Conidium Production by Higher Fungi within Thin Layers of Liquid Paraffin: a Slide-culture Technique

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SUMMARY

A new slide culture technique is described in which 18 of 34 higher fungi tested sporulated asexually within a thin layer of liquid paraffin, some normally, some more or less abnormally. The oil greatly facilitated observation of the developing structures at high magnifications. The technique promises to be of use in studies of development in higher fungi.

INTRODUCTION

Conventional slide cultures (e.g. Smith, 1960) are extremely useful for studying the course of development of fungi but present a number of disadvantages when sustained observation beneath the microscope is proposed. These include the appearance of condensation droplets on the cover glass which seriously obscure vision; the proneness of the small closed system to rapid temperature changes which affect relative humidity and so tend to interfere with the fungus's development; and the difficulty of observing changes inside living cells that are surrounded by air. Observation of the development or behaviour of aquatic fungi is by contrast relatively easy because of the aqueous medium in which such fungi can be immersed while they are being observed. Unfortunately, relatively few subaerial moulds sporulate readily when immersed in aqueous solutions. Submerged sporulation has been recorded in species of the genera *Penicillium, Aspergillus, Fusarium, Curvularia,* and a number of other moulds under narrowly defined conditions (Vézina, Singh & Sehgal, 1965, and references therein; Capellini & Peterson, 1965), but Vézina *et al.* noted that in general a medium that is found to be optimal for sporulation of one species is not necessarily good for another, even when closely related. The absence of a fairly general method for obtaining submerged sporulation has prevented the development of a convenient general slide culture technique based on sporulation of moulds in liquids.

Consideration was therefore given to the possibility that moulds might sporulate if the immersion liquid was hydrophobic. An obvious candidate for such trials was liquid paraffin, or light mineral oil. Many records testify to its general innocuousness. It has been widely used for the preservation of stock cultures of bacteria and moulds and has also been advocated for the conservation of plant tissue cultures (Caplin, 1959). Stock cultures of moulds beneath oil may even sporulate during storage (Buell & Weston, 1947). Certain other micro-organisms whose reproductive development is normally subaerial are able to fructify when covered by liquid paraffin, for example, the cellular slime mould *Dictyostelium mucoroides* (Potts, 1902) and the myxobacterium *Chondromyces crocatus* (Quinlan & Raper, 1965). In experiments on the phototropism

of sporangiophores of *Phycomyces blakesleeanus* (Buder, 1918; Banbury, 1952) and on the growth of conidiophores of *Aspergillus giganteus* (Trinci & Banbury, 1967) specialized hyphae were immersed in liquid paraffin and still continued to grow. A layer of liquid paraffin is not a barrier to respiratory gas exchange. Edwards, Buell & Weston (1947) obtained results which suggested that even 1 cm. depth of mineral oil permits sufficient respiration of *Sordaria fimicola* for it to survive a long time, and showed that beneath 5 mm. of oil its respiration rate was still more than one fifth of the rate in controls. Further, Caplin (1959) has pointed out that the solubility of oxygen in light mineral oil at 38° is four times that in water at the same temperature, and that the solubilities are probably in the same proportion at lower temperatures.

Attempts were therefore made to devise a simple system in which a good range of subaerial higher fungi would sporulate in mineral oil in a culture cell of such a construction that high power microscope objectives could be used for critical observation of the details of development.

METHODS

The mineral oil used in these experiments was liquid paraffin, light, specific gravity 0·830–0·870 (British Drug Houses, Ltd., Poole, Dorset). It was sterilized in approximately 15 ml. lots in capped test tubes by autoclaving at 15 lb./in.² for 20 min. and was left for several weeks before use. The Cellophane was type PT 300 manufactured by British Cellophane Ltd., Bridgwater, Somerset. Transparent polythene sheeting was 60 μ thick, and was derived from the polybags in which sterile disposable Petri dishes are supplied. The fungi used were isolates held in the Botany Department, University of Bristol.

Technique for preparing mineral oil slide cultures

After preliminary tests, the culture cell illustrated in Fig. 1 was developed. The requirements which are met in its design are: (1) that the fungus should be visible through a thin, plane cover-glass; (2) that there should be no air space between the developing organism and the cover glass that would hamper observation; (3) that aeration should be provided by arranging the shortest possible diffusion path to an air space; (4) that the culture cell should be stable enough to withstand normal handling; (5) that the fungus and its substrate should not be able to dry during protracted incubation.

The procedure for preparing slide cultures of the type figured is as follows. (1) Wash, polish, and flame-sterilize slide (76 × 25 mm.) and cover-glass (50 × 22 mm.). (2) Apply a worm of petroleum jelly around cover-glass, close to the side edges, but set back about 5 mm. from each end to facilitate handling with forceps; the worm is delivered from a hypodermic syringe fitted with a cannula about 1·3 mm. internal diameter. (3) Place a small drop (about 5 mm. diam.) of molten nutrient agar (e.g. malt extract or potato extract) about 17 mm. from one end of the cover-glass with a sterile glass rod. (4) Inoculate the agar drop centrally with the fungus. (5) Use a sterile glass dropper to place one or two small drops of sterile liquid paraffin on the agar drop. (6) Cover the oil and agar with a 16 mm. square of either Cellophane or polythene sheeting (see below) which has been sterilized by immersion in boiling water. Adherent water droplets must be shaken off before the membrane is laid over the oil. Avoid trapping air bubbles. (7) Place a 13 mm. square of damp filter paper on the slide with its centre

about 20 mm. from one end. (8) Invert the cover-glass over the slide and gently press it down to complete the petroleum jelly seal which should be about 1 mm. thick. An air space must remain between the membrane and the slide beneath.

Squares of membrane filter have been tested for use as membranes in these cultures but proved unsatisfactory. The immersion of Cellophane squares in boiling water to sterilize them served additionally to wash out a constituent that was inhibitory to the sporulation of certain fungi.
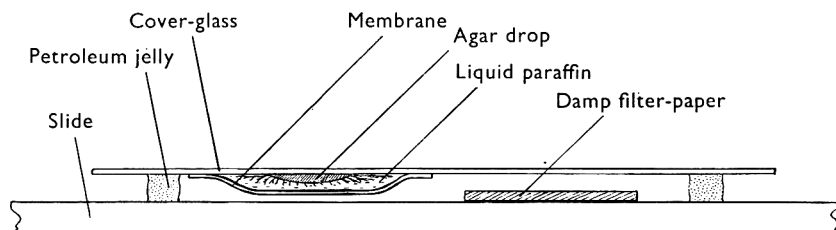
Fig. 1. Diagrammatic median section of slide culture (not to scale).

RESULTS AND DISCUSSION

The mineral oil slide-culture technique was applied to a series of fungi with the results shown in Table 1. The cultures were incubated at room temperature and examined frequently over a period of up to 2 weeks. Asexual spores were formed by 18 of the 34 fungi tested, usually when the culture was only a few days old. Negative results recorded in Table 1 may not signify that sporulation of the species in question is unobtainable with this technique, for all combinations of the two media and the two gas-permeable membranes were not applied to all species, nor was the range of possible cultural conditions explored. Other conditions of nutrition, temperature, illumination, and even of relative humidity within the culture cells might prove suitable for other species.

The reason for a membrane of a particular composition being suitable for sporulation of some species but not others is not clear from the results to hand. One possibility is that a given membrane leads to a critical favourable degree of aeration in the slide culture, but a more likely one is that cellophane acts as a nutrient for certain of the fungi and interferes with their sporulation. *Alternaria brassicicola* and *Alternaria* sp. 1 were both seen to grow through the cellophane membrane, beneath which neither sporulated. However, at least the former sporulated when the membrane was one of polythene.

In most of the fungi in which asexual sporulation occurred it appeared to be essentially normal (see, for example, Pl. 1, fig. 1-6). The conidia of *Acrospeira levis* developed normally at first, but the appearance of a gas phase within almost all the spores as they aged was a slightly unusual feature (Pl. 1, fig. 8). Old and dry spores of this species from conventional agar slant cultures sometimes contain a gas phase that rapidly disappears after the spores are re-wetted, but they are a minority. A similar gas phase that appeared normally within drying ascospores of *Sordaria fimicola*, *Pleurage curvicolla* and *Hypoxylon fuscum* was reported by Ingold (1956). More pronounced morphological abnormalities were shown by some conidiophores of some of the aspergilli tested. It was not uncommon for a smaller or greater number of

Table 1. *The production or otherwise of asexual spores by different higher fungi culti-vated beneath liquid paraffin in slide cultures in which different combinations of culture media and gas-permeable membranes were employed*

MA = 3% malt extract agar PA = potato extract agar
C = Cellophane membrane P = polythene membrane

Species	Sporulation	Medium/membrane combinations allowing sporulation	Medium/membrane combinations not allowing sporulation
Acrospeira levis	+	MA/C, PA/P	
Alternaria brassicicola	+	PA/P	MA/C, PA/C
Alternaria brassicicola (albino mutant)	−		MA/C
Alternaria sp. I	+	MA/C	PA/P
Alternaria sp. II	−		MA/C, PA/C
Aspergillus flavus	+	MA/C	
Aspergillus niger	+	MA/P	MA/C
Aspergillus ochraceus	−		MA/C
Aspergillus oryzae	−		MA/C
Aspergillus repens	+	MA/C, MA/P	
Aspergillus tamarii	+	MA/C, PA/P	
Aspergillus versicolor	−		MA/C, MA/P
Botrytis cinerea	−		MA/C, PA/C, PA/P
Cephaliophora tropica	−		PA/C
Cephalosporium sp.	+	MA/C	
Ceratostomella adiposa	+	MA/P	MA/C
Fusarium roseum	−		MA/C
Fusarium solani	+	MA/C	
Fusarium sp.	−		MA/C
Neurospora crassa			
Macroconidia	−		MA/C, MA/P, PA/C, PA/P
Microconidia	+	MA/P, PA/C	MA/C, PA/P
Oedocephalum roseum	−		PA/C
Penicillium brevicompactum	+	PA/C	MA/C
Penicillium cyclopium	+	MA/C	
Penicillium frequentans	−		MA/C
Penicillium janthinellum	−		MA/C
Penicillium luteum	−		PA/C
Penicillium notatum	+	MA/C, PA/C	
Penicillium sp.	+	MA/C	
Sclerotinia fructigena	−		MA/C, MA/P
Stachybotrys atra	−		MA/C, MA/P, PA/P
Stemphylium sp.	+	MA/C	
Trichoderma viride	−		MA/C
Trichothecium roseum	+	MA/C	
Wardomyces pulvinata	+	MA/C	

phialides on a conidiophore to proliferate as hyphae (Pl. 1, fig. 7) or even as small aberrant conidiophores. Spores in *Penicillium brevicompactum* and *P. notatum* were formed singly or in only small numbers on each phialide, and the conidiophores were only sparsely branched. *Botrytis cinerea* produced no conidia but formed many sclerotial initials whose processes of development were clearly displayed (cf. Townsend & Willetts, 1954). Three additional sclerotial or microsclerotial species were tested for growth beneath oil in MA/C cultures (see Table 1 for abbreviations), namely *Rhizoctonia solani*, *Sclerotium rolfsii* and *Papulaspora rubida*, but none formed sclerotia. *Neurospora crassa* (Lindegren stock, Lr6a) formed microconidia but not macro-

conidia beneath oil. Protoperithecia were formed, most abundantly in MA/C and PA/P cultures, but no attempts were made to spermatize them and observe their further development. Three Zygomycetes also were grown in MA/C cultures, namely *Rhizopus sexualis, Conidiobolus villosus*, and *Thamnidium elegans* but none formed spores.

Oil-immiscible droplets of a presumably watery exudate were sometimes formed on hyphae or conidiophores growing through the liquid paraffin, or on developing spores or sclerotial initials. Some can be seen in Pl. 1, fig. 4, and fig. 6 shows a thin layer of exudate that invests the spores joined together in a single chain. Trinci & Banbury (1967) observed similar droplets on conidiophores of *Aspergillus giganteus* when immersed in liquid paraffin, but were uncertain whether the droplets played a specific role in the growth of the conidiophores. They suggested that they were comparable with guttation droplets formed on the leaves of plants under conditions of high turgor in a saturated atmosphere.

Tests of this slide-culture technique thus suggest that the ability to sporulate asexually beneath thin layers of mineral oil is probably fairly common among higher fungi, and therefore that the technique is likely to prove useful in studies of their development, especially where photomicrographic records are required, as for example in quantitative studies of developmental processes.

REFERENCES

BANBURY, G. H. (1952). Physiological studies in the Mucorales. Part I. The phototropism of sporangiophores of *Phycomyces blakesleeanus*. *J. exp. Bot.* **3**, 77.

BUDER, J. (1918). Die Inversion des Phototropismus bei Phycomyces. *Ber. dt. bot. Ges.* **36**, 104.

BUELL, C. B. & WESTON, W. H. (1947). Application of the mineral oil conservation method to maintaining collections of fungous cultures. *Am. J. Bot.* **34**, 555.

CAPLIN, S. M. (1959). Mineral oil overlay for conservation of plant tissue cultures. *Am. J. Bot.* **46**, 324.

CAPPELLINI, R. A. & PETERSON, J. L. (1965). Macroconidium formation in submerged cultures by a non-sporulating strain of *Gibberella zeae*. *Mycologia* **57**, 962.

EDWARDS, G. A., BUELL, C. B. & WESTON, W. H. (1947). The influence of mineral oil on the oxygen consumption of *Sordaria fimicola*. *Am. J. Bot.* **34**, 551.

INGOLD, C. T. (1956). A gas phase in viable fungal spores. *Nature, Lond.* **177**, 1242.

POTTS, G, (1902). Zur physiologie des *Dictyostelium mucoroides*. *Flora, Jena* **91**, 281.

QUINLAN, M. S. & RAPER, K. B. (1965). Development of the myxobacteria. In *Encyclopedia of Plant Physiology*. Ed. by W. Ruhland, vol. 15, part 1, p. 596. Berlin, Heidelberg and New York: Springer-Verlag.

SMITH, G. (1960). *An Introduction to Industrial Mycology*, 5th ed. London: Edward Arnold (Publishers) Ltd.

TOWNSEND, B. B. & WILLETTS, H. J. (1954). The development of sclerotia of certain fungi. *Trans. Br. mycol. Soc.* **37**, 213.

