1, fig. 10, arrows) and none was folded; possibly all were at some stage of germination and therefore might have had softer walls. This would have allowed the fixative and embedding material to penetrate better, and account for the clearer cytological details of these spores compared with those of the hard-walled top spores. Disintegrating hyphae were only occasionally found.

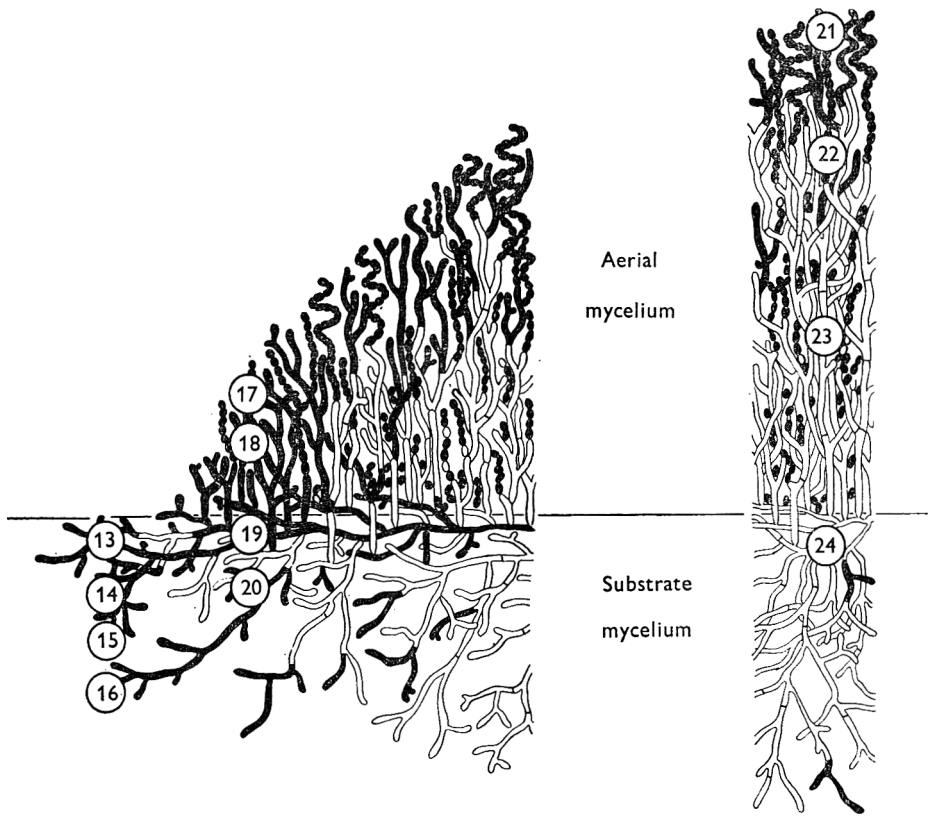

Fig. 1. Idealized diagram of a vertical section through the centre of a colony at the climax of sporulation. Black represents intact cells and white disintegrating or completely lysed cells. Numbers indicate the positions where the pictures of Pl. 2 were taken.

Grey-brown aerial mycelium (Pl. 2, fig. 13–24, and Fig. 1). At this stage the colony had reached its climax of sporulation. Impressions contained practically only spores. In sections the mycelium appeared as a complicated and heterogeneous network of intact and disintegrating hyphae and all stages of sporulation. The spatial organization of the colony was studied in both the wild type and the auxotrophic mutant. Morphologically they were identical except that mutant colonies grown on minimal medium were much smaller. Vertical sections of parts of a mutant colony are shown in Plate 2. The regions photographed are indicated in Fig. 1. On the top, near the centre of the aerial mycelium, most hyphae were intact (Pl. 2, fig. 21 and 22), but with increasing distance from the top, more disintegrating hyphae were encountered (Pl. 2, fig. 23). In the centre of the colony, below 50 μm. from the top, hardly any intact cells were found. (Pl. 2, fig. 24.) At the edge of the colony, however, most hyphae appeared intact,

including those of the substrate mycelium (Pl. 2, fig. 17–20). The edge of the aerial mycelium was rather sharp and the network of hyphae still very dense. In contrast, the edge of the substrate mycelium was very sparse and the number of hyphae decreased radially (Pl. 2, fig. 13–16). Although the colony was growing centrifugally the number of decaying hyphae was surprisingly high at the edge. The proportion of lysing cells increased towards the centre, where hardly any intact cells were found.

Old aerial mycelium: grey-brown with white patches. Old colonies which showed many symptoms of senescence consisted mainly of disintegrated hyphae and mature spores. The white patches were nests of young aerial mycelium which probably fed on the decaying part of the old colony over which they grew.

Spore forming and non-sporulating hyphae. The features seen in vertical sections of colonies of different ages suggest that the hyphae of the aerial mycelium have two alternative fates: spore formation and lysis. In negatively stained impressions of old colonies very many disintegrating hyphae were observed. Such hyphae were characterized by a lower electron density. Furthermore, pieces of liberated membrane were often seen outside the cell wall. Occasionally spore chains were attached to a non-sporulating part of a hypha (Pl. 3, fig. 25), the latter frequently showing signs of lysis. From these findings it is concluded that there are entirely sterile hyphae, and partly sterile hyphae, in which a non-sporulating 'stalk' bears spores at its tip, or in side branches along its axis. Sporulation was often initiated by coiling of the hyphal tip, but the formation of such helices did not seem obligatory since undisturbed impressions viewed with the phase-contrast microscope showed young straight spore chains alongside helical ones. It seems unlikely that the straight spore chains were produced by stretching due to the pressure of the coverslip, since Hopwood (1960) observed such a chain in a colony grown in an air space.

While the hyphal tip was coiling (Pl. 3, fig. 26) its cells were divided into spore-sized compartments by cross walls formed at regular distances (Pl. 3, fig. 27). Later the spores began to separate, the first sign of this being the formation of constrictions between the spore compartments (Pl. 3, fig. 28). Mature spore chains were held together by only a delicate envelope probably consisting of the fibrous sheath (surrounding the aerial hyphae) and parts of the parent cell wall (Wildermuth & Hopwood, 1970). Therefore they easily broke into single spores. The number of spores within one chain was very variable. In impressions, short fragments containing only a few spores were found beside long rows sometimes consisting of more than 80 spores.

DISCUSSION

Spore germination in situ. This study revealed that spores developed immediately after the appearance of the aerial mycelium and that some of them began to germinate straight away. This behaviour of the young spores was repeatedly, although not always, observed. When the aerial mycelium turned pale grey the germinating cells were found under a top layer of 'resting' spores which was very thin in sections but might have been much thicker in reality since spores which were not trapped between hyphae tended to float off during fixation. At later stages no germinating spores could be observed. The reason for the immediate germination of the first batch of spores is unknown; possibly the relative humidity very close to the medium was high enough to initiate germination.

Similarity with multicellular eukaryotes. Grey sporulating colonies were still growing radially; in a relatively thin surface layer numerous hyphal tips sporulated while overgrown by young hyphae. From the beginning the surface of the aerial mycelium was a steadily moving front of sporulating hyphae, leaving an enlarging zone of disintegrating cells behind them. Some of the lysing cells were basal 'stalks' of sporulating hyphae, but many had never borne spores.

These observations suggest that in Streptomyces colonies there are cells responsible for reproduction (spores) and hyphae which have a function only in an individual colony and have a limited lifespan. Therefore the colonies are differentiated into a soma and germ line comparable with those of multicellular eukaryotes. The fact that the colonies consist of at least three cell types (substrate hyphae, sterile and fertile aerial hyphae) organized in a mycelium makes the similarity even more obvious.

Dense granules. A striking feature of ageing cells was the appearance of large dense granules. These bodies, which were occasionally observed by Glauert & Hopwood (1961), are referred to as volutin granules. Glauert & Brieger (1955) demonstrated that such dense bodies in *Mycobacterium phlei* were identical with metachromatic granules seen in the light microscope. They contained a large amount of metaphosphate, but their significance was not clear. In *Streptomyces coelicolor* they were related to cell lysis, although occasionally they were also found in healthy-looking cells, even in germinating spores. Baldacci, Gilardi & Amici (1956) found similar granules in unstained whole mount preparations of disintegrating hyphae of Streptomycetes. However it is uncertain whether these corresponded to the granules observed in thin sections. During this study round dense granules were often seen in unfixed and unstained spores, but never in sections of spores. It is believed that the density of the granules seen in sections is due to uptake of osmium during fixation. Large 'osmiophilic granules' of about the same size appear in great numbers in chloroplasts of senescing leaves of Elodea, Phaseolus and Cucurbita (Ikeda & Ueda, 1964; Barton, 1966; Butler, 1967). In spite of the similarity in morphology and appearance of the granules in these different organisms it is not certain whether they have the same significance; more has to be learned about their development and chemistry.

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EXPLANATION OF PLATES

PLATES 1 AND 2

Electron micrographs of parts of vertical sections of colonies of various physiological age. Note population density, intact and disintegrating cells, 'resting' and germinating spores. The scale mark represents 1 μm.

PLATE 1

Fig. 1–4. Selected parts of the same section of a young colony, consisting only of substrate mycelium. Note disintegrated cell (arrow).

Fig. 5–8. Young colony with white aerial mycelium shortly after its appearance. Note vacuoles (Fig. 5, arrows) and boundary between substrate and aerial mycelium (Fig. 8, arrows).

Fig. 9–12. Colony with pale grey aerial mycelium. 'Resting' spores are mostly found in the top layer (Fig. 9), while germinating spores are seen in deeper zones (Fig. 10, arrows). Fig. 11 was taken at the boundary between the two mycelia and Fig. 12 represents substrate mycelium.

PLATE 2

Colony of autotrophic mutant 504 with grey brown aerial mycelium. The positions where the pictures were taken are shown in Fig. 1 in text.

Fig. 13–16. Substrate mycelium beyond the aerial mycelium.

Fig. 17–20. Edge of the aerial mycelium.

Fig. 21–24. Centre of the colony.

PLATE 3

Electron micrographs of negatively stained impressions of sporulation stages. The scale mark represents 1 μm.

Fig. 25. Spore-chain with adjacent 'stalk' which is beginning to disintegrate.

Fig. 26. Coiled hyphal tip before septation.

Fig. 27. Hyphal tip after formation of sporulation septa. The constrictions between spores are not yet visible.

Fig. 28. Chain of mature spores. Note constrictions.

Fig. 29. Part of a spore chain from the top of white aerial mycelium, showing a germinating spore.

PLATE 4

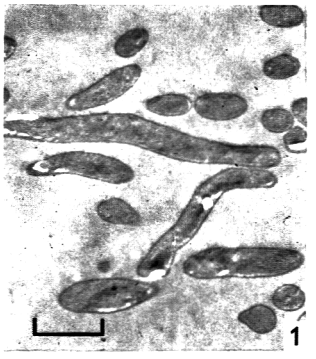
Electron micrographs of sections of aerial hyphae at various stages of disintegration. The scale mark represents 0·5 μm.

Fig. 30. Intact cell. *CW* = cell wall; *FS* = superficial layer (fibrous sheath); *C* = cytoplasm; *N* = nuclear material; *PM* = plasma membrane.

Fig. 31. Aerial hypha showing the first signs of lysis. Note condensed fibrils of the nuclear material (*N*), replacement of the cytoplasm by large vacuole-like spaces (*S*) and the gaps between membranes.

Fig. 32. Lysing cell with dense bodies.

Fig. 33. Advanced stage of lysis. *N* = nuclear material; *S* = vacuole-like spaces.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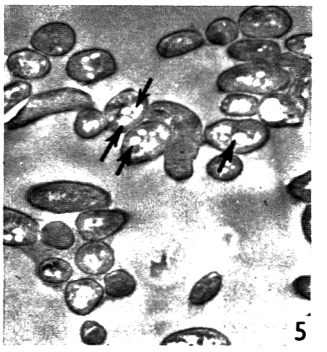
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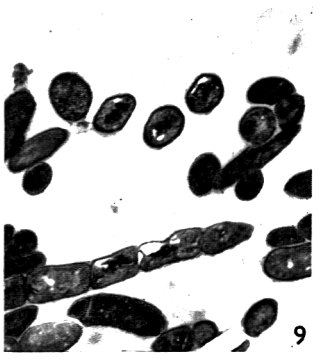
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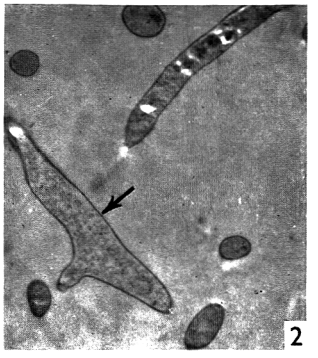
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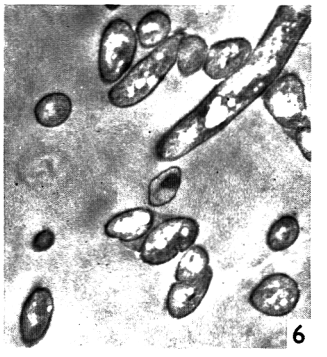
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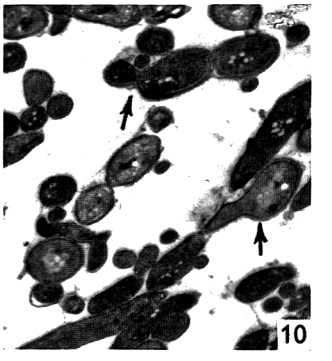
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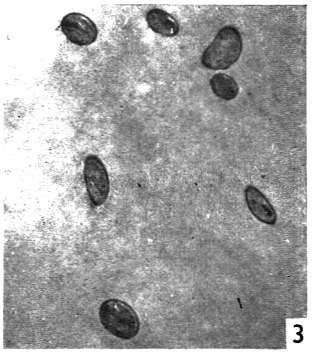
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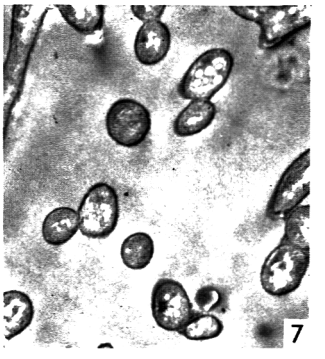
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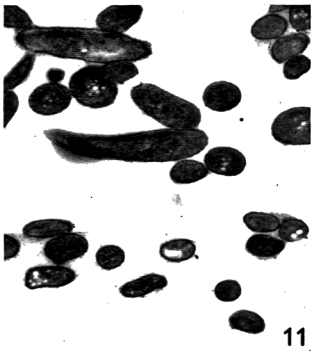
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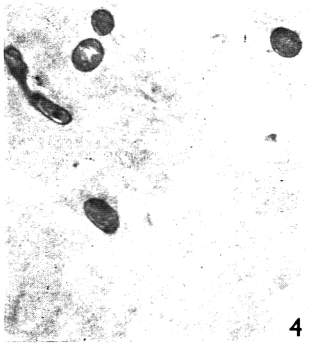
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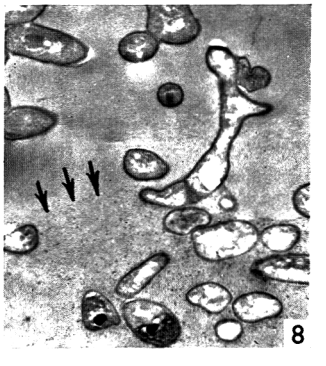
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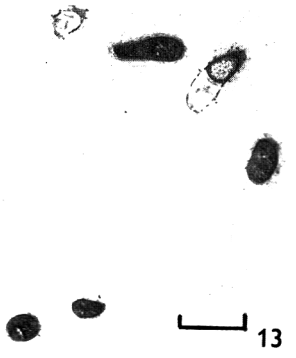
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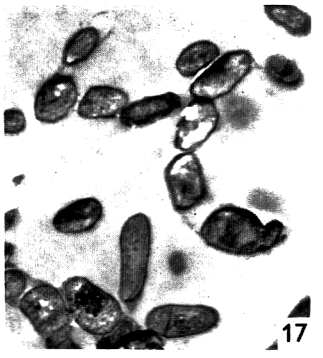
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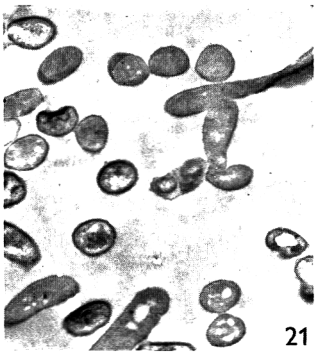
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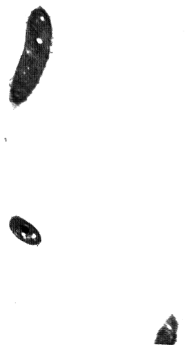
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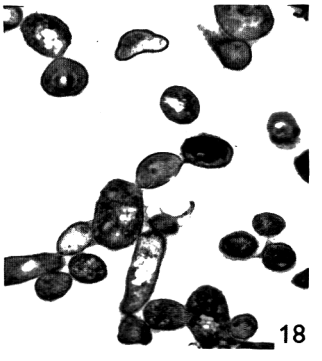
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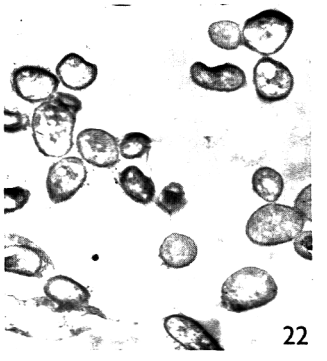
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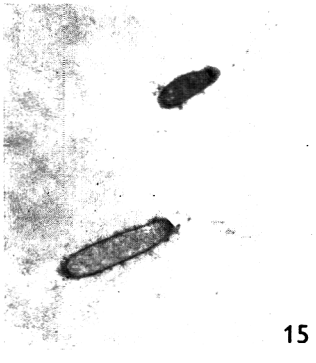
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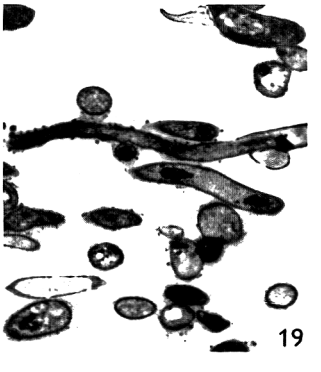
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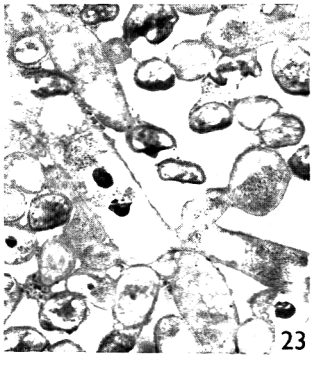
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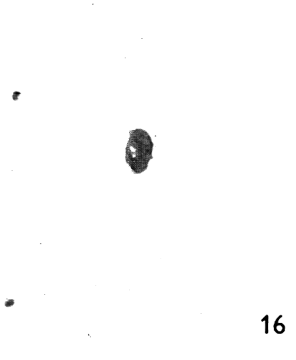
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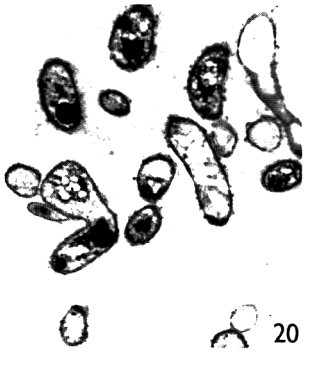
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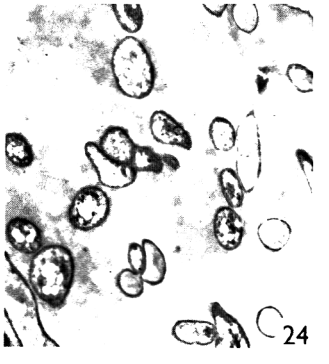
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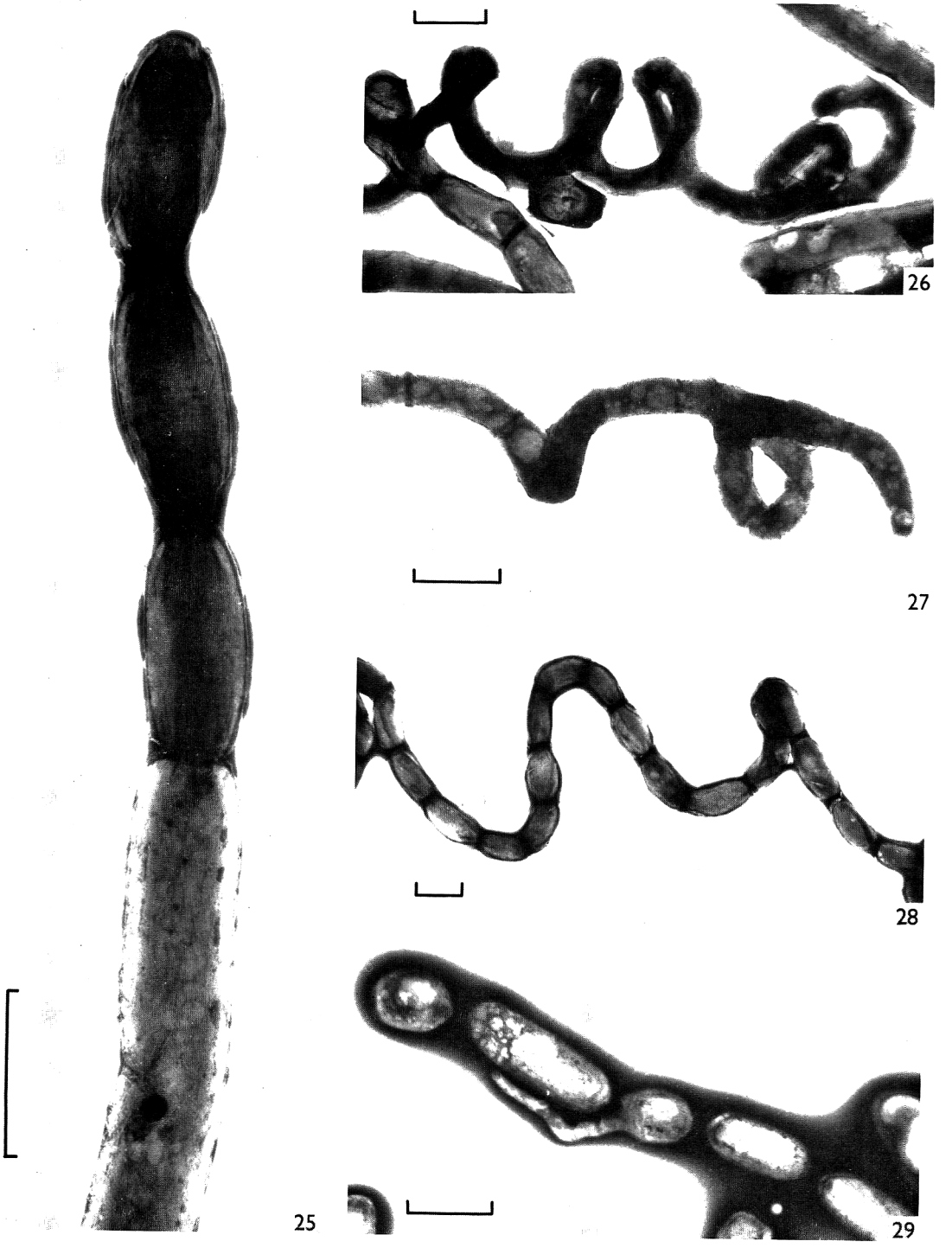

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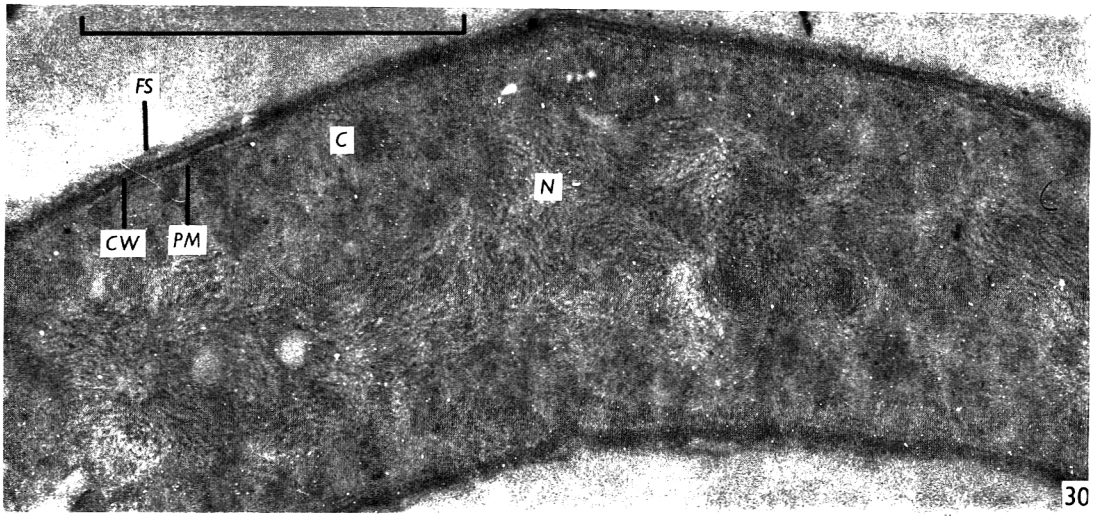
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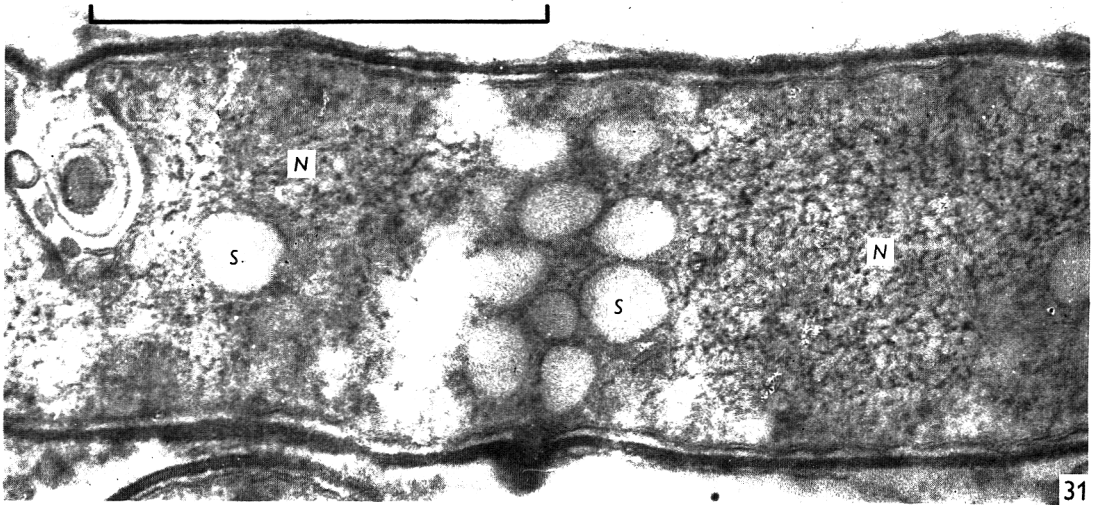
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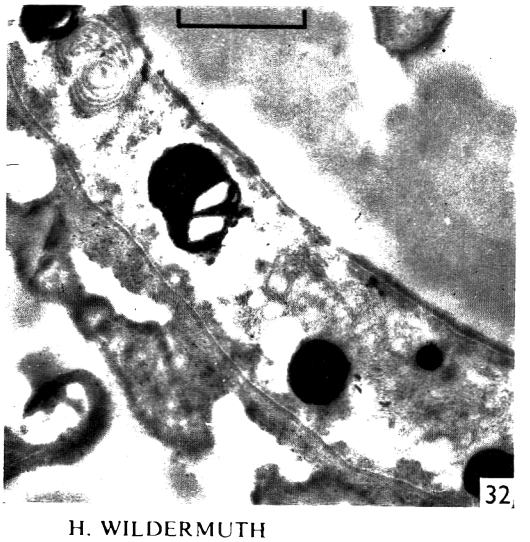
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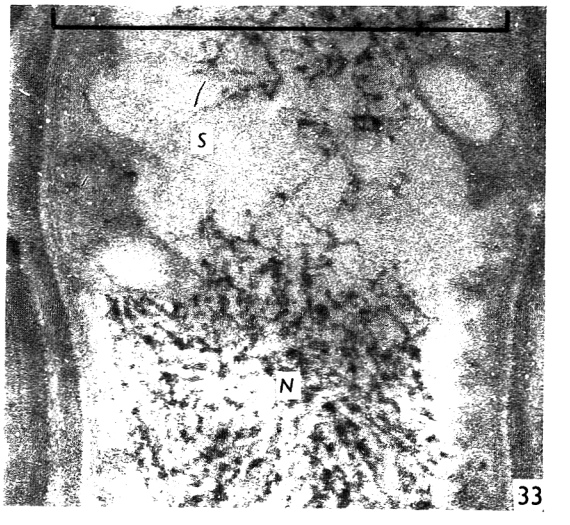
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Septation During Sporulation in *Streptomyces coelicolor*

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SUMMARY

The fine structure of the morphological events during sporulation in *Streptomyces coelicolor* grown on chemically defined medium was studied in electron micrographs of thin sections. During vegetative growth the young hyphae were divided into long cells by cross-walls which formed in a way comparable to those in certain other Gram-positive bacteria. Sporulation was initiated by coiling of hyphal tips, which were then divided into spore-sized compartments by special cross-walls, the 'sporulation septa'. These were laid down as double rings attached to the hyphal wall and extending centripetally. After completion of the septa the walls thickened uniformly and the individual spores began to round off. The new wall material on the long side of the spores appeared to be deposited underneath the old parent wall; the old cell wall probably formed part of the spore wall.

INTRODUCTION

New information about sporulation in Streptomycetes became available during the last decade when thin sections were studied in the electron microscope. According to Glauert & Hopwood (1961) sporulation in *Streptomyces coelicolor* begins by separation of the hyphal wall into an outer and inner layer. Annular ingrowths from the inner layer then extend towards the centre of the hypha to divide it into spore-sized units. The inner parent wall, together with the new septum material, constitutes the wall of the young spore, to which further layers are added as the spore matures. Rancourt & Lechevalier (1964) and Bradley & Ritzi (1968) came to similar conclusions for *S. viridochromogenes* and *S. venezuelae* respectively.

The aim of the present study was to obtain more information about the morphological changes during spore formation in wild-type *Streptomyces coelicolor* as a prerequisite for a study of the genetic control of sporulation, a morphogenetic problem which is being investigated in this laboratory by the study of mutants defective in sporulation (Hopwood, Wildermuth & Palmer, 1969). Since the lesion in many of the mutants is in the division of the aerial hyphae into spore-sized units, attention was focused on this process in the wild-type.

METHODS

The strain was wild-type A3(2) of *Streptomyces coelicolor* (*S. violaceoruber*) according to Kutzner & Waksman (1959). Stock cultures were maintained on a complex medium ('complete medium' of Hopwood, 1967). Well sporulating colonies were grown on plates of chemically defined 'minimal medium' (Hopwood, 1967) incubated at 30°. Colonies were fixed on the second day after the appearance of

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a grey-brown coloration on the aerial mycelium; such colonies contained all stages of sporulation, including mature spores (Wildermuth, 1969). The colonies were cut out of the medium on small blocks of agar and prepared for electron microscopy as described in an accompanying paper (Wildermuth, 1969).

RESULTS

The developmental steps in spore formation could not be experimentally synchronized in a growing culture as can be done in Bacillus (see for example Ryter, 1965). This, and the fact that the colonies of filamentous Streptomycetes are morphologically complex, made structural analysis of the process more difficult; in sections and impressions of sporulating aerial mycelium all the different stages of spore formation occurred together. A prerequisite for the present work was therefore a study of the development and spatial organization of the colonies (Wildermuth, 1969) which aided the reconstruction of the developmental sequence in spore formation.

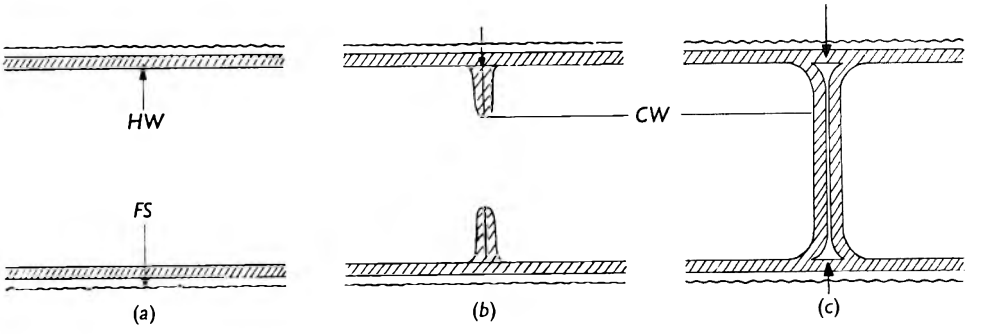

Fig. 1. Diagram of cross-wall formation in an aerial hypha. At the start of the process (*a*) the cell envelope consists of the hyphal wall (*HW*) and the fibrous sheath (*FS*). New wall material is deposited (*b*) in the form of an annulus of double thickness, separated at first from the old hyphal wall by a dark line (arrow). Later (*c*) the cross-wall fuses with the old hyphal wall, leaving a piece of old wall material (arrow); the cross-wall (*CW*) is now distinctly double.

Cross-wall formation in vegetative aerial hyphae. Although this process is not part of sporulation it provided a comparison for the morphologically different septation in aerial hyphal tips that led to the formation of spores. Stages in cross-wall formation were only rarely observed. The process was initiated by annular ingrowth of a *double* layer of wall material, beginning at the inside of the old hyphal wall (Pl. 1, fig. 1). First (Fig. 1) the young cross-wall was separated from the old hyphal wall by a dark line (Pl. 1, fig. 1, and Fig. 1*b*: arrow); later the new cross-wall fused with the original hyphal wall, leaving a spare piece of old wall material which sometimes broke when the cells rounded off slightly at their ends. The cross-walls separating adjacent cells were double (Pl. 1, fig. 1, 2; Pl. 2, fig. 3); this doubtless accounts for the fact that the aerial hyphae can fragment into viable single cells able to initiate new colonies (Hopwood, 1960). The cross-walls appeared in unbranched regions of the hyphae, but also frequently at branch points (Pl. 2, fig. 3); branching without septation was also found.

The lengths of cells in the aerial hyphae were measured in negatively stained

preparations in which cross-walls were clearly visible. Non-apical cells of sporulating hyphae were 3 to 16 μm. long, while the apical cells of young hyphae which had not yet begun to sporulate were much longer; some could be followed for 50 μm. without showing any cross-walls. It is believed that young aseptate hyphae were divided into long segments by hyphal cross-walls, the apical cells subsequently being partitioned into shorter units (1·5 to 2·0 μm.), each of which gave rise to a spore. The actual length of sporulating apical cells could not, however, be determined since in negatively

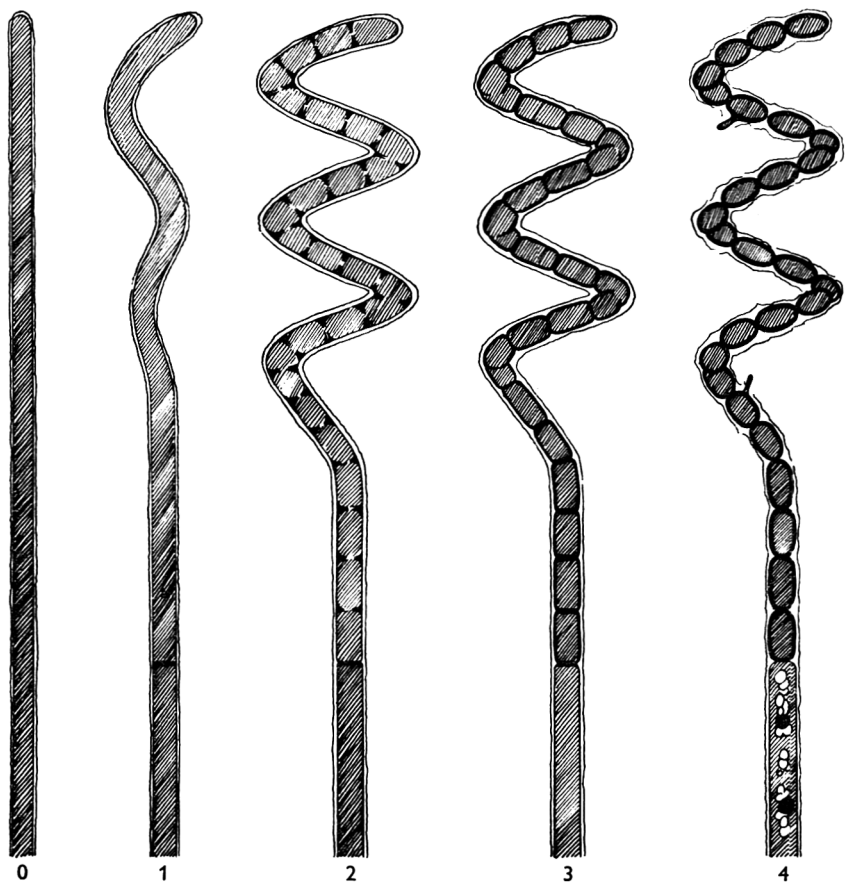

Fig. 2. Diagram of sporulation stages in *Streptomyces coelicolor*. After a phase of growth (0) the sporulating hyphae are divided into long cells by ordinary cross-walls and the tips begin to coil (1). The apex is then partitioned into spore-sized compartments by sporulation septa (2). The cell walls thicken and constrictions appear between the young spores (3). As spores mature, they round off and separate (4). Some spores begin to germinate immediately after maturation.

stained specimens it was not possible to distinguish between the hyphal cross-walls and the septa which divided the cells into spore-sized compartments. Therefore it was not certain whether sporulation was confined to apical cells or occurred also in sub-apical ones.

Fine structural changes during spore formation. The steps in sporulation are represented diagrammatically in Fig. 2; they have been classified into a number of stages

as in *Bacillus subtilis* (Ryter, 1965) and *Clostridium histolyticum* (Bayen, Frehel, Ryter & Sebald, 1967).

Thin sections of aerial mycelial cells at stage 0 are shown in Pl. 2, fig. 3. The typical features of such cells were described by Glauert & Hopwood (1959, 1960, 1961) and Hopwood & Glauert (1960*b*) and are briefly summarized here. The cell wall (*CW*), which had the appearance characteristic of a Gram-positive wall, was overlain by the superficial fibrous sheath (*FS*). The interior of each cell was bounded by the plasma membrane (*PM*) and was occupied by membranes of the mesosome system (*M*), 'vacuoles' (*V*), and fibrous nuclear material (*N*) mainly in long zones corresponding with the elongated chromatic bodies seen in the light microscope (Hopwood & Glauert, 1960*a*); ribosomes, which presumably would have been revealed by lead staining, were barely visible in these sections because of their low contrast.

Plate 3, fig. 4 represents a longitudinal section of a hypha which had coiled at stage 1 and was therefore ready for the formation of the special 'sporulation septa'. The nucleoplasm was still in long zones, which only later became divided into smaller portions, each allocated to one spore compartment. The orientation of the nuclear fibrils was not random; as noted by Hopwood & Glauert (1960*b*), most of the fibrils ran longitudinally in some regions of the hyphae and transversely in others.

At stage 2 (Pl. 3, fig. 5 and Pl. 4, fig. 6) the sporulation septa (*SS*) were formed at regular intervals, their development being synchronized over a certain length of hypha. Each septum developed as a double annulus growing towards the centre of the hypha. The two adjacent layers of the septum were separated from the beginning by a gap which was very obvious at the inner edge of the annulus (Pl. 3, fig. 5, arrow). At the same time the nuclear material (*N*) appeared to be constricted at the sites of the growing spore septa (Pl. 3, fig. 5) and was fully divided just before the septa were completed (Pl. 4, fig. 6). Occasionally, the young septa could be seen to be accompanied by mesosomes, but usually these were more obvious at the next stage of spore formation (Pl. 4, fig. 7, *M*). Sections through the top of the aerial mycelium of a colony revealed few early stages of septum formation, but many later stages; hence septation must have been rapid. The hyphal wall at stages 1 and 2 always consisted of *one* layer, still 10 to 12 nm. thick; the double-layered hyphal wall described by Glauert & Hopwood (1961) was not observed.

Late stage 2 (2b) (Pl. 4, fig. 7 and Pl. 5, fig. 8) was characterized by completed septum formation. Whereas in early stage 2 (2a) there was more material at the base of the septa than on their inner edge, at the end of the process the septa were uniformly thick and each layer of the double septum was no thicker than an average hyphal wall (10 to 12 nm.). Spore wall material was initially deposited on the long sides of spore walls only at the margin of the sporulation septa, where a boundary between the spore wall material and the old hyphal wall could be identified (Pl. 3, fig. 5, *B*). Later, after completion of the septa, new wall material was gradually added to the septum and to the inner side of the parent hyphal wall. These processes led to stage 3, which is defined by a thickened wall (20 to 25 nm.) and the appearance of marginal constrictions between the spore compartments (Pl. 6, fig. 9). Sometimes the spore compartments also began to separate centrally and were then held together by only a thin annulus consisting of the old parent wall (Pl. 6, fig. 9, *PW*) and the fibrous sheath (*FS*), a thin superficial layer which surrounded the aerial hyphae and

spores (Hopwood & Glauert, 1961). Except where adjacent spores abutted, it was difficult to distinguish between the old hyphal wall and the new spore wall: the only detached layer was the superficial fibrous sheath.

Structures in the interior of the cell appeared indistinct at this stage; in most sections it was not even possible to identify the nuclear material. Mesosomes were only rarely found and the cytoplasm was full of vacuoles with ill-defined boundaries and of very low electron density (Pl. 6, fig. 9: *P*). At stage 4 the spores were mature (Pl. 6, fig. 10 and Pl. 7, fig. 11) and were held together by only a fragile envelope consisting of the fibrous sheath and disintegrating parts of the old hyphal wall; the chains easily broke into separate spores. The spore wall had reached its final thickness (30 to 50 nm.). The mature spores were very hard and consequently were extremely difficult to section; most of the spores were folded during cutting. Cytoplasm and nuclear material looked much the same as at stage 3. Mesosomes were found in both sections and negatively stained preparations of spores.

In colonies just beginning to sporulate a number of newly formed spores were already germinating (Wildermuth, 1969). In negatively stained impressions of such colonies germinating spores were even found within chains of spores. In sections of young colonies many spores appeared better preserved (Pl. 7, fig. 12). It seems likely that these spores were germinating (although the germ tube was not seen in the section) or at least in a preparative stage for the process.

The spore wall occasionally appeared multilayered, this being more obvious in germinating spores which mostly exhibited two distinct layers. However since the stratification showed great variation it must be interpreted with caution (see Discussion).

DISCUSSION

This study of thin sections of the aerial mycelium of *Streptomyces coelicolor* has revealed two different processes of cross wall formation, one occurring in vegetative aerial hyphae and the other in sporulating hyphal tips. The difference between these two processes has been confirmed in a study of mutants defective in sporulation (Hopwood *et al.* 1969); some classes of mutants are unable to form the sporulation septa but produce normal cross walls in the aerial hyphae and in the substrate mycelium.

The cross-walls in the non-sporulating aerial hyphae (and probably also in the substrate hyphae) were formed in the manner typical of Gram-positive bacteria, as for example in Bacillus (Glauert, 1962; Ellar, Lundgren & Slepecky, 1967): by annular deposition of new wall material centripetally. In Bacillus the young wall is about twice as thick as the normal cell wall; it splits after completion and the daughter cells separate. In the filamentous Streptomycetes the adjacent cells normally did not separate, but could be separated artificially; the isolated hyphal cells were viable and able to initiate new colonies (Hopwood, 1960). In sections of both complete and unfinished cross-walls a dark central line could be seen. This boundary was not observed in young cross-walls of Bacillus (see for example Ellar *et al.* 1967) but was found in *Microbacterium thermosphactum* (Davidson, Mobbs & Stubbs, 1968).

In contrast to the hyphal cross-walls the sporulation septa were deposited synchronously over a certain range at regular short intervals. The wall material was less compact in the beginning, wider at its base and regularly split at the inner edge of the

annulus. This unusual behaviour for bacterial cross-walls was also found in *Streptomyces venezuelae* (Bradley & Ritzi, 1968) but not in *Streptomyces viridochromogenes* (Rancourt & Lechevalier, 1964).

According to Glauert & Hopwood (1961) the parent hyphal wall separated into an outer and an inner latter, the latter extending towards the centre of the cell by annular ingrowth, thus forming the spore septum. This new cross-wall together with the inner parent wall constituted the young spore wall to which more material was added as the spore matured. Rancourt & Lechevalier (1964) and Bradley & Ritzi (1968), who studied sporulation in *Streptomyces viridochromogenes* and in *Streptomyces venezuelae* respectively, followed essentially Glauert and Hopwood's interpretation. However it is doubtful whether such a behaviour of the parent hyphal wall can be deduced from their micrographs, which show fewer details than those of Glauert & Hopwood (1961).

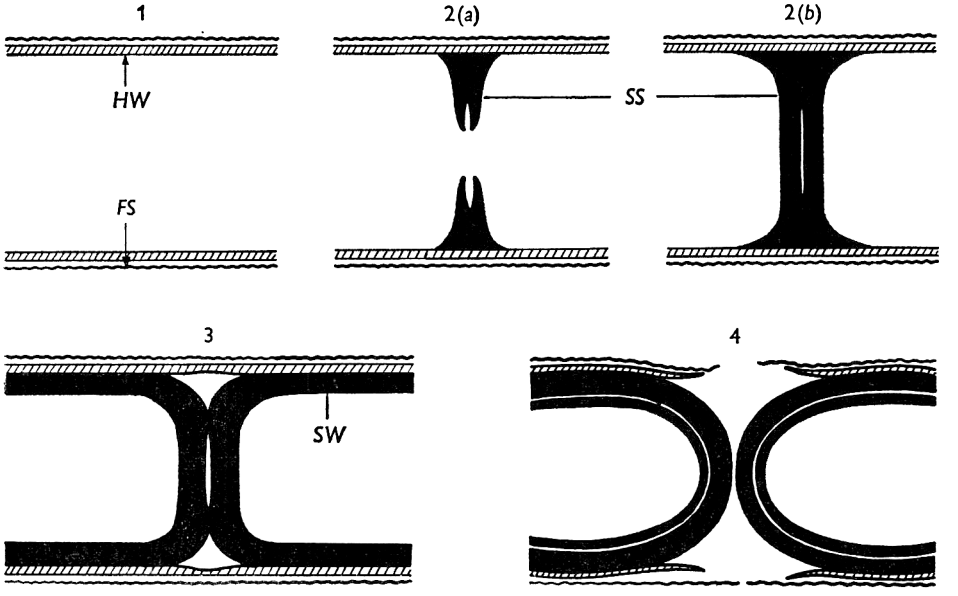

Fig. 3. Diagram of spore-wall formation. At the beginning of sporulation (Stage 1) the cell envelope consists of the hyphal wall (*HW*) and the fibrous sheath (*FS*). During stage 2 the sporulation septa (*SS*) are formed. At stage 3 the future spore-wall (*SW*) is laid down. While the spores round off and begin to separate, they are maturing (stage 4) and the spore-wall is still thickening.

In the sections described in this paper the separation of the parent hyphal wall into an outer and an inner layer by a dark line could not be confirmed and the thickness of the wall remained the same until completion of the cross-wall. According to these findings it is believed that the material of the future spore walls was deposited inside the parent hyphal wall, and probably a completely new coat was synthesized for the spore (Fig. 3). However the old hyphal wall presumably remained attached to the spore and formed part of the wall, leaving detached ends only at the site of the sporulation septa. A clear boundary between the old and the new wall was not generally seen. Therefore Streptomyces spores cannot be considered as endospores, especially since all the nuclear material and cytoplasm was included in the spore, in

contrast to the situation in Bacillaceae and to the recently discovered situation in certain thermophilic actinomycetes (Cross, Walker & Gould, 1968).

The walls of the mature spores seemed to be multilayered; several distinct layers were described by Hagedorn (1960) in *Streptomyces griseus* and by Glauert & Hopwood (1961) in *Streptomyces coelicolor*. In the present study the walls of 'resting' spores occasionally exhibited two layers. In germinating spores, whose walls were apparently softer, the stratification was more obvious. However it was less clear than in Bacillus (Ryter, 1965, Ellar & Lundgren, 1966, Leadbetter & Holt, 1968) or Clostridium (Hoeniger, Stuart & Holt, 1968, Bayen *et al.* 1967) and probably depends on factors such as fixation, sectioning and the physiological state of the spore. Therefore these structures have to be interpreted with caution. The same applies to the interior of mature spores which appeared smooth and without any details. This is seen in all spore-forming bacteria so far examined, including Actinomycetes (Dorokhorva, Agre, Kalakoutskii & Krassilnikov, 1968), but it remains possible that this is due to insufficient penetration of the fixative and stains.

The mesosomes, which were conspicuous during sporulation, behaved as in other spore-forming bacteria: they were associated with the young septa. Like the other structures they became less clear during the late stages of sporulation, but they definitely persisted in spores. This could be demonstrated not only in germinating spores (Mach, 1965) but also in sections (Pl. 7, fig. 11) and negatively stained preparations of 'resting' mature spores.

Part of this work was carried out at the Institute of Genetics, University of Glasgow. It is a pleasure to thank Audrey M. Glauert for her helpful criticisms of the manuscript during its preparation.

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EXPLANATION OF PLATES

Electron micrographs of thin sections of *Streptomyces coelicolor* aerial hyphae and spores. The scale mark represents 0·25 μm.

PLATE 1

Fig. 1. Cross-wall formation in an aerial hypha during the vegetative phase. The arrow indicates the boundary between the outer cell wall and the young cross-wall.

Fig. 2. Longitudinal section through a vegetative part of an aerial hypha, showing the double complete cross-wall. Note the gap (*G*) within the double wall. *M*, mesosome.

PLATE 2

Fig. 3. Section through vegetative aerial hyphae, showing the typical features of a Streptomyces cell. *CW*, cell wall; *FS* fibrous sheath; *M*, mesosome; *N*, nuclear material; *PM*, plasma membrane; *V*, vacuole. Note the double cross-wall.

PLATE 3

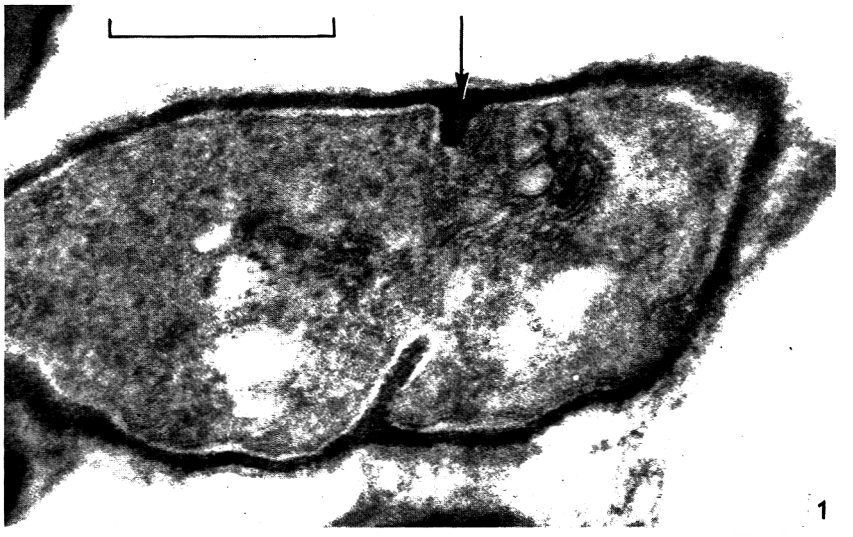
Fig. 4. Longitudinal section through an aerial hypha at sporulation stage 1. The cell is curved and the nucleoplasm (*N*) forms an elongated body.

Fig. 5. Aerial hypha at early sporulation stage 2 (2a). The sporulation septum (*SS*) appears as a double annulus, showing a gap at the growing margin (arrow). The nucleoplasm is constricted at the site of the septum. Note the boundary between the old parent wall and the septum material (*B*).

PLATE 4

Fig. 6. Stage 2a, showing a spore compartment bounded by two sporulation septa. The left septum appears almost complete because it was tangentially sectioned. The nuclear bodies (*N*) appear to be completely separated.

Fig. 7. Late stage 2 (2b). The sporulation septa are complete. Note the mesosomes (*M*) associated with a newly formed septum.



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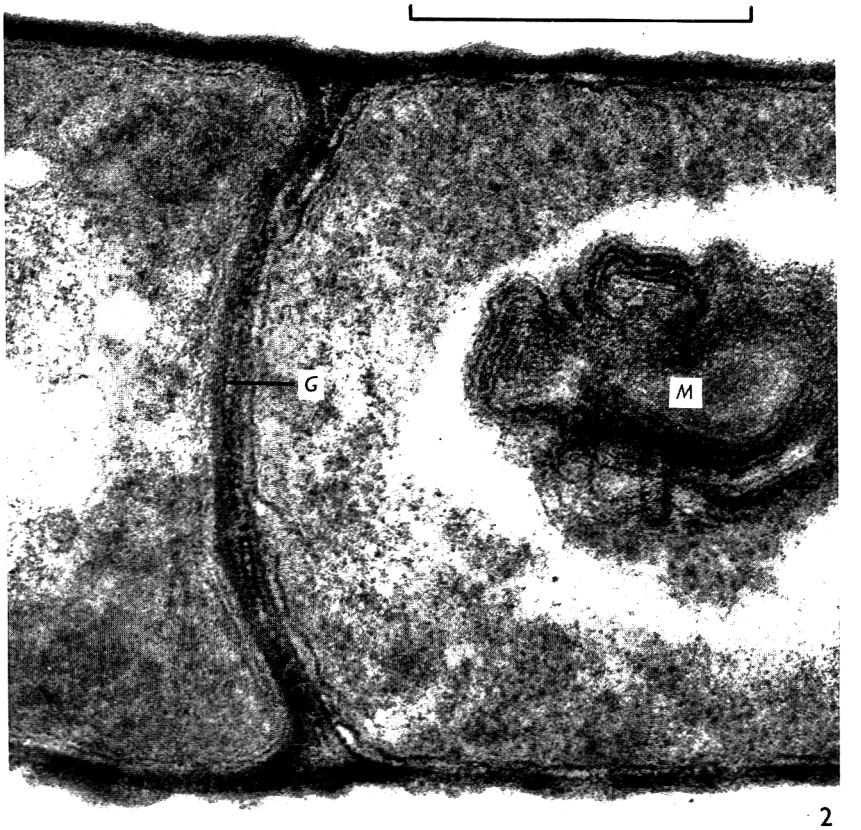

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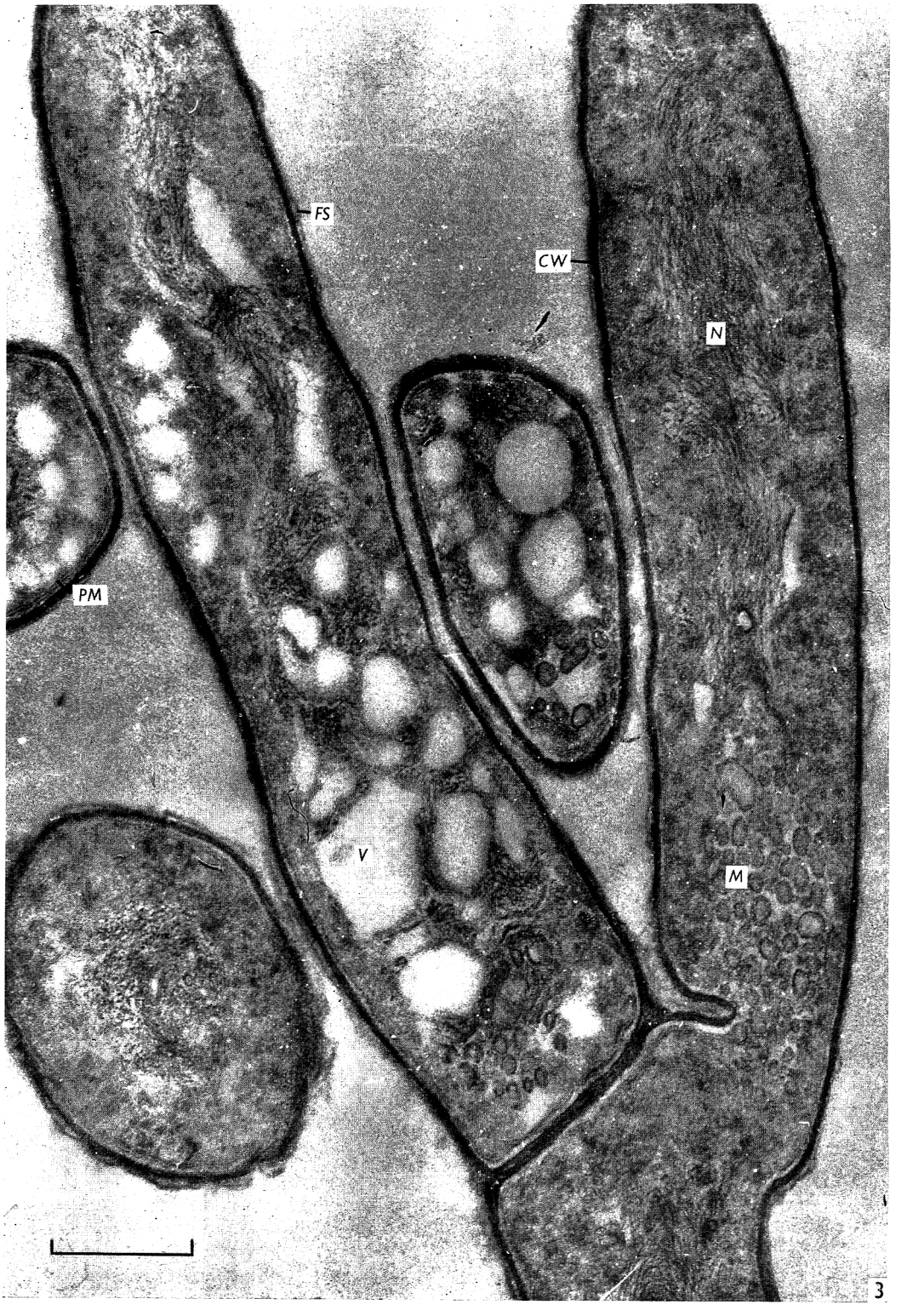

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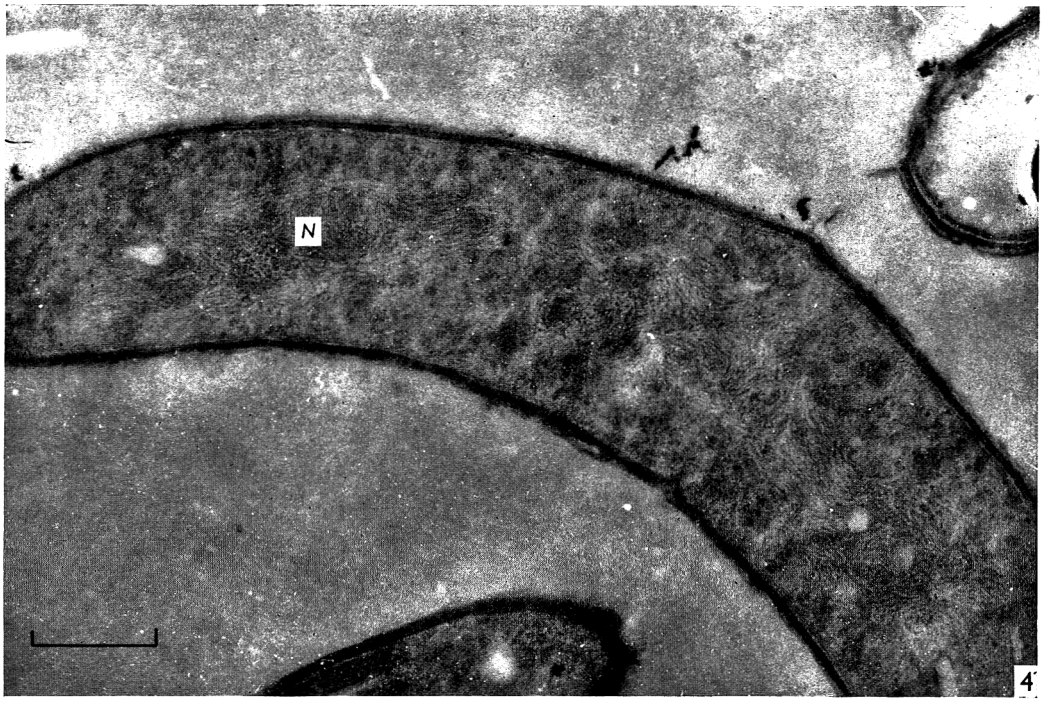
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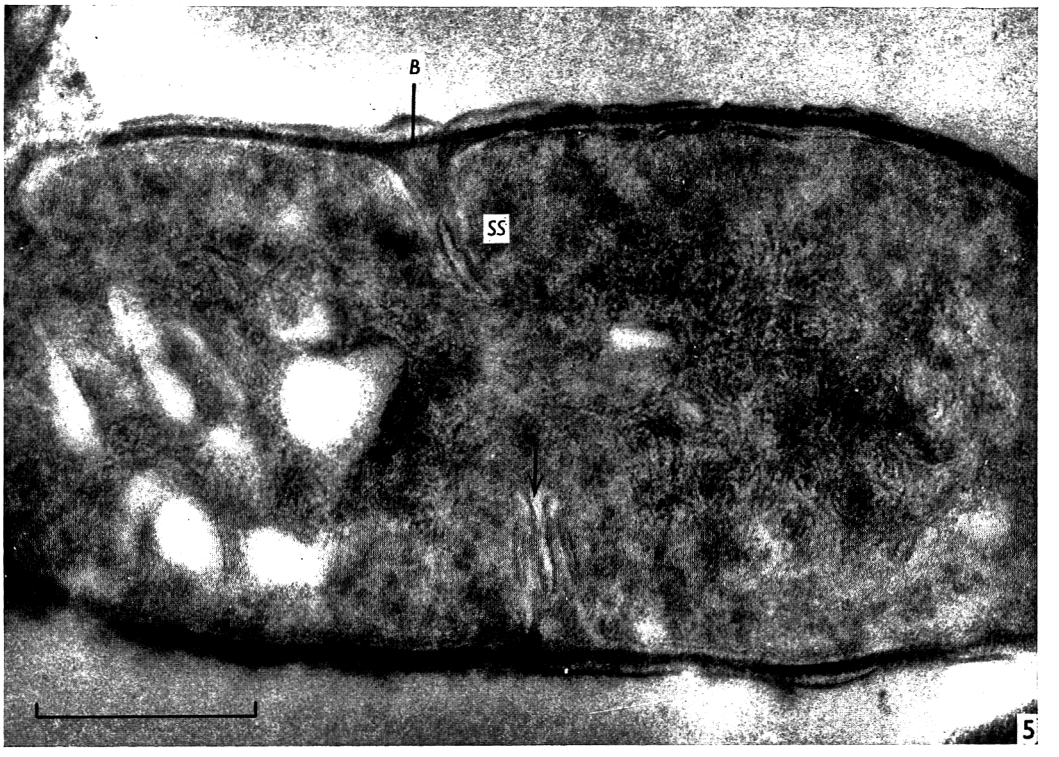
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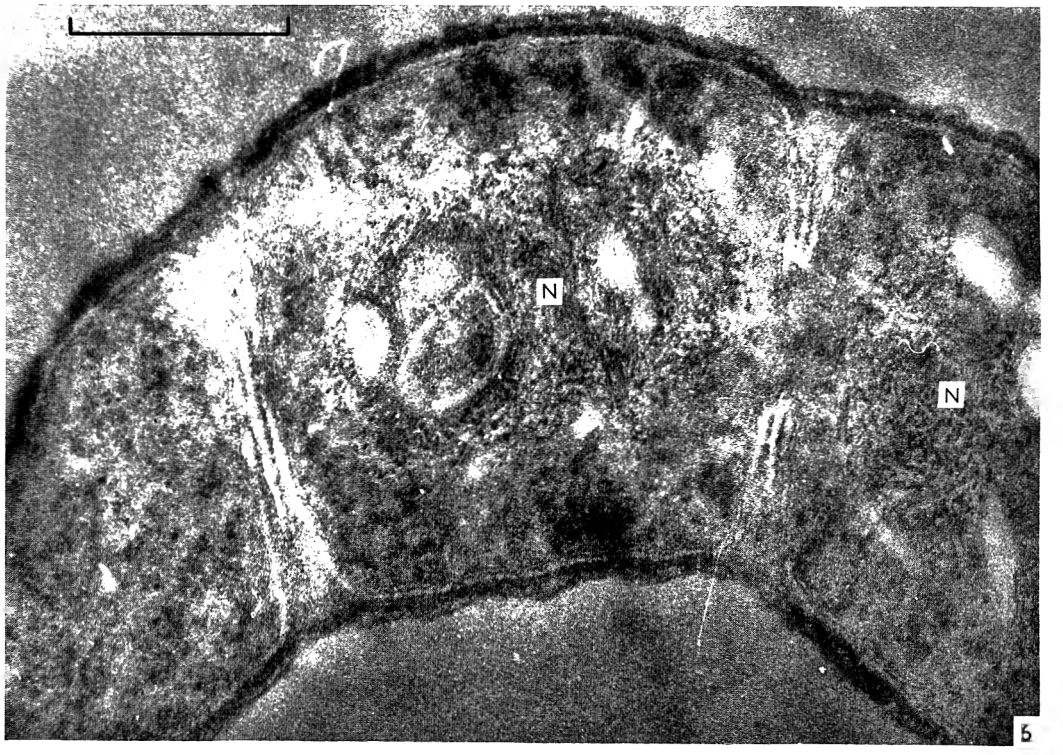
SS
5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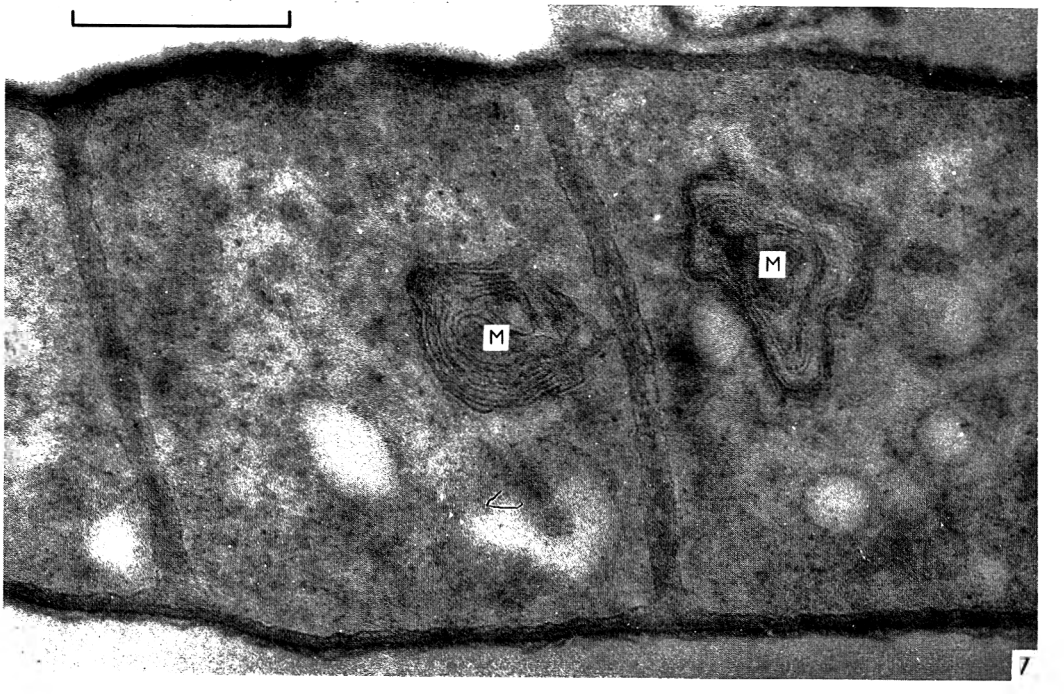
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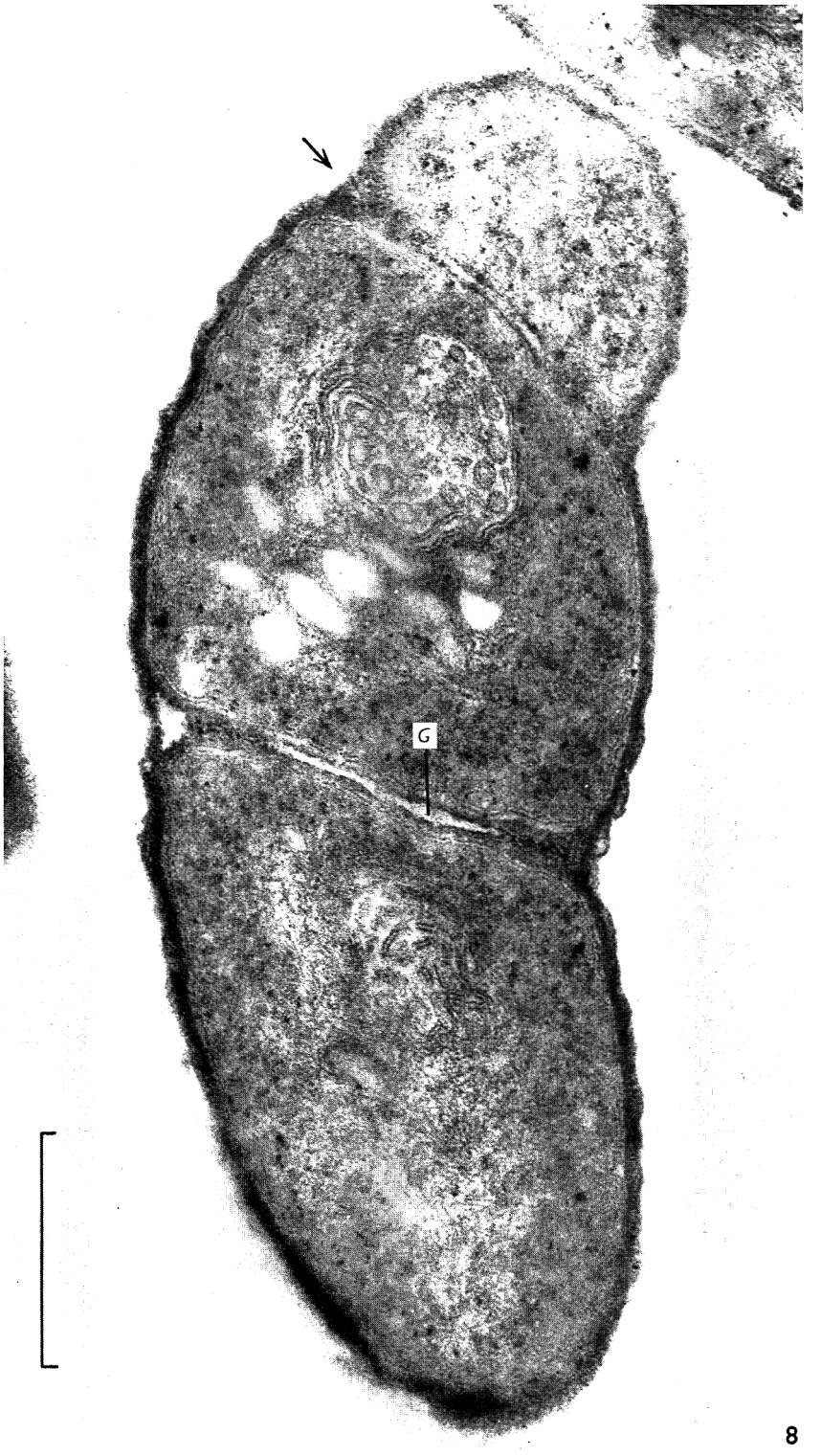
7