TRINCI, A. P. J. & BANBURY, G. H. (1967). A study of the growth of the tall conidiophores of *Aspergillus giganteus*. *Trans. Br. mycol. Soc.* **5**, 525.

VÉZINA, C., SINGH, K. & SEHGAL, S. N. (1965). Sporulation of filamentous fungi in submerged culture. *Mycologia* **57**, 722.

EXPLANATION OF PLATE

Fig. 1. *Trichothecium roseum.*   × 600.

Fig. 2. *Ceratostomella adiposa.*   × 700.

Fig. 3. *Alternaria* sp. I.   × 870.

Fig. 4. *Alternaria* sp. II.   × 350.

Fig. 5. *Wardomyces pulvinata.*   × 1040.

Fig. 6. *Aspergillus tamarii.*   × 700.

Fig. 7. *Aspergillus repens.*   × 335.

Fig. 8. *Acrospeira levis.*   × 870.
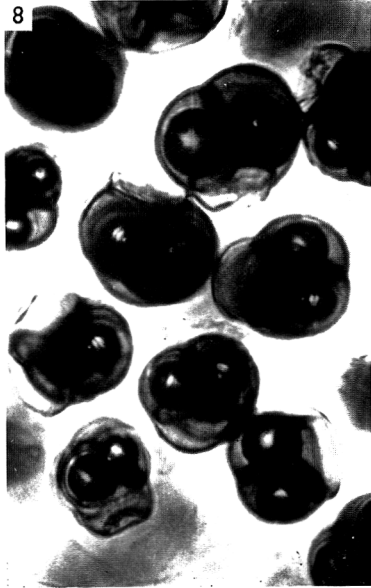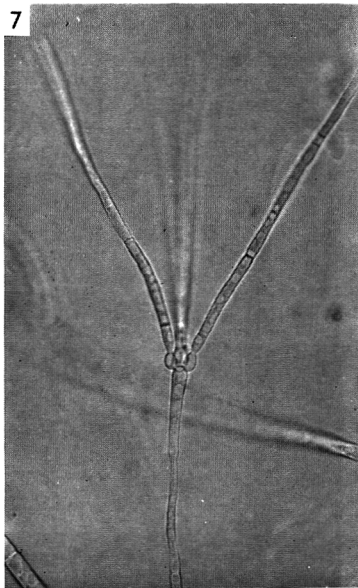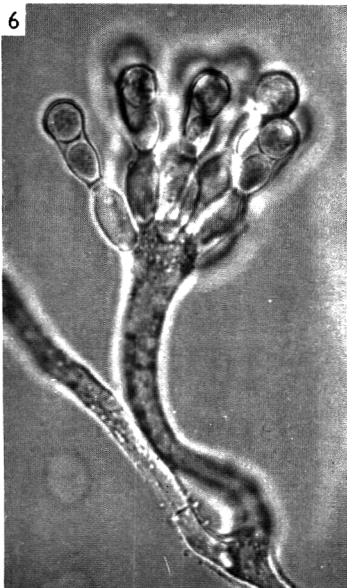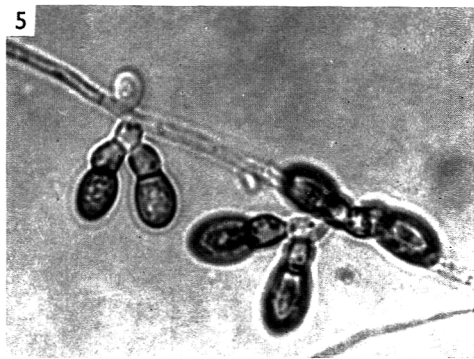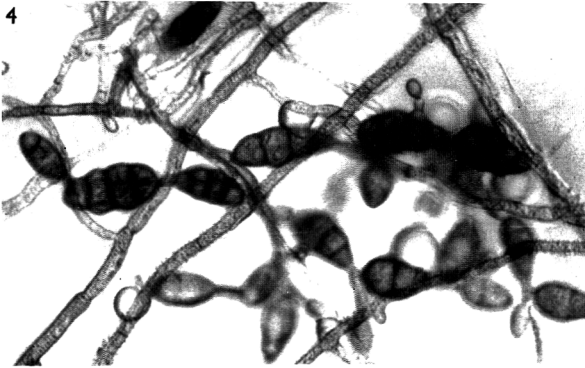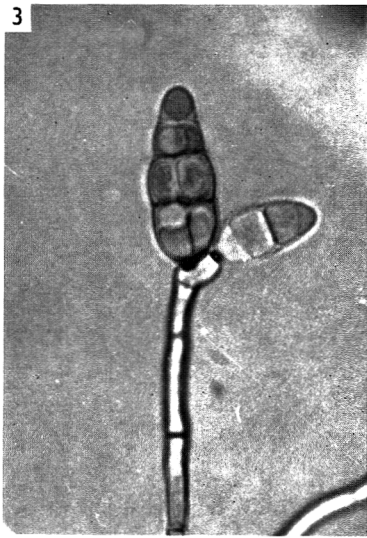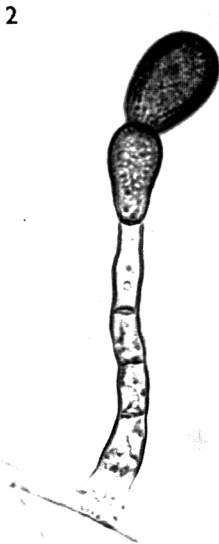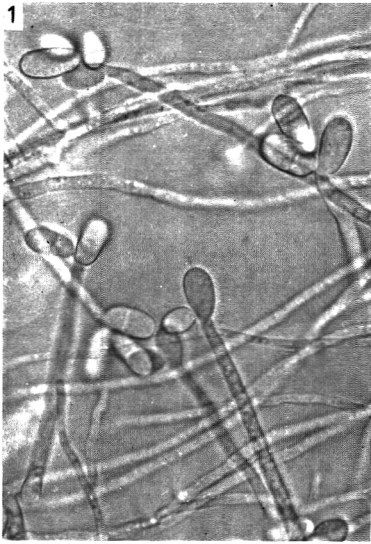
A Study of the Cell Envelope of the Halobacteria

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SUMMARY

Electron microscopy on thin sections of three different extremely halophilic Halobacterium species showed that their cell envelopes were of similar general construction: an inner membrane and an outer layer. The outer layer stains most strongly in the outermost part. When the NaCl concentration of the environment was lowered from the optimal of 4·3 M to 2·2 M the outer layer of the cell envelope of *Halobacterium salinarium* strain 1 became frayed; in many cells a release of material from the outer layer appeared to take place. When the cells were exposed to distilled water the outer layer of the envelope appeared to dissolve completely and the cell membrane disintegrated into tiny flakes. Fragments of the cell envelope produced by mechanical disintegration of the cells in 4·3 M-NaCl formed closed vesicles very rapidly; some of the cytoplasmic material became trapped inside the vesicles. Detergents appeared to slow down the closing of the vesicles and also to cause a release of material from the outer layer of the cell envelope. The cell envelope vesicles were mainly composed of protein and lipid; their content of amino sugar was low compared with the cell envelope of other Gram-negative bacteria. The cell envelope vesicle also contained nucleic acids; most of these were probably parts of the cytoplasmic material trapped inside the vesicles. The amino acid composition showed that the protein of the cell envelope vesicles was quite acidic, consistent with the contention that high concentrations of sodium ions stabilize the cell envelopes of these organisms by neutralizing the negative charges of the protein. Upon centrifugation at high speed of the lysate obtained by dialysis of the cell envelope vesicles against distilled water, the fragments of the cell membrane sedimented whereas most of the protein, presumably from the outer layer of the cell envelope, stayed in the supernatant fraction. Carotenoids and cytochromes were contained in the sediment with the membrane fragments. Most of the amino sugar-containing components stayed in the supernatant fraction; in the presence of 10–25 mM salt most of the amino sugar-containing components sediment with the membrane fragments.

INTRODUCTION

Stoeckenius & Rowen (1966, 1967) and Cho, Doy & Mercer (1967) showed with improved techniques for electron microscopy that the anatomy of the cell envelope of *Halobacterium halobium* is more complex than indicated by earlier studies (Larsen, 1967). The envelope appears to be composed of an inner membrane having the appearance and the dimensions of a 'unit membrane', and an outer 2-layered structure, 75–150 Å in thickness, which was shown by Stoeckenius & Rowen (1967) to be

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proteinaceous in nature. The studies of Stoeckenius & Rowen (1967) have also led to a more detailed insight into the process of lysis of *H. halobium* in hypotonic solutions. Upon dilution with water of the strongly saline (4–4·5 M-NaCl) environment in which the halobacteria live, protein is released from the outer layer of the cell envelope; at higher dilutions the cell membrane disintegrates to fragments of varying sizes.

In the present work the anatomy of the cell envelopes of three other extremely halophilic bacteria of the Halobacterium type has been studied. One of them, *Halobacterium salinarium*, strain 1, appears to contain a less complex system of membranes than *H. halobium* and was selected for chemical studies of the cell envelope and the pattern of its disintegration in hypotonic solutions. A short account of some of the results has already been given (Steensland, 1967).

METHODS

Organisms. Halobacterium salinarium strain 1 (Mohr & Larsen, 1963) and *Halobacterium* sp. strain 5 (Larsen, Omang & Steensland, 1967) are slender regular rods when grown in liquid cultures. The latter organism produces gas vacuoles; the former does not. The third organism used in the present work was kindly given to us by Dr G. Penso; he assigned this organism to the genus *Amoebobacter* Winogradsky and named it *Amoebobacter morrhuae* (Penso, 1947). This organism bears, however, little relation to the genus *Amoebobacter*. It is an extremely halophilic organism of the Halobacterium type (Larsen, 1962), but in contrast to the two organisms mentioned above the organisms are irregular in shape.

Cultivation. The organisms were grown at 30° on a reciprocating shaker (84 oscillations/min. of excursion 5 cm.) in 500 ml. flasks containing 120 ml. of a medium of the following composition (%, w/v): 25, solar salt (Trapani); 1, $MgSO_4.7H_2O$; 0·5, KCl; 0·02, $CaCl_2.6H_2O$; 0·5, tryptone (Oxoid); 10 (v/v), yeast autolysate (Mohr & Larsen, 1963); in tap water; pH 7. The salts and the organic nutrients were sterilized separately, the salt solution after being filtered through cotton wool. The cultures were harvested at the end of the exponential growth phase.

Chemicals. Except for the solar salt used in the growth medium all chemicals were of the highest purity available commercially.

Preparation of cell envelope fragments of H. salinarium strain 1. The contents of many culture flasks were pooled and kept aerated until fed into a Sharples (open type motor drive) supercentrifuge at − 10°. The subsequent operations were done at 0°. The packed organisms were disintegrated mechanically by stirring the paste with a glass rod as described by Holmes, Dundas & Halvorson (1965). The viscous mass obtained by this treatment was suspended in 5 vol. of 4·3 M-NaCl and homogenized in a MSE Homogenizer at full speed for 30 sec. The suspension was centrifuged (Servall SS-3) at 4000 g (10 min.). The sediment was discharged and the supernatant fluid recentrifuged at 27,000 g (1 hr). The new sediment containing the cell envelope fragments was washed 2–4 times and suspended in 5 vol. of 4·3 M-NaCl. The washed suspension was stored at − 8°. In some of the preparations a solution containing 4·3 M-NaCl and 0·025 M-$MgCl_2$ was used for suspending and washing the cell-envelope fragments.

For the preparation of cell-envelope fragments with detergents, packed organisms from the Sharples centrifuge were suspended in 5 vol. of 4·3 M-NaCl containing 2 % Tween 80 (Koch–Light, England) or 0·005 % Triton X 100 (Serva, West Germany);

3 vol. of glass beads (Ballotini, no. 12) were added and the suspensions homogenized for 3 min. in the MSE Homogenizer run at full speed. The glass beads were removed by passing through a coarse sintered glass filter (no. 1) and the suspension was centrifuged in the Servall centrifuge as described above; 4·3 M-NaCl was used to wash and suspend the cell-envelope fragments.

Preparations for electron microscopy

Thin sections. 1 vol. of culture or cell-envelope suspension was mixed with 1 vol. of the Glauert & Thornley (1966) glutaraldehyde fixative in 4·3 M-NaCl (5 % glutaraldehyde in 0·1 M-Na cacodylate-HCl pH 7·1, 0·01 M-CaCl$_2$, 4·3 M-NaCl) and kept in the cold for 1 hr. The organisms were sedimented at 1000 g (5 min.) and the cell-envelope fragments at 27,000 g (15 min.) The sedimented material was washed three times by centrifugation at 1 hr intervals in the cold in 0·1 M-cacodylate + 4·3 M-NaCl. The sediments were treated in the cold overnight with the Kellenberger, Ryter & Séchaud (1958) osmium tetroxide fixative (without tryptone) in 4·3 M-NaCl. The subsequent operations were done at room temperature. The sediments were washed with 6 vol. of the Kellenberger *et al.* (1958) acetate + veronal buffer in 4·3 M-NaCl and then treated with the Kellenberger *et al.* (1958) uranyl acetate washing fluid in 4·3 M-NaCl (30 min.). The preparations of whole organisms were embedded in 2 % agar (Difco) in 4·3 M-NaCl; the preparations of cell-envelope fragments were not. Both types of preparations were dehydrated in ethanol + water mixtures saturated with NaCl, taken up in acetone and embedded in Vestopal 310 (Chemische Werke Hüls A.-G., West Germany), using 1 % Trigonox CM-50 and 0·03 % Co-naphthenate (Noury & van der Lande, Holland) as initiator and accelerator, respectively. The blocks were sectioned in a Leitz Ultramicrotome with a glass knife. The sections were stained with 2 % uranyl acetate (15 min.) and 0·002 % lead citrate (3 min.). The preparations were examined with the Siemens Elmiskop at 80 kV.

In some experiments the NaCl concentration in cultures and suspensions of cell-envelope fragments was decreased to 2·2 M by dialysis 3 times (1 hr) against 100 vol. of 2·2 M-NaCl, before preparation for electron microscopy. The preparations for sectioning were made as described above, with the modification that all solutions contained 2·2 M-NaCl instead of 4·3 M-NaCl.