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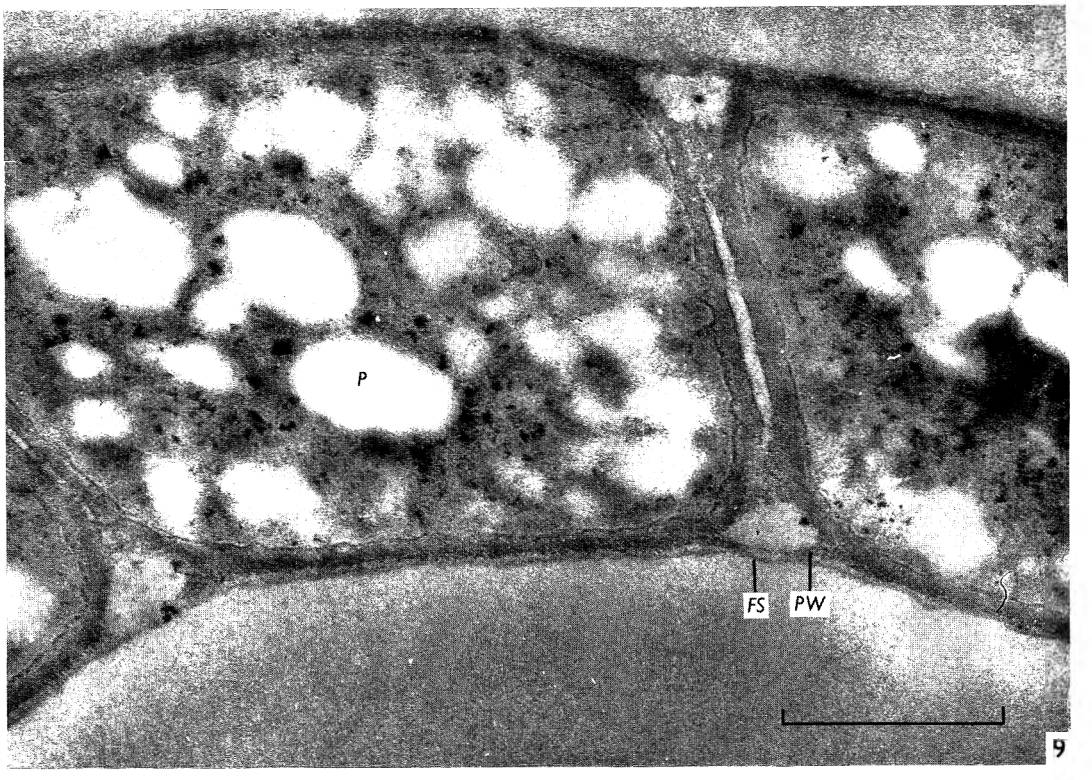
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FS
PW
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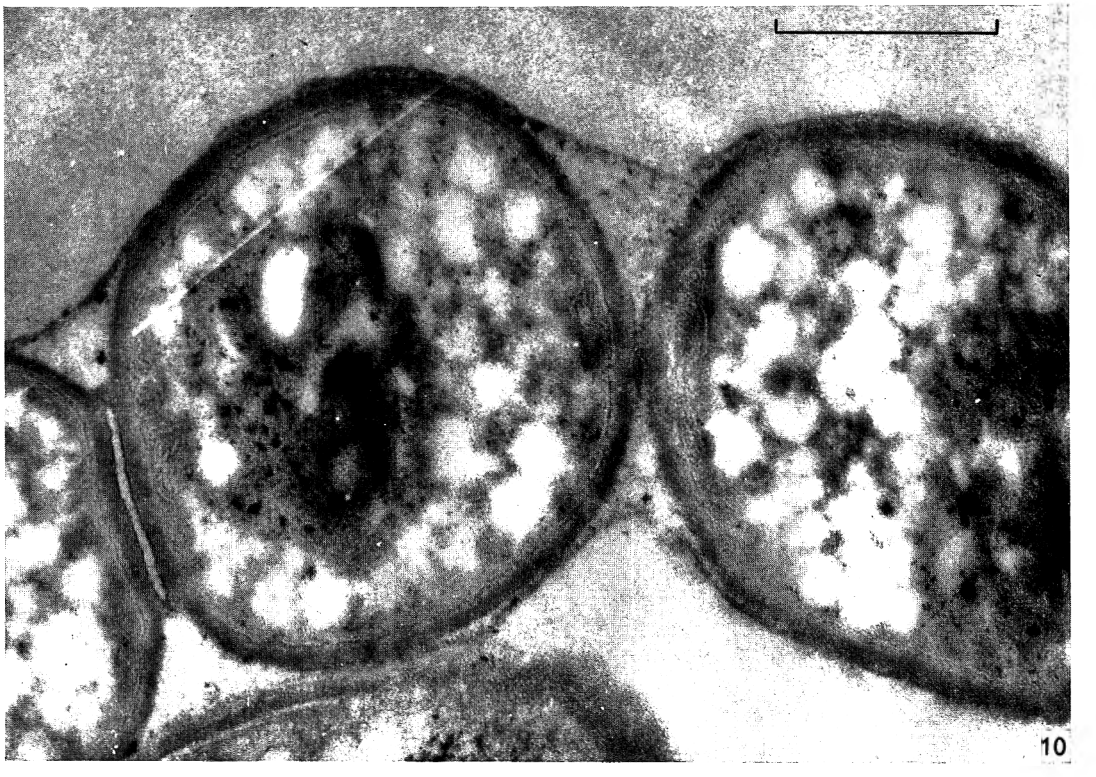
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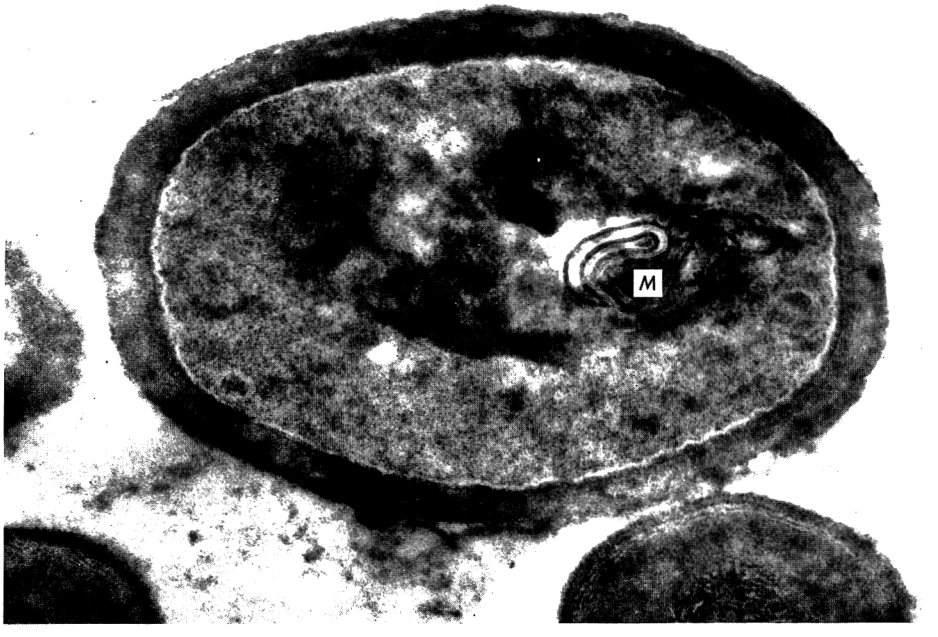

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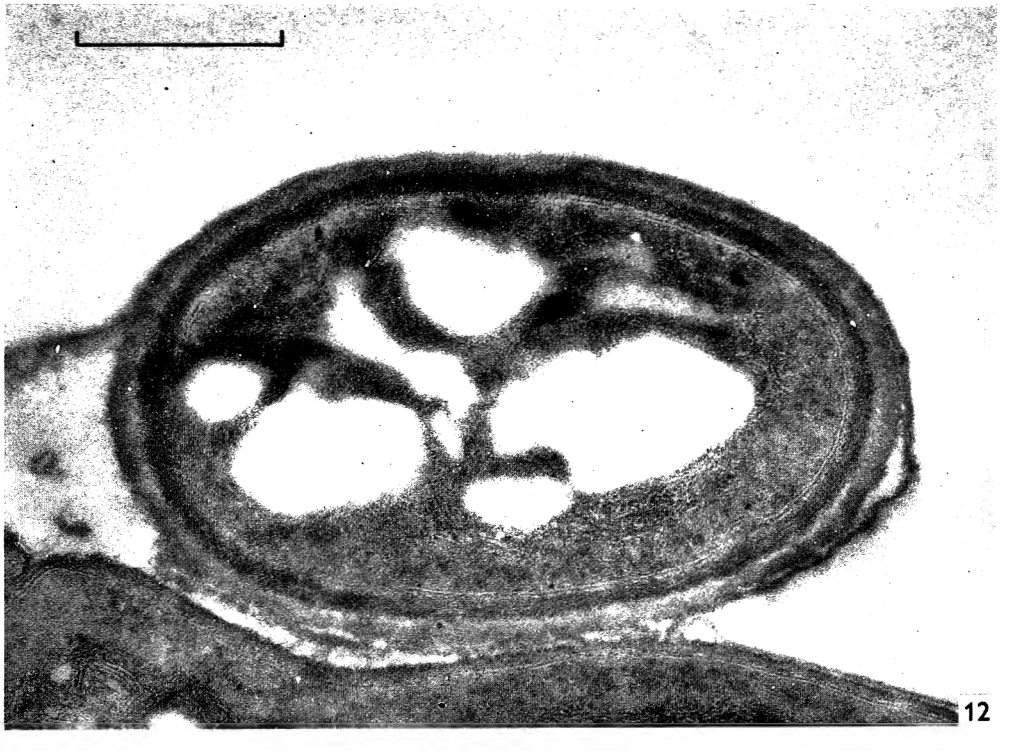

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PLATE 5

Fig. 8. Stage 2b, showing the double septa. The gap (*G*) and rounding corners (arrow) indicate the transition to stage 3.

PLATE 6

Fig. 9. Stage 3. The spore wall has noticeably thickened from 12 to about 25 nm. and the individual spores, which are still held together by remnants of the parent hyphal wall (*PW*) and the fibrous sheath (*FS*), begin to round off. The contents of the cell appear blurred with vacuole-like patches (*P*).

Fig. 10. Part of a chain of maturing spores (stage 4).

PLATE 7

Fig. 11. Typical features of a mature spore. The mesosome (*M*) in this specimen is exceptionally well visible.

Fig. 12. Mature spore, probably before or during germination. Details such as nucleoplasm and plasma membrane are better preserved than in the specimen shown in fig. 11. The spore-wall appears double-layered.

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The Presence of N_2-fixing Bacteria in the Intestines of Man and Animals

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SUMMARY

Cultures of N_2-fixing bacteria have been isolated from the intestinal contents of humans, pigs and guinea-pigs. Acetylene-reduction tests indicate that N_2-fixation may be occurring in the intestines. Best N_2-fixation was by cultures provisionally identified as *Klebsiella aerogenes*, but other genera were also involved. All cultures fixed more N_2 anaerobically than aerobically but some fixation occurred when 20% O_2 was present.

INTRODUCTION

Recent studies have shown consistent negative nitrogen balances in sweet potato-eating people in the highlands of New Guinea (Luyken, Luyken-Koning, Pikaar & Blom, 1964; Oomen & Corden, 1969). The apparent deficit in nitrogen was about equal to the daily dietary intake of N (about 2·0 g.). It seemed possible that fixation of atmospheric N_2 in the intestines of these people living on a diet of very high C:N ratio, could result in high levels of faecal-N and so give spurious nitrogen balance results. This communication establishes that N_2-fixing bacteria can occur in the intestines of man and animals.

METHODS

Nutrient agar (Oxoid or Difco) and Burk N-free agar (Burk & Lineweaver, 1930) were used for maintenance of cultures. N-free broth cultures were grown on Burk medium under an atmosphere of N_2. Yeast extract (Oxoid), 150 mg./l., was used to supplement Burk medium for some cultures. Media for biochemical tests were those listed by Carpenter, Lapage & Steel (1966). N_2-fixation was measured by increase in culture total-N, by $^{15}N_2$ incorporation and by the acetylene-reduction method (Hardy, Holsten, Jackson & Burns, 1968). ^{15}N was analysed as previously described (Bergersen, 1962) using Kjeldahl digestion of cultures, distillation of the NH_3 and oxidation to N_2 by NaOBr; mass 29:28 ratios were measured with an Atlas M 86 mass spectometer. The reduction of acetylene to ethylene was measured in rubber-capped bottles of 13·5 ml. capacity with a gas phase containing 20% (v/v) acetylene, or on cultures on agar in McCartney bottles fitted with perforated caps and thick rubber seals to enable gassing and sampling through hypodermic needles. Ethylene production was measured in 200 μl. gas samples using a Philips 4000 gas chromatograph equipped with 2 m. × 2 mm. columns of Poropak R at 50°. Control tests without acetylene did not give any detectable ethylene production with faecal material or cultures. In the guinea-pig experiment there was no ethylene production before the introduction of acetylene into the chamber.

RESULTS AND DISCUSSION

N_2-fixation in human faecal material

Samples of about 1 g. (fresh weight) of freshly voided faeces from five Europeans in Canberra were homogenized in 2 ml. 1·0% (w/v) glucose and incubated for 24 hr at 37° under N_2. The gas was then replaced with acetylene-argon mixture and incubated for 2 hr. One sample (H) showed a small but significant production of ethylene amounting to about 1·5 mμmole. Later, a further sample was obtained from the same donor and examined in the same way together with samples from four other European donors. Again H was the only positive reaction observed. Five serial sub-cultures of H faeces in Burk liquid medium under N_2, followed by plating on Burk agar, yielded a pure culture of a Gram-negative, lactose-fermenting, non-motile bacterium, which grew well anaerobically with N_2 as the sole nitrogen source (culture H 1). A similar culture was obtained from the same donor by the same procedure several months later (culture H 2).

On two occasions, samples of faecal material from sweet potato-eaters were flown to Canberra, packed in ice, from the Morobe district, Territory of Papua–New Guinea (T.P.N.G.). These were examined for acetylene reduction, and on both occasions all specimens showed slight but significant activity. Cultures were obtained from all of the second set of samples, which were able to grow well on Burk medium supplemented with traces of yeast extract. These cultures were obtained from high dilutions of the original faeces (10^{-6} to 10^{-8}), indicating that the organisms were present in high numbers (cultures H 3 to H 6). On another occasion cultures were isolated from diluted fresh human faeces at Baiyer River, T.P.N.G. Data are presented for two of these (H 46, H 55).

Animal experiments

A guinea-pig which had been fed on a low-N diet for several days was placed in a large desiccator of about 6 l. equipped with an O_2 metering device and a CO_2-absorbing by-pass. The atmosphere was slowly replaced by one containing 20% (v/v) O_2, 10% acetylene, and the balance was argon. A cooked potato diet and drinking water were supplied. The gas in the chamber was analysed at intervals for ethylene. Slight but progressive ethylene production was observed over a period of 22 hr, finally amounting to about 75 mμmoles. After removal of the animal, some ethylene production continued but when the faeces were removed, this ceased. Diluted caecal contents of a guinea-pig on low-N diet yielded discrete grey colonies when incubated anaerobically under N_2 on Burk medium (culture P 5 B). Attempts to demonstrate nitrogen fixation in the intestine of these animals by means of the acetylene method failed because of the difficulty of maintaining the acetylene concentration in a gas bubble injected into the lumen of the gut, for more than a few minutes.

A pig was fed on a diet of raw potato for a week before slaughter. Very watery colonies of a motile bacterium were obtained on Burk medium, supplemented with yeast extract, from diluted caecal contents obtained after slaughter (culture P 11). On subculture the nature of the colonies changed to a small, dense, raised type.

Table 1. *Properties of cultures isolated from human faeces and animal intestinal contents*

Culture	Biochemical tests§														N_2 fixation test*			
															Aerobic		Anaerobic	
Culture	Motility‡	MacConkey broth	Methyl red	Voges-Proskauer	Indole	Urease	Citrate	Glucose	Sucrose	Lactose	Dulcitol	Inositol	Salicin	Provisional identification	Growth†	Atoms % ^{15}N excess	Growth†	Atoms % ^{15}N excess
H 1	−	+	−	+	+	+	+	AG	+	+	−	+	+	*Klebsiella aerogenes*	+	0·453	+ + +	15·496
H 2	−	+	−	+	−	+	+	AG	+	+	sl+	sl+	+	*K. aerogenes*	+	0·882	+ + +	22·186
H 3	+	sl+	−	+	−	−	+	AG	+	±	−	−	sl+	*Enterobacter cloacae*	+	0·324	±	0·775
H 4	−	o	−	−	−	−	−	o	−	o	o	o	o	?	+ + +	0·059	+	0·151
H 5	−	o	o	o	o	o	−	o	−	o	o	o	−	?	+ + +	0·048	+	0·163
H 6	+	o	−	−	−	−	−	−	−	±	o	o	−	?	+ + +	0·022	+	0·067
H 46	−	+	−	+	−	+	−	AG	+	+	+	+	+	(?) *K. aerogenes*	+	0·245	+ +	40·473
H 55	+	o	+	−	−	−	−	A	+	+	−	−	+	(?) *Escherichia coli*	+	1·492	+ +	9·116
P 5 B	−	+	−	+	−	+	+	AG	+	+	sl+	+	+	*K. aerogenes*	+	0·410	+ + +	15·465
P 11	+	+	+	−	−	−	−	AG	−	+	−	−	+	*E. coli*	+	0·061	+	0·521

* Gas mixtures contained 20% $^{15}N_2$ (80 atoms %) + 80% argon or 60% argon and 20% O_2. The temperature was 30°.

† Rating of turbidity of growth washed off agar slope in 5 ml. H_2O prior to digestion and ^{15}N analysis.

‡ Motility was examined in MR/VP broth after 16 hr at 30°.

§ Tests given, o, were those where no growth occurred; −, indicates growth but no reaction; ±, indicates growth with a weak reaction; +, indicates a positive reaction which, for the sugars, was either acid or acid and gas production in parallel to the results given for glucose. A = acid, AG = acid + gas. Tests given sl+ were those which were + only after more than 48 hr at 30°. Indole and methyl red/Voges–Proskauer reactions were done after 72 hr at 30°.

Properties of the N_2-fixing cultures

The biochemical tests done on the cultures are summarized in Table 1. Cultures H4, H5 and H6 were Gram-negative rods but they failed to grow in most of the simple media used. This may have been due to a growth factor requirement, since all three cultures required traces of yeast extract for growth on Burk medium. They were also yellow-pigmented on nutrient agar. Six other cultures were members of the Enterobacteriaceae, being Gram-negative, facultative, anaerobic rods producing acid and gas from glucose and growing on media containing bile salts. Their provisional identification is shown on the basis of the criteria given by Carpenter, Lapage & Steel (1966). Those provisionally identified as *Klebsiella aerogenes*, H1, H2 and P5B, were differentiated from *K. pneumoniae* by their methyl red Voges–Proskauer reactions but their properties are similar to those of N_2-fixing strains of *K. pneumoniae* isolated from sugar cane by Nunez & Colmer (1968) and identified from the classification of Edwards & Ewing (1962). Isolate H46 may be a non-citrate-utilizing strain of *K. aerogenes*. The cultures H3, H55 and P11 were distinct from the Klebsiella cultures in being motile. If their identification as *Enterobacter cloacae* and *Escherichia coli* is confirmed, they represent new examples of organisms able to fix N_2.

The ability of these cultures to fix N_2 has been confirmed in a number of separate tests using $^{15}N_2$ and in the C_2H_2 reduction test. Table 1 gives the result of one experiment in which cultures were grown on Burk agar, supplemented with yeast extract, in McCartney bottles with atmospheres containing $^{15}N_2$. After 24 hr the growth was washed off the agar in 5 ml. water and digested for nitrogen analysis. Cultures H1, H2, H46, H55 and P5B grew better and fixed more N_2 anaerobically than aerobically. Culture P11 grew equally well under aerobic and anaerobic conditions but fixed more N_2 anaerobically. Cultures H4, H5 and H6 appeared to grow as well aerobically as the best N_2-fixing cultures grown anaerobically, but they fixed only small amounts of N_2. Culture H1 produced H_2 and CO_2 in the ratio of 1:2 when grown anaerobically with glucose as energy source in a shaken liquid culture in which 11·7 μg.N/ml. culture was fixed in 48 hr.

The results presented show that N_2-fixing bacteria can be isolated from the faeces of man and the intestinal contents of pigs and guinea-pigs. The best nitrogen-fixers appeared to be *Klebsiella aerogenes* but at least three other genera also occur. It has not been established that N_2-fixation occurs *in situ*, but the fact that several cultures have been obtained from high dilutions of intestinal contents and faeces indicates that substantial populations of these organisms may be present. Positive acetylene reduction tests have been obtained with fresh faeces and with the guinea-pig chamber experiment. This provides some evidence which supports the possibility of N_2-fixation occurring in the intestines. Further work is being done in identifying the bacteria which occur and in determining the parts of the intestine in which they are most numerous.

We thank Dr L. A. Malcolm, Regional Medical Officer, Lae, and Dr J. Crotty, Commonwealth Health Laboratory, Canberra, for active co-operation in this work. Mr G. L. Turner did the ^{15}N analyses and Mrs L. Hush provided technical assistance.

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Neisseria elongata sp.nov., a Rod-shaped Member of the Genus *Neisseria*. Re-evaluation of Cell Shape as a Criterion in Classification

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(*Accepted for publication 6 October 1969*)

SUMMARY

A rod-shaped, non-motile, Gram-negative, oxidase-positive and asaccharolytic organism found in the human nasopharynx is allotted to genus *Neisseria* and named *Neisseria elongata*. The strain M 2, which is the only isolate, is proposed as the type strain. The average guanine+cytosine (G+C) content of its DNA is 53 mole %. Genetic transformation of streptomycin resistance reveals a comparatively high compatibility with *N. meningitidis* and a strain designated *N. flava*, with ratios of interstrain to autologous transformation frequency in the range 0·01 to 0·1. On the other hand, there is no affinity in streptomycin-resistance transformation between *N. elongata* and members of genus *Moraxella*, including the old concepts *N. catarrhalis* and *N. ovis*. The family Neisseriaceae now appears to consist of two genera, *Neisseria* and *Moraxella*, each containing both coccal and rod-shaped species, which makes cell shape questionable as a highly weighted criterion in the construction of bacterial genera and higher taxa.

INTRODUCTION

Previous investigations by means of genetic transformation and determination of DNA base composition (Bøvre, 1967*b*; Bøvre, Fiandt & Szybalski, 1969) revealed strong indications of relationships between rod-shaped moraxellas and a group of coccal species within the family Neisseriaceae (*Neisseria catarrhalis*, *N. caviae*, *N. ovis*). This observation, which was later corroborated by RNA-to-DNA hybridization (K. Bøvre, unpublished), led Henriksen & Bøvre (1968) to propose that these 'false neisserias' (Baumann, Doudoroff & Stanier, 1968) be grouped together with the rod-shaped moraxellas in the genus *Moraxella*, proposed to belong in Neisseriaceae. The revised genus *Moraxella* would then consist of both rod-shaped and coccal species, with guanine+cytosine (G+C) contents of their DNA in the range 40 to 45 mole %. The remaining species of the family, which had G+C contents in the neighbourhood of 50 mole % and which were interrelated in terms of genetic transformation (Catlin & Cunningham, 1961; Bøvre, 1965, 1967*b*) and isolated from rod-shaped moraxellas and 'false neisserias' in these terms (Catlin & Cunningham, 1961; Bøvre, 1965, 1967*a*,*b*), were retained in the genus *Neisseria* ('true neisserias'). The genus *Neisseria* seemed at the time to consist exclusively of coccal organisms: *N. gonorrhoeae*, *N. meningitidis*, *N. sicca*, *N. subflava* (*N. flava*, *N. perflava*, *N. subflava*), *N. flavescens*, *N. cinerea* (Henriksen & Bøvre, 1968). The present report provides evidence that this genus also contains a rod-shaped species.

METHODS

Isolation. The rod-shaped bacterial strain M2 was isolated from the nasopharynx of a presumably healthy person in northern Norway. It evoked interest initially because a few streptomycin-resistant transformants were elicited in the strain by DNA of *Neisseria meningitidis*. This finding was a result of a screening for competence of transformation (undertaken by E. H.) of oxidase-positive strains isolated from the upper respiratory tract of military personnel.

Morphological, cultural and biochemical tests. With the modifications mentioned below, these were identical with the procedures previously described (Bøvre & Henriksen, 1967*b*). Heat resistance was studied in a water bath at 45° and 56°. The amino acids+biotin medium used was as described by Baumann *et al.* (1968). The basal medium used for study of growth on different carbon sources was identical with the mixture of salts used in a defined fluid medium for meningococci by Jyssum (1965); all organic substances were omitted. Ammonium chloride was the sole source of nitrogen. Before use, glucose, maltose, acetate, citrate or lactate was added from a sterile 10% (w/v) solution to a final concentration of 1%. The inoculum for the two last-mentioned media consisted of 0·01 ml. from a 20-hr broth culture in 10 ml. medium. The cultures were observed for 3 days of incubation with continuous shaking. Apparent growth was checked by subculture in the same medium. Acid production from carbohydrates was tested for in Mueller-Hinton broth with 1% glucose, maltose, sucrose, lactose, fructose, arabinose, galactose, xylose or mannitol, respectively, and with phenol red as indicator (Beno, Devine & Larson, 1968). Cultures were incubated for 4 weeks, and examined daily during the first 10 days. When otherwise not specified, incubation was at 37° and in most cases also at 33°.

Buoyant density determination of DNA. Preparation of DNA and analytical CsCl density gradient centrifugation were done according to methods previously described (Bøvre *et al.* 1969).

Transformation methods. In addition to the strain M2, the following strains were used either as source of transforming mutant DNA or as recipients in the experiments designed to characterize the genetic relations of the new isolate in a quantitative way: *Neisseria meningitidis* B8152/66; *N. meningitidis* M6; *N. flava* ATCC 14221; *Moraxella nonliquefaciens* 4663/62; *M. nonliquefaciens* NCTC 7784; *M. nonliquefaciens* 6121/68; *M. osloenisis* A1920; *M. phenylpyrouvica* 2863; *M. kingii* 4177/66; *N. catarrhalis* NE11; *N. ovis* 199/55. The strains (except *N. flava* ATCC 14221) have previously been characterized in intraspecies and interspecies transformation studies, which have been extensively referred to recently (Baumann *et al.* 1968; Bøvre, 1967*b*; Bøvre *et al.* 1969). The asaccharolytic *N. meningitidis* strain B8152/66 was studied in transformation a short time ago (Bøvre, 1969), as was also the strain *M. nonliquefaciens* 6121/68 (Henriksen & Bøvre, 1969). Most of the strains have also been characterized in terms of DNA base composition (Bøvre *et al.* 1969). The representativeness of the strains as regards their respective species designations is therefore well established. The old designation 'Neisseria' for the 'catarrhalis' and 'ovis' strains is used throughout this paper instead of 'Moraxella', which according to Henriksen & Bøvre (1968) is the correct genus for these species. This is done because these designations were used in the papers referred to. We also use the name *N. flava*, which probably should be included in *N. subflava*, as deduced by Henriksen & Bøvre from the transformation

results of Catlin & Cunningham (1961). *Neisseria flava* ATCC 14221 was found to produce yellow pigment on coagulated serum medium and to produce acid from glucose, maltose and fructose, but not from sucrose or lactose.

The procedure for screening of competence (see section on isolation), which revealed that strain M2 was possibly a unique rod-shaped organism as regards genetic relations, was described by Jyssum & Lie (1965). It was done by the streptomycin underlayering technique introduced by Bøvre & Henriksen (1962) and described further by Bøvre (1964). The principles of mutant selection, preparation of transforming DNA and transformation techniques have been described in connexion with application of the methods for moraxellas and neisserias (Bøvre, 1964, 1967*a*, *b*). The genetic marker used was exclusively one-step mutation to high streptomycin resistance (> 1000 μg. streptomycin/ml.). Briefly, two principal methods were used in the present study: (1) a strictly quantitative procedure with short-term (20 min.) DNA exposure, discontinued by means of DNase; (2) a more sensitive but less accurate (semiquantitative) procedure where the DNA exposure was continued (DNase treatment omitted) after plating the fluid DNA + recipient mixture. In the latter modification, the increasing number of colonies containing transformants was limited by the addition of streptomycin by the underlayering technique at the end of the period allowed for phenotypic expression of transformants (after 5 to 7 hr incubation). The only modification of the previous methods was that the recipients of strains M2, *N. meningitidis* B8152/66 and *N. flava* ATCC 14221, were stored for 20 to 30 min. at $+4°$ before use in transformation, instead of at $-20°$. The quantitative transformation procedure was done with bacteria in the lag phase. A few other experiments indicated that results obtained with lag-phase cultures were of the same order as with bacteria in the exponential phase of growth.

RESULTS

Strain M2

Morphological, cultural and biochemical characters. Microscopically the strain M2 appeared as short and slender rods, often arranged as diplobacilli or in short chains. The length of the organisms was not always uniform in the same preparation, some of the organisms being almost coccoid. However, stained smears made from the margin of the inhibition zone around a penicillin depot showed a quantitative change of the organisms into long filaments; such filaments were also occasionally seen in ordinary cultures. The micromorphology in the absence or presence of penicillin is given in Pl. 1, fig. 1, 2. For comparison, the normal micromorphology of *Moraxella nonliquefaciens*, at identical magnification, is shown in Pl. 1, fig. 3. Organisms of strain M2 were distinctly smaller than *M. nonliquefaciens*. They were Gram-negative, with a slight tendency to retention of the first stain. No capsules were observed in Indian ink preparations. The strain was non-motile.

After incubation on blood agar for 20 hr, the colonies were 1 to 1·5 mm. in diameter, even, shiny, low hemispherical, and had an entire edge. They were non-pigmented, as confirmed by culture on coagulated serum medium, greyish white, opaque. There was a tendency towards growth into the blood agar, with a resulting slight pitting of the agar surface. The colony consistency was clay-like and coherent; sometimes adherence to the medium was observed. The organisms were not emulsifiable in saline. No haemolysis was seen. There was no anaerobic growth. Slight growth

occurred at room temperature (20 to 22°). Optimal growth temperature was 33 to 37°. The organisms survived exposure to 45° for 30 min., but were killed at 56° in 5 min. The ability to survive on blood agar at room temperature was limited, cultures becoming sterile in 5 to 10 days. Growth on nutrient agar was almost as good as growth on blood agar. Elevated carbon dioxide tension enhanced the growth slightly. On simple peptone media such as the nitrate and indole media used, growth was good although somewhat slow. There was also relatively good growth in Hugh & Leifson medium, 1 cm. downwards from the surface, without production of acid, but with the appearance of small amounts of a brownish pigment along the stab. The organism grew well in amino acids + biotin medium. No growth occurred in the basal medium with ammonium ions as nitrogen source and glucose, maltose, acetate, citrate or lactate as carbon source, nor in Koser citrate medium. The catalase reaction was weakly positive. Strongly positive oxidase reactions were observed with both the tetramethyl- and the dimethyl-*p*-phenylenediamine reagents. No acid was produced from glucose, maltose, sucrose, lactose, fructose, arabinose, galactose, xylose or mannitol. Nitrate was not reduced to nitrite. Nitrate did not disappear on the addition of Zn powder. Gelatin and coagulated serum were not liquefied. There was no production of indole or hydrogen sulphide. Urea was not decomposed. There was no oxidative deamination of phenylalanine or tryptophan.

The organism was very sensitive to penicillin G, streptomycin, chloramphenicol, oxytetracycline and erythromycin.

DNA base composition. The buoyant density of DNA of strain M2 wild type was determined in two different analytical ultracentrifuges and found to be 1·7131 g./cm.^{3} and 1·7128 g./cm.^{3}, respectively. The DNA gave one very sharp peak, with a gaussian distribution. The average base composition of the DNA, i.e. the mole percentage of guanine + cytosine (mole % G + C), was calculated from the buoyant density by using the simplified formula: mole % (G + C) = $1000 \times (\rho - 1{\cdot}660)$, where ρ = buoyant density of DNA (g./cm.^{3}) measured versus the density of *Escherichia coli* DNA, assumed to be 1·710 g./cm.^{3} and to contain 50 mole % (G + C). This calculation led to 53 mole % (G + C) for the strain M2.

Transformation reactions. The strain M2 was used as donor and as recipient against each of the strains *Neisseria meningitidis* B 8152/66 and *N. flava* ATCC 14221 in quantitative transformation (short-term DNA exposure). The results are shown in Table 1. Ratios of interstrain to autologous transformation of the respective recipients were all in the range from $9{\cdot}2 \times 10^{-3}$ to $1{\cdot}2 \times 10^{-1}$, with the highest ratios observed when the coccal species were used as recipients. The ratios were somewhat higher between strain M2 and *N. flava* than between strain M2 and *N. meningitidis*. In a complete additional set of experiments similar to those of Table 1 the highest relative variation of ratio value was in the combination of recipient strain M2 and donor *N. flava* ATCC 14221, which revealed the ratio $1{\cdot}4 \times 10^{-2}$ instead of $2{\cdot}1 \times 10^{-2}$. By using a dense recipient population and at the same time omitting DNase treatment before plating, plates crowded with 1000 to 10,000 or more heterologous transformant colonies were easily obtained in all these recipient + donor combinations, when at the same time controls without DNA revealed less than 10 streptomycin-resistant colonies/plate. Although assayed at 50 μg. streptomycin/ml. the four kinds of heterologous transformants were uniformly able to grow well at 1000 μg. streptomycin/ml., as shown by replica-plating to blood agar plates with that concentration of antibiotic.

Transformant colonies examined microscopically all revealed the morphology (rod or coccus) of the recipient in question.

Semiquantitative transformation experiments (without DNase termination of the transformation process) with highly competent recipients of *Moraxella nonliquefaciens* NCTC 7784, *M. osloensis* A 1920, *N. catarrhalis* NE 11 and *N. ovis* 199/55, revealed no transformation with the donor strain M2, nor with the donors *N. meningitidis* B 152/66, *N. meningitidis* M6 and *N. flava* ATCC 14221, which were included for comparison and confirmation of previous data. The results of these experiments are collected in

Table 1. *Compatibility in streptomycin-resistance transformation between strain M2 and coccal Neisseria species*

In each experiment identical samples of the recipient were exposed simultaneously to the respective donor DNA samples (20 or 40 μg./ml.). Following DNA exposure for 20 min. at 33°, DNase was added and 0·1 ml. spread on each of 2 to 4 plates, which were incubated at 33° for 5–6 hr. Then streptomycin was added from below the agar and the incubation continued for 3 to 4 days before assay of transformants. The donor mutants had been selected at 500 μg. streptomycin/ml.; the transformants were assayed at 50 μg/ml. Methods: Bøvre (1964).

Recipient strain; colony count/ml.	Donor strain	Transformants/ml.	Ratio of interstrain to autologous transformation
Strain M2	*N. meningitidis* B8152/66	$2{\cdot}3\times10^{2}$	$9{\cdot}2\times10^{-3}$
$(5{\cdot}8\times10^{8})$	*N. flava* ATCC 14221	$5{\cdot}3\times10^{2}$	$2{\cdot}1\times10^{-2}$
	Strain M2	$2{\cdot}5\times10^{4}$	
N. meningitidis B 8152/66	Strain M2	$3{\cdot}6\times10^{2}$	$4{\cdot}9\times10^{-2}$
$(1{\cdot}1\times10^{9})$	*N. meningitidis* B8152/66	$7{\cdot}3\times10^{3}$	
N. flava ATCC 14221	Strain M 2	$1{\cdot}3\times10^{3}$	$1{\cdot}2\times10^{-1}$
$(7{\cdot}0\times10^{9})$	*N. flava* ATCC 14221	$1{\cdot}1\times10^{4}$	

ATCC = American Type Culture Collection.

Table 2. The donors *N. ovis* 199/55, *M. phenylpyrouvica* 2863, *M. nonliquefaciens* 4663/62 and *M. osloensis* A 1920, were chosen as sources of reference transforming DNA because they had low, but still distinct, transforming activities on the respective recipients, as measured in previous short-term experiments where the autologous mutant DNA of the recipients had been included (see legend to Table 2). Using the affinities of the reference donors as parameters in the continuous DNA exposure experiments, it was possible to calculate that the upper limit of any possible transformation between the M2 donor and the four recipients had to be as low as about 10^{-8}, autologous transformation of each recipient taken as unity (Table 2). This resolution would have been technically impossible by direct comparisons with the high-frequency autologous reaction in continuous DNA exposure, since this procedure did not allow differential dilutions to be made in the parallels to be compared. The rationale for its use was its very high sensitivity in the detection of low transformation activities. A series of semiquantitative experiments (not tabulated) were also made with the strain M2 as recipient and the following donors: *M. nonliquefaciens* 6121/68, *M. osloensis* A 1920, *M. phenylpyrouvica* 2863, *M. kingii* 4177/66, *N. catarrhalis* NE 11, *N. ovis* 199/55. In no case were transformants observed. These experiments were not sensitive as regards very low transformation compatibilities (minimum

detectable ratio of interstrain to autologous transformation in the order of 10^{-5} for the donors of *M. phenylpyrouvica* and *M. kingii*, and in the order of 10^{-4} for the other donors).