Fragments of cell membranes obtained upon removal of NaCl by dialysis were sedimented in the Spinco model L ultracentrifuge at 140,000 g and prepared for sectioning by treating the sediment with half-strength glutaraldehyde fixative; otherwise as described above, but NaCl was omitted from all solutions.

Carbon replicas of whole organisms and cell-envelope fragments were made as described by Mohr & Larsen (1963).

Analytical methods

Salt-free dry weight was determined in preparations dialysed against water, by drying a sample at 105° to constant weight and subtracting the ash remaining after heating to 400° for 2–4 hr.

Total nitrogen was determined by the micro-Kjeldahl method (Reiner, 1941). N × 6·25 was taken as a measure of protein.

Amino acids. For the determination of amino acid composition of proteins the preparations were hydrolysed in 6 N-HCl at 110° (40 hr). Particles in the hydrolysate

were removed by filtration and HCl removed by evaporation under reduced pressure over solid NaOH. The amino acids were chromatographed according to Moore, Spackman & Stein (1958) and estimated according to Yemm & Cocking (1955). Expected loss of serine (20 %) and threonine (10 %) during hydrolysis was corrected for (Tristram & Smith, 1962). Cysteine and half-cystine were determined according to Moore (1963), tryptophan according to Noltmann, Mahowald & Kuby (1962). To test for diaminopimelic acid the hydrolysate was chromatographed on paper according to Rhuland, Work, Denman & Hoare (1955). The part of the paper where diamino-pimelic acid should collect, was cut out and eluted separately. The eluate was chromato-graphed according to Moore *et al.* (1958).

Total lipid was extracted by the method of Folch, Lees & Stanley (1957) and deter-mined gravimetrically.

Amino sugar. The preparations to be analysed were hydrolysed in 4 N-HCl at 105° (4 hr). The hydrochloric acid was evaporated under reduced pressure and amino sugar was determined by the method of Rondle & Morgan (1955) and expressed as gluco-samine.

Carotenoids were extracted in acetone + water (2 + 1, v/v), transferred to ether and estimated spectrophotometrically (Zeiss PMQ II) at 499 mμ; $E_{1cm}^{1\%}$ = 2620 (Liaaen Jensen, 1960).

Cytochromes. Difference spectra were obtained with the Zeiss PMQ II spectro-photometer by running two cuvettes containing the same amount of preparation against each other, some crystals of $Na_2S_2O_4$ being added to one of the cuvettes. An estimate of the amount of cytochrome was obtained from the difference between the maximum and the minimum optical densities in the Soret region of the spectrum.

In separation experiments using the phase distribution method (Albertsson, 1960) the two-phase system contained 5 % dextran (D_{500}), 4 % polyethylene glycol (PEG_{6000}) in 0·02 M-K-phosphate (pH 6·8) and 0–0·05 M-NaCl. The polymers were kindly given to us by Professor Albertsson. Rough estimates were made of nucleic acids and protein according to the method of Warburg & Christian (Layne, 1957).

RESULTS

Observations in the electron microscope

Organisms kept at optimal NaCl concentration. Plate 1, fig. 1 and 2 show thin sections of *Halobacterium salinarium* strain 1 and *Halobacterium* sp. strain 5, respectively. Care had been taken to keep the organisms at optimal (4·3 M) NaCl concentration during the preparations for the sectioning. The cells appear to have an outer layer varying in thickness (60–150 Å) from cell to cell. This layer stained unevenly and the outermost part most strongly, indicating a two-layered structure. Many sections of *Halobacterium* sp. strain 5 showed a dented appearance of the cell surface, and with a spacing of about 140 Å (Pl. 1, fig. 2). This structure presumably relates to the pattern of hexa-gonally arranged pebble-like structures observed in shadowed preparations of halo-bacteria (Larsen, 1967). In sections of *H. salinarium* strain 1 the dented appearance of the surface was not so clearly recognizable (Pl. 1, fig. 1) although replicas of the surface show that this organism possesses the hexagonal surface patten (Mohr & Larsen, 1963).

Plate 1, fig. 1 and 2 reveal an electron transparent layer of about 30 Å in thickness

next to the outer layer, and bonded by thin stained layers. This indicates a membrane structure. The membrane structure was not, however, clearly recognizable in most sections prepared of cells kept at optimal NaCl concentration, and its significance became clear only from the experiments reported below in which the cells were exposed to hypotonic conditions.

Sections of the halobacterium of Penso gave the same general picture of the fine structure of the cell envelope as described for the two organisms above.

Organisms exposed to half strength NaCl concentration. Upon lowering the NaCl concentration in the cultures of *Halobacterium salinarium* strain I from the optimal of 4·3 M to 2·2 M, thin sections of the cells appeared as shown in Pl. I, fig. 3 and 4. Some of cells were disrupted and partly emptied of their content; others were not disrupted. In the whole cells the outer layer appeared characteristically frayed (Pl. I, fig. 3) as compared to cells not exposed to hypotonic conditions (Pl. I, fig. I), and the membrane structure stood out quite clearly in most of the cells. In the disrupted cells most of the outer layer was missing (Pl. I, fig. 4); the material appeared to be released. The three-layered membrane structure, however, could be clearly recognized but appeared broken. The membrane measured about 80 Å in thickness. Upon examination of a number of sections it appeared quite striking that the undisrupted cells possessed the outer layer whereas the disrupted cells had lost most of this layer.

Replicas of the surface showed that all cells exposed to the hypotonic condition, regardless of whether they were disrupted or not, had lost the hexagonal pattern so characteristic of the cells kept at optimal NaCl concentration.

Cell-envelope fragments kept at optimal NaCl concentration. Plate 2, fig. 5 shows a section of cell envelope fragments of *Halobacterium salinarium* strain I. The NaCl concentration was kept at 4·3 M during the preparation of the fragments. The difference in magnification from the figures on Plate I should be noted. The cell-envelope fragments all appear as closed vesicles. When liberated by the mechanical rupture of the cells the fragments of the cell envelope apparently close themselves rapidly by fusion along the line of shear. Some of the cytoplasmic material is trapped inside the vesicles and cannot be removed by washing in the centrifuge. In Pl. 2, fig. 5 the cytoplasmic membrane can be seen as part of the envelope structure. The outer layer of the envelope does not appear as distinct as in sections of whole cells. However, in replicas of the surface the hexagonal pattern was clearly seen, indicating that the outer layer is indeed present in the washed fragments of the cell envelope kept in 4·3 M-NaCl.

Plate 2, fig. 6 and 7 show sections of cell-envelope fragments treated with Tween 80 and Triton X 100, respectively. By this treatment the vesicles appear completely emptied of cytoplasmic material indicating that the detergents slowed down the closing of the vesicles or possibly prevented the vesicles from closing completely. Treatment with detergents also removed most, or possibly all, of the outer layer of the envelope, leaving the three-layered membrane structure. Another result of the treatment with detergents was the rather frequent finding in the sections of one or more vesicles inside another (Pl. 2, fig. 7).

Cell-envelope fragments exposed to hypotonic solutions. Upon decreasing the NaCl concentration in the suspension of cell-envelope fragments of *Halobacterium salinarium* strain I from 4·3 to 2·2 M by dialysis, replicas of the surface of the fragments revealed that the hexagonal pattern was lost. Sections of the fragments showed that little was left of the outer layer of the cell envelope in most of the fragments, but the

cytoplasmic membrane remained apparently intact. Most of the fragments still appeared in the sections as vesicles, but they were emptied of cytoplasmic material which indicated that they were ruptured. In many of the vesicles gaps were observed as visible evidence of disruption; some of the vesicles even appeared completely unfolded. Decreasing the NaCl concentration from 4·3 to 2·2 M thus appeared to result in a dissolution of material from the outer layer and an opening of the vesicles.

Cell-envelope fragments suspended in a solution of 4·3 M-NaCl+0·025 M-MgCl₂ were dialysed against 0·025 M-MgCl₂ to remove NaCl. The sediment obtained by centrifugation at 140,000 g (1 hr) appeared in sections as shown in Pl. 2, fig. 8. Only the membrane appeared to be left of the organized structure. A disruption of most of the membranes had taken place. Upon dialysis of the cell-envelope fragments against distilled water a sediment was obtained in the ultracentrifuge which appeared in sections as shown in Pl. 2, fig. 9. An extensive disruption of the membrane had taken place by the latter treatment; the membrane appears as tiny flakes.

Chemical studies

The chemical studies were done on *Halobacterium salinarium* strain 1.

Composition of cell-envelope vesicles. Cell-envelope fragments, isolated in 4·3 M-NaCl and forming vesicles as illustrated in Pl. 2, fig. 5, were dialysed against water. The opaque suspension became transparent by this treatment. Nitrogen was not lost from the dialysis bag according to Kjeldahl analyses. Table 1, column 2 shows that

Table 1. *Chemical composition of cell envelope vesicles and 'cell membranes' of Halobacterium salinarium strain 1*

Results are expressed as % of salt free dry weight of the cell envelope vesicles and 'cell membranes', respectively.

	Cell envelope vesicles	'Cell membranes'
Protein (N × 6·25)	66	51
Lipid	21	49
Amino sugar (as glucosamine)	1·3	0·4
Carotenoids	0·13	0·34

the bulk of the dialysed material is made up of protein (N × 6·25) and lipid. The amount of amino sugar (estimated as glucosamine) was low compared to cell envelope preparations of other types of bacteria, but fits quite well with the data given for other halobacteria (Brown & Shorey, 1963; Kushner *et al.* 1964; Brown, Shorey & Turner, 1965).

The components given for cell-envelope vesicles added up to somewhat less than 90 % of the salt free dry weight. The bulk of the remainder consisted of nucleic acids, both RNA and DNA. This was evidenced by spectrophotometric data and by gel filtration on Sephadex G 100 of the dialysed cell-envelope material after treatment with RNase and DNase. Each of the nucleases gave rise to low molecular nucleotidic material that was separated from the protein and lipid on the Sephadex column. The latter components filtered quantitatively through the column with no retention. At first the nucleic acids seemed rather firmly bound to the cell envelope in their native

state since they could not be removed by repeated washing of the cell envelope fragments in the centrifuge. However, using the phase distribution method of Albertsson (1960) on dialysed preparations of the cell-envelope fragments the nucleic acids were separated from protein and lipid. Somewhat later the vesicular nature of the cell-envelope fragments was realized and it then appeared probable that the nucleic acids, or at least a good deal of them, were contaminants from the cytoplasmic material trapped in the vesicles.

Table 2. *Amino acid content of cell-envelope vesicles of*
Halobacterium salinarium strain 1

	Anhydro amino acid/100 mg. salt-free dry weight (mg.)	Anhydro amino acid/100 mg. salt-free dry weight (μmole)	Mole (%)
Aspartic acid	10·5	91	14·0
Glutamic acid	9·8	76	11·6
NH₃*	(1·3)	(94)	—
Lysine	1·8	14	2·2
Arginine	2·7	17	2·7
Histidine	0·9	6	1·0
Glycine	3·6	62	9·6
Alanine	4·2	59	9·1
Valine	5·6	57	8·7
Leucine	5·3	47	7·2
Isoleucine	3·3	28	4·4
Serine	4·6	56	8·5
Threonine	6·2	61	9·4
Cystine/2 ⎫ Cysteine ⎭	0·3	2	0·3
Methionine	1·7	13	1·9
Proline	2·3	24	3·6
Hydroxyproline†	—	—	—
Phenylalanine	2·9	20	3·0
Tyrosine	2·8	17	2·6
Tryptophan	0·4	2	0·3
Sum	68·9	652	100

* Not included in total.   † Undetectable amounts.

The amino acid content of the dialysed cell-envelope material was determined after treatment with RNase and DNase and removal of the nucleotides on Sephadex G 100 (Table 2). The amino acid residues added up to about 69 % of the salt-free dry weight; this figure was a measure of the protein content of the preparation. The ammonia probably stemmed almost entirely from the amide group of the aspartic and glutamic acids since the lipid was low in nitrogen (less than 0·3 %; Urdahl, unpublished) and this nitrogen could not easily give rise to ammonia in the hydrolysis of the preparation. The contribution to ammonia from amino sugar must also be small. The data thus suggest that 50–55 % of the aspartic and glutamic acids were present in the cell-envelope vesicles as amides. Diaminopimelic acid was not detected in the eluate from the ion exchange column. In order to detect small amounts of this compound it was enriched for on paper as described under Methods and the proper fraction tested on the ion exchange column. Diaminopimelic acid could still not be detected. Hence, if

present, diaminopimelic acid would make up less than 0·001 % of the salt-free dry weight of the cell envelope vesicles.

Composition of sedimentable and non-sedimentable fractions of the cell-envelope vesicles after removal of NaCl. Cell-envelope vesicles suspended in 4·3 M-NaCl were dialysed against water, the dialysate was treated with RNase and DNase and centrifuged at 140,000 g (3 hr). A red transparent sediment was formed; the supernatant fluid appeared colourless. The distribution of the components of the material between sediment and supernatant fluid is given in Table 3. It appears that the non-sedimentable fraction was enriched in protein and amino sugar; the sedimentable fraction was strongly enriched in lipid and contained practically all the carotenoid. The nucleotides liberated by the nuclease treatment remained in the supernatant fluid.

Table 3. *Distribution between sediment and supernatant fluid of the components of the cell-envelope vesicles after dialysis against water and centrifugation at 140,000 g (3 hr)*

Results are expressed as % of salt free dry weight of the cell envelope vesicles.

	Sediment	Supernatant
Protein (N × 6·25)	19	47
Lipid	16	7
Amino sugar (as glucosamine)	0·25	1·0
Carotenoids	0·11	0·002
Sum	35·3	55·0

About one-third of the dialysed material of the cell-envelope vesicles sedimented in the ultracentrifuge. The sediment contained the cell membranes in the form of tiny flakes as illustrated in Pl. 2, fig. 9, whereas the supernatant fluid presumably contained the bulk of the material of the outer layer of the cell envelope. The membrane flakes were washed twice in the ultracentrifuge with water containing a small amount of Mg^{2+} which facilitated their sedimentation. The washed preparation had a composition as shown in Table 1, column 3. The high content of lipid should be noted. In addition to the carotenoids, the cytochromes of the cell-envelope vesicles appeared to collect quantitatively in the sedimented membrane fraction. Both these components were contained in the membrane fraction at a concentration about 3 times as high as in the cell-envelope vesicles, and their content in the membrane fraction became constant by washing in the centrifuge. The difference spectrum of the cytochromes in the washed membrane fraction showed absorption maxima at 560, 525 and 430 mμ typical of cytochromes of the b-type.

Sedimentability of amino sugar-containing cell-envelope components at low salt concentrations. After dialysis of the suspension of cell-envelope vesicles in 4·3 M-NaCl against water, only about one-fifth of the amino sugar-containing components sedimented with the cell membrane flakes in the ultracentrifuge (Table 3). The rest remained in the supernatant fluid even after prolonged centrifugation. Mg^{2+} in relatively modest concentrations greatly facilitated the sedimentation of the membrane flakes, and the sediment under these conditions contained a considerably higher proportion of the amino sugar-containing components (Table 4). When the suspension of cell-envelope vesicles in 4·3 M-NaCl is dialysed against various salts in the concentration range 11–25 mM, 60–75 % of the amino sugar-containing components of the cell-envelope

vesicles apparently sedimented in the ultracentrifuge with the cell-membrane flakes. When the vesicles were dialysed against 2 mM-MgCl$_2$ (i.e. the concentration of MgCl$_2$ used for the activation of DNase in the experiments described above) or against distilled water, only about 20 % of the amino sugar-containing components sedimented.

Table 4. *Sedimentability of amino sugar-containing components after dialysis of cell-envelope vesicles in 4·3 M-NaCl against various dilute salt solutions*

The centrifuge was run at 144,000 *g* until the red pigment (i.e. presumably the membrane flakes) had quantitatively sedimented.

	Time of centrifuga-tion (hr)	Total amino-sugar sedimented (%)
0·01 M-K-phosphate, pH 5·9	9	74
0·01 M-K-phosphate, pH 7·6	9	69
0·025 M-MgCl$_2$	1	70
0·012 M-MgCl$_2$	1	60
0·02 M-NaCl	5	59
0·002 M-MgCl$_2$	8	23
Distilled water	5	23

DISCUSSION

The electron microscope studies on the fine structure of the cell envelope of the three different halobacteria reported in the present work agree in essence with those reported earlier for *Halobacterium halobium* by Stoeckenius & Rowen (1966, 1967) and Cho *et al.* (1967); an inner membrane having the appearance and dimensions of a 'unit membrane' and an outer layer staining most heavily in the outermost part seems to be a structure common to the cell envelopes of halobacteria.

Halobacterium salinarium strain 1 was affected by hypotonic conditions in much the same way as Stoeckenius & Rowen (1967) reported for *H. halobium*. A decrease of the NaCl concentration caused a release of the outer layer of the cell envelope; upon further dilution of the environment with water the cell membrane disintegrated to tiny flakes. However, Stoeckenius & Rowen observed little change in the fine structure of *H. halobium* until a dilution of 1·0 M-NaCl was reached. In the present studies, *H. salinarium* strain 1 showed characteristic changes in the fine structure of the cell envelope upon dilution of the NaCl concentration to 2·2 M-NaCl. In sections the outer layer appeared frayed; a good proportion of the cells were ruptured and in these cells it could be observed that a release of the outer layer had taken place. It thus seems that *H. salinarium* strain 1 is more halophilic than *H. halobium* in this respect, and resembles *H. cutirubrum* for which a significant release of protein from cell envelope preparations was reported to take place at NaCl concentrations of 2·0–3·0 M (Onishi & Kushner, 1966).

Halobacterium salinarium strain 1 appears to be equipped with a less complex system of membranes than *H. halobium*. In the latter organism Stoeckenius & Rowen (1967) identified three different types of membranes, one 'intracytoplasmic', one 'purple coloured' and one 'red-to-orange coloured'. The latter two types of membranes could be separated by centrifugation of lysates of the cells in pure water; the purple membrane sedimented easily, the red-to-orange membrane only at very high

forces of gravity. Intracytoplasmic membranes were not observed in sections of *H. salinarium* strain 1 and no evidence was found for an easily sedimentable membrane in lysates of the cells. The only type of membrane identified in sections of this organism was the membrane of the cell envelope and this appears to correspond, both in colour and sedimentation character, to the red-to-orange membrane of *H. halobium*.

By mechanical disintegration of *Halobacterium salinarium* strain 1 in 4·3 M-NaCl the cell-envelope fragments formed closed vesicles immediately. Vesicle formation by fragments of biological membranes is a well-known phenomenon, and it was also observed by Stoeckenius & Rowen (1967) in their work on cell-envelope fragments of *H. halobium*. Possibly this phenomenon is a characteristic of all halo-bacteria, but it has been overlooked in earlier work on their cell envelope. It is clear that the formation of closed vesicles makes it difficult to prepare 'pure' envelopes or envelope fragments by the customary procedure which involves breaking the cells by mechanical means and fractionating the envelope material in the centrifuge; cytoplasmic material is apt to be trapped inside the vesicles. In the experiments on *H. sali-narium* strain 1 treatment with detergents gave cell-envelope vesicles appearing 'clean' inside. However, the detergents also seemed to release material from the outer layer of the cell envelope.

Brown (1963) first pointed out that lysis of the cell envelopes of halobacteria in hypotonic solutions might be due to the acidic nature of the envelope protein. At high concentrations of NaCl the negative charges of the protein would be neutralized by the sodium ions; upon removal of NaCl the negative charges would cause a dissolu-tion of the cell envelope. The present work on *Halobacterium salinarium* strain 1 supports this general idea. Though the amino acid analysis was done on the cell-envelope vesicles and thus on a preparation contaminated with some cytoplasmic material, the bulk of the protein of the preparation stemmed from the cell envelope. The analysis shows an excess of acidic over basic amino acids of about 20 mole % (not corrected for amide) or at least 5 mole % (corrected for amide). Cell-envelope preparations of *H. cutirubrum* (Kushner & Onishi, 1966) and *H. halobium* (McClare, 1967) gave similar figures. For comparison it should be mentioned that analysis of cell-envelope preparations of non-halophilic enterobacteria (Howe, Featherston, Stadel-man & Banwart, 1965) and a marine pseudomonad (Brown, 1963) gave an excess of acidic over basic amino acids of only about 9 mole % and 5 mole %, respectively, uncorrected for amide.

Cell-envelope vesicles of *Halobacterium salinarium* strain 1, dialysed against distilled water so that the structures disintegrated, yielded cell membrane flakes in the sediment after ultracentrifugation. The supernatant fluid contained the bulk of the protein, presumably acidic protein from the outer layer of the cell envelope. The sedimented fraction is probably not a 'pure' preparation of cell membrane material even after washing; it might be contaminated with, for instance, ribosomal material trapped inside the cell-envelope vesicles during their preparation. The washed sediment can, however, be considered highly enriched in the cell-membrane material. This sediment contains about 50 % lipid, a considerably higher figure than those reported for purified cell membranes of Gram-positive bacteria and mycoplasmas (Salton, 1967). It is difficult to imagine that the contaminating material will contribute significantly to the lipid content of the membrane fraction. On the other hand it is indeed possible that protein has been released from the cell membrane also upon removal of the salt

by dialysis, thus leaving a simpler membrane structure with a higher lipid content than in its native state.

The carotenoids and cytochromes collect in the membrane fraction and are probably constituents of the native cell membrane, as in the case of the Gram-positive bacteria. The amino sugar-containing component can be assumed to be a part of the cell envelope rather than the cytoplasmic material contaminating the cell-envelope vesicles. The amino sugar units are probably building blocks in polysaccharide structures. Some of these structures seem to be attached to bigger structures, possibly the cell membrane, and released only when the salt concentration becomes very low.

REFERENCES

ALBERTSON, P.-Å. (1960). *Partition of Cell Particles and Macromolecules*. Stockholm: Almqvist and Wiksell.

BROWN, A. D. (1963). The peripheral structure of Gram-negative bacteria IV The cation-sensitive dissolution of the cell membrane of the halophilic bacterium, *Halobacterium halobium*. *Biochim. biophys. Acta* **75**, 425.

BROWN, A. D. & SHOREY, C. D. (1963). The cell envelopes of two extremely halophilic bacteria. *J. cell Biol.* **18**, 681.

BROWN, A. D., SHOREY, C. D. & TURNER, H. P. (1965). An alternative method of isolating the membrane of a halophilic bacterium. *J. gen. Microbiol.* **41**, 225.

CHO, K. Y., DOY, C. H., & MERCER, E. H. (1967). Ultrastructure of the obligate halophilic bacterium *Halobacterium halobium*. *J. Bact.* **94**, 196.

FOLCH, J., LEES, M. & STANLEY, G. H. S. (1957). A simple method for the isolation and purification of total lipids from animal tissues. *J. biol. Chem.* **226**, 497.

GLAUERT, A. M. & THORNLEY, M. J. (1966). Glutaraldehyde fixation of Gram-negative bacteria. *Jl R. microsc. Soc.* **85**, 449.

HOLMES, P. K., DUNDAS, I. E. D. & HALVORSON, H. O. (1965). Halophilic enzymes in cell-free extracts of *Halobacterium salinarium*. *J. Bact.* **90**, 1159.

HOWE, J. M., FEATHERSTON, W. R., STADELMAN, W. J. & BANWART, G. J. (1965). Amino acid composition of certain bacterial cell-wall proteins. *Appl. Microbiol.* **13**, 650.

KELLENBERGER, E., RYTER, A. & SÉCHAUD, J. (1958). Electron microscope study of DNA-containing plasma. *J. biophys. biochem. Cytol.* **4**, 671.

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

KUSHNER, D. J., BAYLEY, S. T., BORING, J., KATES, M. & GIBBONS, N. E. (1964). Morphological and chemical properties of cell envelopes of the extreme halophile, *Halobacterium cutirubrum*. *Can. J. Microbiol.* **10**, 483.

LARSEN, H. (1962). Halophilism. In *The Bacteria*. Ed. by I. C. Gunsalus & R. Y. Stanier, vol. 4, p. 297. New York: Academic Press.

LARSEN, H. (1967). Biochemical aspects of extreme halophilism. In *Advances in Microbial Physiology*. Ed. by A. H. Rose & J. F. Wilkinson, vol. 1, p. 97. London: Academic Press.

LARSEN, H., OMANG, S. & STEENSLAND, H. (1967). On the gas vacuoles of the halobacteria. *Arch. Mikrobiol.* **59**, 197.

LAYNE, E. (1957). Spectrophotometric and turbidimetric methods for measuring proteins. In *Meth. Enzym.* **3**, 447.

LIAAEN JENSEN, S. (1960). Bacterial carotenoids. VI. A note on the constitution of bacterioruberine α. *Acta chem. scand.* **14**, 950.

McClare, C. W. F. (1967). Bonding between proteins and lipids in the envelopes of *Halobacterium halobium*. *Nature, Lond.* **216**, 766.

Mohr, V. & Larsen, H. (1963). On the structural transformation and lysis of *Halobacterium salinarium* in hypotonic and isotonic solutions. *J. gen. Microbiol.* **31**, 267.

Moore, S. (1963). On the determination of cystine as cysteic acid. *J. biol. Chem.* **238**, 235.

Moore, S., Spackman, D. H. & Stein, W. H. (1958). Chromatography of amino acids on sulphonated polystyrene resins. *Analyt. Chem.* **30**, 1185.

Noltmann, E. A., Mahowald, T. A. & Kuby, S. A. (1962). Studies on adenosine triphosphate transphosphorylases. *J. biol. Chem.* **237**, 1146.

Onishi, H. & Kushner, D. J. (1966). Mechanism of dissolution of envelopes of the extreme halophile *Halobacterium cutirubrum*. *J. Bact.* **91**, 646.

Penso, G. (1947). Il rosso dei bacallari-Etiologia, commestibilità, bonifica e prevenzione. *Rc. Ist. sup. Sanità* **10**, 563.

Reiner, M. (1941). *Manual of Clinical Chemistry*. New York: Interscience.

Rhuland, L. E., Work, E., Denman, R. F. & Hoare, D. S. (1955). The behaviour of the isomers of α,ϵ-diaminopimelic acid on paper chromatograms. *J. Am. chem. Soc.* **77**, 4844.

Rondle, C. J. M. & Morgan, W. T. J. (1955). The determination of glucosamine and galactosamine. *Biochem. J.* **61**, 586.

Salton, M. R. J. (1967). Structure and function of bacterial cell membranes. *A. Rev. Microbiol.* **21**, 417.

Steensland, H. (1967). Electron microscope studies of the surface layers of halobacteria. *Abstr. 4th Meeting Federation Europ. Biochem. Soc.* p. 49. Oslo: Universitetsforlaget.

Stoeckenius, W. & Rowen, R. (1966). Fine structure of the cell envelope and internal membranes of *Halobacterium halobium*. *Abstr. 6th int. Congr. Electron Microscopy, Kyoto*, vol. 2, p. 273. Tokyo: Maruzen Co.

Stoeckenius, W. & Rowen, R. (1967). A morphological study of *Halobacterium halobium* and its lysis in media of low salt concentration. *J. cell Biol.* **34**, 365.

Tristram, G. R. & Smith, R. H. (1962). The amino acid composition of some purified proteins. In *Advances in Protein Chemistry*. Ed. by C. B. Anfinsen, M. L. Anson & J. T. Edsall, vol. 18, p. 227. New York and London: Academic Press.

Yemm, E. W. & Cocking, E. C. (1955). The determination of amino-acids with ninhydrin. *Analyst, Lond.* **80**, 209.

EXPLANATION OF PLATES

PLATE 1

Fig. 1. Section of *Halobacterium salinarium* strain 1. × 140,000. Organism kept at optimal NaCl concentration (4·3 M).

Fig. 2. Section of *Halobacterium* sp. strain 5. × 140,000. Organism kept at optimal NaCl concentration (4·3 M).

Fig. 3–4. Sections of *H. salinarium* strain 1. × 140,000. Organism exposed to half strength (2·2 M) NaCl. Fig. 3, unbroken cells. Fig. 4, broken cells.

PLATE 2

Fig. 5–7. Sections of cell-envelope fragments of *H. salinarium* strain 1. × 50,000. Fragments kept at optimal NaCl concentration (4·3 M). Fig. 5, no detergent. Figures 6 and 7, cells disintegrated in the presence of 2% Tween 80 and 0·005% Triton X 100, respectively.