Table 2. *Degree of incompatibility in streptomycin-resistance transformation between strain* M 2 *and Moraxella nonliquefaciens, M. osloensis and the 'false neisserias' (N. catarrhalis and N. ovis)*

Methods as described in legend to Table 1, except that DNase was not added for termination of DNA exposure (Bøvre, 1967*a*) and that incubation times before and after streptomycin addition were 5 to 7 hr and 4 to 5 days.

Recipient strain	Donor strain	Sum of resistant colonies on 2 or 4 plates	Ratio of interstrain to autologous transformation*
M. nonliquefaciens NCTC 7784	No DNA	0	
	N. meningitidis B 8152/66	0	
	N. flava ATCC 14221	0	$< 7 \times 10^{-8}$
	Strain M 2	0	
	N. ovis 199/55	413	$< 2{\cdot}5 \times 10^{-5}$ (*a*)
M. osloensis A 1920 = ATCC 19976 = NCTC 10465	No DNA	1	$< 3 \times 10^{-8}$
	N. meningitidis B 8152/66	0	
	N. flava ATCC 14221	0	$< 3 \times 10^{-8}$
	Strain M 2	1	
	M. phenylpyrouvica 2863 = ATCC 23333 = NCTC 10526	277	6×10^{-6} (*b*)
N. catarrhalis NE 11	No DNA	23	
	N. meningitidis M 6	25	$< 2 \times 10^{-8}$
	Strain M 2	18	$< 1 \times 10^{-8}$
	M. nonliquefaciens 4663/62 = ATCC 19975 = NCTC 10464	6800	$6{\cdot}7 \times 10^{-5}$ (*c*)
N. ovis 199/55	No DNA	3	
	N. meningitidis M 6	4	$< 2 \times 10^{-8}$
	Strain M 2	5	$< 4 \times 10^{-8}$
	M. oslosenis A 1920 = ATCC 19976 = NCTC 10465.	1069	2×10^{-5} (*d*)

* Autologous transformation = intrastrain transformation of the recipient.

a, *b*, c, *d*: Reference donor activities, basis for calculation of ratios in the other parallels. *a*, *c*: quantitative determinations (Bøvre, 1967*a*); *b*: semiquantitative estimate (Bøvre & Henriksen, 1967*b*) with another recipient of *M. osloensis*; *d*: derived from semiquantitative data of Bøvre, (1967*a*). Other donors than M 2 and the reference donors were included in parallel for comparison.

ATCC = American Type Culture Collection; NCTC = National Collection of Type Cultures, London.

DISCUSSION

The micromorphological, cultural and biochemical characters of strain M 2 distinguish this organism from all known moraxellas. The species which has the closest resemblance is *Moraxella nonliquefaciens* (Bøvre & Henriksen, 1967*a*). Strain M 2 differs from *M. nonliquefaciens* by its small dimensions, distinct colony consistency, growth in Hugh & Leifson medium and in the amino acids + biotin medium (see Baumann *et al.* 1968), and a poor ability to survive on blood agar at room temperature. The strain M 2 shows a pattern of affinities in genetic transformation and a G+C % content never observed with oxidase-positive rods. It has no compatibility with known rod-shaped moraxellas (e.g. *M. nonliquefaciens* and *M. osloensis*), nor with the 'false neisserias' (e.g.

N. catarrhalis and *N. ovis*). On the other hand, strain M2 is distinctly compatible in transformation with the 'true' Neisseria group (e.g. *N. meningitidis*, *N. flava*), with which it shares a high G+C % content, unique for an oxidase-positive non-motile rod. If the organism had been a coccus, there would have been no reason to discuss its designation as a Neisseria. Since it is a rod, the relative value of this classically high-weighted character must be considered against molecular composition of DNA and genetic affinities. It is quite possible that cell shape (rod or coccus) may be a genetically more complex character than most single phenotypic traits. However, the very clear difference in terms of G+C % content and the transformation incompatibility (with a 'ribosomal' marker) between strain M2 and other oxidase-positive rods, taken together with the similarity in G+C % content and genetic compatibility with coccal species, probably characterize the basic relations of strain M2 better than its shape. We therefore feel that the strain M2 should be placed in the revised, restricted genus *Neisseria* (Henriksen & Bøvre, 1968). The alternative construction of a new genus for the organism is not favoured, because of the relatively high genetic affinity to Neisseria species. However, since most of the studies of heterologous transformation in genus *Neisseria* were made with another technique than the one used here (Catlin & Cunningham, 1961), we hesitate to draw firm conclusions about the exact degree of high compatibility between strain M2 and the coccal species of the genus, as compared with the mutual relations of the latter. Since there is no known case of genetically stable intraspecies variation from coccus to rod or *vice versa*, we consider it advisable to give the strain M2 rank as a separate species without testing the genetic affinities to all species within genus *Neisseria*.

We propose the name *Neisseria elongata* for the new species, the specific epithet expressing the rod shape, distinguishing it from all other known members of genus *Neisseria*. The strain M2, the only isolate as yet, is proposed as the type strain. It has been deposited in the National Collection of Type Cultures, London, and in the American Type Culture Collection, as NCTC 10660 and ATCC 25295.

As a consequence of the existence of *Neisseria elongata*, the revised definition of the Family Neisseriaceae Prévot, 1933, Genus I, *Neisseria* Trevisan, 1885, as proposed by Henriksen & Bøvre (1968), should be emended by changing the first sentence to 'Organisms coccal or rod-shaped'. That this family now appears to contain two genera, each with coccal and rod-shaped species (loc. cit.), may warrant a general reconsideration of cellular shape as a master criterion in bacterial classification at genus and higher level.

A condensed description of *Neisseria elongata* follows.

Description of Neisseria elongata sp.nov.

Micromorphology. Small slender rods, often occurring as diplobacilli or in short chains. Some organisms may be coccoid, which change uniformly into long threadlike filaments by culture in the presence of low concentrations of penicillin. Gram-negative, with some tendency to resist decolorization. Not encapsulated. Non-motile.

Colonies. Greyish white, opaque, 1 to 1·5 mm. in diameter after incubation of blood agar cultures for 20 hr. Even, shiny, low-hemispherical with an entire edge. Clay-like, coherent consistency. Some pitting of the agar. No haemolysis.

Relation to oxygen. Aerobe.

Temperature. Slight growth at 20 to 22°; optimal growth at 33 to 37°, survives 45° for 30 min., but is killed at 56° in 5 min.

Viability. Blood agar cultures are not viable after 5 to 10 days at 20 to 22°.

Growth requirements. Growth on nutrient agar almost as good as on blood agar. Good but slow growth in peptone media. Growth in Hugh & Leifson medium in the upper 1 cm. Growth in amino acids + biotin medium. No growth in basal medium with ammonium ions as nitrogen source and glucose, maltose, acetate, citrate or lactate as carbon source.

Biochemical reactions. Weakly positive catalase reaction. Oxidase reaction strongly positive with tetramethyl- and dimethyl-*p*-phenylenediamine reagents. No acid produced from glucose, maltose, sucrose, lactose, fructose, arabinose, galactose, xylose or mannitol. Nitrate not reduced to nitrite. Gelatin and coagulated serum not liquefied. No production of indole or hydrogen sulphide. No decomposition of urea. No oxidative deamination of phenylalanine or tryptophan.

Antibiotic sensitivity. Highly sensitive to penicillin G, streptomycin, chloramphenicol, oxytetracycline and erythromycin.

Habitat. Human nasopharynx.

Pathogenicity. Unknown.

DNA base composition. One strain: 53 mole % guanine + cytosine, as calculated from buoyant density in CsCl gradient (1·713 g./cm.^{3}).

Genetic affinities. Incompatible in streptomycin-resistance transformation with species of genus *Moraxella*, as defined by Henriksen & Bøvre (1968). Compatible with members of genus *Neisseria* (loc. cit.), with ratios of interstrain to autologous transformation in the order of 0·01 to 0·1.

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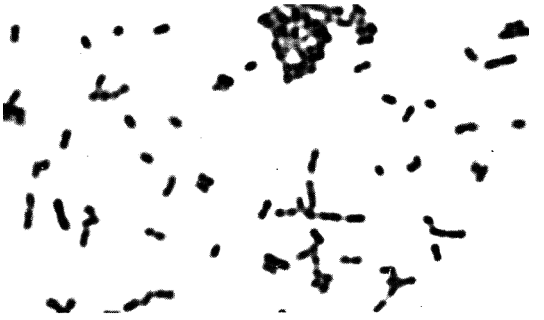

Fig. 1

Fig. 2

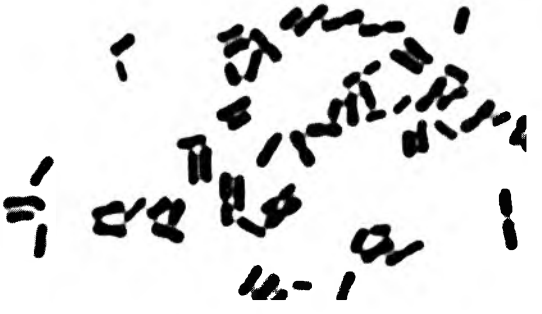

Fig. 3

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EXPLANATION OF PLATE

Fig. 1. *Neisseria elongata*, strain M2 (type strain). Blood agar culture. Stained with Loeffler methylene blue. × 2300.

Fig. 2. Same strain as in Fig. 1 after growth on blood agar with low penicillin concentration. Stained with Loeffler methylene blue. × 2300.

Fig. 3. *Moraxella nonliquefaciens* 4663/62 = ATCC 19975 = NCTC 10464. Blood agar culture. Stained with Loeffler methylene blue. × 2300.

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The Host–Parasite Relationship between Freshwater Crayfish and the Crayfish Disease Fungus *Aphanomyces astaci*: Responses to Infection by a Susceptible and a Resistant Species

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SUMMARY

In studies of the responses to infection by the crayfish disease fungus *Aphanomyces astaci* of the susceptible European crayfish *Astacus astacus* and the resistant Western American species *Pacifastacus leniusculus*, the latter was consistently more resistant to infection by injection or via aquarium water; the difference in resistance was more striking in the second method. The American crayfish was only slightly less sensitive than the European crayfish to the toxic action of large numbers of dead spores of *A. astaci* and to infection by living spores of another phycomycete, *Entomophthora apiculata*, which is not a natural parasite of crayfish. *A. astacus* acquired a significantly higher degree of resistance to infection by injection or via the ambient water as a result of two previous exposures to sublethal numbers of spores of the pathogenic fungus.

The two strains of *Aphanomyces astaci* tested behaved identically within the cuticular layer of the exoskeleton of both crayfish species, showing strain-dependent degrees of ability to penetrate and grow within the cuticle. The epicuticle offered a major barrier to penetration in both hosts. The fungus was unable to establish growth within the musculature and developed only sparsely along the ventral nerve cord of living animals of both species; fungal growth was luxurious shortly before and after the death of both crayfish. Aggregation of injected spores and their encirclement by haemocytes occurred rapidly in living *Astacus astacus* as well as in *Pacifastacus leniusculus*, but melanization of the spore aggregates and hyphal elements was considerably more pronounced in the resistant crayfish, *P. leniusculus*. The observations suggest the participation of at least some active processes of native and acquired resistance of crayfish to the crayfish disease fungus, particularly in the internal tissues and the epicuticle.

Evidence has been found for the existence of specifically acquired means of defence against microbial disease in invertebrates, but there is little information as to whether such animals can respond with reactions to foreign macromolecules akin to the immunological response of vertebrates (Smith, Miescher & Good, 1966; Pathology Society Symposium, 1967; Seaman & Robert, 1968). Recognition mechanisms which

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permit an organism to distinguish between closely related substrates in the process of phagocytosis, including the discrimination between self and non-self substances, have been shown in invertebrates (Boyden, 1963). The relationship between capacity to discriminate in phagocytosis and acquisition of heightened resistance to microbial pathogens, and the relative importance of acquired defence reactions in ability to withstand invasion and disease from the numerous potentially parasitic micro-organisms in the environment, also remain largely unknown. Most of the attempts to discover in invertebrates mechanisms similar to the antibody response of all animals phylogenetically above the primitive fish have been in the direction of searching for immunoglobulin-like factors against antigens bearing no relation to the natural environment of the animal. It is not impossible, however, to conceive that invertebrate species can mount antibody-like reactions to alien substances, but in a more restricted range, and perhaps only or largely against foreign chemical determinants of agents which pose threats to survival. It would be surprising if as complex a family of molecules as the vertebrate immunoglobulins had arisen *de novo* at the level of the hagfish or lamprey eel; and related though less evolved molecules, still capable of some of the biological activities of antibodies, would not be an unexpected component of the host responses of the more highly developed invertebrates.

To explore further the occurrence, nature and biological significance of specific recognition mechanisms in the response of invertebrates to foreign molecules, a systematic study was undertaken of the host–parasite relationship between two closely related species of freshwater crayfish and an aquatic phycomycete. The European crayfish *Astacus astacus* is highly susceptible to the fungus *Aphanomyces astaci* Schikora, the causative organism of 'Krebspest' or crayfish plague or disease, whereas the west coast American crayfish *Pacifastacus leniusculus* Dana is highly resistant.

It is apparent that crayfish are endowed with resistance mechanisms markedly effective against some micro-organisms but not against others. The outer surface of their exoskeleton is exposed to, and frequently occupied by, a variety of micro-organisms which are incapable of invading the internal living tissues, not even through small wounds, although they have the enzymic equipment for penetration and are able to do so readily, spreading quickly through the body, after death of the host. On the other hand, crayfish are very susceptible to several varieties of protista. This differential refractoriness, and the large variation in resistance to a given micro-organism, by otherwise very similar species of host, suggested the crayfish–Aphanomyces system as a good model for the study of specificities in the behaviour of invertebrates towards foreign chemical configurations.

The crayfish disease appeared in Italy during the 1860s (Seligo, 1895), and swept through large parts of Europe in succeeding decades, eradicating the population of *Astacus astacus* wherever it erupted. It appears that no members of this species survived in attacked waters; at least, resistant populations have not been reported. Similar plagues have not been described among non-European crayfish species.

Schäperclaus (1935, 1954) and Nybelin (1936) showed that the causative agent of this disease was *Aphanomyces astaci*, and they described the characteristic disease picture. Fungal hyphae are invariably present in and on limited areas of the exoskeleton of infected crayfish, commonly in the soft areas of joints and abdominal segments where penetration usually occurs; involvement of the cuticle is often limited and difficult to detect. After penetrating the skeleton, the hyphae are frequently seen to

grow along the ventral nerve cord and brain ganglion, but growth is often restricted largely to the vicinity of the cuticular penetrations; the tissues around these foci sometimes become yellow or brown and necrotize. Hyphae are occasionally seen in the eye, but seldom in other organs. Late in the disease, hyphae grow outwards from foci of infection on the skeleton, forming zoosporangia and zoospores in the surrounding water. Death of the host occurs within several days to weeks after infection with symptoms of neurotoxicity, the animals failing to respond to external stimuli during the last days of life.

Aphanomyces astaci has not been isolated as a free-living organism, and may be incapable of surviving for long away from a host (Rennerfelt, 1936; Unestam, 1969). European freshwater crayfish are its only known natural hosts. The fungus does not attack *Orconectes limosus* Rafinesque (*Cambarus affinis*), an American freshwater crayfish which was introduced to Europe and now inhabits water formerly occupied by *Astacus astacus* (Schäperclaus, 1954), or any other American species tested, but it does cause a frequently fatal infection of a Japanese species and of the crab *Eriocheir sinensis* Milne Edwards (Benisch, 1940) under laboratory conditions. No special growth requirements by the fungal mycelium have been found (Unestam, 1965). The present communication reports the findings of experiments to determine quantitatively the differential degree of susceptibility to this fungus by crayfish of the two species, to ascertain whether survivors of a sublethal infection acquire a heightened resistance to a second challenge, and to delimit the major areas of interaction between host and parasite.

METHODS

Crayfish. Specimens of *Pacifastacus leniusculus* were obtained by trapping from Lake Tahoe, in the Sierra Nevada mountains between California and Nevada, and from Alpine Lake, in Marin Country, north of San Francisco. *Astacus astacus* came from Lake Ullvättern in Värmland, Sweden. Sample specimens of both species are shown in Pl. 1, fig. 1.

The animals were maintained in aquaria at 5° for storage. During experimentation, they were kept in groups of 15 or fewer in aquaria containing 7 l. pond water, at $16{\cdot}5 \pm 0{\cdot}3°$. In experiments where fungal spores were added to the aquarium water, doubly glass-distilled water was used in place of pond water. The aquaria were under continuous aeration. The crayfish were fed once weekly with carrot slices, which were removed after 1 day if not eaten; however, when spores were added to the water, no food was given to the animals for several weeks. In most experiments, intermolt crayfish of 70 to 90 mm. body length were used; smaller animals were occasionally used in duplicate trials. In any one experiment, care was taken to use animals of only very similar size.

Fungi. The origin of the strains of *Aphanomyces astaci* here studied, J1 and D1, and the culture methods and techniques for obtaining spores have been described previously (Unestam, 1965, 1966*b*). Zoospores obtained by exposure of mycelial suspensions to a mixture of pond and distilled water overnight were separated from the hyphal elements by filtration through cheesecloth. The spore suspensions were concentrated by low-speed centrifugation, and the concentrations of spore suspensions in homologous sera or water were determined by haemocytometer counts. The viability of spores was tested by spreading samples of suspension over peptone glucose agar;

degree of germination was ascertained by direct microscopic examination after incubation for 20 hr at 23° in a 10% (v/v) CO_2 in air atmosphere. All *A. astaci* spore suspensions showed a germination frequency of 10 to 20%.

Some other fungi were also tested: *Aphanomyces laevis* de Bary, a saprophyte obtained from the Centraalbureau voor Schimmelcultures, Baarn, Holland; an Aphanomyces species shown by Fowles (1967) to act as a wound parasite of freshwater dolphin, kindly made available by Dr B. Fowles (Department of Biology, Colby College, Waterville, Maine, U.S.A.; *Entomophthora apiculata* Thaxter, an insect parasite, kindly sent us by Dr S. R. Dutky (Entomology Research Division, U.S.D.A. Agricultural Research Service, Beltsville, Md.). The different Aphanomyces species were grown in the same manner as the *A. astaci* cultures; their spore preparations consistently exhibited germination frequencies of 50 to 90%. The Entomophthora was grown on agar containing 0·013 M-phosphate buffer (pH 5·5), 1% Difco Bacto-Peptone, 5% glucose and 0·1% $MgSO_4 7\ H_2O$; the conidiospores were collected from the lids of the culture dishes, and showed germination frequencies of the order of 30%.

Infection experiments. For infection by injection spores were suspended in pooled sera of the corresponding host species, obtained by permitting the bloods to coagulate and removing the clots by light centrifugation. Samples (0·05 ml.) of the serum + spore suspensions were injected ventrally into the subabdominal haemocoel, between the third and fourth abdominal segments, left of the nerve cord and very close to it, Serum was used to suspend the spores in order to decrease physiological disturbance to the animals; injections of saline have been considered to be harmful (Crowley, 1964). In other experiments, infection was via the environment, by adding known numbers of spores to the aquarium water.

The animals were usually observed for 2 months after infection, but death occurred in most instances within the first 3 weeks. Death was ascribed to the infection only when living hyphae were found in the tissues or exoskeleton on post-mortem examination. When infection was via the aquarium water, the entire skeleton was examined for the presence of fungal growth if there was no gross involvement of the abdominal cuticle or the central nervous tissue. LD50 values were calculated by the method of Reed & Muench (1938).

The brown or yellow-brown pigment designated as 'melanin' was readily soluble in N-NaOH at 60°, but not in cold NaOH or in 95% H_2SO_4, 10 M-HCl, acetone, xylene, chloroform or dimethyl ether.

RESULTS

Differential susceptibility of Astacus astacus and Pacifastacus leniusculus to living and killed spores of Aphanomyces astaci and other phycomycetes

The production of toxins by Entomophthora species and other fungal pathogens of arthropods has been described by several investigators (MacLeod & Loughheed, 1965; Prasertphon, 1967; Yendol, Miller & Behnke, 1968), and it seemed possible, therefore, that *Aphanomyces astaci* toxins could also play a role in the crayfish-fungus interaction. Preliminary experiments indicated that zoospores of this fungus could induce rapidly manifested toxic effects in crayfish when injected in large quantities, in addition to initiating progressive infection. Experiments were therefore con-

ducted in both species of crayfish with spores killed by acetone or by freezing and thawing (exposure to $-20°$ for 30 min.), to determine whether there existed a differential degree of susceptibility to toxic action by the parasite. The results of a typical experiment with freeze-killed spores of *A. astaci* strain J1 are presented in Table 1. It appears from Table 1 that freeze-killed spores had a similar degree of toxicity for *Astacus astacus* and *Pacifastacus leniusculus*, with the former showing slightly greater susceptibility, both in terms of the number of spores constituting one LD50 dose and of length of survival after infection. Identical findings were obtained in repeated experiments.

Table 1. *Susceptibility of crayfish Astacus astacus and Pacifastacus leniusculus to injection of spores of Aphanomyces astaci strain J1, killed by freezing and thawing**

Number of spores of *A. astaci* injected	Animals dead/total		Mean time to death (days)	
	P. leniusculus	*A. astacus*	*P. leniusculus*	*A. astacus*
6×10^6	8/9	10/10	1·8	1·0
2×10^6	4/9	7/9	5·5	3·3
6×10^5	0/9	1/9	—	1·0
LD50	$2{\cdot}5 \times 10^6$	1×10^6	—	—

* Spores suspended in homologous serum.

Table 2. *Susceptibility of crayfish Astacus astacus and Pacifastacus leniusculus to injection of living spores of Aphanomyces astaci strain J1**

Number of spores of *A. astaci* injected	Animals dead/total		Mean time to death (days)	
	P. leniusculus	*A. astacus*	*P. leniusculus*	*A. astacus*
6×10^5	8/8	—	4·2	—
2×10^5	2/9	8/8	5·0	9·9
6×10^4	0/9	8/9	—	9·4
2×10^4	—	4/8	—	13·5
6×10^3	—	1/5	—	15·0
LD50	3×10^5	2×10^4	—	—

* Spores injected in homologous serum.

Spore-free filtrates of suspensions of acetone-killed zoospores of *Aphanomyces astaci* proved not to be toxic, suggesting that the toxic properties of the spores were bound firmly within, or on, the spore structure.

Similar experiments were now initiated with living spores of *Aphanomyces astaci*, using numbers of zoospores below the gross toxic threshold. Most of the crayfish given such numbers of spores remained seemingly unaffected for several days. All the animals which died thereafter showed some degree, and sometimes even a considerable amount, of fungal spread and growth; deaths commonly occurred within the first 15 days after challenge. The results of a representative experiment with strain J1 are shown in Table 2. As seen from Table 2, the European crayfish was significantly more susceptible to challenge with living spores of the crayfish disease fungus. This finding was obtained in repeated similar experiments with this strain, the difference in susceptibility between the two host species ranging from 10 to 20 times the number of living spores constituting one LD50 dose. An identical experiment was made with living spores of strain D1 of the Aphanomyces pathogen; the LD50 dose for *Astacus astacus* was 5×10^4 spores and for *Pacifastacus leniusculus* 2×10^6 spores.

To test the specificity of the different resistances of the two species of crayfish to the pathogenic fungus, groups of the animals were now challenged with living spores of the insect parasite *Entomophthora apiculata* (not a natural parasite of crayfish, though injection of large numbers of conidiospores can cause intoxication or progressive infection leading to death). The observations recorded in this experiment are seen in Table 3. At the highest spore concentration, $1{\cdot}5 \times 10^5$, Entomophthora rapidly killed all the animals of both species of host; penetration of the exoskeleton from within and some hyphal development were none the less seen in many of the crayfish at death. With smaller numbers of spores, there was a suggestion of somewhat greater susceptibility by *A. astacus*, the dying animals all showing typical symptoms of infection, but the difference between the European and American crustaceans in relation to the naturally parasitic *A. astaci* was not clear here. Injection of 10^6 living spores of two other phycomyces species not naturally pathogenic to crayfish, the Aphanomyces dolphin wound organism and *Aphanomyces laevis*, resulted neither in death nor in any evidence of hyphal growth or penetration of the cuticle, despite the fact that these fungi are also active chitinase producers (Unestam, 1966*a*).

Table 3. *Susceptibility of crayfish Astacus astacus and Pacifastacus leniusculus to injection of living spores of a fungus parasitic of insects Entomophthora apiculata**

Number of spores of *E. apiculata* injected	Animals dead/total		Mean time to death (days)	
	P. leniusculus	*A. astacus*	*P. leniusculus*	*A. astacus*
$1{\cdot}5 \times 10^5$	9/9	7/7	1·9	1·6
3×10^4	1/7	4/7	2	4·2
6×10^3	0/7	0/7	—	—
LD50	6×10^4	$2{\cdot}5 \times 10^4$	—	—

* Spores injected in homologous serum.

Table 4. *Susceptibility of crayfish Astacus astacus and Pacifastacus leniusculus to infection with living spores of Aphanomyces astaci strain* D 1 *in aquarium water*

Number of spores/ml. aquarium water	Animals dead/total		Mean time to death (days)	
	P. leniusculus	*A. astacus*	*P. leniusculus*	*A. astacus*
$2{\cdot}5 \times 10^4$	2/4	—	8·5	—
$2{\cdot}5 \times 10^3$	0/4	7/7	—	6
$2{\cdot}5 \times 10^2$	0/5	7/7	—	8·1
$2{\cdot}5 \times 10^1$	—	3/7*	—	14·0
LD50†	$2{\cdot}5 \times 10^4$	3×10^1	—	—

* All of the remaining four crayfish died, but relatively late after challenge: one on day 28 and three on day 36; these animals showed slight symptoms of fungal infection. They may have succumbed to secondary infection by an unrelated micro-organism, or to late infection with Aphanomyces spores.

† LD50 values expressed in terms of number of spores/ml. aquarium water.

An experiment was now made to assay the susceptibility of the crayfish *Astacus astacus* and *Pacifastacus leniusculus* to infection by *Aphanomyces astaci* via the natural route, i.e. by introduction of spores into aquarium water. As evident from Table 4, only a few spores/ml. water were necessary to induce lethal infection of *A. astacus*, whereas the LD50 dose for the American crayfish remained high, $2{\cdot}5 \times 10^4$ spores/ml.

The difference in susceptibility of the two crayfish species was thus even greater when infection was by the natural route than when it was established by injection into the haemocoel.

Acquisition of specifically heightened resistance by Astacus astacus

Attempts were made to determine whether prior contact with sublethal numbers of living spores of *Aphanomyces astaci* could induce heightened resistance in *Astacus astacus* towards subsequent lethal infection. A single exposure to 3×10^3 living spores, by injection, failed to provide conspicuous immunity against challenge with 4×10^4 spores 2·5 weeks later. In contrast, two prior injections of 3×10^3 living spores each, 2·5 weeks apart, bestowed a significant degree of heightened resistance against challenge 2·5 weeks later with 4×10^4 spores (Table 5). Six of the surviving crayfish (*A. astacus*) of the immunized group were then placed in an aquarium with a living spore content of 800/ml. water (strain D 1), 17 days after challenge with 4×10^4 spores. A group of nine control crayfish was placed into the same aquarium; all of these control animals developed progressive infection and died, with a mean survival time of 10·9 days; in contrast, only three of the six previously exposed crayfish succumbed to the superinfection from outside, with a mean survival time of 18·3 days.

Table 5. *Acquisition of heightened resistance by crayfish Astacus astacus against injection with living spores of Aphanomyces astaci as result of previous sublethal exposure*

	Number of spores* injected, on days			Animals dead/total	Mean time to death (days after last infection)
Animals	1	19	37		
Immunized	3×10^3	3×10^3	4×10^4	1/15	(7)
Control	0 (serum)	0 (serum)	4×10^4	7/14	10·6

* Spores of *Aphanomyces astaci* strain J 1, were used; the spores were suspended inhomologous serum. All animals dying showed considerable fungal involvement.

Table 6. *Acquisition of increased resistance by crayfish Astacus astacus against infection with living spores of Aphanomyces astaci strain J 1 as result of previous sublethal exposure; repeat experiment*

	Number of spores injected*			Animals dead/ total	Mean time to death (days after last infection)
Animals					
Immunized	10^4	10^4	5×10^4	2/8	10
Control	0	0	5×10^4	8/9	11

* 2·5 Weeks between each injection.

A repeat experiment on the acquisition of heightened resistance by *A. astacus* yielded similar results (Table 6). Studies are now in progress to determine the specificities of origin and direction of this acquired resistance of *A. astacus* to the crayfish disease fungus. The results here reported indicate at least that these invertebrates can acquire a considerable degree of heightened resistance against a naturally parasitic micro-organism under circumstances very similar to those which pertain to specific immunization of higher animals.

Spore germination and mycelial growth of Aphanomyces astaci in crayfish blood in vitro

Experiments were made to determine whether the blood of normal crayfish of the two species possessed different capacities for supporting mycelial growth and spore germination of the plague fungus. The spore germination incidence of both strains of *Aphanomyces astaci* was observed after incubation for 20 hr in pooled blood of both crayfish species, and the weight of the mycelial growths at 48 and 68 hr was determined. Both fungus strains behaved similarly in the blood of *Astacus astacus* and *Pacifastacus leniusculus*, thus indicating that the differential susceptibility of the two species of crayfish did not lie with any differential humoral ability to support growth and germination of the pathogen.

Behaviour of Aphanomyces astaci within the tissues of Astacus astacus and Pacifastacus leniusculus

Infection by injection. The events in *A. astacus* and *P. leniusculus* following injection of *Aphanomyces astaci* zoospores appeared to be qualitatively and quantitatively very similar: shortly after introduction into the haemocoel, the spores were seen to be aggregated in clumps on the surface of the ventral nerve cord, encapsulated by large numbers of blood cells (Pl. 1, fig. 2*a*, *b*). Such clumping of spores was rarely seen elsewhere. Melanization of the clumps was evident within several hours. Hyphae grew out of the masses only when these were composed of large numbers of spores; the escaping hyphae were rapidly surrounded by more blood cells, thereby increasing the size of the original aggregates. The accumulation of blood cells around growing hyphae was often so dense that the fungal structures became difficult to see (Pl. 1 fig. 3). *In vitro* studies showed no clear evidence of chemotactic attraction of the blood cells to spores or hyphae; their localization around the fungus may have been the result, at least initially, of random collision. On the other hand, the melanization reaction around the spores and hyphae aggregated on the nerve cord was considerably more rapid and intense with *P. leniusculus* than with *A. astacus* (Pl. 2, fig. 4*a*, *b*, *c*). The melanin deposits were also more pronounced and distinct around living than around killed spores. It seemed that deposits of melanin offered mechanical barriers to the spread of the fungal mycelium; thus, heavy melanization around a hyphal tip was frequently seen to result in a breaking through of hyphal branches subapically, where the melanin deposits were usually lighter (Pl. 3, fig. 5). In both host crayfish, the processes of melanization, blood cell aggregation, and clumping of fungal structures on the nerve cord were on the whole very similar *vis-à-vis* the other Aphanomyces species as well, and towards *Entomophthora apiculata*.

When spores of *Aphanomyces astaci* were injected directly into the musculature of either *Astacus astacus* or *Pacifastacus leniusculus*, germination and some hyphal development were often seen at first. The outgrowing hyphae were rapidly and heavily melanized, however, and progressive mycelial development was never seen within the muscles.

The active nature of the retardation or inhibition of spore germination and mycelial growth was evident throughout this study; fungal growth and spread usually became marked shortly before or after death of the host. Even *Entomophthora apiculata*,

which rarely initiated extensive mycelial growth as long as the crayfish were alive, was able to do so from the spore aggregates on the nerve cord within hours after the animals died (Pl. 3, fig. 6). It was obviously difficult to measure the observations on the behaviour of the crayfish disease fungus and other phycomycetes in *Astacus astacus* and *Pacifastacus leniusculus*. The only clear and repeatedly evident difference between the susceptible and resistant crayfish in the reaction to the pathogen within the tissue was the more marked melanization response of the American crayfish. The gross appearance of both crayfish in the later stages of the disease was the same as in animals infected via the aquarium water (see below).

Infection via the aquarium water. Hyphal growth was invariably seen in the exoskeleton of *Astacus astacus* infected from outside, although at low spore concentrations in the water only small areas, sometimes limited to the joint membrane of a single leg or pleopod, showed frank involvement. Mycelial growth along the ventral nerve cord was usually sparse, and could sometimes not be detected at all at post-mortem examination; encapsulation of fungal structures on the cord was limited in most animals. There was no invasion of muscular tissues. The dorsal side of attacked segments often discoloured before death. Paralysis of the entire abdomen was frequently observed 1 or 2 days before death, and the pathological picture was consistent with the suggestion that neurotoxic effects were the major cause of death in the crayfish (see Nybelin, 1936; Schäperclaus, 1954).

Behaviour of Aphanomyces astaci in the exoskeleton of Astacus astacus and Pacifastacus leniusculus

Infection by injection. Once germination had begun from numbers of zoospores sufficient to initiate progressive disease, the events in both host species were very similar. In most instances, hyphae growing out from spores of *Aphanomyces astaci* strain J 1 injected into the haemocoel eventually penetrated the exoskeleton from inside and developed within the cuticular layer, but almost always to a limited extent. In both types of crayfish, the penetrating hyphae were surrounded by heavy melanin deposits in the cuticular layer close to the epidermis and in the layer adjacent to the epicuticle, but not in the intermediate cuticle; melanization was even more pronounced with *Entomophthora apiculata*. With *A. astaci* strain D 1, penetration and growth in the cuticle was heavy, in both *A. astacus* and *P. leniusculus*. Although the LD50 dose of injected spores of *A. astaci* strain D 1 was higher than that for strain J 1, strain D 1 was much more virulent when introduced into the ambient water. Further studies are under way to analyse this suggested correlation between virulence of infection by the natural route and ability to grow well within the cuticular structure of the host.

In further experiments, sections of the subabdominal exoskeleton of normal crayfish were disinfected with ethanol, cut out, and set in sterile Petri dishes. Spore suspensions were then placed either on the epicuticular surface or on the inner surface from which muscle and epidermis had been removed. Spores from strains J 1 and D 1 germinated and penetrated the cuticle from the inner surface. The first hyphae reached the epicuticle from inside and broke through it after about 40 hr, in sections from both host species (Pl. 3, fig. 7). Penetration never occurred from the epicuticular surface with either strain of fungus, unless the epicuticle was first pricked with a needle or peeled although normally shaped hyphae grew along this surface. Similar results were

obtained with disinfected sections of exoskeleton in continuously aerated distilled water containing 7×10^3 spores/ml. Penetration took place only from the endocuticular surface. After several days, the sections of exoskeleton in infected, but not in spore-free, waters became soft and lost resistance to mechanical stress, very possibly because of chitinase attack on the cuticular matrix (Unestam, 1966*a*). Swelling of the hyphal tip was commonly seen when hyphae reached the epicuticle from inside; this was never the case when hyphae encountered inert mechanical obstacles on an agar surface. No obvious differences were observed in the behaviour of excised exoskeleton sections derived from *Astacus astacus* and *Pacifastacus leniusculus*.

Infection via the aquarium water. As described above, the sites of attack even in the susceptible crayfish were usually difficult to find during the first several days after the animals were placed in infected water, even when large numbers of spores (10^4/ml.) of the virulent *Aphanomyces astacus* D 1 strain were used. Penetration was most commonly evident in the area of joints and body openings. This was consistent with *in vitro* experiments which showed the undamaged epicuticular surface to be highly resistant to attack. It thus appears that successful fungal penetration may well be conditional on localized absence of the epicuticular membrane or on accidental damage or strain to it, as in the joint areas where cuticular folding occurs. When penetration did take place, hyphal branching was seen within the cuticle itself in less than two days; melanization was evident around the puncture, near the surface of the epicuticle, but not deeper. In the resistant crayfish, penetration of the exoskeleton was never seen within 23 days in water containing 10^4 spores/ml. In several instances, hyphae were found growing along the outside surface of the exoskeleton, heavily melanized, perhaps as result of some 'etching' of the epicuticular layer.