Fig. 8–9. Sections of cell membranes of *H. salinarium* strain 1. × 100,000. Membranes obtained by dialysis against 0·025 M-MgCl$_2$ (Fig. 8) and by dialysis against water (Fig. 9).

Plate 1

H. STEENSLAND AND H. LARSEN
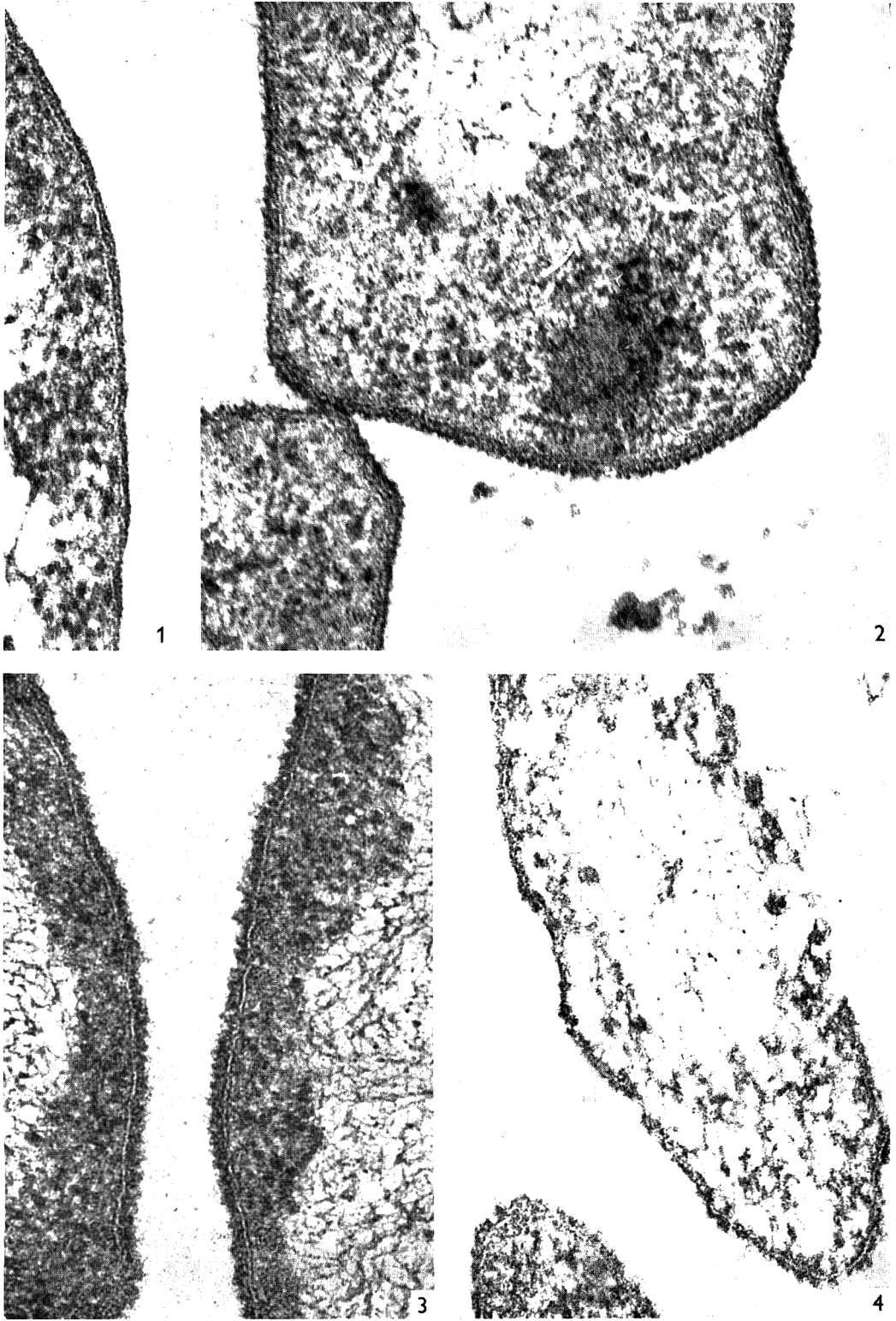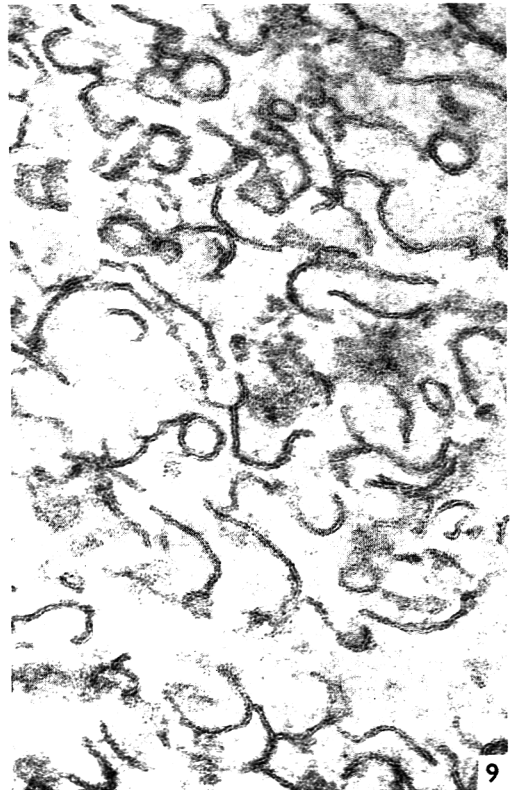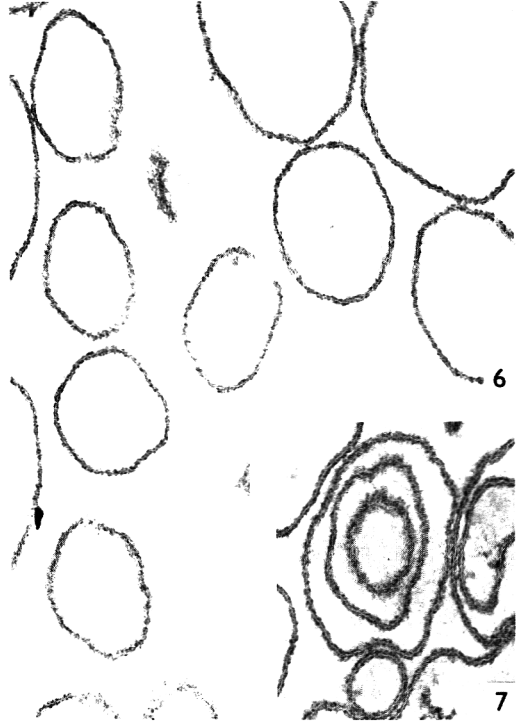
Growth and Pigmentation of *Micrococcus radiodurans*

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SUMMARY

Micrococcus radiodurans grew well between 25° and 40° with an optimum at 25° to 30°. At 35° pigmentation lagged behind growth after 3 days, growth and pigmentation showed similar maxima at 25°. The bacterium appeared to synthesize five carotenoids, at least two of which have the same chromophore and are keto-carotenoids.

INTRODUCTION

Micrococcus radiodurans is the radiation-resistant micro-organism isolated from samples of supposedly sterile meat by Anderson, Nordan, Cain, Parrish & Duggan (1956). Mosely & Schein (1964) reported good growth and pigmentation on agar containing 0·2 % (w/v) aspartic acid along with tryptone, glucose and yeast extract at pH 7·2. This is consistent with the observation of Goodwin (1963) that the addition of intermediates of the citric acid cycle enhances pigmentation in many bacteria. The general characters of the organism have been published by Anderson *et al.* (1956), Thornley (1963), Krabbenhoft, Anderson & Elliker (1965), and Kaplan & Zavarine (1965).

The present work reports studies on rates of growth and pigmentation for this bacterium at various temperatures and outlines qualitative data on the nature of the pigments. The latter are compared with the results of Bamji & Krinsky (1966) who examined the pigments of a radiation-resistant micrococcus.

METHODS

Micrococcus radiodurans was a gift from Dr B. E. B. Moseley, Molteno Institute, Cambridge. It was grown either in aspartate broth or on aspartic agar plates where the medium had the following composition: tryptone (Oxoid Ltd.) 0·5 % (w/v); glucose 0·1 % (w/v); yeast extract (Difco) 0·3 % (w/v); aspartic acid 0·2 % (w/v); pH to 7·2 with NaOH. If required, 2 % (w/v) agar no. 3 (Oxoid Ltd.) was added before autoclaving.

Relationship of growth and temperature. Twelve bottles of aspartic broth were inoculated with 2 ml. of a 2-day broth culture and were incubated in pairs at 15, 20, 25, 30, 35 and 40° in shaking incubators. One ml. samples were withdrawn with a sterile syringe at intervals.

Relationship of pigmentation with time at 35°. Bacteria were grown on aspartic agar plates which were inoculated by flooding with 2-day broth cultures. At daily intervals up to 6 days, the bacteria from an equal number of plates were harvested and suspended

in 95 % (v/v) methanol. They were subjected to ultrasound for a total of 5 min. in a Mullard ultrasonicator. The suspension was brought to the boil under a reflux condenser and immediately cooled. After centrifugation, the bacterial sediments were re-extracted until colourless. The carotenoids were purified by the lipid precipitation technique of Blessin (1962) followed by saponification in 10 % (w/v) KOH in methanol overnight at room temperature in an atmosphere of nitrogen. The unsaponifiable material was recovered in the usual way, to taken dryness, dissolved in methanol and read at 478 mμ on a spectrophotometer. The bacterial sediments were taken to dryness and constant weight and the optical density of the pigments expressed per unit weight of bacterial sediments.

Variation of pigmentation with temperature at 3 days. Bacteria were grown as for pigmentation above, but at 15, 20, 25, 30, 35 and 38° and they were harvested after 3 days. The pigments were extracted and the bacterial sediments treated as described.

Carotenoids. Bacteria were grown at 35° on aspartate agar and harvested after 3 days. The carotenoids were extracted and purified as described. They were concentrated and separated by preparative thin layer chromatography on 250 μ layers of silica gel G (Merck) using light petroleum (b.p. 60–80°)/acetone (50/50; v/v) as solvent. The material was strip loaded on the plates using the Desaga applicator (Camlab (Glass) Ltd.) and dried under a stream of nitrogen. The plates were developed in glass tanks gassed out with nitrogen. After development, the plates were viewed with the naked eye, under ultra-violet illumination and after spraying with a saturated solution of antimony trichloride in chloroform (Morton, 1942). The R_f values of the resolved fractions were determined and the absorption spectra of the two most abundant fractions, eluted into methanol, were read. The presence of keto groups in these fractions was investigated by two techniques: (i) formation of 2,4,dinitrophenyl-hydrazones (Fieser, 1955), (ii) reduction with LiAlH$_4$ (Goodwin, 1956).

RESULTS

Growth and temperature. The organism grew well at temperatures between 25 and 40° with an optimum between 25 and 30° (Fig. 1). Bacterial numbers declined fairly rapidly after reaching their peak, a result which is different from that found with *Sarcina flava* (Thirkell, Strang & Carstairs, 1965).

Pigmentation with time at 35°. Pigmentation reached a maximum of 1·6 O.D. units/mg. organism after 3 days and hence lagged behind growth which at 35° reached a peak in bacterial numbers at about 48 hr and remained steady for a further 24 hr. After 3 days, both growth and pigmentation showed similar maxima at 25° (Fig. 2). In a duplicate experiment omitting aspartic acid from the medium, only slight pigmentation was observed though good growth was supported.

Nature of the pigments. The total carotenoid extract resolved into five components which differed in colour from pale yellow to bright red-orange. Fraction 5, the least polar, was pale yellow and was only detected when the plates were very heavily loaded; fraction 4 was present in the greatest concentration. Satisfactory spectra were recorded only for fractions 2 and 4 after elution into methanol, and both of these fractions appear to be keto-carotenoids. The data obtained for the fractions are summarized in Table 1.
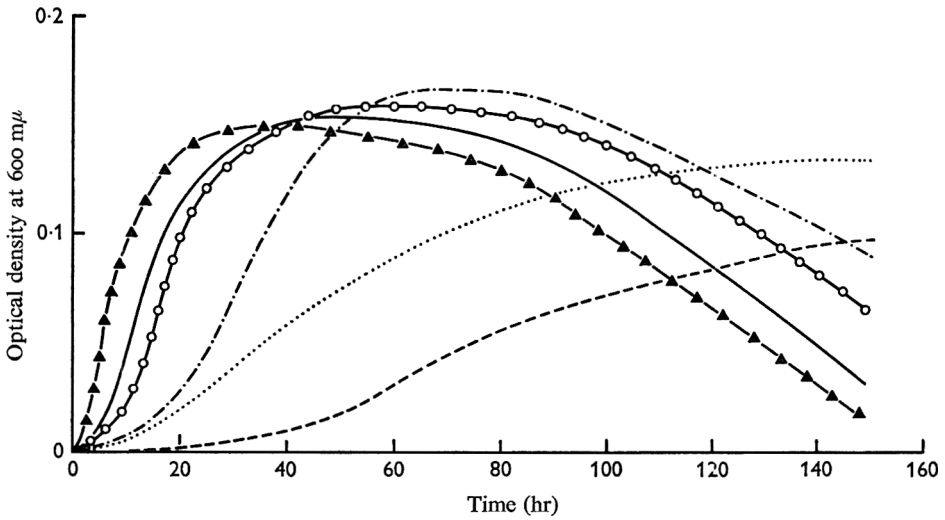
Fig. 1. Growth of *M. radiodurans* at different temperatures. Samples were withdrawn at intervals up to 159 hr incubation from shaken cultures. Optical density values were recorded at 600 mμ. – – – – 15°; ······ 20°; —·—·— 25°; O—O—O 30°; ——— 35°; ▲—▲—▲ 40°.
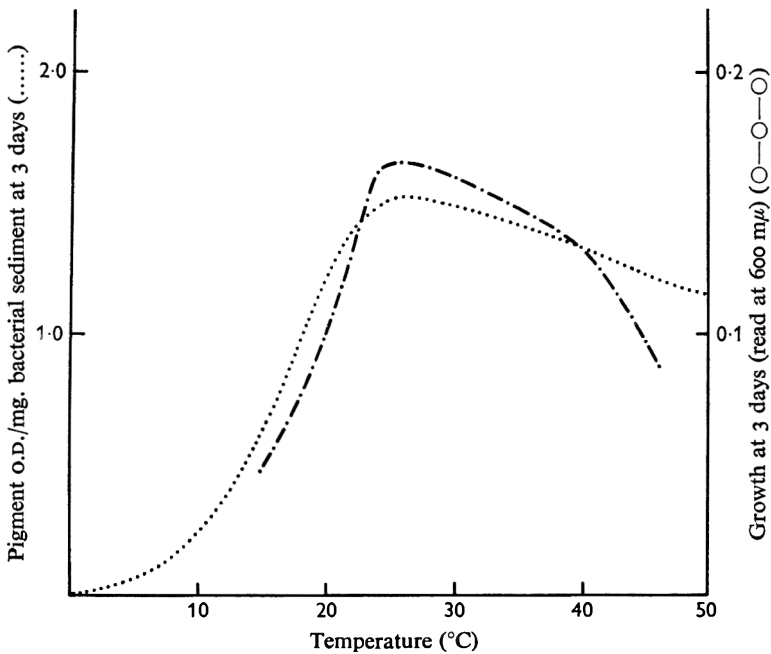

Fig. 2. Growth and pigmentation of *M. radiodurans* at various temperatures after 3 days incubation.