The importance of the epicuticular membrane in the crayfish's defence against *Aphanomyces astaci* became further evident in an experiment in which this layer (and probably some of the immediately adjacent cuticle) was peeled away from one intersegmental abdominal membrane of a number of *Pacifastacus leniusculus*. Of four such animals placed into water containing a sub-LD 50 number of living spores (*A. astaci* strain D 1), 7×10^3/ml., three died within 10 days with heavy involvement of the naked areas. Neither intact *P. leniusculus* put in such water, nor specimens similarly peeled and placed in non-infected water, were adversely affected. The resistant crayfish could similarly be made susceptible to fatal outside infection in water containing 7×10^3 spores/ml. of *A. astaci* strain D 1 by merely pricking the abdominal intersegmental membranes with a fine needle in several places, with fungal penetration clearly limited to the wounds. In parallel experiments, it was found that a majority of *Astacus astacus* could be made to succumb to otherwise sublethal numbers of spores of *A. astaci* strain J 1 in the surrounding water by previous peeling of small areas of the intersegmental membranes. Once penetration had occurred in either host species, hyphae again grew outwards through the exoskeleton. In later stages of the disease in heavily infected animals, but especially in *A. astacus*, sections of the exoskeleton became brown and necrotic, and brown pigment stained the underlying muscles despite their freedom from fungal growth.

DISCUSSION

It appears that the first and a major defensive barrier of crayfish against the crayfish disease fungus consists of the non-chitinous proteolipid epicuticular membrane and, perhaps, of the most nearly adjacent cuticular stratum which is often also damaged in experiments designed to remove or injure the epicuticle alone. The defensive role of the epicuticular membrane has also been suggested for insects (Koidsumi, 1957; Sussman, 1951*a*, *b*). In intact animals of the susceptible European crayfish, fungal penetration occurred only in limited regions, largely where the epicuticle was missing, damaged or presumably strained; it was not seen at all in the resistant Western American species even when the infected waters contained as many as 10^4 virulent spores/ml. Following experimental breaking of this membrane, however, even the resistant crayfish *Pacifastacus leniusculus* exhibited susceptibility to fatal infection in heavily contaminated water.

It is possible that the epicuticle offers resistance to fungal penetration in part for mechanical reasons, and that trapping of the spores in ducts, folds and areas of injury facilitates invasion at these sites. That at least some active and specific processes also participate in epicuticular resistance is indicated by the melanization reactions prominent in this layer, and by the differential pathogenicity of *Aphanomyces astaci* strains J1 and D1 by the exterior route. The differences in host resistance between the crayfish *Astacus astacus* and *Pacifastacus leniusculus*, and the state of heightened resistance which could be elicited in the former may also be expressed at least partly at the level of the epicuticle. The observation that penetration and involvement of the cuticle by hyphae growing from within the crayfish following injection of the parasite, and from the inner surface of isolated fragments of exoskeleton *in vitro*, was very similar in both species is in line with this suggestion. On the other hand, the fact that fungal invasion from outside is much more common at sites of epicuticular absence or injury indicates that the considerably greater native resistance of *P. leniusculus* and the increased acquired resistance of the European crayfish must also depend on mechanisms functioning within deeper layers of the exoskeleton, the internal tissues and the areas of the body openings.

The ability of hyphae of Aphanomyces to transverse the epicuticular membrane in both crayfish species from inside does not argue against the important defensive role of this structure: there may be significant differences in the morphological and physiological characteristics of the membrane presented at its interior and exterior surfaces, and, in addition, the already developing hyphae which reach the membrane from the endocuticular side undoubtedly pose a very different challenge than do isolated spores impinging from without.

Although the interaction between *A. astaci* and crayfish of both species within the cuticle itself appeared to be identical, at least grossly, once progressive infection was under way, it was observed (T. Unestam, to be published) that the blood of the resistant crayfish *P. leniusculus* was more capable of inhibiting the extracellular chitinase activity of the fungus than was the blood of *Astacus astacus*. Death often occurred in infected crayfish of both species with little fungal growth either within or without the cuticle, perhaps caused by toxic substances liberated by the fungus in the cuticular stratum or produced as a result of host reactions against the invader. The ability to retard fungal enzymic action and/or proliferation could thus be imagined

to constitute a second line of defence, within the cuticle, should epicuticular penetration occur. The internal tissues of the living crayfish appear to constitute a poor environment for the development of the parasite: large numbers of spores must be injected to initiate progressive infection even in the susceptible crayfish *A. astacus*; there is very little fungal involvement of most internal tissues; and, where fungal growth does occur (along the ventral nerve cord and ganglion), it is very limited. The involvement of the nerve cord in some of these studies might have been at least partially the consequence of deposit by injection of large numbers of spores in its vicinity, in the abdominal haemocoel. Moreover, rapid clumping of spores and hyphal elements and encirclement by large numbers of blood cells was a ubiquitous event in both species.

The difference in susceptibility to injection of spores between the European and American crayfish was not as great as the difference in susceptibility to extrinsic infection, but it was still marked. The greater internal resistance of *Pacifastacus leniusculus* was accompanied by a considerably more pronounced melanization of spore and hyphal aggregates on, and in the vicinity of, the nerve cord. The pertinence of this observation to resistance, and whether melanin deposition is, indeed, cause or effect of microbial inhibition, remain unknown. Melanin formation has been suggested by a number of investigators as an arthropod defence mechanism against microbial attack, but its causal role in resistance states awaits confirmation (Briggs, 1964). Recent studies (Kuo & Alexander, 1967; Metlitskii & Ozeretskovskaya, 1968) have indicated an inhibitory effect by melanin and other phenolic compounds on proteases, chitinases and other extracellular enzymes, and there might thus exist a relationship between the larger melanin response of *P. leniusculus* and the greater chitinase inhibition of its body fluids. Whether it is melanin formation or another mechanism, active defence processes apparently come into play against the crayfish disease fungus on the level of the internal tissues as well, once penetration through the exoskeleton has taken place. The active nature of these defences was also apparent from the luxurious fungal growth supported by these tissues of the crayfish shortly before and after death. The cuticular pore channels which connect the epidermis with the epicuticular region in the crayfish may continue to function in the less heavily mineralized parts of the exoskeleton even after moulting (Dennell, 1960; Travis & Friberg, 1963). It is possible that by virtue of this communication system some of the same humoral or cellular resistance mechanisms which are manifested towards injected spores are also operative at the level of the cuticular and epicuticular strata towards spores introduced from outside and towards mycelial elements.

A significant degree of increased resistance was evoked in *Astacus astacus* as result of preceding sublethal exposure to living spores of *Apharomyces astaci*. Experiments are now in progress to determine whether this acquired resistance of the European crayfish, and the natively higher refractoriness of *P. leniusculus*, reside in humoral or in cellular factors or in both, and to isolate these. From these studies it should eventually become apparent whether the natural and acquired resistance phenomena here described are related to classical immunological mechanisms.

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EXPLANATION OF PLATES

PLATE 1

Fig. 1. The European freshwater crayfish, *Astacus astacus* (left), and the Western American variety, *Pacifastacus leniusculus* (natural size).

Fig. 2*a*. Nerve cords of two specimens of *A. astacus* 4 days after injection into the abdominal haemocoel of a sublethal dose of spores of *Aphanomyces astaci*. The spores are clumped on the surface of the nerve cords and are surrounded by aggregates of blood cells. × 7.

Fig. 2*b*. Encapsulated spores in a clump adhering to the nerve cord of a specimen of *A. astacus* 4 days after sublethal infection with *Aphanomyces astaci*. Surrounding the spores (dark) can be seen large numbers of haemocytes. × 150.

Fig. 3. Hyphae of Strain J1 of *Aphanomyces astaci* growing out into the haemocoel of a specimen of *A. astacus* from a spore clump on the surface of the ventral nerve cord, 5 days after infection by injection. The hyphae are surrounded and almost obscured by aggregates of haemocytes. × 110.

PLATE 2

Fig. 4*a*. Ventral nerve cords of specimen of *P. leniusculus* (left) and *A. astacus* (right) 45 hr after injection of 5×10^5 spores of Strain J1 of *Aphanomyces astaci* into the abdominal haemocoel. Melanization in the attached blood cell–spore aggregates is considerably more pronounced in Pacifastacus. (Nerve cords fixed in alcohol and mounted in Canada balsam to minimize light scattering in the nerve tissue and to emphasize the melanin.) × 2.

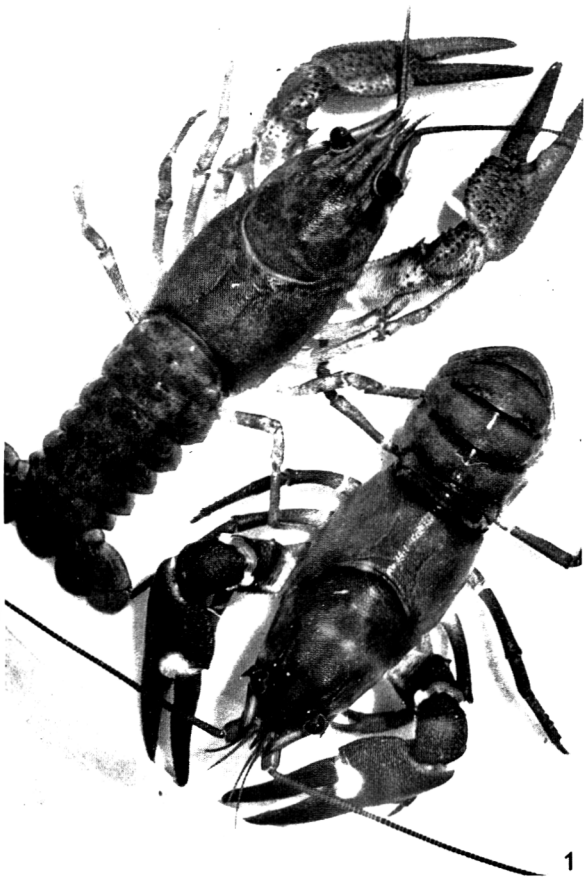
Fig. 4*b*, 4*c*. Spores melanized within two of the clumps shown in Fig. 4*a*. *P. leniusculus* specimen on the left, *A. astacus* on the right. × 250.

PLATE 3

Fig. 5. Melanized hyphal tip of *Aphanomyces astaci*, Strain J1, in clotted blood within abdominal cavity of a specimen of *P. leniusculus*. A hyphal branch is seen developing subapically. × 1100.

Fig. 6. Outgrowth of hyphae of *Entomophthora apiculata* from a spore–haemocyte clump on ventral nerve cord of *P. leniusculus*, 3 hr after death of the host. × 95.

Fig. 7. Outer, epicuticular surface of a section of intersegmental membrane of a specimen of *P. leniusculus*, showing fungal hyphae penetrating to the surface from the cuticular layer within. The membrane fragment was infected *in vitro*, on the inner surface of the cuticle, with spores of Strain D1 of *Aphanomyces astaci*, 40 hr previously. Swelling of the hyphal tips was a typical phenomenon when hyphae reached the epicuticle of either crayfish species from within, *in vitro* as well as *in vivo*. × 260.



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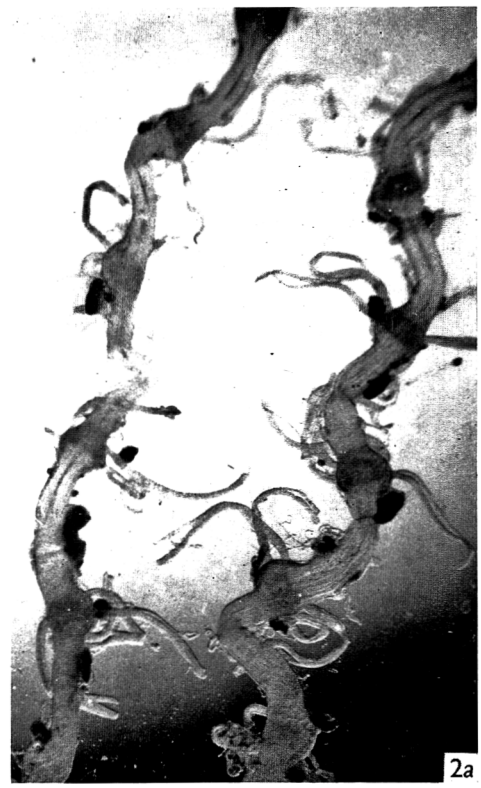

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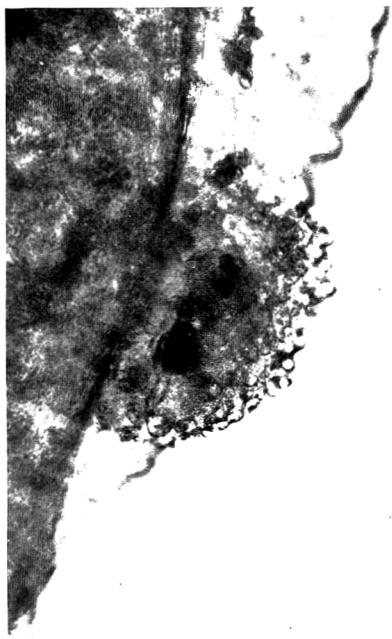

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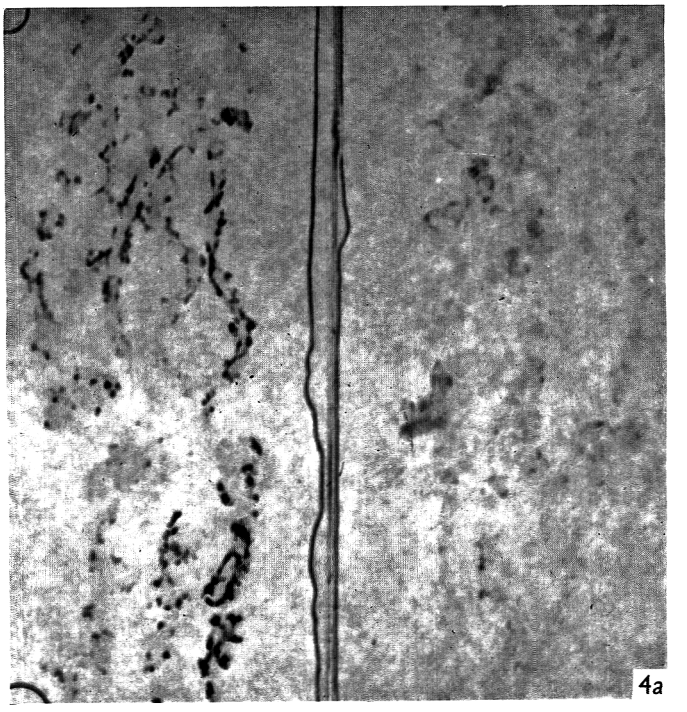
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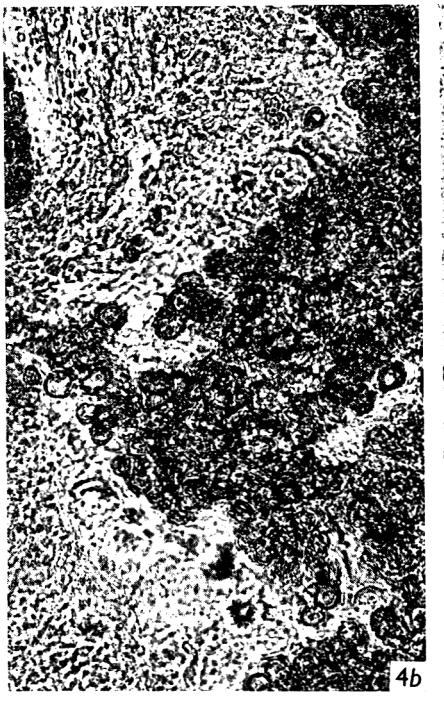
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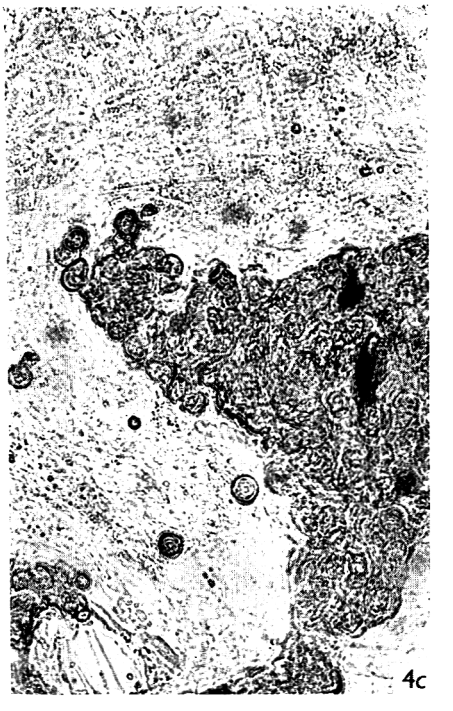
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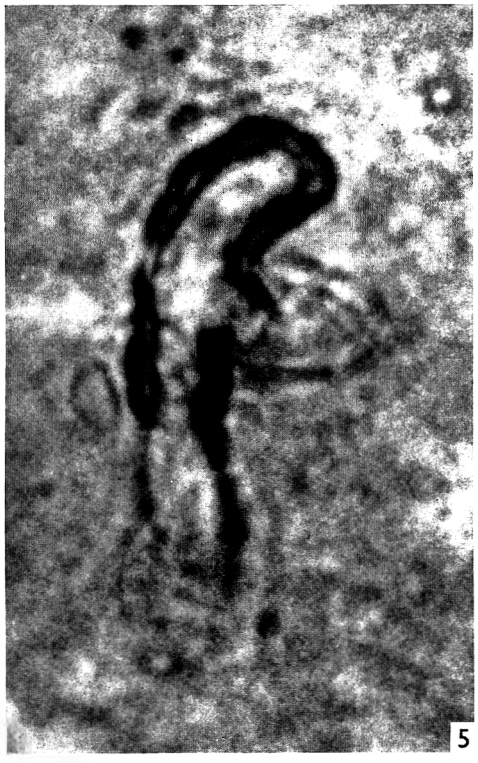

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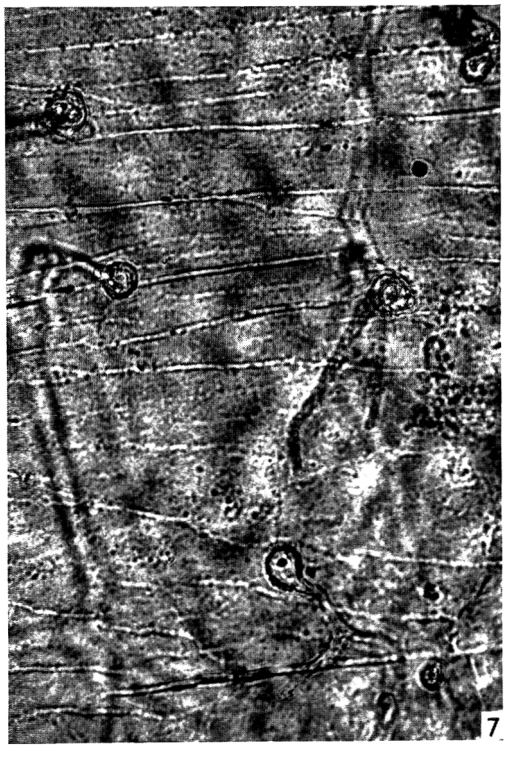

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A New Gene Cluster *rfe* Concerned with the Biosynthesis of Salmonella Lipopolysaccharide

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SUMMARY

A new class of rough mutants has been found in both *Salmonella montevideo* and *S. minnesota*. The mutants resemble phenotypically *rfb* mutants, having a complete lipopolysaccharide core but no O-specific material in lipopolysaccharide or as free hapten. The site of these *rfe* mutations is near the isoleucine and valine gene *ilv*, and is thus separate from the known lipopolysaccharide gene clusters *rfa* for the core, *rfb* for the O-specific side-chains, and *rfc* for O side-chain polymerization. The function of the *rfe* gene(s) is not known. It is suggested that they might participate in the utilization of the antigen carrier lipid for lipopolysaccharide synthesis, or that they might regulate the activity of *rfb* genes. The gene determining phosphorylation of the heptose in the lipopolysaccharide core of *S. minnesota* was identified as one within the *rfa* cluster.

INTRODUCTION

The O-antigenic lipopolysaccharide of Salmonella is thought to have a central core which is common to all Salmonella species. Attached to it in an unknown way are the O side-chains specific for each O-antigenic type (Fig. 1). Mutants in which the synthesis of lipopolysaccharide is defective are culturally rough (R). Three classes of rough mutants have been described (Subbaiah & Stocker, 1964; Naide *et al.* 1965; Beckmann, Subbaiah & Stocker, 1964). *rfa* mutants have a defective core, and produce a low molecular weight 'hapten' with O specificity. The *rfa* mutations are located in a part of the chromosome near *xyl* (Fig. 2). Later many, although not all, of the *rfa* mutations were shown to be cotransducible with *pyrE* and *cysE* between *xyl* and *ilv* (Kuo & Stocker, 1968). The hapten is thought to consist of polymerized O-specific repeating units as precursors of O side-chains which cannot be attached to the unfinished core (Kent & Osborn, 1968). *rfb* mutants on the other hand have a complete core, but produce no O-specific material. Their mutations map in a tight cluster near *his*. The third class of mutants, *rfc*, are unable to polymerize the O-specific repeating units. Their sites of mutations are located between *trp* and *gal*.

However, some R mutants do not fit into this scheme. These have a complete core with *N*-acetylglucosamine, and also O-specific hapten (Beckmann, Subbaiah & Stocker, 1964; Gemski & Stocker, 1967), which they apparently cannot attach to the core. Some of these (*rfbT*) have mutations that map in the *rfb* cluster. Other mutations

called *rfaL* resemble *rfa* in being cotransducible with *pyrE* (Kuo & Stocker, 1968). The LPS core in both cases is also complete in the sense that it can accept O side-chains, when incubated with cell-free preparations of *rfb* bacteria for example. It is therefore assumed that both *rfbT* and *rfaL* mutants might lack a component of a ligase or 'translocase' specified by these genes. Cell envelope preparations of *rfaL* mutants have been shown to be unable to catalyse the translocation step (Cynkin & M. J. Osborn, unpublished results quoted by Osborn, 1969).

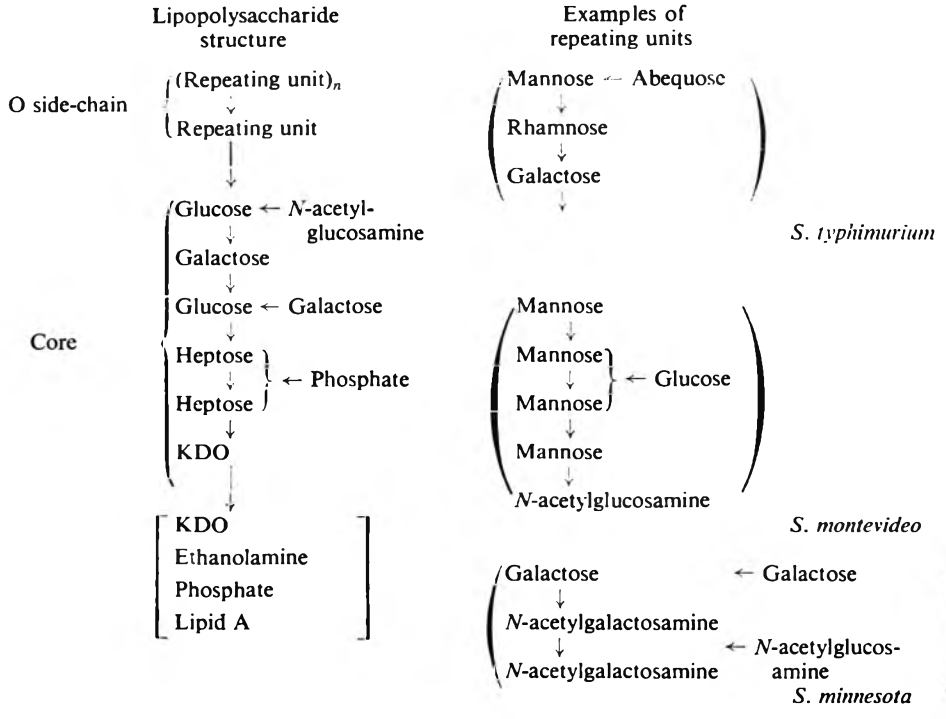

Fig. 1. Schematic structure of the Salmonella lipopolysaccharide (Lüderitz, Jann & Wheat, 1968; Fuller & Staub, 1968; Osborn, 1969). The innermost part of lipopolysaccharide contains KDO (= ketodeoxyoctonate), ethanolamine, phosphate and lipid A. To this are attached polysaccharide side-chains composed of a core and an O side-chain. The O side-chain is built up of repeating units, which carry the O-specific antigenic determinants characteristic of each Salmonella O group. Examples of the structure of the repeating unit in different Salmonella species, belonging to different O groups, are shown on the right.

Rough mutants have so far been analysed genetically only in *Salmonella typhimurium* strain LT2. While studying rough mutants in two other Salmonella species, *S. montevideo* (O antigens 6, 7) and *S. minnesota* (O antigen 21), we found a new class of R mutants (*rfe*) which produce a complete core, but no O-specific hapten. Their sites of mutation are located close to *ilv* and are clearly separate from *rfa*, *rfb* and *rfc*. The symbol *rfe* is preferred to *rfd*, which would be similar to the *rouD* used earlier to describe leaky *rfa* mutants (Gemski & Stocker, 1967). Typical *rfa* mutations in these species, as in *S. typhimurium*, occur close to *xyl*.

METHODS

Bacterial and phage strains. Salmonella typhimurium strain LT2 and various rough (R) mutants of it were obtained from Dr B. A. D. Stocker, Stanford University, California, as were the O- and R-specific phages (Wilkinson & Stocker, 1968)

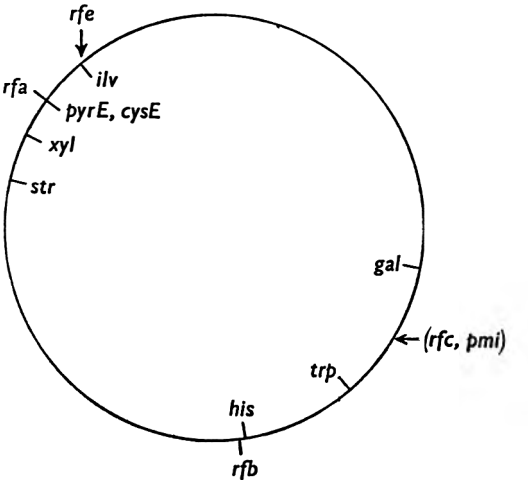

Fig. 2. Part of the chromosome map of Salmonella (after Sanderson, 1967; Mäkelä & Stocker, 1969). Positions not accurately known are indicated by arrows, and loci whose order is not known are bracketed. Genes labelled outside circle affect lipopolysaccharide synthesis. Gene symbols as follows: *gal* = galactose utilization, *trp* = tryptophan biosynthesis, *his* = histidine biosynthesis, *str* = resistance to streptomycin, *xyl* = xylose utilization, *pyrE* = orotidylic acid pyrophosphorylase, *cysE* = cysteine biosynthesis, *ilv* = biosynthesis of isoleucine and valine, *pmi* = phosphomannoseisomerase, *rfa*, *rfb*, *rfc*, *rfe* = biosynthesis of the O-antigenic lipopolysaccharide.

Salmonella montevideo was strain no. 129 of Edwards & Bruner (1942), used earlier by Mäkelä (1966); its mutants used are listed in Table 1. *Salmonella minnesota* was strain S99 obtained from Dr F. Kauffmann, State Serum Institute, Copenhagen, Denmark (Lüderitz *et al.* 1965); its R mutants mR3 and mR592 were those described by Lüderitz *et al.* (1966). It turned out that these mutants had more than one R mutation. Single R-mutant derivatives were therefore isolated from them as recombinants in crosses with the smooth parent strain first made F^+; they are listed in Table 1. Nutritional, fermentation or streptomycin-resistant mutants were selected from these strains by standard procedures (Lederberg, 1950). R mutants were selected as morphologically rough colonies on complete media or as clones resistant to the Felix-01 phage. Diethylsulphate was the mutagen used (10 mg./ml. added to an overnight broth culture, and incubation continued for 30 min. at 37°, after which the culture was diluted 1/100 in fresh medium and incubated overnight before plating out). The gene symbols are explained in the legend for Fig. 2.

Bacteriological techniques. In the bacterial crosses, F^+ strains with the F factor derived from *Escherichia coli* K12 (Mäkelä, Lederberg & Lederberg, 1962) were used as donors. The donors were streptomycin-sensitive and streptomycin (1 mg./ml.) was added to the selective medium. All R strains were identified by cultural

Table 1. *Salmonella strains used*

Gene symbols are described in the legend of Fig. 2; + is the wild type allele for each gene; mutant alleles are described by mutant numbers or by − or R (in the case of *str* indicating mutant allele conferring resistance to streptomycin).

Species	Strain no.	Mating type	Relevant genotype							Source
			rfa	*rfb*	*rfe*	*his*	*ilv*	*xyl*	*str*	
S. typhimurium line LT 2	SL 1027	·	+	+	+	·	·	·	·	From B. A. D. Stocker
	SL 748	·	+	−	+	·	·	·	·	From B. A. D. Stocker
	SL 758	·	−	+	+	·	·	·	·	From B. A. D. Stocker
	SL 1060	·	−	+	+	·	·	·	·	*rfaH* (Wilkinson & Stocker, 1968)
S. montevideo line 129 (Edwards & Bruner, 1942)	SW 829	F^+	+	+	+	+	+	+	+	F from *E. coli* K 12
	SH 1675	F^-	+	+	3623	−	−	−	R	
	SH 1676	F^-	+	3795	3624	−	−	−	R	
	SH 1679	F^-	+	+	3627	−	−	−	R	
	SH 1688	F^-	+	+	3636	−	−	−	R	
	SH 1711	F^-	3646	3610	+	−	−	−	R	From SH 1622, *his ilv xyl str* (*rfb* 3795 is very leaky)
	SH 1717	F^-	3652	3618	+	−	−	−	R	
	SH 1723	F^-	3658	3625	3796	−	−	−	R	
	SH 1724	F^-	3659	3626	+	−	−	−	R	
	SH 1732	F^-	3667	3624	+	−	−	−	R	
	SH 3269	·	3652	+	+	+	−	−	R	From cross SW 829 × SH 1717*
S. minnesota line S 99 (Lüderitz *et al.* 1965)	SH 309	F^+	+	+	+	+	+	+	+	F from *E. coli* K 12
	MR 3†	F^-	3791, 3792	+	3793‡	−	−	−	R	Lüderitz *et al.* 1966
	MR 592†	F^-	3789	+	3790‡	−	−	−	R	Lüderitz *et al.* 1966
	SH 1756	·	+	+	3793	−	−	+	R	From cross SH 309 × MR 3
	SH 1757	·	+	+	3790	−	−	+	R	From cross SH 309 × MR 592
	SH 3237	·	3791, 3792	+	+	−	+	−	R	From cross SH 309 × MR 3
	SH 3264	·	3789	+	+	−	+	−	R	From cross SH 309 × MR 592

* Crosses are written as donor × recipient.

† *his*, *ilv*, *xyl* and *str* mutations were selected in the original MR 3 or MR 592, checking the phage sensitivity and agglutination pattern of the strains at each step. However, as new R mutations might have accumulated during this selection, the *rfx* genotype of the original MR 3 and MR 592 was later checked by making them F^+ and then using them as donors to a smooth *his ilv xyl str* recipient strain; the same *rfa* and *rfe* mutations were recovered as reported

‡ is paper (Jousimies & P. H. Mäkelä, to be published).

in th*rfe*-3793 may be identical with *rfe*-3790.

characteristics, slide agglutination (4% saline; anti-O sera diluted in 0·2% saline), and sensitivity to O and R-specific phages (Wilkinson & Stocker, 1968). The methods and media were otherwise as described earlier (Mäkelä, 1966).

Chemical methods. Lipopolysaccharides were prepared by the phenol–water procedure followed by centrifugation at 105,000 *g*. Lipopolysaccharide was obtained as the sediment, while the supernatant represents the L1 fraction and contains the O-specific hapten (Beckmann, Subbaiah & Stocker, 1964). The analyses of these fractions were performed as described previously (Risse *et al.* 1967). The examination of phosphateless (P−) mutants was performed as described by Dröge, Ruschmann, Lüderitz & Westphal, 1968). Haemagglutination inhibition by lipopolysaccharide was examined according to Beckmann, Lüderitz & Westphal (1964).

RESULTS

Characterization of R mutants with bacteriophages

Wilkinson & Stocker (1968) found that the lysis of rough *Salmonella typhimurium* mutants by selected phages correlated well with the chemical composition of the defective lipopolysaccharide. Some of these phages do not attack *S. montevideo* or *S. minnesota* at all, but others produce lytic patterns comparable to those in *S. typhimurium* (Table 2). We found sensitivity or resistance to the Felix-01 phage (called FO in this paper) particularly useful. In *S. typhimurium* sensitivity to this phage has been shown to depend on the presence of *N*-acetylglucosamine—that is, the complete core—in the lipopolysaccharide (Lindberg, 1967). Thus smooth bacteria and R mutants that have a complete core are FO-sensitive, while core mutants are FO-resistant.

Table 2. *Phage sensitivity patterns of smooth and rough strains of Salmonella typhimurium, S. montevideo and S. minnesota*

Action of phage (clear lysis = +; no lysis = −) was determined by applying the phage dilutions (10^8 plaque-forming units/ml) as drops on nutrient agar plates spread with an overnight broth culture of the bacterial strain and dried for 30 min. (Wilkinson & Stocker, 1968).

				Sensitivity to phages			
		Strain	Mutant	FO (= Felix-01)	Br 60	Ffm	C 21
Smooth							
	*S. typhimurium**	SL 1027	·	+	−	−	−
	S. montevideo	SW 829	·	+	−	−	−
	S. minnesota	SH 309	·	+	−	−	−
Rough							
FO-sensitive	*S. typhimurium**	SL 748	*rfb*	+	+	+	−
	S. montevideo	SH 1675	*rfe*†	+	+	+	−
	S. minnesota	SH 1756	*rfe*†	+	+	+	−
FO-resistant	*S. typhimurium**	SL 758	*rfa*	−	+	+	−
	S. montevideo	SH 3269	*rfa*†	−	+	+	−
	S. minnesota	SH 3237	*rfa*†	−	+	+	−
	*S. typhimurium**	SL 1060	*rfaH*	−	+	+	+
	S. minnesota	SH 3264	*rfa*†	−	+	+	+

* According to Wilkinson & Stocker (1968 and personal communication), repeated in this series of experiments.

† Classification according to the data in the present paper.

Table 3. *Frequencies of rfe⁺ among different classes of recombinants in crosses between a smooth streptomycin-sensitive F⁺ donor and streptomycin-resistant xyl ilv rfe recipients*

Crosses were made between exponentially growing broth cultures of donor and recipient, mixed in equal proportions (SW 829 and SH 309 with *S.montevideo* and *S. minnesota* recipients respectively). The mating mixture was kept undisturbed at 37° for 2 hr, then plated for selection of *xyl⁺str^R*, *ilv⁺str^R* or *xyl⁺ilv⁺str^R* recombinants, which were recovered at a rate of *c.* 10^{-7} (= 10 recombinants per plate spread with 0·1 ml. of the mating mixture). The recombinants were streaked on nutrient agar plates, from which single colonies were tested for their nutritional characteristics and LPS character. For the latter purpose colony morphology, agglutinability in 4% saline and in the correct anti-O serum, and phage sensitivity pattern (according to Table 2) were determined.

Recombinant classes	Recipient strains: *S. montevideo* SH 1675 *rfe⁺**	SH 1676 *rfe⁺*	SH 1679 *rfe⁺*	SH 1688 *rfe⁺*	Total *rfe⁺*	% *rfe⁺*	*S. minnesota* MR 3† *rfe⁺*	MR 592‡ *rfe⁺*	Total *rfe⁺*	% *rfe⁺*
xyl⁻ ilv⁺	9/18	4/9	2/5	2/6	17/38	45		55/121	55/121	45
xyl⁺ilv⁻	1/18	1/11	0/27	0/36	2/92	2	1/87	4/78	5/165	3
xyl⁺ilv⁺	4/5	4/5	7/9	11/11	26/30	87	104/120	30/50	134/170	78
ilv⁺					18/39	46			61/131	47
xyl⁺					11/102	11			103/340	30
For comparison, frequency of *xyl⁺* among *ilv⁺*					1/39 = 3%				42/342 = 12%	
For comparison, frequency of *ilv⁺* among *xyl⁺*					10/102 = 10%				131/340 = 38%	

* *rfe* strains were culturally rough and agglutinated in 4% saline; *rfe⁺* were culturally smooth and agglutinated in the corresponding anti-O serum.

† MR 3 is also *rfa⁻*; *rfe⁺* could be scored only in recombinants that had become *rfa⁺*, and the figures given for *rfe⁺* are only approximate.

‡ MR 592 has an *rfa* mutation as well as *rfe*, but the *rfa* is so leaky that *rfe⁺* could be scored by agglutination, although the *rfa⁻rfe⁺* recombinants were still resistant to FO.