DISCUSSION

The optimum temperature for the growth of *Micrococcus radiodurans* agreed with that quoted by Anderson *et al.* (1956), but good growth was supported over a fairly wide range of temperature. Omission of aspartate from the medium reduced pigmenta-

tion significantly though good growth was still supported. Other experiments in this laboratory showed that nutrient agar enriched with 1 % (w/v) glucose suppressed pigmentation.

Table 1. *Data for the pigments of Micrococcus radiodurans*

Fraction	R_f	$\lambda_{max.}$ (Methanol) mμ	Colour (vis.)	Tests for keto group(s)
1	0·12	—	Orange	—
2	0·21	475	Orange-red	+
3	0·71	—	Orange	—
4	0·84	475	Orange-red	+
5	0·88	—	Pale yellow	—

All pigments gave a dark blue colour with SbCl$_3$.
All pigments were absorbent under ultraviolet illumination.

The finding that pigmentation and growth showed similar maxima at 25° after three days is interesting since many bacteria show a lower temperature for optimum pigmentation than for growth (Goodwin, 1963).

The fifth pigment, as against the four reported by Bamji & Krinsky (1966), was only observed when certain plates were overloaded. All the fractions reported by Bamji & Krinsky (1966) were keto-carotenoids, but only fractions 2 and 4 were available in sufficient concentrations to demonstrate this in this work. Again, all four pigments reported previously had the same chromophore, and although satisfactory spectra of fractions 1 and 3 were not determined, they had a different visible colour which would suggest that they had a lower $\lambda_{max.}$. The low absorption readings which were obtained, however, suggested that the spectra had no fine structure and therefore fractions 1 and 3 are probably also keto-carotenoids.

REFERENCES

ANDERSON, A. W., NORDAN, H. C., CAIN, R. F., PARRISH, G. & DUGGAN, D. (1956). Studies on a radio-resistant micrococcus, I. Isolation, morphology, cultural characteristics and resistance to γ-radiation. *Food Technol.* **10**, 575.

BAMJI, M. S. & KRINSKY, N. I. (1966). The carotenoid pigments of a radiation-resistant micrococcus species. *Biochim. biophys. Acta* **115**, 276.

BLESSIN, C. W. (1962). Carotenoids of corn and sorghum. *Cereal Chem.* **39**, 236.

FIESER, L. F. (1955). *Experiments in Organic Chemistry.* Boston: Heath and Co.

GOODWIN, T. W. (1956). Studies in carotenogenesis. *Biochem. J.* **63**, 481.

GOODWIN, T. W. (1963). *Biosynthesis of Vitamins and Related Substances.* London: Academic Press.

KAPLAN, H. S. & ZAVARINE, R. (1965). Correlation of bacterial radiosensitivity and DNA base composition. *Biochem. biophys. Res. Comm.* **8**, 432.

KRABBENHOFT, K. L., ANDERSON, A. W. & ELLIKER, P. R. (1965). Ecology of *M. radiodurans. Appl. Microbiol.* **13**, 1030.

MORTON, R. A. (1942). *Absorption Spectra Applied to Vitamins and Hormones.* London: A. Hilgar.

MOSELEY, B. E. B. & SCHEIN, A. H. (1964). Radiation resistance and DNA base composition of *M. radiodurans. Nature, Lond.* **203**, 1298.

THIRKELL, D., STRANG, R. H. C. & CARSTAIRS, E. A. (1965). Experiments with *Sarcina flava.* I. Relationship of time and temperature with growth and pigmentation. *Phytochemistry* **4**, 155.

THORNLEY, M. J. (1963). Radiation resistance among bacteria. *J. appl. Bact.* **26**, 334.

An Evaluation of the Pathways of Metabolism of Glucose, Gluconate and 2-Oxogluconate by *Pseudomonas aeruginosa* by Measurement of Molar Growth Yields

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SUMMARY

Pseudomonas aeruginosa, ATCC 9027, was grown in a simple ammonium salts medium with limiting glucose, gluconate or 2-oxogluconate as the sole carbon and energy source. There was little difference in molar growth yield between glucose and gluconate but with 2-oxogluconate the yield was signi-cantly less. Measurement of Y_{O_2} values (g. dry weight of cells produced/mole of oxygen consumed) during growth on the three substrates indicated most efficient oxygen utilization with 2-oxogluconate. The results are discussed in relation to the relative metabolic importance of the various pathways of of glucose metabolism in the organism.

INTRODUCTION

Several initial pathways of glucose metabolism are possible in *Pseudomonas aeruginosa*. The presence of a direct oxidative pathway via gluconate and 2-oxogluconate has been recognized for many years (for a review, see De Ley, 1960) and evidence has been presented that these oxidations are not linked to nicotinamide or flavin nucleotides (Ramakrishnan & Campbell, 1955; Campbell, Ramakrishnan, Linnes & Eagles, 1956). The presence of enzymes characteristic of phosphorylative pathways, such as hexokinase, gluconokinase and glucose 6-phosphate dehydrogenase, has also been demonstrated in cell-free extracts of glucose-grown *P. aeruginosa* (Claridge & Werkman, 1953, 1954). In the present investigation, molar growth yield determinations (Bauchop & Elsden, 1960) were carried out with glucose, gluconate and 2-oxogluconate as the sole carbon and energy sources in an attempt to gain some insight into the relative metabolic importance of the various pathways, the assumption being made that glucose metabolism via the direct oxidative pathway would make less energy available to the organism than a pathway via glucose 6-phosphate and 6-phosphogluconate in which the oxidative step is nicotinamide nucleotide-linked. Campbell *et al.* (1956) did report that when *P. aeruginosa*, strain ATCC 9027, was grown on limiting equi-molar amounts of glucose, gluconate or 2-oxogluconate identical growth yields were obtained, and they concluded that no energy was gained in the oxidative steps from glucose to 2-oxogluconate. Unpublished observations in our laboratory with a dif-ferent strain of *P. aeruginosa*, 2F32, indicated that there were some differences in molar growth yield with the three substrates and the present work was undertaken to re-examine the observations of Campbell *et al.* (1956) with strain ATCC 9027.

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METHODS

Organism. Pseudomonas aeruginosa, strain ATCC 9027, was maintained on nutrient agar slopes at 30°. It was subcultured at monthly intervals and stock slopes were stored at 4°.

Media. For molar growth yield determinations, the following medium was used: KH_2PO_4, 9 g.; $(NH_4)_2SO_4$, 2 g.; nitrilotriacetic acid, 0·48 g.; $Na_2MoO_4.2H_2O$, 0·004 g.; the pH was adjusted to 7·1 with 5 N-NaOH and the volume made up to 1 l. with distilled water. Three volumes of this solution were diluted with two volumes of carbon source solution (see below) and 5 ml. of each of the following two salts solutions added per litre of complete medium. Salts solution 1 contained: concentrated HCl, 13·4 ml.; $CaCO_3$, 2 g.; ZnO, 0·406 g.; $FeCl_3.6H_2O$, 5·4 g.; $MnCl_2.4H_2O$, 0·99 g.; $CuCl_2.2H_2O$, 0·17 g.; $CoCl_2.6H_2O$, 0·238 g.; H_3BO_3, 0·062 g.; distilled water up to 1 l. Salts solution 2 contained: concentrated HCl, 45 ml., MgO, 10·08 g.; distilled water up to 1 l. Details of these salt solutions were originally provided by Mr D. W. Tempest. All carbon sources were sterilized by filtration through a Millipore membrane (Millipore Filter Corporation, Bedford, Massachusetts) and, with the exception of 2-oxo-gluconate, were added to the medium at 2, 3, 4, and 5 mM final concentration. 2-Oxo-gluconic acid was prepared by passing a solution of the commercial calcium salt through a column of Dowex 50 cation exchange resin in the hydrogen form. The free acid was eluted from the column with distilled water and its concentration determined by titration against standardized 0·01 N-NaOH in a Conway microburette using phenol red as indicator. CO_2-free air was bubbled through the titration mixture. The acid was neutralized with NaOH and added to the medium at known concentrations approximating to those used for the other carbon sources.

Growth experiments. Molar growth yield determinations were carried out at 30° and 37° in two ways:

1. In 12·5 ml. medium contained in 50 ml. Erlenmeyer flasks shaken in reciprocating water baths at 80 strokes/min. with an amplitude of 1·5 in. Growth was followed turbidimetrically at 570 mμ with a Unicam SP 600 spectrophotometer (Unicam Instruments, Cambridge) by using flasks fitted with test-tube side arms. When growth was complete, the cultures were made up quantitatively to 25 ml. in volumetric flasks with 0·067 M-phosphate buffer, pH 7·1, for measurement (see later).

2. In 3 ml. medium in Barcroft flasks (without side arms but with centre wells) on the Warburg apparatus. Oxygen consumption was followed until it ceased to be exponential, at which point the flasks were removed and the cultures made up to 10 ml. with phosphate buffer prior to measurement. Barcroft flask centre wells contained 0·2 ml. of the CO_2-buffer, diethanolamine (60%, w/v) containing thiourea (0·1%, w/v), a solution based on the reagent of Pardee (1949). The use of KOH as centre well fluid resulted in extremely long lag phases (often greater than 24 hr) before growth began. Inocula for growth yield experiments were prepared as follows: the organism was subcultured from a nutrient agar slope into 12·5 ml. of medium in which the carbon source concentration was 0·8% (w/v). Two further subcultures were carried out under these conditions before transfer to a medium in which the carbon source was present in limiting amount (5 mM). The organism was subcultured at least three times in limiting medium before use in growth yield experiments to ensure adequate training of the cells on the particular energy source and no carry-over of unused

energy source. The organism was not grown on excess 2-oxogluconate but trained through several subcultures on limiting concentrations of 2-oxogluconate, cells grown on gluconate being used for the initial inoculum. Erlenmeyer flasks were inoculated with 0·1 ml. and Barcroft flasks with 0·05 ml. of a limiting culture (5 mM energy source) which had just reached the stationary phase.

Growth was measured in two ways. In the first, the extinction of suspensions was measured at 570 mμ in the Unicam SP 600 spectrophotometer and the dry weight was read from a standard calibration curve relating extinction to bacterial dry weight over the range 0·05–0·5 mg. cells/ml. In the second, a modification of the method of Johnson (1949) for the colorimetric determination of organic matter was used. Cell suspensions were mixed with an equal volume of 0·8 M-perchloric acid, in glass centrifuge tubes, centrifuged and washed once with a volume of 0·4 M-perchloric acid equal to twice the volume of the original suspension. The precipitates were suspended in 1 ml. of distilled water and 3 ml. of the oxidizing reagent, $K_2Cr_2O_7$ (0·18 %, w/v) in concentrated H_2SO_4 (95 %, v/v), added. The tubes were closed with glass condensers and heated at 100° for 20 min. The solutions were cooled, made up quantitatively to 10 ml. with distilled water and the extinctions measured at 440 mμ against a reagent blank, reduced chemically by addition of 0·2 ml. $Na_2SO_3.7H_2O$ (20 %, w/v). A calibration curve relating extinction at 440 mμ to bacterial dry weight over the range 0·1–0·8 mg. cells/ml. was prepared. All spectrophotometric measurements of bacterial growth were made with 4 ml. glass spectrophotometer cells with a 1 cm. light path. For preparation of calibration curves, large batches of cells were grown to stationary phase at 30° or 37° under conditions of forced aeration with limiting glucose (5 mM) as carbon source. Dry weight measurements were made by heating concentrated cell suspensions to constant weight in a hot air oven at 104°.

Isotopic experiments with [U-¹⁴C]glucose. 30 ml. of medium were prepared such that the glucose concentration was 4 mM and its specific activity was 0·168 μc/μmole. This medium was sterilized by membrane filtration and 12·5 ml. amounts dispensed into 50 ml. Erlenmeyer flasks fitted with side arms. These were inoculated with 0·05 ml. of a limiting culture as previously described, stoppered with rubber bungs and incubated until growth was complete. The suspensions were made up quantitatively to 25 ml. with distilled water for measurement of turbidity. The radioactivity of (*a*) the original medium, (*b*) the cell suspension, and (*c*) the growth supernatant solution (obtained by centrifuging a sample of the cell suspension at 14,000 *g* for 15 min.) was determined by spreading portions (0·1 and 0·2 ml.) of suitably diluted solutions on clean nickel planchets having an effective raised area of 1·77 cm.² and drying under an infra-red lamp. In (*b*) and (*c*) the pH was adjusted to at least 7·6 with 0·2 N-NaOH to prevent loss of steam-volatile acids. All estimations were made at infinite thinness using an Ekco Automatic Scalar, Type N530G, and corrections were applied for background. Counts were collected for a period sufficient to ensure that the counting error was less than ± 4 %.