Genetic analysis of rfe mutants

Most randomly isolated rough mutants of *Salmonella montevideo* were sensitive to FO and rough-specific phages. Most of these mutations mapped near *his*, as does *rfb*, but a number did not. All of these mutants whose mutations did not map in the *rfb* area were isolated from an *xyl ilv* parent strain which was streptomycin-resistant. To locate the site of these mutations each mutant was crossed with an F^- streptomycin-sensitive smooth donor strain of *S. montevideo* (SW 829). The frequency of smooth recombinants among xyl^-ilv^+, xyl^+ilv^- and xyl^+ilv^+ recombinants is given in Table 3. The number of recombinants analysed in each cross is small because of low fertility in F^- crosses with this strain. However, the trend was the same in each case: nearly half the xyl^-ilv^+ recombinants were smooth, compared with two out of 92 xyl^+ilv^- recombinants. Of the 30 xyl^+ilv^+ recombinants 26 were smooth, confirming that the R mutation is in the *xyl-ilv* region of the chromosome.

In these crosses the unselected xyl^+ donor allele appeared in about 3% of the ilv^+ recombinants, and the unselected ilv^+ donor allele in 10% of the xyl^+ recombinants, as shown in the lower part of Table 3. The 45% linkage found between the R mutation and *ilv* suggests that this R mutation is much closer to *ilv* than is *xyl*. We shall call the gene or genes affected in these mutants *rfe*.

A similar R mutation was found in two *Salmonella minnesota* strains. The right half of Table 3 shows some results of mapping this mutation. In both MR 3 and MR 592 the mutation had a similar location to *rfe* in *S. montevideo*, with 45% linkage to *ilv*. The mutants MR 3 and MR 592 were originally isolated by a complex procedure (J. Schlosshardt, personal communication), and have turned out to contain *rfa* mutations in addition to *rfe*. In MR 592 the *rfa* mutation was so leaky that rfe^+ could be recognized by smooth colony form and smooth-type agglutination in the corresponding anti-O serum, although the recombinants that were still rfa^- gave a very slight agglutination in 4% saline, and retained the FO-resistant phage sensitivity pattern of the MR 592 parent. The *rfa* in MR 3 was not leaky—the strain has been demonstrated to have two separate biochemical defects in core synthesis (see p. 101) and hence probably has two independent *rfa* mutations. With this strain the *rfe* genotype could be scored only in recombinants that had become rfa^+. The single *rfe* mutant derivatives obtained in these crosses from MR 3 and MR 592 as xyl^+rfa^+ were phenotypically similar to *rfb* mutants, e.g. in sensitivity to FO (Table 2, strain SH 1756). Genetically, however, they were very different, as typical *his*-linked *rfb* mutants could be easily isolated in the *S. minnesota* strain (unpublished observations). Because of the isolation method of MR 3 and MR 592 we do not know whether the *rfe* mutation occurred independently in these two strains or not.

Genetic analysis of rfa mutants

We studied a number of other rough mutants that were resistant to the phage FO. These included five independent mutations of *Salmonella montevideo* and two of *S. minnesota*. As shown in Table 4, all these mutations mapped closer to *xyl* than to *ilv* (34 versus 6% linkage for the *S. montevideo* mutants, 67 versus 8% for the *S. minnesota* mutants). The rfa^+ allele was scored in all recombinants as giving rise to an FO-sensitive phage pattern regardless of the possible presence of an *rfe* or *rfb* mutation in the recipients and recombinants.

Table 4. *Frequencies of rfa⁺ among different classes of recombinants in crosses between a smooth streptomycin-sensitive F⁺ donor and streptomycin-resistant xyl rfa ilv recipients*

	Recipient strain										
	S. montevideo							*S. minnesota*			
Recombinant classes	SH 1711 *rfa**	SH 1723 *rfa⁺*	SH 1724 *rfa⁺*	SH 1732 *rfa⁺*	SH 1717 *rfa⁺*	Total *rfa⁺*	% *rfa⁺*	MR 3 *rfa⁺*	MR 592 *rfa⁺*	Total *rfa⁺*	% *rfa⁺*
xyl⁻ilv⁺	0/22	3/41	5/62	2/25	0/32	10/182	6	12/167	12/121	24/288	8
xyl⁺ilv⁻	13/30	15/56	33/79	7/24†	6/30†	74/219	34	87/131	53/78	140/209	67
xyl⁺ilv⁺	20/24	34/50	26/29	7/7	—	77/110	70	120/123	47/50	167/173	97

Crosses were made as in Table 3.
* *rfa⁺* characterized as FO-sensitive.
† many reversions of the recipient.

Relative order of the xyl, rfa, ilv and rfe genes

The relative position of the loci *xyl*, *rfa*, *ilv* and *rfe* could be more closely examined in the two *Salmonella minnesota* mutants MR 3 and MR 592 that have mutations in both *rfa* and *rfe* genes. (It is probable that MR 3 has two separate mutations, in two

Table 5. *Frequencies of different recombinant classes in crosses between a smooth streptomycin-sensitive F⁺ donor and xyl⁻ rfa⁻ ilv⁻ rfe⁻ str^r recipients of S. minnesota*

Crosses were made as in Table 3, *rfe* and *rfa* characterized as in Tables 3 and 4. Numbers of recombinants belonging to each class are listed in columns 5 and 6 (N.D. = not determined). In the last four columns are given numbers of the recombinants whose genotype could be produced only by four or more cross-overs. (The + after some numbers means that there might be a few more recombinants belonging to these groups which were not identified. Their number is certainly small—less than five—and would affect all columns nearly equally.) Different recombinant classes fall into this category when different orders of the four relevant genes are assumed, permitting conclusions of the most likely gene order. This appears to be the first one, *xyl-rfa-ilv-rfe*, which would necessitate quadruple cross-overs in not more than eleven recombinants.

Genetic constitution of recombinants				Recipient strain		Minimum number of quadruple cross-overs required to produce the recombinants observed, assuming gene order			
xyl	*rfa*	*ilv*	*rfe*	MR 3	MR 592	*xyl-rfa-ilv-rfe*	*xyl-rfa-rfe-ilv*	*rfa-xyl-ilv-rfe*	*rfa-xyl-rfe-ilv*
+	−	−	−	N.D.	23	—	—	—	—
+	−	−	−	86	51	—	—	—	—
+	−	+	−	17	19	—	36	—	36
+	−	+	+	103	28	—	—	—	—
−	+	+	+	12	8	—	—	20	20
−	−	+	+	167	47	—	—	—	—
−	−	+	−		62	—	—	—	—
+	−	+	−	3	1	6	6	—	1+
+	−	+	+		2				
+	−	−	+	N.D.	2	2+	2+	2+	—
+	+	−	+	1	2	3	—	3	—
−	+	+	−	0	4	—	4	4	4
Totals				389	249	11+	48+	29+	61+
Recombinants with at least two donor markers (excluding *ilv⁺rfe⁺* with the recipient MR 3)				222	164				

different *rfa* genes. In this genetic analysis they were never separated, and we feel justified in treating them as a single mutation.) These mutants were crossed with SH 309 (smooth, F⁺), and all *xyl⁺* or *ilv⁺* recombinant classes analysed (Table 5). Taking into account the fairly close linkage between *ilv* and *rfe*, and between *xyl* and *rfa*, the four loci can be assigned four different orders as shown in the last four columns of Table 5. Assuming the gene order *xyl–rfa–ilv–rfe* we see that most of the recombinant classes can be produced through a double cross-over, integrating the donor alleles in the recipient chromosome as a single block. The four classes *xyl⁺rfa⁻ilv⁺rfe⁻*, *xyl⁺rfa⁻ilv⁺rfe⁺*, *xyl⁺rfa⁻ilv⁻rfe⁺* and *xyl⁺rfa⁺ilv⁻rfe⁺*, however, cannot be produced in this simple way but require at least four cross-overs. Eleven representatives of these classes were found among the 386 recombinants that had at least two donor alleles and therefore could contribute to the analysis. Assuming any of the other three gene orders, a larger number of recombinants (48, 29 and 61

Table 6. *Composition of the lipopolysaccharide of rfa and rfe mutants of S. montevideo and S. minnesota*

Lipopolysaccharide was isolated by phenol-water extraction followed by centrifugation at 105,000 *g* (Beckmann, Subbaiah & Stocker, 1964). Polysaccharide was prepared from lipopolysaccharide by removing the lipid A (Dröge *et al.* 1968); phosphate and glucosamine were determined from the polysaccharide. The analyses followed previously described methods (Risse *et al.* 1967) and the results were calculated as moles/2 (or 1) moles of heptose. For the expected lipopolysaccharide structure see Fig. 1.

		Relevant genotype		Mole content of the various lipopolysaccharide constituents, with heptose normalized as 2 or 1							
Species	Strain	*rfa*	*rfe*	KDO	Heptose	Phosphate in LPS†	Phosphate in PS	Glucose	Galactose	Glucosamine in PS	Galactosamine* or mannose
S. montevideo	SH 3269	3652	+	1·3	2		1·8	1·2	0·7	< 0·15	Trace
	SH 1675	+	3623	1·4	2	3·4	2·3	1·2	1·2	0·99	0
	SH 1679	+	3627	1·3	2	3·1	2·4	1·0	1·1	0·78	0
S. minnesota	SH 3237	3791, 3792	+	2·4	1		0·5	0	0	0	0
	MR 3	3791, 3792	3793	1·4	1	2·2	0·7	0	0	0	0
	MR 592	3789	3790	2·3	2	2·3	0·8	1·3	0·6	0·09	0
	SH 1756	+	3793	1·3	2	3	1·5	0·73	1·2	0·29	0
	SH 1757	+	3790	1·7	2	3	2·1	1·4	1·6	1·4	0
Expected of a strain with a complete core (Fig. 1)				2	2	3	2	2	2	1	

* The O-specific sugars are mannose in *S. montevideo* and galactosamine in *S. minnesota* (Fuller & Staub, 1968; Lüderitz *et al.* 1966).
† LPS, lipopolysaccharide; PS, polysaccharide.

respectively) would require quadruple cross-overs, and therefore the first-mentioned order appears the most likely one.

These results thus locate the *rfa* cluster of *Salmonella minnesota* between *xyl* and *ilv*, as it is in *S. typhimurium*. To make its homology with the *rfa* in *S. typhimurium* more certain one would like to test its cotransducibility with, for example, *pyrE*, which is between *xyl* and *ilv* and cotransducible with *rfa* in *S. typhimurium*. The *rfe* locus remains clearly separated.

Chemical composition of the lipopolysaccharide and LI *fraction of the mutants*

For studying the chemical composition of the mutant lipopolysaccharide we prepared separate *rfa* or *rfe* mutant strains from the original double or triple mutants by crossing these with smooth donors of the same species. Thus SH 3269 (Table 6) is a recombinant from the cross between SW 829 as the smooth donor and SH 1717 as the recipient; it was selected as *his*$^+$*rfb*$^+$, and has the *rfa*-3652 mutation of SH 1717. Similarly, a cross between SH 309, the smooth F$^+$ *Salmonella minnesota* strain and MR 3 gave the *rfa* mutant SH 3237 as an *ilv*$^+$*rfe*$^+$ recombinant, and the *rfe* mutant SH 1756 as an *xyl*$^+$*rfa*$^+$ recombinant. The other single *rfe* mutant SH 1757 was prepared as *xyl*$^+$*rfa*$^+$ from a similar cross between SH 309 and MR 592.

The monosaccharide composition of the lipopolysaccharide prepared from these mutants was determined, and the results are given in Table 6. *N*-acetylglucosamine was analysed from the polysaccharide (PS) obtained from lipopolysaccharide after removal of lipid A (Dröge *et al.* 1968) because lipid A also contains glucosamine. Phosphate was determined in both lipopolysaccharide and polysaccharide. The molar proportions of these constituents found in strains with the complete lipopolysaccharide core are given as the bottom line of the Table (see also Fig. 1, for the structure of the core).

Let us first examine the *rfe* mutants. Two of these were studied in each species: SH 1675 and SH 1679 of *Salmonella montevideo* and SH 1756 and SH 1757 (which may or may not represent the same *rfe* mutation—see p. 97) of *S. minnesota*. The sensitivity of these mutants to the phage FO strongly suggests that they contain the complete lipopolysaccharide core. All the core constituents were indeed found in the lipopolysaccharide prepared from them, and the polysaccharide contained the expected amount of *N*-acetylglucosamine. SH 1756 was the only exception, with a lower value for *N*-acetylglycosamine.

The serological specificity of the *rfe* lipopolysaccharide was studied by a haemagglutination inhibition technique (Beckmann, Lüderitz & Westphal, 1964). When red blood cells are coated with lipopolysaccharide of various R mutants they become agglutinable by the specific anti-R serum prepared against this sort of R lipopolysaccharide; this agglutination can be specifically inhibited by the same sort of lipopolysaccharide but not by others. In this test the *rfe* lipopolysaccharide could inhibit the haemagglutination by anti-*rfaL* of cells coated with *rfaL* lipopolysaccharide and by anti-*rfb* of cells coated with lipopolysaccharide from several *rfb* mutants. The *rfe* lipopolysaccharide thus resembles that of *rfaL* and *rfb* mutants, both of which are known to contain the complete core (see Introduction).

Two *rfa* mutants SH 3269 and SH 3237 were examined. They had no or very little *N*-acetylglucosamine in the polysaccharide, indicating deficient synthesis of the core. The defect in SH 3269 is apparently leaky, permitting some core stubs to be completed

and then capped by the O-specific side-chains, as indicated by the presence of some *N*-acetylglucosamine and traces of O-specific sugars. This strain behaves like a leaky R mutant also in its reactivity with the specific anti-O serum.

Strain SH 3237 (and its parent mR 3) has a more defective core lacking the second heptose and all other constituents distal to it. Strain mR 3 has been described as a mutant deficient in the transfer of the second heptosyl residue to the first heptose of the core (Risse *et al.* 1967). Later experience (unpublished observations) has shown that it also lacks the ability to phosphorylate lipopolysaccharide heptose. These two defects probably have their basis in two separate mutations, provisionally designated as *rfa*-3791 for the defect in heptosyl transfer, and *rfa*-3792 for the defect in phosphorylation. In the genetic analysis we have so far been unable to separate the two hypothetical mutations, which might indicate that they represent a deletion encompassing at least parts of two genes; however, no deletion mutations have so far been found in the *rfa* cluster (Mäkelä & Stocker, 1969). Strain mR 3 has been shown to possess at least one enzyme participating in core synthesis, namely the transferase for the first glucose residue (Risse *et al.* 1967), which in *Salmonella typhimurium* is determined by a gene *rfaG* in the *rfa* cluster (Wilkinson & Stocker, 1968; Osborn, 1968). The hypothetical deletion could thus not extend to this gene. The presence of either two separate mutations or a deletion would also account for the apparent non-leakiness of the defect in core synthesis.

The *rfa*-3789 of the *Salmonella minnesota* mutant mR 592 was not examined chemically because of its leakiness. *rfa*-3789 *rfe*$^+$ derivatives of mR 592 were nearly smooth in cultural behaviour and O-specific agglutination although they retained a rough FO-resistant phage sensitivity pattern (SH 3264 in Table 2). A conspicuous feature of the *rfa rfe* double mutant mR 592 itself is the decreased amount of phosphate in both lipopolysaccharide and polysaccharide, indicating a lack of the phosphate normally attached to the core (Table 6). This type of mutant lipopolysaccharide has been described as P− as opposed to the normal P+ (Dröge *et al.* 1968). The P− lipopolysaccharide is characterized by (1) less phosphate in the lipopolysaccharide and polysaccharide than in P+ strains; (2) no heptosephosphate obtained on hydrolysis of lipopolysaccharide performed according to Slein & Schnell (1953); and (3) a peak of methylated heptoses obtained after methylation and methanolysis, not seen with P+ lipopolysaccharide. The lack of phosphorylation prevents the completion of the core by preventing the transfer of galactose (Mühlradt, Risse, Lüderitz & Westphal, 1968). The defect in phosphorylation in mR 592 is probably very leaky, as high amounts of glucose and galactose and even some *N*-acetylglucosamine are found in the lipopolysaccharide and polysaccharide. The absence of O-specific sugars in the lipopolysaccharide preparation is probably accounted for by the *rfe* mutation in mR 592.

When the *rfa* of either mR 3 or mR 592 was replaced by the *rfa*$^+$ allele, the resulting *rfe* recombinants SH 1756 and SH 1757 had, as shown above, gained the ability to synthesize the complete core. The P+ character of their lipopolysaccharide was specifically confirmed by all the three criteria mentioned above. Thus it has been demonstrated that the phosphorylation of lipopolysaccharide heptoses is determined by a gene belonging to the *rfa* cluster.

As *rfa* mutants synthesize an incomplete core, they are unable to attach O-specific side-chains to their lipopolysaccharide. In *Salmonella typhimurium rfa* mutants the

Table 7. *Paper chromatographic analysis of the monosaccharide constituents in the L 1 (hapten) fraction of the rfa and rfe mutants*

The L fraction was obtained as the supernatant after centrifugation (100,000 *g*) of the phenol-water extract to precipitate the lipopolysaccharide (Beckmann, Subbaiah & Stocker, 1964). This fraction is expected to contain the O-specific hapten.

		Relevant genotype		Monosaccharides in the L 1 fraction					
Species	Strain	*rfa*	*rfe*	Heptose	Glucose	Galactose	Glucosamine	Galactosamine*	Mannose*
S. montevideo	SH 3269	3652	+	−†	+†	−	+	−	+
	SH 1675	+	3623	−	+	−	−	−	−
	SH 1679	+	3627	−	+	−	−	−	−
S. minnesota	SH 3237	3791, 3792	+	−	−	+	+	+	−
	MR 3	3791, 3792	3793	−	+	−	−	−	−
	MR 592	3789	3790	−	+	−	−	−	−
	SH 1756	+	3793	−	+	−	−	−	−
	SH 1757	+	3790	−	+	−	−	−	−

* The O-specific sugars are mannose in *S. montevideo*, galactosamine in *S. minnesota* (Fuller & Staub, 1968; Lüderitz *et al.* 1966).
† Stands for a large amount of the sugar detected, − for none; no intermediate amounts were detected.

O-specific material is found as haptenic polysaccharide in the so-called L 1 fraction after phenol-water extraction and high-speed centrifugation, which removes lipopolysaccharide (Beckmann, Subbaiah & Stocker, 1964). We isolated the L 1 fraction from our *S. montevideo* and *S. minnesota* mutants in the same way, and analysed its monosaccharide content by paper chromatography (Table 7). The L 1 fraction of the *S. montevideo rfa* mutant SH 3269 contained large amounts of glucose, mannose and glucosamine, which are sugars constituting the O-specific side-chains in this species (Fuller & Staub, 1968). Of these sugars mannose is 'O-specific', occurring only in the O side-chains in the bacterium. Similarly the *rfa* mutant SH 3237 of *S. minnesota* contained in its L 1 fraction galactose, glucosamine and galactosamine, which are the constituents of its O side-chains (Lüderitz, Jann & Wheat, 1968), and of which galactosamine is O-specific. None of the four *rfe* mutants contained O-specific sugars in the L 1 fraction. Glucose was found in most of the L 1 fractions; it probably derives from cell-wall glucans. The original *S. minnesota* mutants MR 3 and MR 592 have no hapten no doubt because of their *rfe* mutation—when the *rfe* was replaced by the *rfe*+ allele (to obtain SH 3237 from MR 3), the strain gained the ability to synthesize hapten as expected of an *rfa* mutant.

DISCUSSION

Mutations at two separate locations in the *xyl*, *ilv* region of *Salmonella montevideo* and *S. minnesota* resulting in defects in the synthesis of lipopolysaccharide were demonstrated. One group corresponds to the *rfa* gene cluster described in *S. typhimurium*, which determines the synthesis of the lipopolysaccharide core, including its phosphorylation. The other group, the *rfe* mutants, has not been detected in *S. typhimurium*, and its function is unknown. The *rfe* mutants resemble phenotypically the *rfb* and *rfaL* mutants of *S. typhimurium*. All of these are sensitive to FO and several rough-specific phages, and they all synthesize a core lipopolysaccharide with *N*-acetylglucosamine. The *rfaL* mutants contain O-specific hapten and the *rfaL* gene is apparently concerned with the translocation of the O side-chain from its lipid carrier to the lipopolysaccharide core (Osborn, 1969). The *rfb* mutants are defective in the synthesis of O-specific sugars or in their assembly into the O side-chains, and therefore have no hapten (Beckmann, Subbaiah & Stocker, 1964; Nikaido, Nikaido, Subbaiah & Stocker, 1964; Nikaido, Levinthal, Nikaido & Nakane, 1967).

The *rfe* mutants of *Salmonella montevideo* and *S. minnesota* synthesize no hapten either (Table 7), and therefore it seems most likely that their mutation interferes with the biosynthesis of O-specific material. The *rfb* cluster is generally known to harbour most of the genes whose products participate in the synthesis of O-specific repeating units (Stocker, Wilkinson & Mäkelä, 1966), both the genes for the synthesis of the O-specific monosaccharides and the genes for the transferases assembling the repeating units. This was specifically shown to be true of the *rfb* cluster of *S. montevideo*. When the *rfb* region of the *S. montevideo* chromosome was introduced, by conjugation, into the cells of *S. typhimurium*, these started synthesizing a lipopolysaccharide of the *S. montevideo* type (Mäkelä, 1966; Nikaido, Nikaido & Mäkelä, 1966). It was concluded that all the information necessary for the synthesis of *S. montevideo*-specific lipopolysaccharide, and not shared by *S. typhimurium*, was located in the *rfb* gene cluster. Thus if the *rfe* gene(s), remote from *rfb*, specify a product necessary for the

synthesis of O side-chains, this function must be common to *S. montevideo* and *S. typhimurium*. What could such a common function be? The only monosaccharide found in the O side-chains of both these species is mannose (Fig. 1). However, the enzymes participating in the synthesis of mannose (as its guanosine-diphosphate nucleotide) are specified by *rfb* genes, or by a gene *pmi* (for phosphomannoseisomerase) between *trp* and *gal* (Stocker, Wilkinson & Mäkelä, 1966; Nikaido *et al.* 1967). Furthermore, mannose is not present in the O side-chains of *S. minnesota*, in which *rfe* gene(s) were also identified. The antigen carrier lipid (Wright, Dankert & Robbins, 1965) on which the O side-chains are assembled, is very likely to be common to all Salmonella species. The possible role of *rfe* in antigen carrier lipid synthesis now seems to deserve serious investigation. There are, however, immediate objections to this hypothesis: a similar antigen carrier lipid is involved in the synthesis of the cell-wall peptidoglycan (Higashi, Strominger & Sweeley, 1967). Bacteria with an impaired synthesis of peptidoglycan would not be viable under the normal growth conditions used in the isolation and study of all our R mutants. Therefore we might rather think that the antigen carrier lipid will have to be modified in a certain way to mark it for either peptidoglycan or lipopolysaccharide (or some other) synthesis. This modification could be a slight chemical change or perhaps rather a fixed localization of the lipid in association with the enzymes participating in the relevant synthesis. On the other hand, the *rfe* gene(s) might have a regulatory function, e.g. for the *rfb* genes. So far no genetic regulation of the *rfb* genes has been found, and the *rfb*-determined enzymes have shown the same levels of activity whatever the growth conditions of the bacteria (Nikaido, 1968).

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The Effect of Temperature on the Metabolism of Baker's Yeast growing on Continuous Culture

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SUMMARY

Glucose-limited cultures of baker's yeast growing at 25° had a maximum growth rate, saturation constant and yield constant of 0·22 hr^{-1}, 129 μg./ml. and 0·225, respectively, whereas when growing at 38° the corresponding values were 0·25 hr^{-1}, 300 μg./ml. and 0·204. In continuous culture, with the dilution rate fixed at 0·1 hr^{-1} there were no differences observed in viability, incidence of respiratory deficient mutants, cytochrome spectra or mean cell dry weights, between cultures grown at 25 and 38°. The culture grown at 25° had a smaller mean cell volume, greater yield value and nitrogen utilization. Ethanol, pyruvate and α-ketoglutarate were secreted to a greater degree in cultures grown at 38°. Yeast grown at 25° had a smaller capacity to produce carbon dioxide but greater ability to take up oxygen. Enzymes associated with glycolysis, alcohol production, tricarboxylic acid cycle and respiratory chain in organisms cultured continuously at 25 and 38° showed few important differences. The most obvious ones were those involving α-ketoglutarate as a substrate, especially α-ketoglutarate dehydrogenase. There were only small differences in adenosine phosphates and nicotinamide nucleotides. At 25° the ratio NAD/NADH was 1·5 but for organisms grown at 38° the ratio was 1·1.

INTRODUCTION

The growth and metabolic activities of micro-organisms are profoundly affected by the temperature at which they grow. For example, with Saccharomyces it has long been known that the rate of alcohol production increases with temperature up to 40° (Brown, 1914). Again fusel alcohol production is stimulated by increasing temperature with top-fermenting yeasts but not with bottom-fermenting yeasts (Hough & Stevens, 1961) and Wolter, Lietz & Beubler (1966) showed an increase in ethyl acetate production with increases in incubation temperature.

There are many reports that the synthesis of enzymes are affected by the growth temperature (Knox, 1955). Christopherson (1967) demonstrated with *Candida pseudotropicalis* that the activity of glucose-6-phosphate dehydrogenase was lower when grown at 37° than when grown at 20°. In a similar experiment the alcohol dehydrogenase activity of *Saccharomyces cerevisiae* was two- to three-fold different between organisms grown at 15 and 37°. The induction of the enzyme catalase in *S. cerevisiae* also has been reported to be temperature sensitive (Sulebele & Rege, 1967).

In continuous culture the metabolic activities of a micro-organism vary with growth rate. Thus Tempest & Herbert (1965) demonstrated with glucose-limited continuous

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cultures of *Candida utilis* that growth rate markedly affected respiration rate. But the growth rate in batch culture varies with temperature and thus there is always doubt as to whether reported effects of temperature on metabolism are direct or arise from an alteration in growth rate. An assessment of the effect of culture temperature on the metabolism of a micro-organism, therefore, is best carried out under conditions where the growth rate is constant and independent of temperature. This is readily achieved with continuous culture.

In the present study, a detailed comparison of the metabolism of baker's yeast has been carried out with continuous cultures maintained at 25 and at 38°, but with the rate of growth kept constant. The object was to obtain information which would explain why, at higher temperatures of cultivation, cell production is decreased but the rate of ethanol production is enhanced (Hough & Rudin, 1958).

METHODS

Organism and media. The yeast used was a strain of *Saccharomyces cerevisiae* isolated from a commercial sample of baker's yeast and employed in earlier studies (Brown & Hough, 1965, 1966). It was maintained on a solidified malt extract medium (Wickerham, 1951) and subcultured monthly. For experimental work the liquid synthetic medium of Cutts & Rainbow (1950) was modified in that the lactate buffer was replaced by citric acid monohydrate (1·13 g./l.) plus trisodium citrate dihydrate (4·44 g./l.).

Culture conditions. Batch cultures (50 ml.) were grown in 150 ml. conical flasks in a thermostatically-controlled incubator at temperatures from 25 to 40°. The flasks were shaken at 100 strokes/min. with an amplitude of 4·5 cm. Continuous cultures were established in a single-stage glass culture vessel of 150 ml. working volume. The dilution rate was controlled, generally at 0·1 hr^{-1} (equivalent to a residence time of 10 hr) using a peristaltic metering pump and the temperature regulated by pumping water from a thermostatically-controlled water-bath through a jacket surrounding the vessel. Filtered sterilized air was injected below liquid level in the vessel, at a rate of 50 ml./min. This procedure gave reasonably high levels of dissolved oxygen, indicated by similar yields when various oxygen and air mixtures at the same flow rates were used. The culture was inoculated and sampled by means of a device described by Heatley (1950). When the culture optical density (measured at 625 nm., using a Unicam SP500 spectrophotometer) was constant for 3 days, equilibrium was established and samples taken for detailed analyses.

Growth constants. Yeast, previously grown in glucose-limited continuous culture, was inoculated into shake flasks containing a complete medium but with glucose concentration ranging from 0·1 to 10 mg./ml. In each series of experiments the inoculum yeast was grown at a corresponding temperature. Readings of absorbance, at 625 nm., were taken at short intervals during early stages of growth of the yeast Results were calculated from the ratio of $\log_2$ O.D. to time.

Viability measurements. Both a staining method (Lindegren, 1949) and slide-culture technique (Gilliland, 1959) were employed.

Respiratory-deficient mutants. The method of Ogur, St. John & Nagai (1957) was used for detection of the respiratory deficient organisms.

Distribution of yeast cell sizes. Organisms suspended in sodium chloride solution

(0·9%), containing maltose (10%) to minimize aggregation, were examined in a Coulter electronic particle counter fitted with 70 μm. orifice.

Growth factor requirements. These were determined by the method of Shultz & Atkin (1947).

Manometry. Organisms were separated from the cultures by centrifugation washed with citrate buffer (50 mM) plus potassium dihydrogen phosphate (5 mM) at pH 5·0 and resuspended in the same buffer solution at a known concentration of about 2 mg./ml. Oxygen uptake and carbon dioxide output under aerobic conditions were measured using a Braun Rotary Warburg Manometer.

Samples for analyses. Samples were transferred to ice-cooled tubes and centrifuged (3000 *g* for 3 min.) at 2°. The samples for cytochrome spectra and assays of enzyme and coenzyme estimations were then treated as indicated below. For all other analyses, the yeast pellets were washed with water (at 2°) and then dried under reduced pressure until of constant weight. They were then stored until required in a desiccator (at 4°).

Cytochrome spectra. The method of Linnane, Biggs, Huang & Clark-Walker (1968) was used.

Table 1. *List of enzymes and coenzymes investigated*

Enzyme or coenzyme	E.C. number	Reference for assay
Phosphofructokinase	2.7.1.11.	Viñuela, Sales & Sols, 1963
Pyruvate kinase	2.7.1.40	Rose, 1960
Pyruvate dehydrogenase enzyme system	—	Alvarez, Vanderwinkel & Wiame, 1958
Pyruvate decarboxylase	4.1.1.1.	Holzer & Goedde, 1957
Alcohol dehydrogenase	1.1.1.1.	Bücher & Redetzki, 1951
Aconitate hydratase	4.2.1.3.	Racker, 1950
Isocitrate dehydrogenase (NAD specific)	1.1.1.41.	Kornberg, 1955*a*
Isocitrate dehydrogenase (NADP specific)	1.1.1.42.	Kornberg, 1955*b*
Ketoglutarate dehydrogenase enzyme system	—	Holzer, Hierholzer & Witt, 1963
Succinate dehydrogenase	1.3.99.1.	Hauber & Singer, 1967
Fumarate hydratase	4.2.1.2.	Racker, 1950
Malate dehydrogenase	1.1.1.37.	Bergmeyer, 1963
NADH oxidase enzyme system	—	Green & Ziegler, 1963
NADH: cytochrome-*c* oxidoreductase	1.6.2.1.	Polakis, Bartley & Meek, 1964
Cytochrome-*c*: oxygen oxidoreductase	1.9.3.1.	Polakis, Bartley & Meek, 1964
Succinate:cytochrome-*c* oxidoreductase	—	Mackler *et al.* 1962
Glutamate:NAD oxidoreductase	1.4.1.2/3.	Holzer & Schneider, 1957
Glutamate:NADP oxidoreductase	1.4.1.3/4.	Holzer & Schneider, 1957
Aspartate:ketoglutarate aminotransferase	2.6.1.1.	Holzer, Gerlach, Jacobi & Gnoth, 1958
ATP, ADP, AMP	—	Bergmeyer, 1963
NAD	—	Bergmeyer, 1963
NADH	—	Polakis & Bartley, 1965

Assays for enzyme and coenzyme estimations. Yeast samples (100 to 200 mg. dry weight) were washed twice with 50 mM-potassium phosphate buffer (pH 7·0), resuspended in 6 ml. of this buffer and disintegrated in a Mickle tissue disintegrator (15 min. using 1 mm. diam. glass beads). After centrifugation (10 min. at 3000 *g*, 2°) the clear supernatant fraction contained the soluble enzymes and enzymes of the mitochondria which were then assayed immediately. All stages in the extraction were carried out at 2°. The assays used are given in Table 1. Protein was estimated by the Folin–Lowry method (Lowry, Rosebrough, Farr & Randall, 1951).

Keto-acids. Pyruvate and α-ketoglutarate were estimated enzymically by the methods described by Bergmeyer (1963).

RESULTS

The effect of temperature on the growth characteristics of Saccharomyces cerevisiae

The growth factor requirements of this yeast when growing at 25° or at 38° were identical. There was an absolute requirement for biotin, pantothenate, inositol and pyridoxine and a partial requirement for nicotinic acid and thiamine. Comparison of growth constants of yeast cultured at 25 and 38° are, therefore, not complicated by any changes in growth factor requirements. The saturation constants (K_s) and max. growth rates (μ_{max}) for glucose-limited cultures at these temperatures were calculated from experimental results obtained with batch cultures containing a range of glucose concentrations (Herbert, Elsworth & Telling, 1956). For cultures incubated at 25° the μ_{max} value was 0·22 hr^{-1} and K_s value 129 μg./ml. whereas for cultures incubated at 38°, μ_{max} was 0·25 hr^{-1} and K_s equal to 300 μg./ml. (Fig. 1). The yield constants for these cultures grown at 25 and 38° were 0·225 and 0·204, respectively.

Table 2. *Growth characteristics of S. cerevisiae*

Temperature of continuous culture	Glucose utilized (%)	Cell yield mg./ml. medium	Ethanol produced μg./mg. yeasts	Nitrogen utilized (%)
25°	100	3·2	193	41
38°	99	2·6	468	37

The viability of the yeast populations were measured over a range of dilution rates from 0·07 to 0·20 hr^{-1} when growing in a glucose-limited chemostat. With the growth temperature at 25° the percentage of viable cells ranged from 92 to 98, while with cultures maintained at 38°, viability varied from 90 to 98. The incidence of respiratory deficient mutants was less than 0·2% irrespective of the growth temperatures and, therefore, like viability could be ignored when interpreting subsequent results.

Again, the cytochrome spectra of organisms grown continuously (glucose-limited, $D = 0{\cdot}1$ hr^{-1}) were similar and showed peaks at 524, 530, 551, 562 and 600 nm. These probably were due to the presence of cytochromes $c+c_1$, $b+b_2$ and $a+a_3$ respectively. There was a difference, however, between the cultures grown at 25 and 38° with respect to their mean cell volumes; organisms grown at 38° were slightly larger than those grown at 25° although their mean cell dry weights were identical.

Cultures grown at 25 and 38° in a glucose-limited medium ($D = 0{\cdot}1$ hr^{-1}) were found to differ in several respects (Table 2). The yield of organisms was greater at the lower growth temperature and slightly more nitrogen was utilized. In contrast, the ethanol production was less than half that observed with cultures grown at 38°, despite the complete utilization of glucose.

A further difference was the higher levels of α-keto acids excreted into the medium by cultures grown continuously at 38°. The steady state levels of the pyruvate and α-ketoglutarate attained in glucose-limited ($D = 0{\cdot}1$ hr^{-1}) when the temperature was varied from 20 to 42° are shown in continuous culture (Fig. 2). The level of α-ketoglutarate was higher than that of pyruvate at all temperatures below 40°. Between the growth temperature of 30 and 35° the level of α-ketoglutarate in the medium rose from 3 to 11 μg./ml. At 35° the rate of excretion of α-ketoglutarate was maximal. At higher temperatures the level of pyruvate in the medium rose sharply and at 41°

exceeded 150 μg./ml. Other metabolities of the tricarboxylic acid cycle (malate, oxaloacetate and isocitrate) could not be detected in the medium, nor were amino acids, fatty acids and lactic acid excreted.

Respiratory activities. Manometric studies were carried out in order to examine the effect of various growth temperatures upon the respiratory activities of the yeast. In one experiment, the growth-limiting substrate was glucose (10 mg./l.) and in another DL-lactate (15 mg./l.). The dilution rate was held constant (0·1 hr^{-1}) and the temperature raised progressively in small steps from 25 to 39°. Organisms were removed

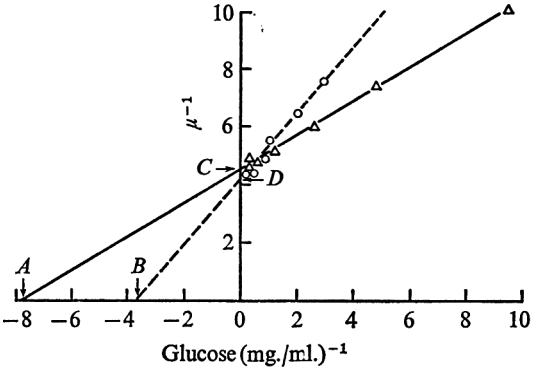

Fig. 1

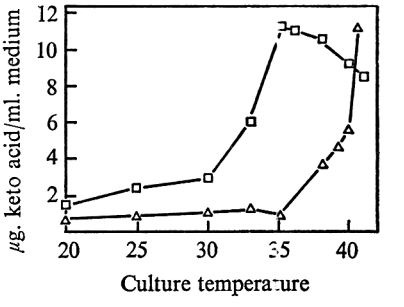

Fig. 2

Fig. 1. Plot of reciprocal of specific growth rate (μ) against the reciprocal of the glucose concentration for a series of shaken batch cultures of *S. cerevisiae*. Solid line and triangles refer to 25° cultures, broken line and circles to 38° cultures. A and B indicate values for $-1/K_s$ from which the values of the saturation constant for 25 and 38° respectively were calculated. *C* and *D* indicate values for maximum specific growth rates for 25 and 28° respectively. Growth was limited by glucose concentrations which ranged from 0·1 to 10 mg./ml.

Fig. 2. Levels of α-ketoglutarate (square symbols) and pyruvate (triangular symbols) present in the effluent from a glucose-limited chemostat culture of *S. cerevisiae* maintained at various temperatures at a dilution rate of 0·1 hr^{-1}. Glucose concentration in feed 10 mg./ml.

from the chemostat cultures when equilibrium conditions prevailed and were washed and transferred to Warburg flasks with an excess of glucose as substrate and incubation at 25°. With glucose as growth-limiting substrate in the chemostat and aerobic conditions in the respirometer, the carbon dioxide output was found to be higher as temperature at which the organisms were cultured increased from 25 to 39°: in contrast, the oxygen uptake remained fairly constant between 25 and 32° and then fell steadily with increasing temperature (Fig. 3). The respiratory quotient at 25° was 1·15 but at 39° it was 8·0 indicating a substantial change in the metabolic pattern of the yeast.

With DL-lactate (15 mg./ml.) as growth-limiting substrate in the chemostat and glucose in excess in the respirometer the oxygen uptake increased as the temperature of the culture was increased from 25 to 34°. With cultures grown at 34 to 39° the Q_{O_2} (glucose) declined. When lactate was substituted for glucose in the respirometer the Q_{O_2} results were generally similar although slightly greater in the range 25 to 35° (Fig. 4).

In order to determine whether, at higher temperatures, the synthesis of respiratory

enzymes was inhibited or their activity impaired the following experiments were performed. Yeasts grown in a glucose-limited continuous culture at 25° were transferred to a respirometer maintained at various temperatures between 22 and 45°. The oxygen uptake increased with temperature up to 42°. Above this temperature (which incidentally, is the maximum permitting growth of the yeast) the respiratory ability fell sharply. This contrasts strongly with the effect of temperature or growth and suggests that it is the synthesis of respiratory enzyme that is inhibited by elevated growth temperatures rather than the activity of the preformed enzymes.

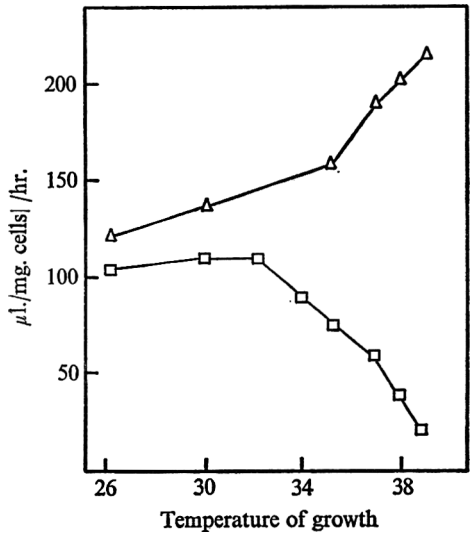

Fig. 3

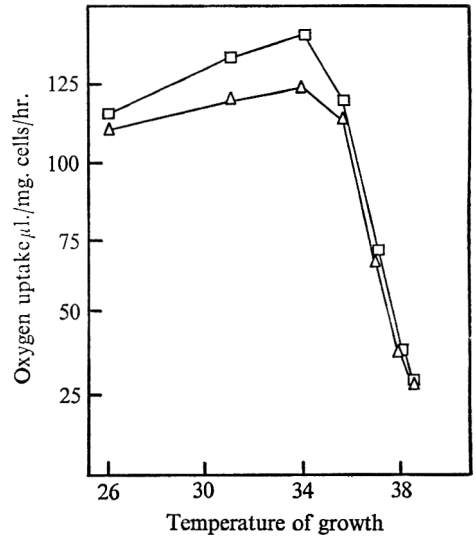

Fig. 4

Fig. 3. Relationship between oxygen uptake (square symbols), carbon dioxide output (triangular symbols) by *S. cerevisiae* and the temperature of growth using continuous culture and a dilution rate of 0·1 hr^{-1}. Glucose concentration in the feed was 10 mg./ml. and growth-limiting. Gas exchange was measured at 25° after samples of yeasts from the chemostat were withdrawn, washed and placed in Warburg respirometers with glucose as substrate.

Fig. 4. Relationship between oxygen uptake by washed suspensions of *S. cerevisiae* and temperature of growth in continuous culture. Dilution rate was 0·1 hr^{-1}, DL-lactate concentration in the feed was 15 mg./ml. and growth-limiting. Gas exchange was measured as for Fig. 3 with glucose (triangular symbols) and lactate (square symbols) as substrate.

Intracellular enzyme and metabolite levels

A comparison of the enzyme complements of yeast cells grown at 25 and 38° (Table 3) revealed little difference, particularly for the enzymes concerned with glycolysis and alcohol production. Yeasts grown at 38° had a slightly smaller content of TCA cycle enzymes: exceptions were succinate dehydrogenase and fumarate hydratase which were similar in the two types of yeast. Activities of most of the respiratory enzymes measured were slightly greater in the organisms grown at 38° but succinate: Cytochrome-*c* oxidoreductase was different in that it was lower.

The greatest differences in enzyme contents were found with enzymes reacting with α-ketoglutarate (Table 4). Only one of the enzymes in this group (aspartate: α-ketoglutarate aminotransferase) was unaffected by the temperature at which the

organisms were grown. The enzyme most influenced was α-ketoglutarate dehydrogenase; the levels within organisms grown at 25° were low but the extracts of the organisms grown at 38° had no detectable activity. The yeast cultured at 38° had slightly greater levels of α-keto acids. Pyruvate and α-ketoglutarate were 7·5 ± 0·2 and 3·1 ± 0·2 nmole/mg. yeast material. For yeasts grown at 25° the corresponding values were 5·8 ± 0·2 and 2·1 ± 0·2.

Table 3. *Comparison of activities of enzymes associated with glycolysis, alcohol production, tricarboxylic acid cycle and respiratory chain*

Enzyme	E.C. number	Yeasts grown continuously at 25°	Yeasts grown continuously at 38°
Phosphofructokinase	2.7.1.11.	73 ± 3·0 (3)	77 ± 3·7 (3)
Pyruvate kinase	2.7.1.40.	6·1 ± 0·5 (3)	6·3 ± 0·4 (3)
Pyruvate dehydrogenase enzyme system	—	3·0 ± 0·4 (5)	2·7 ± 0·3 (4)
Pyruvate decarboxylase	4.1.1.1.	734 ± 5·0 (4)	509 ± 4·4 (4)
Alcohol dehydrogenase	1.1.1.1.	224 ± 3·7 (4)	224 ± 5·7 (4)
Aconitate hydratase	4.2.1.3.	115 ± 1·4 (4)	93·0 ± 1·7 (4)
Isocitrate dehydrogenase (NAD specific)	1.1.1.41.	6·6 ± 0·2 (3)	5·6 ± 0·3 (4)
Isocitrate dehydrogenase (NADP specific)	1.1.1.42.	15·9 ± 0·5 (4)	12·2 ± 0·5 (3)
Succinate dehydrogenase	1.3.99.1.	34·2 ± 1·0 (4)	35·7 ± 0·6 (4)
Fumarate hydratase	4.2.1.2.	27·8 ± 0·6 (3)	28·0 ± 1·5 (3)
Malate dehydrogenase	1.1.1.37.	613 ± 8·0 (4)	517 ± 3·5 (3)
NADH oxidase enzyme system	—	13·7 ± 0·3 (4)	16·4 ± 0·4 (4)
NADH:cytochrome-*c* oxidoreductase	1.6.2.1.	41·5 ± 0·8 (3)	52·8 ± 0·5 (3)
Cytochrome-*c*:oxygen oxidoreductase	1.9.3.1.	18·3 ± 0·4 (3)	22·7 ± 0·5 (3)
Succinate:cytochrome-*c* oxidoreductase	—	12·1 ± 0·2 (3)	7·9 ± 0·2 (4)

Levels of enzyme activities in yeast grown in glucose-limited continuous culture. (Glucose concentration 10 mg./ml.; dilution rate 0·1 hr^{-1}.) Results are mean values expressed as mμmole substrate utilized/min./mg. protein ± S.E.M. The numbers of observations are given in parenthesis.

Table 4. *Comparison of activities of enzymes in yeast which involve ketoglutarate as a substrate*

Enzyme	E.C. number	Yeasts grown continuously at 25°	Yeasts grown continuously at 38°
α-Ketoglutarate dehydrogenase enzyme system	—	2·2 ± 0·4 (8)	0·3 (14)
Glutamate:NAD oxidoreductase	1.4.1.2/3.	13·5 ± 0.3 (4)	27·5 ± 0·3 (3)
Glutamate:NADP oxidoreductase	1.4.1.3/4	668 ± 8·2 (4)	532 ± 7·6 (4)
Aspartate:ketoglutarate aminotransferase	2.6.1.1.	13·7 ± 0·2 (3)	13·1 ± 0·3 (3)

Total adenosine phosphate levels for yeasts grown at 38 and 25° were, respectively, 6·4 and 7·1 nmole/mg. yeast material. This small difference was reflected in the corresponding values for ATP and AMP; levels of ADP were slightly greater for the 38° culture. There were no major differences between the levels of NAD at the two

temperatures and of reduced NAD. In each case the value was in the range 1·1 and 1·6 nmole/mg. yeast material. NAD:NADH ratios were 1·1 for yeasts grown at 38° and 1·5 for those cultured at 25°.

DISCUSSION

Many reports on the effect of temperature upon micro-organisms are complicated by the fact that not all the other environmental parameters were controlled. This is particularly true for batch culture in which the environment invariably changes continuously, leading to changes in enzymic composition of the organisms. For instance, the level of succinate-cytochrome C oxidoreductase in yeast cells varied 100-fold during growth in a batch culture (Ephrussi, Slonimski, Yotsuyanagi & Tavlitski, 1956). The effect of temperature on the metabolism of growing organisms is, therefore, more easily studied when they are growing in a constant environment with their growth rate determined by the rate of supply of growth-limiting nutrient; that is, in continuous culture.

The chemical environments provided by the media of the yeast culture growing in glucose-limited chemostat 25 and 38° were very similar. This was emphasized by the overall constancy in the levels of both the tricarboxylic acid cycle enzymes and the enzymes of the respiratory chain between the cells grown at the two temperatures. However, a marked decrease occurred in the respiratory capacity of the yeast grown at the higher temperature. Yeast grown in lactate-limited cultures was affected to the same extent indicating that the effect was not restricted to organisms metabolizing glucose as the sole carbon source. Neither was the effect due to an enrichment of the population with respiratory-deficient mutants nor to a decrease in viability nor to a significant change in the level of aeration. The decrease in the respiratory capacity of yeast grown at the higher temperature was accompanied by a decrease in yield and by an increase in the amount of ethanol produced. These effects were accompanied by an increase in the levels of α-keto acids in the growth medium. With α-ketoglutarate the levels rose in that range of culture temperature (30 to 35°) in which the respiratory capacity began to decline. At growth temperatures above 35° the level of pyruvate in the medium increased sharply as the respiratory activity continued to fall (compare Fig. 2 with Fig. 3, 4). In contrast the intracellular levels of α-keto acids showed a much smaller increase at the higher growth temperature. A similar situation has been reported by Suomalainen & Ronkainen (1963) for baker's yeast grown first under aerobic and then anaerobic conditions. In their system, despite a considerable increase in the levels of α-keto acids in the growth medium, the intracellular levels remained relatively constant.

The increase in the level of pyruvate from 5·8 nmole/mg. in the organisms grown at 25° to 7·5 nmole/mg. in those grown at 38° may well favour fermentation without any change in the rate of oxidation of pyruvate. Taking results of Polakis & Bartley (1965) for internal cell volume, the corresponding internal mean concentrations were about 1·9 and 2·5 mM. Holzer (1961) reported that a yeast pyruvate oxidase system became saturated at the level of 1 mM-pyruvate whereas pyruvate decarboxylase was only saturated at 20 mM-pyruvate.

A comprehensive enzymic survey of this yeast revealed that the increased level of α-ketoglutarate in the growth medium may be correlated with the level of α-keto-glutarate dehydrogenase present in the cells. In cell-free extracts of organisms grown

at 25° the activity of this enzyme was low but in extracts of organisms grown at 38° the level was greatly reduced. Vitols & Linnane (1961) also found a low level of this enzyme in a commercial sample of baker's yeast, presumably cultured at temperatures below 25°. Cell-free extracts of their yeast quickly oxidized citrate and pyruvate plus malate but accumulated α-ketoglutarate.

It is of interest that one enzyme, NAD-specific glutamate dehydrogenase, showed a significant increase in complement (100%) at the higher temperature of culture. In contrast the complement of NADP-specific glutamate dehydrogenase decreased by 20%. Chapman & Bartley (1968) demonstrated a similar reciprocal relationship between the changes in levels of the two glutamate dehydrogenases of yeast during a change from aerobic to anaerobic conditions. The reason for such a reciprocal relationship is not known but may be related to the intracellular compartmentation of α-ketoglutarate. It is suggested that the primary effect on the yeast of the increase in growth temperature was to reduce the level of α-ketoglutarate dehydrogenase within the organisms, probably arising from the inhibition of the synthesis of the enzyme by the elevated temperature. The changes in respiratory capacity, metabolite levels and levels of other enzymes were probably of secondary importance but acted to stimulate fermentative metabolism at the higher temperatures of growth.

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Correlation of Toxic and Non-toxic Strains of *Clostridium botulinum* by DNA Composition and Homology

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SUMMARY

Various toxic and non-toxic *Clostridium botulinum* strains can be grouped according to DNA binding homology but not according to DNA composition. The guanine + cytosine (GC) contents of DNA from six strains were 28 to 30%. DNA binding with radioactive DNA from *C. botulinum* type E, MINN strain, revealed that the non-proteolytic strains type B, 17, type F, 202 and type E, DETROIT are 100% homologous to the E, MINN strain. *Clostridium botulinum* type E, 32, the boticin producing S 5, and the non-toxic 24 NT, 26 NT and 5 i had 58 to 78% homology with the MINN strain. *Clostridium botulinum* type A, 62 and 78, type B, 32, type C, 573, and non-toxic OS/E 1, OS/E 7 and OS/MINN strains had only 6 to 14% homology with type E, MINN. Competition experiments with unlabelled DNA confirmed these results. The studies indicate that strains which are closely related serologically and biochemically are also closely related genetically, and *vice versa*. The data do not support the idea of a common origin of the non-toxic OS strains and *C. botulinum* type E. The toxic type E strains and the toxic type A, B and C strains of *C. botulinum* are genetically heterogenous.

INTRODUCTION

The species *Clostridium botulinum* includes heterogenous strains that produce toxins types A to F. Based on physiological and serological characteristics, the species can be divided into the proteolytic A group, the non-proteolytic E group, and the C group (Smith & Holdeman, 1968, see Table 2). In recent years, non-proteolytic *C. botulinum* types B and F with characteristics of the type E group have been reported (Eklund & Poysky, 1967). Many non-toxic strains, such as 24 NT and 26 NT isolated from the North Sea (Cann *et al.* 1965) and the boticin producing S 5 and 28–2 isolated from the Great Lakes (Kautter, Harmon, Lynt & Lilly, 1966), were reported to have identical characteristics with type E. Non-toxic OS (opaque sporulating) strains were closely associated with type E cultures (Dolman, 1957). The OS strains have different fermentation, serological reactions and spore structure than the type E strains (Hobbs, Roberts & Walker, 1965; Hodgkiss, Ordal & Cann, 1966; Walker & Batty, 1967).

Non-toxic *Clostridium botulinum* strains are difficult to classify because this species is solely defined by toxin formation. Thus all non-toxic *C. botulinum* strains are named by numbers and numerals like 24 NT or 5 i. The characteristics of the strains tested are summarized in Table 1.

The purpose of this study is to examine the genetic relationship of various toxic and non-toxic *Clostridium botulinum* strains with the type E strains. The difference in DNA

base composition can be used as a measure of genetic relatedness (Marmur & Doty, 1962; Schildkraut, Marmur & Doty, 1962; Hill, 1966). McCarthy & Bolton (1963) measured the genetic relatedness of organisms by the binding of complementary DNA base sequences *in vitro*. The DNA composition and the degree of common DNA base sequence of 22 toxic and non-toxic *C. botulinum* strains were measured in this study.

Table 1. *General characteristics of Clostridium botulinum groups A, C and E (Smith & Holdeman, 1968; Roberts & Hobbs, 1968)*

Group	Serological reaction of vegetative cells	Proteolysis	Gas formation in carbohydrate fermentation	Minimum growth temperature	Resistance of spores to heat
A	A, B and F	Strong	Weak	> 10° (type F to 4°)	> 100° 10 min.
C	C and D	Weak	Weak	> 10°	~ 90° 10 min.
E	E and non-proteolytic B and F	Weak	Strong	3°	~ 60° 30 min.
(OS)*	OS	Weak	Weak	5°	~ 90° 10 min.

* (Ajmal & Hobbs, 1967).

METHODS

Cultures. Toxic cultures *Clostridium botulinum* A, 62, A, 78, B, 32, C, 573, E, MINNESOTA (MINN) and E, DETROIT were obtained from K. Ito (National Canners Assoc., Berkeley, Calif.) and *C. botulinum* E (Wellcome # 3324/61) was obtained from A. Johannsen (Lund, Sweden). Non-proteolytic strains *C. botulinum* B, 17 and F, 202 were obtained from M. W. Eklund (Eklund & Poysky, 1967). *C. botulinum* F, LANGELAND was obtained from V. Møller (State Serum Institute, Copenhagen, Denmark). *Clostridium sporogenes* 213 was obtained from L. S. McClung (Indiana U., Bloomington, Ind.). Non-toxic strains 24 NT, 26 NT were isolated by G. Hobbs (Torry Research Station, Aberdeen, Scotland). The boticin producing non-toxic strains S 5 and 28–2 were isolated by Kautter *et al.* (1966). Non-toxic strains OS/E 1 and OS/E 7 were obtained from C. E. Dolman (1957). Non-toxic OS/MINN and 5 i were picked from the *C. botulinum* type E, MINN culture in our laboratory.

Medium and growth conditions. Unlabelled DNA from all strains was isolated from organisms grown in 1 to 2 l. of medium: proteose peptone 20 g., yeast extract 15 g. (Difco), soluble starch 1 g., mercaptoethanol 0·5 ml. K_2HPO_4 1·85 g./l., pH adjusted to 7·2 with NaOH. Mercaptoethanol and $FeSO_4$ generate a Eh of −275 mV in solution (Wood, 1966). Glucose 10 g./l. was sterilized separately. Organisms were grown at 37° for 7 to 24 hr under 25 in. vacuum in a Duravac plastic desiccator (Ace Glass Inc., Vineland, N.J.) which had been flushed with a gas mixture (N_2 88%, CO_2 10%, H_2 2%). *Clostridium botulinum* C, 573 grew only in the following medium: liver infusion 35 g., meat extract (Difco) 15 g., cysteine-HCl 1 g., and glucose (sterilized separately) 6·7 g./l. Radioactive DNA of *Clostridium botulinum* E, MINN was prepared from organisms grown in 1 l. of Edamin medium: Edamin S (Sheffield Chemicals, Norwich, N.J.) 40 g., soluble starch 1 g., mercaptoethanol 0·5 ml., Triton × 155 (Rohm & Hass, Philadelphia, Pa.) 0·4 g./l., pH adjusted to 7·4 with KOH. Glucose 10 g./l. was sterilized separately. The growth conditions were as described above.

After 4 hr of growth, 385 μC of thymidine-^{3}H, which was placed in a floating small plastic cup, was mixed into the culture medium with a magnetic stirrer. The organisms were harvested 2 hr later.

DNA isolation. Lysis of *Clostridium botulinum* can be difficult to accomplish by standard methods but the following procedure was effective in recovering some DNA from all the strains tested. Acetone dried organisms were sonicated for about 6 min. in ethanol in the cold, dried and suspended in 10 to 20 ml. saline+EDTA buffer (NaCl 0·15 M, Na_2EDTA 0·1 M, pH 8·0). The suspension was treated with about 1 mg lysozyme/ml. at 40° for $\frac{1}{2}$ hr. Then about 1 mg. pronase (Calbiochem, Los Angeles, Cal.)/ml. and 1 % sodium lauryl sulphate(SLS) were added and incubated for 1 to 5 hr. The time of lysis of the various strains differed and lysis could occur at any stage of the treatment. The treatment was terminated when the solution became highly viscous. The released DNA was purified by Marmur's (1961) method for Tm and buoyant density analysis and by Miura's (1967) method for the binding and competition studies. *Clostridium perfringens* DNA was purchased from Worthington Biochem. Corp., Freehold, N.J.

Analytical methods. DNA was determined chemically by Burton's (1968) method and spectrophotometrically by increase in optical density upon heating (Hotchkiss, 1957). Molecular weight was measured by the method of Zimm & Crothers (1962) in a Couette viscometer. The GC content of DNA was determined in duplicate samples by the Tm method (Marmur & Doty, 1962) and checked by the buoyant density method with *Micrococcus lysodeikticus* DNA (Miles Lab., Erkhart, Ind.) as reference (Schildkraut *et al.* 1962). DNA binding was assayed by the method of Denhardt (1966). DNA (30 μg.) was applied to each 25 mm. Millipore HA filter and annealed with 2 μg. DNA-^{3}H (10,000 c.p.m.) at 66° for 12 hr. For competition experiments, 875 μg. of *Clostridium botulinum* type E, MINN DNA was applied to a 90 mm. Millipore HA filter. Small 11 mm. circles were punched from the 90 mm. filter. Two 11 mm. filters were annealed with 250 μg. of various unlabelled DNA and 1 μg. DNA-^{3}H (5,000 c.p.m.) in 0·3 or 0·5 ml. double strength citrate (SSC) at 66° for 12 hr. Thermal denaturation of duplex DNA formed on filters was measured by the radioactivity released from two 11 mm. filters during heating in 1 ml. SSC. The heated solution and 1 ml. rinsing SSC were combined and the radioactivity determined in 15 ml. scintillation solution: Toluene 2 l., Triton × 100 (Rohm & Haas, Philadelphia, Pa.) 1 l., Spectroflour (Nuclear–Chicago, Des Plaines, Ill.) 100 ml. (Peterson and Greene, 1965). The radioactivity of dry filters were also counted in 15 ml. of the same scintillation solution.

RESULTS

DNA composition. The GC contents of 6 *Clostridium botulinum* strains are presented in Table 2. The thermal denaturation curve of *C. botulinum* E, MINN DNA is shown in Fig. 1. No satellite band was observed in the buoyant density photographs of DNA from *C. botulinum* E, MINN which produces type E toxin, or DNA from S 5, which produces boticin.

DNA molecular weight, binding and competition. The molecular weight of isolated DNA varied from strain to strain. The mol. wt of DNA from *Clostridium botulinum* type E, 3324/61, which was easy to isolate, was $1{\cdot}1 \times 10^7$. *Clostridium botulinum* type E, MINN DNA degraded during lysis. Heating the cell suspension to 70° for 15 min.,

addition of 0·1 M-EDTA and 1 % SLS did not prevent DNA breakdown. The mol. wt of the unlabelled type E, MINN DNA used in these experiments was $4{\cdot}2 \times 10^6$, and in one batch it was only $2{\cdot}4 \times 10^6$. Strains 5 i and *C. botulinum* F, 202 DNA degraded very rapidly. Lysis of these two strains was accomplished by adding 1 % SLS to unwashed cells in ice for 5 min. and then phenol was added to prevent DNA breakdown. The

Table 2. *The GC content of toxic and non-toxic Clostridium botulinum strains*

Group	Strain	Tm method	Buoyant density method
A	*C. botulinum* A, 62	28·5 %	—
A	*C. botulinum* B, 32	29·3	—
E	*C. botulinum* E, MINN	27·9*	28·8 %
E	S5 (boticin)	28	29·6
E	24 NT	29	—
E?	OS/E 7	30	—

* Average of 4 runs.

Table 3. *Percentage binding and competition of DNA from various strains relative to the Clostridium botulinum E MINN strain*

Group	Toxic strains	Source	Binding*	Competition†
E	*C. botulinum* E, MINN	Fish, Great Lakes	100 %	100 %
E	*C. botulinum* E, DETROIT	Fish, Great Lakes	100	99
E	*C. botulinum* E, 3324/61	Fish, Baltic Sea	68	100
A	*C. botulinum* A, 62	Cow, Nevada	11	0
A	*C. botulinum* A, 78	Spinach, U.S.	14	20
A	*C. botulinum* B, 32	Horse	11	3
E	*C. botulinum* B, 17	Pacific mud, U.S.	100	100
C	*C. botulinum* C, 573	Horse, France	11	—
E	*C. botulinum* F, 202	Pacific mud U.S.	100	100
A	*C. botulinum* F, LANGELAND	Liver, Denmark	19	52
	Non-toxic strains			
E	S5, Boticin	Fish, Lake Erie	78	80
E	28–2, Boticin	Mud, Lake Huron	43	89
E	24NT	Mud, North Sea	69	95
E	26NT	Mud, North Sea	58	65
E?	5i	Toxic E MINN	73	—
E?	OS/E1	Toxic E culture	8	0
E?	OS/E7	Toxic E culture	6	0
E?	OS/MINN	Toxic E culture	9	0
—	*C. perfringens*	—	23	0
A?	*C. sporogenes* 213	U.S.	12	13
A?	P. A. 3679	U.S.	11	—
—	*Micrococcus lysodeikticus*	—	4	—

* Average of six replications. † 1 to 2 replications.

binding of DNA from various strains relative to type E, MINN DNA is presented in Table 3. From 1 to 250 μg. of type E, MINN and S5 DNA was applied to each filter and maximum binding was observed at about 20 μg. level. The DNA duplexes formed on filters were stable and had a similar thermal denaturation curve to the native DNA, but the Tm was lowered by 4° (Fig. 1). The average count and its standard deviation

for 16 replicates of type E, MINN DNA was 2341 ± 481 counts/min. The results of the competition experiments are presented in Table 3. In these experiments the binding was 1000 counts/min. without the competing DNA and the binding was reduced to 100 counts/min. with 250 μg. of its own competing DNA. The competition experiment was limited to 1 or 2 replicates because of the limited supply of DNA.

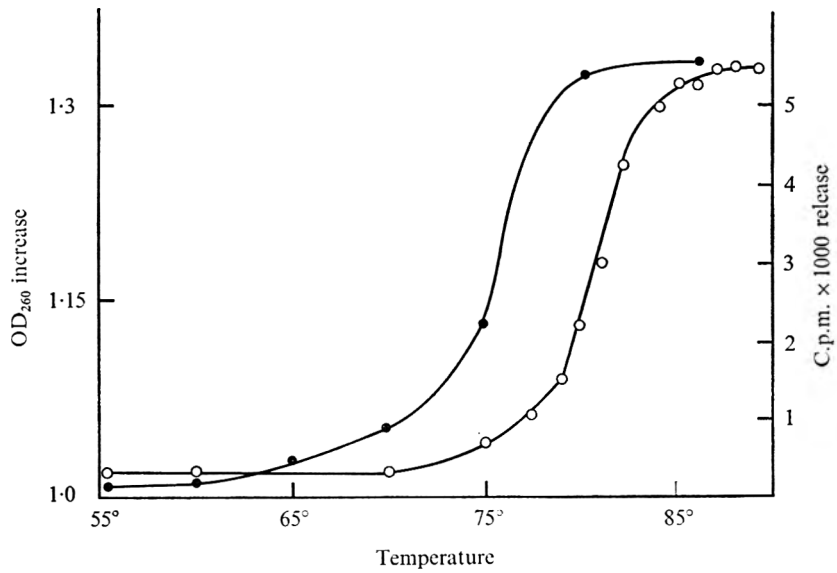

Fig. 1. Thermal denaturation of *Clostridium botulinum* E, MINN native DNA (○) and radioactive duplex annealed on filters at 66° (●).

DISCUSSION

The DNA composition of the examined *Clostridium botulinum strains* (Table 2) was similar to that of *C. perfringens* and *C. sporogenes* (Hill, 1966). Since the six strains tested have similar GC composition, the analysis has no taxonomic value with respect to *C. botulinum* strains.

Results of the DNA binding and competition tests were more revealing. They agreed with the characteristics of three groups of *Clostridium botulinum*. The toxic *C. botulinum*, E, DETROIT, E, 3324/61, B, 17 and F, 202 (Eklund, Poysky & Wieler, 1967) strains were reported to have the characteristics of the E group. These strains had 70 to 1000% DNA binding with the E, MINN strain and hence are closely related (Table 3). The non-toxic S5, 28–2, 24 NT and 26 NT were also reported to be identical to the toxic type E group (Kautter *et al.* 1966; Cann *et al.* 1965). These non-toxic strains have 40 to 80% DNA binding with E, MINN DNA and hence are also closely related to the toxic E group. Both 5i and OS/MINN were isolated from *C. botulinum* E, MINN culture. If E, MINN were the parent of 5i and OS/MINN strains, all three strains should have nearly all common DNA base sequences and nearly 100% DNA binding. The binding of 5i DNA to E, MINN was only 73% (Table 2). The DNA of 5i was always degraded during isolation and this might reduce its binding from the expected 100% to a value of 73%. The binding of OS/MINN to E, MINN DNA was only 8% (Table 3). This means that OS/MINN, like the unrelated control *Micrococcus lysodeikticus* (4%), have

very little common DNA base sequence with the E, MINN (Table 2). No mutations are known to change DNA base sequence so completely. Based on present knowledge, it seems improbable that OS/MINN could have originated by mutation from the E, MINN. The same reasoning applies to the other OS strains and their genetic relatedness to *Clostridium botulinum* type E. The other OS strains had only 8% homology (Table 3) with the E, MINN DNA and thus are not related to the E group. Although these OS strains have close association with the *C. botulinum* type E (Dolman, 1957), many differences in their fermentation serology and structure have been noted (Hobbs *et al.* 1965; Walker & Batty, 1967; Hodgkiss *et al.* 1966).

Many of the characters of toxic *Clostridium botulinum* groups A and C are different from the E group. The binding of *C. botulinum* types A, 62, A, 78, B, 32 and C, 573 DNA to E, MINN DNA was 11 to 14% and therefore we concluded that they are not closely related to the E group (Table 3). *Clostridium botulinum* type F, LANGELAND DNA appeared to have little more common DNA base sequence with E, MINN than the toxic type A, B and C strains tested. Generally the DNA homology among the groups of *C. botulinum* is low compared to the genera of Enterobacteriaceae. The DNA homology of the genera *Escherichia*, *Aerobacter*, *Salmonella* and *Shigella* were found to be 50 to 70% (McCarthy & Bolton, 1963). The species *C. botulinum* seems to include genetically heterogenous strains which are classified together only because they form toxins with similar pharmacological action.

The non-toxic *Clostridium sporogenes* and P.A. 3679 have some characteristics of the toxic *Clostridium botulinum* group A (Kindler, Mager & Grossowicz, 1956; Smith & Holdeman, 1968); *C. sporogenes* 213 and P.A. 3679 had only 10% DNA homology with E, MINN, and so are not related to the E group. We plan to study the DNA homology of *C. botulinum* type A, B and F strains and their related non-toxic strains like *C. sporogenes* and P.A. 3679. Eventually a systematic study of Clostridium DNA homology could lead to a better classification of this genus.

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Isoniazid-resistant Mutants of *Mycobacterium tuberculosis* H37Rv: Uptake of Isoniazid and the Properties of NADase Inhibitor

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SUMMARY

Six independent isoniazid-resistant mutants of *Mycobacterium tuberculosis* were isolated under conditions which largely ensured the selection of one-step mutants. The properties of these mutants with respect to the nicotinamide adenine dinucleotide nucleosidase (NADase) inhibitor, isoniazid uptake and peroxidase activity were studied. The uptake of isoniazid by the mutants and their sensitivity to isoniazid were increased by ethylenediaminetetra-acetic acid (EDTA) and sodium dodecylsulphate. It is suggested that EDTA and sodium dodecylsulphate bring about these effects by altering the cell permeability.

INTRODUCTION

Isoniazid (INH) is specific in its inhibitory action on the growth of mycobacteria; its mechanism of action on this group of organisms has been examined by many workers. Gopinathan *et al.* (1963, 1964, 1966) showed the presence of a heat-labile inhibitor of nicotinamide adenine dinucleotide nucleosidase (NADase) in *Mycobacterium tuberculosis* H37Rv and purified the enzyme and the inhibitor. Bekierkunst (1966) noticed that INH, which had no effect on mycobacterial NADase (Gopinathan *et al.* 1964), caused a decrease in the NAD content of the organism and suggested that the drug might inactivate the inhibitor, thereby enhancing the activity of NADase. Sriprakash & Ramakrishnan (1966) confirmed that INH *in vitro* interacted with NADase-inhibitor complex and thereby enhanced the activity of NADase. It can be argued on the basis of these findings that if the primary mechanism of action of INH is through the inactivation of the inhibitor of NADase, then the inhibitor from INH-resistant mutants might have lost its sensitivity to INH. The results presented in this paper show that resistance to INH is accompanied by an alteration in the permeability of the organism to the drug, while only in a few of these mutants the inhibitor also lost the sensitivity to INH. It is also shown that in both types of mutants, the organism is sensitized to INH by treating it with ethylenediaminetetra-acetic acid (EDTA) or sodium dodecylsulphate. A preliminary communication has been published (Sriprakash & Ramakrishnan, 1968).

METHODS

Chemicals. NAD was obtained from Sigma Chemical Co., St. Louis, U.S.A.; INH from Dumex (India) Private Ltd. Bombay; EDTA from E. Merck, Darmstadt, Germany; phenylethylalcohol from Distillation Products Industries, Rochester, U.S.A.; labelled 7-[^{14}C]INH (10 mc./m-mole) was purchased from the Radiochemical Centre, Buckinghamshire, England. Other reagents were of Analar grade.

Organisms. Mycobacterium tuberculosis H37Rv, originally obtained from National Collection of Type Cultures, London (NCTC 7416) and maintained on Petrik medium, was used in the present studies. To obtain inocula for growth experiments Sauton liquid medium was used. For enzyme studies the organism was grown in the liquid medium of Youmans & Karlson (1947) with glycerol as carbon source and asparagine as nitrogen source. All incubations were at 37°.

Preparation of cell-free extracts. This was done by ultrasonic treatment as described by Murthy, Sirsi & Ramakrishnan (1962).

Enzyme assays. NADase activity was measured by the cyanide addition method (Zatman, Kaplan & Colowick, 1953). The purification and assay of NADase inhibitor were described by Gopinathan, Ramakrishnan & Vaidyanathan (1966). One unit of NADase is defined as that amount of enzyme which breaks down 1 nmole NAD/min. at 37° under the conditions of assay. One unit of NADase inhibitor is defined as that amount of inhibitor which inhibits 1 unit of enzyme by 50% at 37°.

Peroxidase assay was done as follows. One ml. of the reaction mixture contained potassium phosphate buffer (pH 7·0) 100 μmole; hydrogen peroxide 0·1 μmole; pyrogallol, 5 μmole; and enzyme. The reaction was stopped after 10 min. with 3 ml. 5 N-H_2SO_4 and the solution extracted three times with ether. The volume of the ethereal extract was made to 5·0 ml. with ethanol (95%, v/v in water) and the colour measured in a Klett–Summerson Colorimeter with filter no. 42.

Other assays. Total oxidized pyridine nucleotides were estimated by the ethyl-methylketone method of Carpenter & Kodicek (1950) by using a Carl Zeiss Spekol Fluorimeter. Radioactive measurements of the bacteria were done by drying them on tared aluminium planchets and counting in a gas-flow proportional counter. Other radioactivity measurements were made with a Beckman liquid scintillation counter, Model LS-100.