Chemicals. Unless otherwise stated, all chemicals were obtained from Hopkin and Williams Ltd., Chadwell Heath, Essex or British Drug Houses Ltd., Poole, Dorset. Wherever possible, A.R. grade reagents were used. Of the growth substrates, glucose was A.R. and sodium gluconate was Laboratory Reagent quality. Calcium 2-oxogluconate was obtained from Nutritional Biochemicals Ltd., Cleveland, Ohio, U.S.A. The purity of the three growth substrates was checked by paper chromatography

using a modification of the method of Grado & Ballou (1961). The solvent system was ethyl acetate–pyridine–water saturated with boric acid (60:25:40 by volume). On shaking this separated into two layers and the top layer was used as solvent. Descending paper chromatograms (Whatman No. 1) were run for 48 hr, and were developed with the benzidine-periodate reagent described by Smith (1961). Each substrate ran as a single spot.
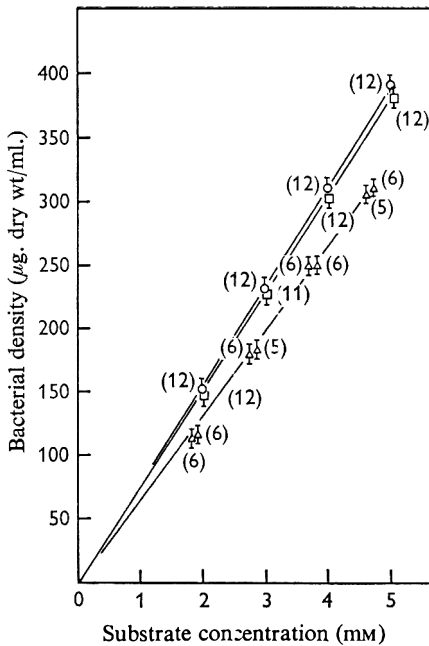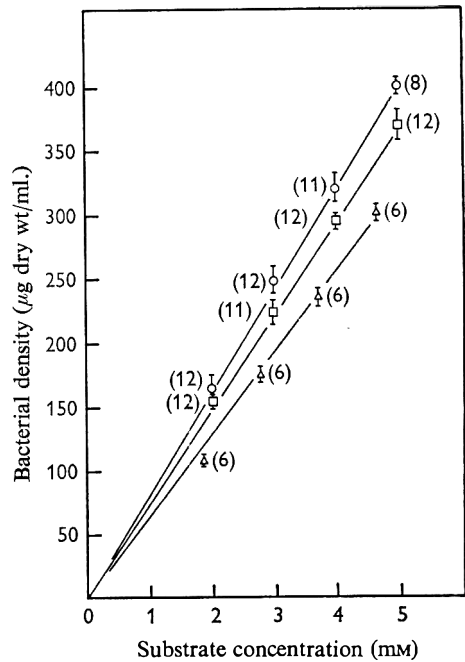
[U-^{14}C]Glucose was obtained from the Radiochemical Centre, Amersham, Bucks.

Fig. 1   Fig. 2

Fig. 1. The relation between growth of *P. aeruginosa* at 30° and substrate concentration. Growth substrates: ○, glucose; □, sodium gluconate; △, sodium 2-oxogluconate. Each point is the mean of the number of estimations shown in parentheses; the standard deviations from the mean are represented by the heights of the vertical bars.

Fig. 2. The relation between growth of *P. aeruginosa* at 37° and substrate concentration. Growth substrates: ○, glucose; □, sodium gluconate; △, sodium 2-oxogluconate. Each point is the mean of the number of estimations shown in parentheses; the standard deviations from the mean are represented by the heights of the vertical bars.

RESULTS

Figure 1 shows the relation between total growth of the organism at 30° in open flasks, as measured by extinction, and substrate concentration. Growth was measured over a range of substrate concentrations to ensure that the energy source was the growth-limiting factor. Similar data were obtained for growth at 37° (Fig. 2). In each case the mean Y values were calculated from the gradients of the straight lines relating growth to substrate concentration and are summarized in Table 1. The results obtained using the method for the estimation of total organic matter were plotted in a similar manner and are also recorded in Table 1.

A summary of the results obtained from manometric growth experiments is given in Table 2. The importance of making turbidimetric growth measurements as soon as oxygen consumption ceases to be exponential is illustrated in Fig. 3 for glucose-grown cells at 37°. The experiment was performed at a single glucose concentration (4 mM) by removing flasks for growth measurement at the times indicated by the triangles. The oxygen consumption which occurs at the end of exponential growth is presumably due to the oxidation of endogenous materials and therefore not a reliable indication of the 'growth curve' of the culture. Similar relationships between oxygen consump-

Table 1. *Summary of molar growth yields obtained with*
Pseudomonas aeruginosa in open flasks

	Method of measurement		
	Turbidimetric		Total organic matter
Substrate	30°	37°	30°
Glucose	77 (48)*	79 (43)	78 (34)
Sodium gluconate	75·5 (46)	74 (47)	77·5 (36)
Sodium 2-oxogluconate	66 (46)	64·5 (24)	—

* Molar growth yields are expressed as g. dry weight of organism per mole of substrate; figures in parentheses represent the total number of determinations made for each substrate.

Table 2. *Summary of results from manometric growth experiments*
with Pseudomonas aeruginosa

Substrate	Growth temperature	Molar growth yield (g./mole)	Percentage of substrate oxidized*	Mean generation time (min.)	Oxygen consumption (mole/mole of substrate)†	Y_{O_2} (g. dry weight/mole of oxygen consumed)†
Glucose	30°	78·1±1·5 (16)	40·6±1·4 (16)	77·6±6 (15)	2·44	32·0
	37°	76·2±2·0 (12)	42·6±1·8 (12)	68·9±8 (12)	2·55	29·9
Sodium gluconate	30°	75·5±1·2 (15)	40·2±1·4 (15)	84·0±4·4 (15)	2·21	34·2
	37°	73·1±1·8 (14)	41·3±1·7 (14)	77·0±5 (14)	2·28	32·1
Sodium 2-oxogluconate	30°	64·6±1·2 (11)	36·0±1·3 (11)	90·8±4 (11)	1·80	35·9
	37°	62·0±2·1 (12)	36·7±2·0 (12)	75·4±6 (12)	1·85	33·8

* Based on the theoretical oxygen consumption for complete oxidation of substrate to CO_2 and water.
† Based on the average values for the percentage of substrate oxidized.
The figures in parentheses represent the number of estimations carried out.

tion and molar growth yield were obtained with gluconate and 2-oxogluconate, also at 4 mM at 30° and 37°. Oxygen consumption subsequent to cessation of exponential growth and the concomitant drop in molar growth yield were always more marked at 37°. The results in Table 2 are expressed in terms of molar growth yields and also as the grams dry weight of cells produced per mole of oxygen consumed (Y_{O_2}). The Y_{O_2} concept was introduced by Whitaker and Elsden (1963) to compare yields of a number of organisms as a function of oxygen consumption under conditions where the energy source concentration was the growth-limiting factor. Of relevance to the problem here were their observations on Y_{O_2} as a measure of the efficiency of oxygen utilization during growth, e.g. they showed that when *Pseudomonas fluorescens* was

grown on benzoate or nicotinate, the Y_{O_2} values were less than those obtained when the organism was grown on equimolar mixtures of the products of ring fission, in agreement with the view that the oxygen used for ring fission does not participate in oxidative phosphorylation. The use of Y_{O_2} as a measure of the efficiency of oxygen utilization during glucose metabolism by *P. aeruginosa* will be discussed later.

To measure the percentage assimilation of glucose by the cells during growth and
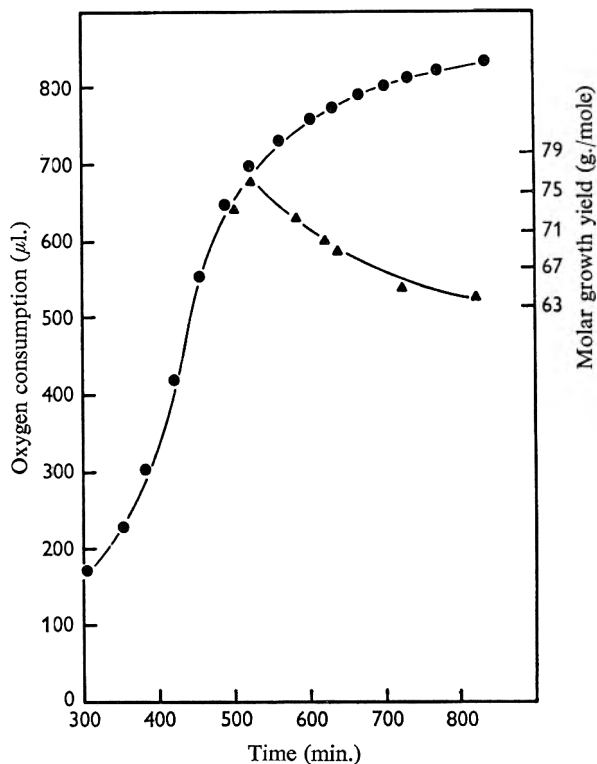
Fig. 3. The relation between oxygen consumption, period of growth and molar growth yield for cultures of *P. aeruginosa* grown on 4 mM glucose at 37°. ●, oxygen consumption; ▲, molar growth yield.

Table 3. *Growth of Pseudomonas aeruginosa on [U-¹⁴C]glucose at 30° and 37°*

Radioactive fraction	30°		37°	
	A	B	A	B
Total counts (a)	1,623,000	100	1,668,000	100
Cells + supernatant (b)	919,700	56·7	931,300	55·8
Supernatant (c)	92,300	5·7	111,800	6·7
Cells (b–c)	827,400	51·0	819,500	49·1
Carbon dioxide (a–b)	703,300	43·3	736,700	44·2

Column A: counts/12·5 ml. medium/min.
Column B: percentage of total counts present.

For growth at 30°, the specific activity of glucose was 31,100 counts/min./μmole and for growth at 37°, 32,800 counts/min./μmole.

The molar growth yield (g./mole measured turbidimetrically) was 77 at 30° and 75·5 at 37°.

to determine whether there was appreciable excretion of materials into the growth medium, the organism was grown at 30° and 37° in the presence of [U-^{14}C] glucose (Table 3). The results agree well with the data from the manometric experiments and show only small amounts of radioactive material in the culture supernatant. In view of its low amount, the nature of this material was not further investigated.

DISCUSSION

Our results indicate that when *Pseudomonas aeruginosa* is grown on limiting glucose or gluconate almost identical molar growth yields are obtained. In contrast with the observations of Campbell *et al.* (1956), the molar growth yield with limiting 2-oxogluconate is significantly less. A direct comparison with the work of Campbell *et al.* is not possible as their growth data were recorded (*a*) as a percentage of light transmission, and (*b*) as mg. protein per 100 ml. of medium. However, in a later paper, Warren, Ells & Campbell (1960) recorded a protein content of 57 % for the organism when grown on 0·2 % glucose, the concentration used in the earlier growth yield work. At 0·2 % glucose, Campbell *et al.* reported a growth yield of 24 mg. protein per 100 ml. medium from which it is possible to calculate a molar growth yield of 37·8, a value approximately one half the figure reported here. $Y_{glucose}$ values have been measured for a number of bacteria grown aerobically in simple medium and have been found to fall in the range 70–90 (e.g. Morris, 1960; Whitaker, 1962; Hadjipetrou, Gerrits, Teulings & Stouthamer, 1964). The $Y_{glucose}$ value reported here for *P. aeruginosa* is thus within this range whereas the figure calculated from the data of Campbell *et al.* is much lower.

In considering the relative metabolic importance of the various pathways of glucose degradation, the fact that glucose and gluconate give similar growth yields indicates that they are probably metabolized by pathways which yield identical amounts of ATP and metabolic intermediates. This would happen if glucose was metabolized to 6-phosphogluconate via gluconate and if the oxidative step from glucose to gluconate was energetically wasteful to the organism. Supporting this concept is the observation that the Y_{O_2} value for gluconate is higher than that for glucose, indicating that a portion of glucose oxidation by the organism is wasteful as regards energy production. The lower molar growth yield with 2-oxogluconate could be interpreted as follows: 2-oxogluconate is phosphorylated to 2-oxo-6-phosphogluconate and then, by analogy with the system operative in *Pseudomonas fluorescens* (Frampton & Wood, 1961), reduced in a nicotinamide nucleotide-coupled reaction to 6-phosphogluconate. Biologically available energy occurs in two forms, ATP and reduced nicotinamide nucleotides, and, since 2-oxogluconate has an energy requirement to initiate its metabolism quite separate from the ATP which all three substrates require for their kinase-mediated phosphorylation, this may explain why $Y_{2-oxogluconate}$ is less than $Y_{glucose}$ and $Y_{gluconate}$. The other possibility, that glucose and gluconate are mainly oxidized via 2-oxogluconate, could only be accepted if there was significant ATP production during the oxidation of gluconate to 2-oxogluconate. This seems unlikely since Ramakrishnan & Campbell (1955) could not detect conventional coenzyme requirements for partially purified gluconic acid dehydrogenase from *P. aeruginosa*.

This interpretation of the results may not, however, be quite as simple, since it does not take into account the presence of hexokinase and gluconic acid dehydrogenase in

extracts of glucose-grown cells. The Y_{O_2} value for 2-oxogluconate is higher than the corresponding gluconate value, indicating the possibility of some uncoupled oxidation with gluconate as well as with glucose.

Molar growth yield determinations were carried out with all three substrates at 30° and 37° to investigate the possibility of a greater maintenance energy requirement by the cells at the higher temperature. Since the Y values for each substrate were indistinguishable at the two temperatures we conclude that this is not the case. Senez (1962) reached a similar conclusion for *Aerobacter aerogenes* grown aerobically on glucose at various temperatures between 23° and 37°.

An interesting feature of the methodology of carrying out molar growth yield determinations in the Warburg apparatus when simultaneous measurement of oxygen consumption is required, is the long lag periods which we encountered with KOH in the centre well. Belief that this could be caused by a CO_2 deficiency led us to substitute a solution based on the CO_2-buffer of Pardee (1949), which ensures the maintenance of a partial pressure of CO_2 in the atmosphere; the lag periods were then eliminated. This prodecure would seem to be of general applicability for measurements of this type.

REFERENCES

BAUCHOP, T. & ELSDEN, S. R. (1960). The growth of micro-organisms in relation to their energy supply. *J. gen. Microbiol.* **23**, 457.

CAMPBELL, J. J. R., RAMAKRISHNAN, T., LINNES, A. G. & EAGLES, B. A. (1956). Evaluation of the energy gained by *Pseudomonas aeruginosa* during the oxidation of glucose to 2-ketogluconate. *Can. J. Microbiol.* **2**, 304.