Isolation of INH-resistant mutants. Six tubes each containing 1 ml. Sauton medium were inoculated with about 10^3 *Mycobacterium tuberculosis* H37Rv organisms and incubated till the colony count was 10^7. Samples (0·1 ml.) from each of the tubes were streaked on to Petrik medium containing 0·1 μg. INH/ml. and incubated till the growth was visible. The isolated colonies were subcultured and maintained on Youmans & Karlson medium containing the same concentration of INH.

RESULTS

INH-resistant mutants. Six independent INH-resistant mutants were isolated. They were all found to be resistant to 1·0 μg. INH/ml., when tested in Youmans medium, in spite of their selection of INH resistance at 0·1 μg./ml. All these mutants were inhibited by INH at 1·5 μg./ml. and above. These mutants are referred to as *inh-r*-1, *inh-r*-2, *inh-r*-3, *inh-r*-4, *inh-r*5, *inh-r*-6.

The effect of INH on NAD concentrations. The results presented in Table 1 indicate that in the INH-resistant strains INH did not cause the depletion of NAD, up to 1 μg. INH/ml., to which concentration these bacilli were resistant. On the other hand, in the wild type NAD was decreased to the extent of 50% with 0·1 μg. INH/ml. These results are consistent with those reported by Bekierkunst & Bricker (1967) for *Mycobacterium tuberculosis* H37Ra.

Effect of INH on NADase. NADase was purified from *Mycobacterium tuberculosis* H37Rv and the mutants *inh-r*-4 and *inh-r*-6, and the effect of INH was studied on the

enzyme. INH had no direct activating effect on NADase of wild type or on either type of INH-resistant bacilli (Table 2).

Table 1. *Effect of INH on NAD concentration in sensitive and resistant tubercle bacilli*

Different concentrations of INH were aseptically added to a 15-day culture which was then further incubated for 24 hr. The bacilli were harvested, washed with cold water, suspended in cold 5 % trichloracetic acid and treated ultrasonically for 5 min. at 0 to 3° in 10 kc. Raytheon Sonic Oscillator. The suspensions were then centrifuged at 13,000 *g* in cold for 30 min. and the supernatant fluid after neutralization with N-NaOH was used for determination of NAD.

Culture	INH (μg./ml.)	μg. NAD(P)/g. wet bacteria
M. tuberculosis H 37 RV	0	220
	0·1	115
	0·5	4
Mutant *inh-r*-4	0	220
	0·1	231
	0·5	217
	1·0	222
	1·5	132
Mutant *inh-r*-6	0	175
	0·1	177
	0·5	168
	1·0	170
	1·5	122

Table 2. *Effect of INH on NADase*

The reaction mixture contained 100 μmole potassium phosphate buffer (pH 6·7); NAD, 0·2 μmole; enzyme, 1 unit; total volume 0·6 ml. The mixture was incubated for 1 hr. INH 1·0 μmole was included in the reaction mixture.

Cultures	NADase activity (Extinction/hr/unit enzyme) No INH	INH 1·7 μmole/ml.
M. tuberculosis H 37 RV	0·105	0·106
inh-r-4	0·105	0·103
inh-r-6	0·105	0·101

Effect of INH on NADase-inhibitor complex. It can be seen (Fig. 1) that the organisms were of two types. The wild type and the mutants *inh-r*-1, *inh-r*-3, *inh-r*-4 and *inh-r*-5 belonged to the first type, where the inhibitor was sensitive to INH. The mutants *inh-r*-2 and *inh-r*-6 belonged to the second type, where the inhibitor was not sensitive to INH. The effect of INH on NADase-inhibitor heterocomplexes was studied to find whether the change had occurred in the inhibitor or the enzyme in mutants *inh-r*-2 and *inh-r*-6.The heterocomplexes were obtained by mixing purified NADase of one strain with the purified inhibitor of a different strain. The results (Table 3) indicated that INH annulment of inhibitor activity was not observed with the heterocomplexes which were composed of the inhibitor of mutant *inh-r*-2 or *inh-r*-6. It was therefore concluded that the inhibitor of these two strains alone was altered.

To investigate whether the alteration of the inhibitors of *inh-r*-2 and *inh-r*-6 resulted in their inability to bind INH, equilibrium dialysis was done with these inhibitors and

labelled INH. Mutants *inh-r*-4 and *inh-r*-6 were chosen as the representatives of two types of mutants. The results (Table 4) showed that the binding of INH by the inhibitor of *inh-r*-6 was insignificant as compared to that by the inhibitors of *inh-r*-4 and wild-type organisms.

Peroxidase activity of Mycobacterium tuberculosis. While the wild type was peroxidase-positive all the mutants were peroxidase-negative. To find whether peroxidase and the NADase inhibitor were one and the same protein the inhibitor was purified up to the alumina Cγ-gel elution stage and both activities were determined at every stage. The ratio of peroxidase activity to the inhibitor activity was almost constant up to the alumina Cγ-gel elution stage of purification (Table 5).

INH uptake by Mycobacterium tuberculosis

Since a change in the sensitivity of NADase inhibitor to INH could explain the resistance to INH of only two of six resistant strains, the uptake of INH by these

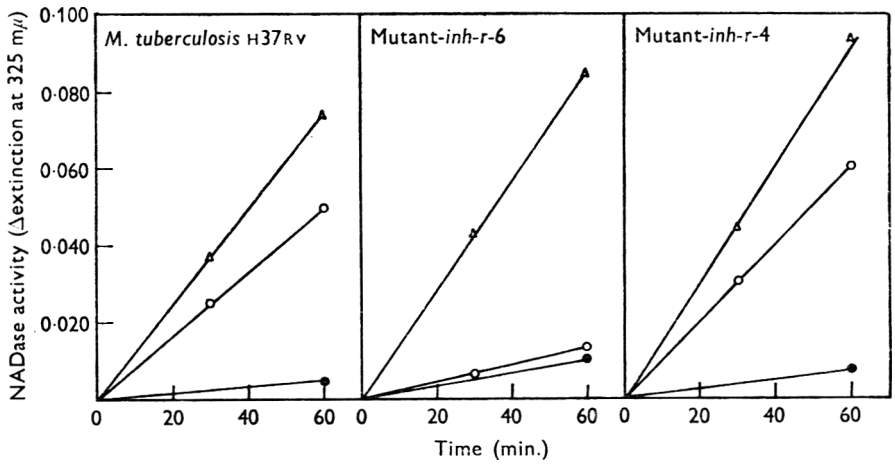

Fig. 1. *The effect of INH on NADase-inhibitor complex.* Incubation mixture (0·6 ml.) contained 100 μmole phosphate buffer (pH 6·7), with or without 1 μmole INH and crude cell-free extract. The reaction mixture was incubated for 20 min. at 37°. NAD (0·25 μmole) was added and further incubated for 1 hr. The total NADase activity was determined by heating the cell-free extract at 80° for 1 min. before adding NAD. Incubation temperature 37°. △, NADase activity of heat-treated cell-free extract; ○, NADase activity of INH-treated cell-free extract; ●, NADase activity of cell-free extract.

Table 3. *Effect of INH on the heterocomplexes*

Purified NADase and inhibitor from different sources were mixed and incubated for 15 min. at 37°. The effect of INH on the heterocomplex was studied by further incubation of this mixture with 1·0 μmole INH for 20 min. at 37° and the NADase activity assayed.

	Source of the inhibitor				
Source of NADase	H 37 Rv	*inh-r*-1	*inh-r*-2	*inh-r*-4	*inh-r*-6
H 37 Rv	+	+	−	+	−
inh-r-1	+	+	−	+	−
inh-r-2	+	+	−	+	−
inh-r-4	+	+	−	+	−
inh-r-6	+	+	−	+	−

+ = Annulment of inhibitor activity; − = no annulment of inhibitor activity.

strains was examined. INH uptake was found to be decreased in all the mutants as compared with the wild type. The results with two of the mutants (*inh-r*-4, *inh-r*-6) are illustrated in Table 6. The effect of certain compounds which have been reported to alter cell permeability of other bacteria, on the uptake of INH was examined. These compounds were incorporated into the Youmans & Karlson medium along with [^{14}C]INH. The results (Table 6) showed that ethylenediaminetetra-acetate and sodium dodecylsulphate increased the uptake of both types of resistant mutants, while phenylethylalcohol inhibited this uptake. These effects were consistent when the experiments were repeated a number of times.

Table 4. *Equilibrium dialysis of NADase inhibitor*

The purified inhibitor was concentrated by lyophilization to about 500 μg. protein/ml. The concentrated inhibitor was dialysed against 0·05 M-potassium phosphate buffer (pH 7·0) containing 0·01 μc. 7-[^{14}C]INH/ml. for 24 hr at 0 to 5° by using Visking tubing, pretreated by boiling in 10^{-3}M-EDTA thrice. The radioactivity inside and outside the Visking tubing was measured.

Source of inhibitor	Counts/min.*	Protein content (μg.)	[^{14}C]INH bound/mg. inhibitor protein
M. tuberculosis H 37 RV	625	500	1250
inh-r-4	725	550	1320
inh-r-6	180	580	310

* Represents the difference in counts between the outside and inside of the dialysis tubing.

Table 5. *Ratio of inhibitor activity to peroxidase activity*

Stage of purification	Units of inhibitor/ml.	Units of peroxidase/ml.	Ratio of inhibitor activity to peroxidase activity
DEAE-eluate	36·0	2·2	16·0
DEAE-eluate dialysed	32·0	1·9	17·0
Alumina Cγ-gel eluate I	27·0	1·8	15·0
Alumina Cγ-gel eluate II	44·0	2·9	15·0
Alumina Cγ supernatant	26·5	1·5	18·0

The effect of increased INH uptake on INH-resistant tubercle bacilli

Experiments were made to determine whether these compounds increased the bacteriostatic effect of INH. Various concentrations of these compounds and INH were incorporated into Youmans & Karlson medium and inoculated with INH-resistant bacilli. The results are given in Table 7. EDTA at 5×10^{-3}M when included in the medium with INH 0·5 μg./ml. completely inhibited the growth of both types of resistant mutant. However, the concentration of EDTA used by itself slightly retarded growth. On the other hand, sodium dodecylsulphate at 0·01 % + INH 0·5 μg./ml. inhibited the growth of both kinds of INH-resistant mutant; sodium dodecylsulphate alone was not inhibitory. Phenylethylalcohol did not supplement the effect of INH on any of the INH-resistant mutants and was itself inhibitory to growth at 0·1 %.

Table 6. *The effect of ethylenediaminetetra-acetate, phenylethylalcohol and sodium dodecylsulphate on the uptake of INH by INH-sensitive and INH-resistant mycobacteria*

The 15-day cultures were inoculated into Youmans & Karlson medium containing 0·05 μC. INH/ml. and incubated for 40 hr. The organisms were then washed with Youmans & Karlson medium containing 10 μg. unlabelled INH/ml., and finally with distilled water.

	M. tuberculosis H 37 RV		Mutant *inh-r*-4		Mutant *inh-r*-6	
Treatment	Counts*/ 5 min./ 10 mg. dry wt	Relative % of [^{14}C]INH uptake	Counts*/ 5 min./ 10 mg. dry wt	Relative % of [^{14}C] uptake	Counts*/ 5 min./ 10 mg. dry wt	Relative % of [^{14}C] uptake
—	420	100·0	70	17	140	33
5×10^{-3}M-EDTA	360	85·0	110	26	220	52
0·01 % Sodium dodecylsulphate	440	105·0	120	29	230	55
0·1 % Phenylethylalcohol	240	57·0	30	7	100	24

* Represents the total counts from which the background (110 counts/5 min.) counts have been subtracted.

Table 7. *Effect of ethylenediaminetetra-acetate, sodium dodecylsulphate and phenylethylalcohol on the growth of Mycobacterium tuberculosis inh-r-6 in the presence of subinhibitory concentration of INH*

The growth was measured by determining the dry weight of organisms after 15 days of incubation. The pattern for mutant *inh-r*-4 was essentially the same.

		Concentration of INH (μg./ml.)			
Compounds used	Concentration	0	0·1	0·5	1·0
		Growth (mg. dry wt)			
—	—	16	17	17	16
Ethylenediamine-tetra-acetate	5×10^{-3}M	15	10	0	0
	10^{-2}M	9	11	0	0
Sodium dodecyl-sulphate	0·005 %	16	15	17	17
	0·01 %	17	17	12	0
Phenylethyl-alcohol	0·01 %	18	16	16	17
	0·05 %	12	10	11	9
	0·1 %	0	0	0	0

DISCUSSION

In this work the mechanism of INH resistance in *Mycobacterium tuberculosis* H 37 RV has been investigated by using independent mutants of the organism selected under conditions that largely ensured the isolation of single-step mutants, by exposing the organism to low growth-inhibitory concentration of INH. Such single-step mutants, unlike resistant strains selected by exposure to graded concentrations of INH, may be expected to have a single biochemical alteration which is solely responsible for resistance. By the above procedure two types of INH-resistant strains were isolated, one in which the NADase inhibitor had lost its sensitivity to INH accompanied by decreased uptake of INH and a loss of peroxidase activity, while in the second type only the uptake of INH and the peroxidase activity were lost. Apparently both types are the results of mutations in the same locus, since the same method of isolation had been used and they were resistant to the same maximum dose of INH. Though the mutants were isolated on media containing 0·1 μg.INH/ml. they were

all resistant to a maximum concentration of 1·0 μg. INH/ml. Evidence for one-step mutation being capable of causing the highest degree of resistance of *M. tuberculosis* to isoniazid was presented by Middlebrook (1957).

If a single mutational step is involved in producing strains which are altered in isoniazid uptake, in the NADase inhibitor and in peroxidase activity, this suggests that all these are functions of the same protein. In partial support of this we have shown that the NADase inhibitor and peroxidase activities go together when the protein is purified. The involvement of peroxidase in INH uptake was suggested by Wimpenny (1967). The existence of mutants altered only in two functions, namely, isoniazid uptake and peroxidase activity, indicates that this protein can be altered so as to change two of its functions, but not the third.

There appears to be direct correlation between the sensitivity of *Mycobacterium tuberculosis* to INH and the depletion of NAD in the organism in the presence of isoniazid. The mechanism of this relationship is not clear. The hypothesis of Bekierkunst (1966) that depletion of NAD is due to the indirect activation of NADase does not appear to be wholly valid since the effect of isoniazid on the NAD content of the resistant mutants is the same whether or not the NADase inhibitor is altered. Two other possible explanations for the difference in the depletion of NAD in the presence of INH between INH-sensitive and INH-resistant strains were disproved by appropriate experiments. INH at 1·5 μmole/ml. had no direct activating effect on the NADase of wild type or INH-resistant mutants of *M. tuberculosis*. On the other hand, INH has inhibitory action on NAD biosynthesis in the extracts of *M. tuberculosis*, but this inhibition is exerted to the same extent in INH-sensitive and INH-resistant strains (Sriprakash & Ramakrishnan, 1969). The possibility that INH brings about the leakage of intracellular NAD is being investigated. However, a simple explanation of this difference may be that the decrease in NAD is a secondary consequence of other damage to the cell by INH, which would be expected to occur only in resistant strains at concentrations of INH higher than those to which these strains are resistant.

The results from the [^{14}C]INH experiments show that alteration in permeability to INH is a major factor in the resistance of *Mycobacterium tuberculosis* to this drug. Evidence of the decreased uptake of INH by resistant tubercle bacilli has been given by many workers (Barclay, Ebert & Koch-weser, 1953; Barclay, Koch-weser & Ebert 1954; Boone, Strang & Rogers, 1957; Youatt, 1958, 1960*a*, *b*; Wimpenny, 1967). It was observed in the present work that compounds such as ethylenediaminetetraacetate and sodium dodecylsulphate which have been shown to act on membranes of other bacteria (Lieve, 1965; Bayer & Anderson, 1965) increased the uptake of INH by the resistant organism. Phenylethylalcohol, another membrane-active agent (Silver & Wendt, 1967), on the other hand, had no such effect. Once INH had accumulated in the resistant organisms, it exerted its bacteriostatic effect irrespective of whether or not the NADase inhibitor was sensitive to INH. This appears to confirm the conclusion that in the mechanism of INH resistance, permeability plays a more important role than the sensitivity of the inhibitor to INH. The emergence of resistance to isoniazid by *M. tuberculosis* appears to be more frequent than that to streptomycin, which has been reported to be 10^{-9} (Szybalski & Bryson, 1952). The frequency of resistance to isoniazid is reported by the above authors to be (1 to 3) $\times 10^{-6}$ by using the statistical method of Delbruck & Luria.

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Note added in proof

The first step INH-resistant mutants were all found to be resistant to 4·0 μg INH/ml. when tested on Petrik medium, though in Youman's medium they were resistant only to 1·0 μg INH/ml.

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SHORT COMMUNICATIONS

Synchronous Nuclear Division and Septation in *Aspergillus nidulans*

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In the uninucleate cells of higher organisms, mitosis and cell division are coordinated both in time and position. In bacteria, similarly, although nuclear division is not an observable event, septation normally occurs only between pairs of nuclear bodies and at a fixed time in relation to DNA synthesis (Cooper & Helmstetter, 1968). In coenocytes such as fungal hyphae it is less obvious that there is any special relationship between nuclear and cell division; however, King & Alexander (1969) have recently found that in *Alternaria solani* the hyphal tip cell is divided up by a number of septa after a wave of nuclear divisions has passed from the tip to the rear of the cell. Rosenberger & Kessel (1967) have shown that nuclear division is also synchronous in spore germlings of *Aspergillus nidulans* if they are grown on rich medium. The results presented here extend this observation to the longer tip cells of mature hyphae and show a pattern of nuclear division and septation very similar to that in Alternaria and Fusarium (Koenig & Howard, 1962).

Observations were made using a culture chamber consisting of a plastic Petri dish with a coverslip laid across a window cut in the base. The coverslip was inoculated with hyphae of a biotin requiring strain of *Aspergillus nidulans* from the Glasgow stocks (Pontecorvo *et al.* 1953), and was then covered with a permeable cellophane sheet (grade PT400, British Cellophane Ltd.) on top of which was poured a 3 to 4 mm. layer of complete medium agar (see Clutterbuck, 1969*b*, for formula). Observations of the hyphae sandwiched between the coverslip and the cellophane were made at 16 to 18° with a Reichert MeF phase-contrast microscope. At this temperature the growth of the hyphae is slow: about 50 μm. per hr (see Fig. 1).

Near the hyphal tips nuclei were visible as less dense areas of the cytoplasm each containing a dense nucleolus, but the nuclear membrane was indistinct. In the central region of the cell, nuclei were usually obscured by vacuoles, etc., but at the rear of the cell there was often a region of less dense cytoplasm in which spherical nuclei with clearly defined nuclear membranes and nucleoli could be seen.

As reported by Rees & Jinks (1952) and King & Alexander (1969), nuclear division was seen first at the hyphal tip and passed in a wave from there to the rear of the cell. During nuclear division, first the nucleolus and then the nuclear membrane became indistinct at a time when the nucleus was showing only slight signs of elongation. No details could then be made out until the smaller daughter nuclei became recognizable approximately 10 min. later. The wave of nuclear divisions took about 20 min. to pass from one end of the cell to the other: a distance of 440 to 700 μm. This length

can be calculated (Clutterbuck, 1969*a*) to contain 60 to 100 nuclei. In the branched hypha shown in the lower part of Fig. 1 the wave of nuclear division started at the tip of branch 'a' 13 min. after the start in the main hypha: in both cases the wave passed only backwards (branch 'b' was not examined at this time). In two cases it was observed that divisions occurred in two separate waves, the first at the tip and the second, somewhat later, at the rear of the cell. This may be taken as the first sign of the breakdown of synchrony that Rosenberger & Kessel (1967) found was the rule on poorer medium.

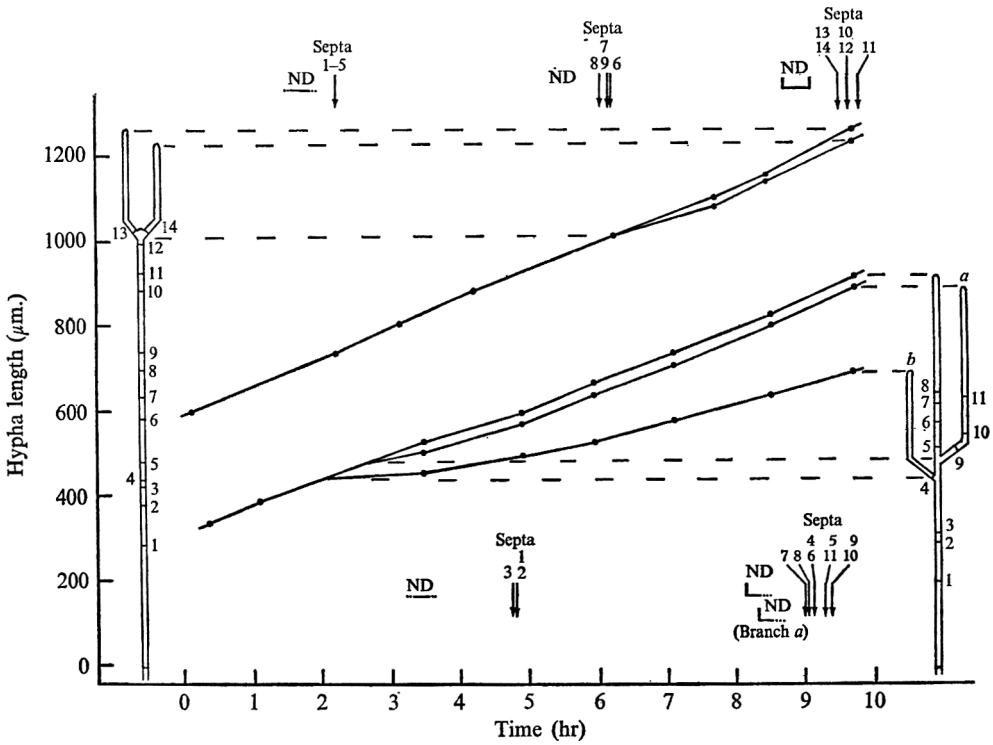

Fig. 1. The timing of nuclear division and septation in *Aspergillus nidulans*. The graphs show the growth rates of hyphal tips: the upper graph and indicators of time of nuclear division (ND) and time of formation of numbered septa refer to the hypha shown diagrammatically at the left, while the lower graph, etc. refer to the hypha at the right.

As can be seen from Fig. 1, the wave of nuclear division was followed after an interval of 20 to 40 min. by the formation of a series of septa at the rear of the tip cell which reduced its length by about half. This sequence of events has now been observed on ten occasions. The septa do not appear to form in any fixed order, although the tipmost one is often the first. The septa grow centripetally and are first seen as blebs on the side walls. On three occasions it has been noticed that two blebs were formed opposite the normal single one; one member of the pair then regressed while the other grew to form the septum. This suggests either that a septum may be initiated from more than one point in the same region or, more likely, that a septum is initiated at one point and spreads round the walls to form an annulus, or in the aberrant cases, a helix. Apart from the frequent formation of a septum at the base of a branch, there

is no indication what determines the sites of septum formation. There is no obvious correlation with the positions of nuclei, although in some tip cells where the nuclei are relatively sparse at the rear end, the septa divide this region into longer units than usual.

Figure 1 also shows that on this medium the hyphal tip may branch to give rise to a lateral or, less frequently, divide into two equal branches. As a rule the formation of a lateral branch does not affect the growth rate of the main hypha, but the branch itself generally takes some time to attain the growth rate of its parent. Where two more or less equal branches are formed the growth rate of both is reduced. Branches appear to arise with equal frequency at all stages of the nuclear division and septation cycle described here.

It is interesting to note that in the sterigmata, which are uninucleate cells of the conidial apparatus, cross-walls are formed in the normal way between pairs of daughter nuclei after nuclear division. The formation of coenocytes can therefore be regarded as due to the suppression of the majority of septa although a relationship between the time of nuclear division and septation is maintained.

I am grateful to Mrs June Baxendale for expert technical assistance.

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The Increased Rate of Loss of Penicillinase Plasmids from *Staphylococcus aureus* in the Presence of Rifampicin

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(*Accepted for publication 9 October 1969*)

There have been a number of reports of the ability of various chemicals to increase the rate of loss of penicillinase and other plasmids from *Staphylococcus aureus*. Hashimoto, Kono & Mitsuhashi (1964) reported that 17 out of 18 staphylococcal strains grown overnight in the presence of 25 μg. acriflavine/ml. showed an increased number of penicillinase-less variants, and Harmon & Baldwin (1964) reported 6·2% penicillin-sensitive cocci in a staphylococcal culture grown overnight in 10 μg. acridine orange/ml. However Novick (1963) and Richmond (1965) were unable to show any curing of staphylococcal strains using acridine dyes, and consequently the effect of these compounds must be regarded as rather variable from strain to strain. Recently Bouanchaud, Scavizzi & Chabbert (1969) have reported the elimination of pencillinase plasmids from certain staphylococcal strains with ethidium bromide, a drug known to intercalate between DNA base pairs, thus indirectly hindering the action of DNA and RNA polymerases (Waring, 1966).

The work presented here follows an observation that rifampicin (a rifamycin derivative—Maggi, Pasqualucci, Ballotta & Sensi, 1966) also has a curative effect on some staphylococcal extrachromosomal elements. This observation is of particular interest since the rifamycins bind directly to the RNA polymerase molecule itself—at least in *Escherichia coli* (Hartmann, Honikel, Knüsel & Staehelin, 1967; Wehrli, Nüesch, Knüsel & Staehelin, 1968; Wehrli, Knüsel, Schmid & Staehelin, 1968) and in *Staphylococcus aureus* (Wehrli, Knüsel & Staehelin, 1968)—rather than acting indirectly by intercalation.

In a preliminary experiment it was found that a culture of penicillin-resistant staphylococci grown at 35° in tryptone/soya broth containing 0·01 μg. rifampicin/ml. contained about 20% of penicillinase-less variants after overnight growth while a culture incubated in the absence of the antibiotic contained 0·2% at most. This concentration of rifampicin was about two-thirds of the growth-inhibitory concentration for the strain used here under these growth conditions.

To investigate this phenomenon further, inocula of various sizes of *Staphylococcus aureus* strain 8325 (α *i*$^{-}$*p*$^{+}$ *cad-r ero-r*)—that is, a staphylococcal culture containing a *Com I* plasmid conferring resistance to penicillin, cadmium ions and to erthyromycin (Richmond, 1969)—were made into batches of tryptone/soya broth each containing a different concentration of rifampicin and the cultures incubated at 35° overnight. A similar culture, inoculated with 10^5 organisms/ml. and incubated in the absence of rifampicin, acted as control. After overnight growth, samples from the cultures were plated onto nutrient agar to give single colonies, and after these colonies had grown

they were replica-plated on to further nutrient agar plates containing either 10^{-4} M-cadmium acetate or 10 μg. erythromycin/ml. to score for the presence of the plasmid *cad-r* and the *ero-r* markers respectively. After replication, the master plates were also stained to test for the presence of penicillinase—that is for the presence of the $i^{-}p^{+}$ gene group. The results of this experiment are shown in the Table. The incidence of penicillinase-less cocci was greatest with an inoculum of 10^{5} organisms/ml. and a concentration of 0·01 μg. rifampicin/ml. and reached a value 100 times higher than the level found in the control. All the penicillinase-less colonies that were detected had also lost the *cad-r* and *ero-r* markers, indicating that the whole penicillinase plasmid (α $i^{-}p^{+}$ *cad-r ero-r*), rather than the penicillinase genes alone, had been lost from the cocci.

Table 1. *The incidence of plasmid-less cocci in a culture of S. aureus 8325 (α $i^{-}p^{+}$ cad-r ero-r) incubated in tryptone/soya broth containing various concentrations of rifampicin*

Inoculum size (cocci/ml.)	Rifampicin concn (μg./ml.)	Incidence of plasmid-less cocci		
		Total no. of colonies tested	No. of plasmid-less colonies	No. of plasmid-less colonies (%)
10^{4}	0·0075	266	66	25
	0·1	No growth		
	0·025	No growth		
10^{5}	0·0075	1011	163	16
	0·01	498	127	
	0·025	861	19	2·2
	Nil	1224	0	<0·1
10^{6}	0·0075	863	69	
	0·01	962	662	45

One possible origin of the high proportion of plasmid-less variants in the treated culture was that rifampicin might select the plasmid-less variants known to occur spontaneously during the growth of this strain in liquid medium (Novick, 1963). To exclude this possibility, the rates of growth of the parent, a spontaneously occurring plasmid-less variant and a plasmid-less variant that had arisen previously in the presence of rifampicin were compared. In no case was there a detectable difference between the growth rates of the strains.

Similar experiments carried out with a *Com II* penicillinase plasmid, carried in this case in strain 147 (Richmond, 1969), have shown that a similar concentration of rifampicin to that used above produced 8·9 % of plasmid-less cocci after overnight growth against 0·11 % found in the control culture.

In view of the primary effect of rifampicin on RNA polymerase, the differential effect of rifampicin on bacterial growth and on plasmid survival suggests that there may be a specific RNA polymerase molecule or group of molecules involved in plasmid replication and plasmid distribution to daughter cells in *Staphylococcus aureus*.

We are very grateful to Professor H. Bein and Dr F. Knüsel of CIBA Ltd., Basle, for a gift of rifampicin; and to Abbott Laboratories Ltd. for a gift of erythromycin. This research was supported by a grant from the Medical Research Council.

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Specificity of Lactate for the Development of Competence in *Haemophilus influenzae*

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(*Accepted for publication 11 October 1969*)

SUMMARY

A number of common carbohydrates and polyols were tested to see if they could replace the lactate requirement for the development of competence in cultures of *Haemophilus influenzae*. Pyruvate, fructose, and glucose, when tested in the presence of inosine, were slightly stimulatory. They gave respectively, 29, 25 and 13 % of the control value obtained with inosine and lactate. Under similar test conditions, mannose, mannitol, galactose, sorbose, sorbitol, ribose, glucosamine, rhamnose, D (−) arabinose, L (+) arabinose, xylose and fucose were inactive.

In combination experiments, glucose, inosine and pyruvate, and fructose, inosine and pyruvate stimulated the development of competence to 50 to 70 % of the inosine and lactate control value. Without inosine, the stimulation due to glucose and pyruvate, and fructose and pyruvate was 3 and 9 %, respectively.

INTRODUCTION

In an earlier report, Ranhand & Herriott (1966) showed that the development of competence in *Haemophilus influenzae* was dependent upon the presence of both inosine and lactate during growth. Lactate could not be fully replaced by certain other three or four carbon acid-salts (β-hydroxybutyrate, alanine, propionate and pyruvate) and inosine could not be fully replaced by other purines or pyrimidines (as free bases or nucleosides). This report extends the list of compounds that cannot replace lactate (when used in conjunction with inosine) and includes hexoses, pentoses and polyols.

METHODS

Organism. The organism used was *Haemophilus influenzae* strain RD described by Goodgal & Herriott (1961).

Transforming deoxyribonucleic acid (DNA). Transforming DNA was prepared according to the method of Goodgal & Herriott (1961) from *H. influenzae* organisms that were resistant to about 2 mg. streptomycin/ml.

Cultural conditions, competence development, and transformation. The methods used for growing organisms as well as the conditions used for the development of competence and transformation have been described previously (Ranhand & Lichstein, 1966).

Growth medium. Because of the nature of the experiments presented here the trypti-

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case growth medium (TCM) described by Ranhand & Lichstein (1966) was modified slightly. Glucose, an original component of the TCM, was omitted unless specifically added. Glycerol however, at 0·3% (v/v) was added to the TCM even when all other carbohydrates listed in Table 1 were present.

Carbohydrates and polyols. All carbohydrates and polyols were obtained commercially. They were sterilized by filtration (Gelman membrane filters, 0·2 μm. in pore size), and were used at a concentration of 0·13% (w/v). Glycerol was sterilized by autoclaving at 121° for 20 min. Inosine was also sterilized by filtration, and was used at a concentration of 0·067% (w/v).

RESULTS AND DISCUSSION

The bacterial cell wall may be the distinguishing feature between competent and non-competent bacterial cells. The experiments of Young, Spizizen & Crawford (1963) and Young (1965) suggest that competent *Bacillus subtilis* cells (those capable of reacting with DNA) do have slight modifications in their cell-wall carbohydrate composition when compared to walls derived from similar but non-competent cells. The Gram-negative bacterial cell wall is a complex structure and its carbohydrate composition can be modified qualitatively by modifying the carbohydrates present in the growth medium (Weidel, Frank & Martin, 1960; Nikaido, 1962). Lactate has been shown to be a precursor of carbohydrates (Krebs, Dierks & Gascoyne, 1964; Lardy, Paetkau & Walter, 1965) and therefore, has the potential of causing such modifications in the cell-wall composition of *Haemophilus influenzae*. In addition, competence can be destroyed with sodium periodate (Ranhand & Lichstein, 1966). It seemed reasonable therefore, to test whether simple carbohydrates could replace lactate's competence promoting activity.

When organisms were grown in the TCM containing inosine and pyruvate, inosine and fructose, or inosine and glucose, transformation (competence development) was stimulated 29, 25 and 13%, respectively when compared to the inosine and lactate control value which was taken as 100% (Table 1). Other hexoses, pentoses and polyols were inactive. The per cent transformation (no. of transformed organisms/ml. × 100 divided by the no. of viable organisms/ml.) obtained with these latter compounds was no greater than the value obtained when inosine was used alone (about 0·01%).

Pyruvate is only 30% as effective as lactate and not 80 to 90% as reported earlier (Ranhand & Herriott, 1966). This difference is probably due to glucose which was present in the TCM at that time. In other combination experiments employing inosine, glucose and pyruvate. or inosine, fructose and pyruvate, the stimulation of competence never exceeded 70 to 80% of the value obtained with inosine and lactate. Fructose and pyruvate, and glucose and pyruvate, in the absence of inosine, were respectively, 9 and 3% as effective as the inosine and lactate control.

When lactate, pyruvate, glucose and fructose were tested singly without inosine, lactate was still the best stimulator for the competent state. It was, however, only 9% as good as the inosine and lactate control value. It appears that the interactions between active or partially active compounds are synergistic in addition to the synergism observed earlier between inosine and lactate (Ranhand & Herriott, 1966).

The data presented in this report show that no single carbohydrate, so far tested,

can substitute for lactate. In addition, one partner of isomeric carbodydrates is partially active for the development of competence whereas the other member is not; for example, glucose and mannose, glucose and galactose, and fructose and sorbose. This result implies that either *Haemophilus influenzae* cannot interconvert these carbohydrates, or that the respective carbohydrates are impermeable to the bacteria. The notion that permeability may be limiting the effectiveness of the other carbohydrates listed in Table 1 was not tested. However, Olitzki & Godinger (1958) showed that certain strains of *H. influenzae* can oxidize pentoses such as arabinose, xylose and ribose.

Table 1. *Effect of carbohydrates and polyols during growth on the development of competence in Haemophilus influenzae*

Addition to the growth medium*	Transformation frequency† (%)	Values relative to inosine and lactate control (%)
Inosine + lactate	1·4	(100)
Inosine + pyruvate	0·41	29
Inosine + fructose	0·35	25
Inosine + glucose	0·18	13
Inosine + mannose	0·027	2
Inosine + mannitol	0·012	1
Inosine + galactose	0·021	2
Inosine + sorbose	0·032	2
Inosine + sorbitol	0·012	1
Inosine + ribose	0·023	2
Inosine + glucosamine	0·014	1
Inosine + rhamnose	0·006	0·4
Inosine + D (−) arabinose	0·008	0·6
Inosine + L (+) arabinose	0·004	0·3
Inosine + xylose	0·007	0·5
Inosine + fucose	0·007	0·5
Inosine + no addition	0·01	0·7
Inosine + glucose + pyruvate	0·67	48
Inosine + fructose + pyruvate	1·0	71
Lactate	0·13	9
Pyruvate	0·013	0·9
Glucose	0·002	0·14
Fructose	0·0007	0·05
Glucose + pyruvate	0·042	3
Fructose + pyruvate	0·13	9
None‡	0·000065	0·000046

* All constituents were added at a concentration of 0·13% (w/v) except for inosine which was added at a concentration of 0·067% (w/v).

† Transformation frequency equals the no. of transformed colonies/ml. × 100 divided by the no. of viable colonies/ml.

‡ TCM contained 0·067% adenosine without which the cells would not grow or grew poorly.

The role played by lactate in the development of competence still remains elusive. However, the data presented here do not rule out its conversion to some cell-wall carbohydrate.

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