CLARIDGE, C. A. & WERKMAN, C. H. (1953). Formation of 2-ketogluconate from glucose by a cell-free preparation of *Pseudomonas aeruginosa*. *Arch. Biochem. Biophys.* **47**, 99.

CLARIDGE, C. A. & WERKMAN, C. H. (1954). Evidence for alternate pathways for the oxidation of glucose by *Pseudomonas aeruginosa*. *J. Bact.* **68**, 77.

DE LEY, J. (1960). Comparative carbohydrate metabolism and localization of enzymes in *Pseudomonas* and related micro-organisms. *J. appl. Bact.* **23**, 400.

FRAMPTON, E. W. & WOOD, W. A. (1961). Carbohydrate oxidation by *Pseudomonas fluorescens*. VI. Conversion of 2-keto-6-phosphogluconate to pyruvate. *J. biol. Chem.* **236**, 2571.

GRADO, C. & BALLOU, C. E. (1961). Myo-inositol phosphates obtained by alkaline hydrolysis of beef brain phosphoinositide. *J. biol. Chem.* **236**, 54.

HADJIPETROU, L. P., GERRITS, J. P., TEULINGS, F. A. G., & STOUTHAMER, A. H. (1964). Relation between energy production and growth of *Aerobacter aerogenes*. *J. gen. Microbiol.* **36**, 139.

JOHNSON, M. J. (1949). A rapid micromethod for estimation of non-volatile organic matter. *J. biol. Chem.* **181**, 707.

MORRIS, J. G. (1960). Studies on the metabolism of *Arthrobacter globiformis*. *J. gen. Microbiol.* **22** 564.

PARDEE, A. B. (1949). Measurement of oxygen uptake under controlled pressures of carbon dioxide. *J. biol. Chem.* **179**, 1085.

RAMAKRISHNAN, T. & CAMPBELL, J. J. R. (1955). Gluconic dehydrogenase of *Pseudomonas aeruginosa*. *Biochim. biophys. Acta* **17**, 122.

SENEZ, J. C. (1962). Some considerations on the energetics of bacterial growth. *Bact. Rev.* **26**, 95.

SMITH, I. (1961). In *Chromatographic and Electrophoretic Techniques*, vol. I, p. 246. Ed. by I. Smith. London: W. Heinemann Ltd.

WARREN, R. A. J., ELLS, A. F. & CAMPBELL, J. J. R. (1960). Endogenous respiration of *Pseudomonas aeruginosa*. *J. Bact.* **79**, 875.

WHITAKER, A. M. (1962). The growth of micro-organisms in relation to their energy supply. *Ph.D. Thesis*. University of Sheffield.

WHITAKER, A. M. & ELSDEN, S. R. (1963). The relation between growth and oxygen consumption in micro-organisms. *J. gen. Microbiol.* **31**, xxii.

The Coupling of Phosphate Accumulation to Acid Production by a Non-growing Streptococcus

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SUMMARY

Accumulation of phosphate by washed, stored suspensions of stationary phase streptococcal strain SL-1 is strictly energy-dependent, essentially unidirectional and coupled stoichiometrically to acid production. At saturating concentrations of extracellular glucose and phosphate, incubation at varying pH values alters the rates of phosphate accumulation and concomitant fermentation of glucose such that coupling remains intact. Extracellular pH, while not affecting the coupling of phosphate accumulation to acid production, determines the total capacity for accumulation of phosphate and consequently the point of uncoupling of these processes. The conditions of cell storage profoundly affect the pH dependency of phosphate accummulation. During phosphate accumulation, the intracellular o-PO_4 pool contracts by more than 50 %. About half of the accumulated phosphate appears in a high molecular weight fraction (i.e. insoluble in cold $HClO_4$) under conditions which contraindicate net nucleic acid synthesis, protein synthesis or the incorporation of exogenous glucose carbon into high molecular weight cell constituents. The data are consistent with the hypothesis that stationary phase streptococcus SL-1 synthesizes inorganic polyphosphates.

INTRODUCTION

A microaerophilic extracellular dextran-producing streptococcus strain SL-1 has been isolated from the mouth of a human and is typical of the HS strains of Fitzgerald & Keyes (1960), which characteristically form heavy bacterial masses adhering to the surface of teeth. They are associated with tooth decay in hamsters.

Organisms of this type may rapidly accumulate phosphate from their environment (Luoma, 1964). The present study describes a stoichiometric coupling of phosphate accumulation to acid production by SL-1. It also presents presumptive evidence that much of the accumulated phosphate is inorganic polyphosphate.

METHODS

Preparation of suspensions. Strain SL-1 was maintained by passage at 10- to 14-day intervals in NIH fluid thioglycollate medium (Baltimore Biological Laboratories, Baltimore, Md., U.S.A.) containing excess $CaCO_3$. For experiments, 5 ml. of a 1-day-old culture was inoculated into 500 ml. of broth (Jordan, Fitzgerald & Bowler, 1960) containing 0·5 % (w/v) glucose and agitated at 37° in 1 l. flasks until the stationary

phase was reached. Cocci were harvested by centrifugation at room temperature and washed 3 times with 50 mM-KCl containing 1 mM-potassium phosphate (pH 6·8). Deviations from this procedure are noted in specific experiments. Wash solution was added to the suspensions so that there were 5–6 mg. cocci/ml.

Incubation procedure. To study phosphate accumulation and acid production, 50 ml. of stored suspension was stirred rapidly at 37° in a titration assembly TTA_3, titrator, TTT_1, and autoburette ABU_{1a} (Radiometer, Copenhagen, Denmark). A constant pH was maintained with N-NaOH and the addition of this titrant was recorded by a Model SRL strip-chart recorder (E. H. Sargent and Co., Chicago, Ill., U.S.A.). After 10 min. temperature equilibration, 2·25 μC. $Na_2H^{32}PO_4$ (New England Nuclear Corp., Boston, Mass., U.S.A.) were added. The specific activity of the final incubation medium was usually 45 mμC./μmole; isotope solution was always less than 0·35 % of the incubation mixture. Unless exogenous glucose was added, cellular levels of radioactivity remained constant from 30 sec. after the addition of isotope to over 2 hr. Ten to 15 min. after the addition of isotope, glucose was added to a final concentration of 0·20 % (w/v), unless otherwise indicated, which diluted the suspension by 1 %. The greatest dilution of the incubation mixture resulting from the addition of titrant was 2·4 %. Five ml. samples of suspension were filtered at timed intervals through 47 mm., 0·45 μ pore diameter Millipore discs (Millipore Filter Corp., Bedford, Mass., U.S.A.). Sampling and filtration required approximately 30 sec.

Throughout these experiments the pH stat incubation flask was open to the air. Incubation mixtures left open to the air, sparged with air, or sparged with argon gave identical values for both phosphate accumulation and acid production.

Acid production was calculated from the recorded addition of titrant to the suspension and from the normality of the titrant.

Phosphate accumulation. Filter discs and samples of filtrate were placed directly into scintillation fluid containing 4 % (w/v) Cab-O-Sil (Cabot Corp., N.Y., N.Y., U.S.A.) (Kinard, 1957; Gordan & Wolfe, 1960). The discs dissolved and the cocci distributed evenly in the gel, no disc-associated quenching occurred. ^{32}P radioactivity evaluations were carried out with a Tri-Carb liquid scintillation spectrometer, series 314 E (Packard Instrument Co., LaGrange, Ill., U.S.A.).

For cold $HClO_4$ extracts of cocci, samples of suspension were filtered through Millipore discs. These were placed into ice cold 0·3 N-$HClO_4$ and extracted for 10 min. with intermittent shaking. This material was filtered again and one volume of filtrate was added immediately to one half volume of 0·8 M-sodium acetate. Orthophosphate (o-PO_4) was analyzed by the Burnham & Hageage (1967) modification of the method of Potter (1947). Perchloric acid treatment and analysis of samples of sodium pyrophosphate, sodium trimetaphosphate, sodium tripolyphosphate, sodium hexametaphosphate (all obtained from The Monsanto Co., St Louis, Mo., U.S.A.) and trisodium adenosine triphosphate (Sigma Chemical Co., St Louis, Mo., U.S.A.) yielded less than 1 % of the absorbance of an equivalent concentration of o-PO_4.

Total phosphate analysis was carried out after digestion of Millipore discs and trapped cocci in 10 N-H_2SO_4 at 150° for 1 hr followed by removal of carbon by treatment with H_2O_2 (Umbreit, Burris & Stauffer, 1957). Then H_2O_2 was destroyed by adding water and boiling. Samples of the digest were analysed for radioactivity. Neither increased quenching nor loss of radioactivity resulted from this procedure.

Radiochemical purity of ^{32}P was evaluated by column chromatography on Dowex-1-

bicarbonate-8x according to the method of Martonosi (1960). Only one radioactive peak was obtained and this peak accounted for 99·6 % of the label eluted from the column. Concentrations of $KHCO_3$ sufficient to regenerate the column did not elute further radioactivity.

Measurement of trapped medium and cell weight. The volume of medium trapped in the Millipore disc was determined by filtering KCl-phosphate wash solution through the disc, weighing the disc, and subtracting the weight of a dry disc. The weight of the cocci in a 5 ml. sample of suspension was determined by filtering this sample, weighing the disc, and subtracting the weight of a wet disc. These procedures were carried out with each experiment. The maximum error in cell weight evaluation due to neglecting the displacement of medium by cocci is 6 %. In calculating the amount of phosphate in cocci, appropriate correction was made for the phosphate contained in the trapped medium.

Evaluation of growth. Growth, as indexed by increased DNA, was examined during incubation. Five ml. samples of suspension were added to an equal volume of 1·0 N-$HClO_4$ at 70° and extracted for 20 min. according to the method of Ogur & Rosen (1950). The extract was analysed for DNA by the method of Burton (1956) employing 2-deoxy-D-ribose standards (Sigma Chemical Co., St Louis, Mo., U.S.A.). Colorimetric chemical analysis was carried out with a Model 300 micro-sample spectrophotometer (Gilford Instrument Laboratories, Inc., Oberlin, Ohio, U.S.A.). No increase of DNA was observed.

RESULTS

Stoichiometry of phosphate accumulation and acid production with varying initial extracellular glucose concentrations. The initial rate of phosphate accumulation (285 μ-moles/g. cocci/hr) was independent of the initial extracellular glucose concentration in the range studied (Fig. 1*a*). By contrast, the total amount of phosphate taken up was a function of the glucose concentration. In the absence of exogenous glucose, no phosphate uptake was detectable.

In the same experiment, the initial rate of acid production was independent of the initial concentration of glucose in the medium and proceded at approximately 5·2 m-equivalents/g. cocci/hr. (Fig. 1*b*). Thus, the rate limiting step in glucose catabolism appeared to be saturated. In the absence of exogenous glucose, acid production from endogenous metabolic stores was very slow.

The stoichiometric relationship between phosphate accumulated at any given time and acid produced at that same time is shown in Fig. 1*c*. The relationship delineated was independent of the initial exogenous glucose concentration. The μmole of phosphate accumulated per m-equivalent of acid produced continuously decreased and approached a limit.

Stoichiometry of phosphate accumulation and acid production with varying initial extracellular phosphate concentrations. As shown in Fig. 2*a*, the initial rate of phosphate accumulation became independent of the initial environmental phosphate level at concentrations of 1 mM or greater. This maximal rate was approximately 520 μmole/g. cocci/hr.

The corresponding initial rate of acid production, at phosphate concentrations of 1 mM or greater, was about 5·1 m-equiv./g. cocci/hr and there was no initial lag (Fig. 2*b*). At lower initial phosphate concentrations acid production rates were slower and a lag was observed with 0·2 mM and 'no' phosphate.

Again, phosphate accumulation and acid production were stoichiometrically related in a progressively decreasing manner (Fig. 2c). At the lower phosphate concentrations (< 1 mM) the ratio of phosphate accumulation to acid production was decreased. Presumably this was due to the lack of saturation of the phosphate accumulation system.

Stoichiometry of phosphate accumulation and acid production wtih varying extracellular pH. A stoichiometric relationship between processes, each occurring at maximal rates, does not necessarily imply coupling of those processes. In order to demonstrate that
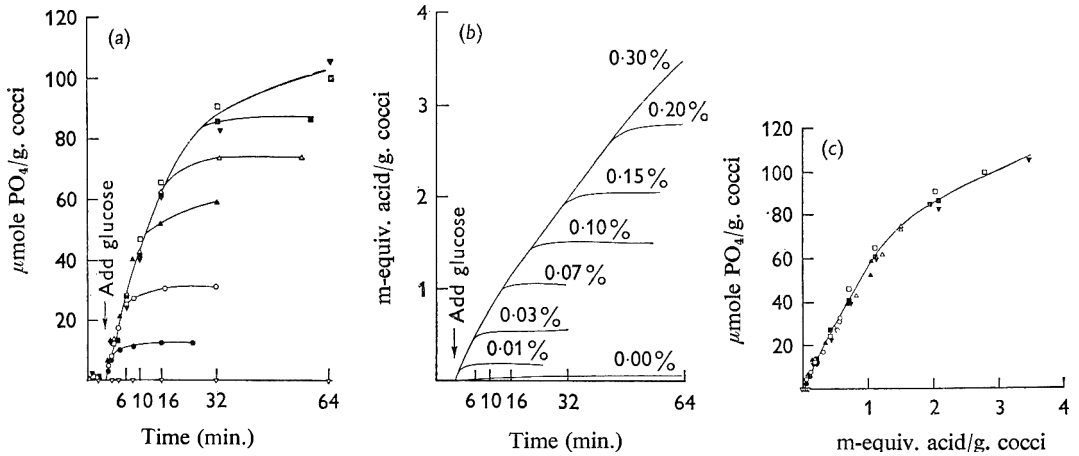
Fig. 1. Relationship of phosphate accumulation (a), acid production (b) and the stoichiometric relationship of phosphate accumulation and acid production (c) to the initial glucose concentration of the incubation medium. Glucose was added to a concentration of 0·00 % (▽), 0·01 % (●), 0·03 % (○), 0·07 % (▲), 0·10 % (△), 0·15 % (■), 0·20 % (□) or 0·30 % (▼) after the addition of 2·25 μC Na₂H³²PO₄.
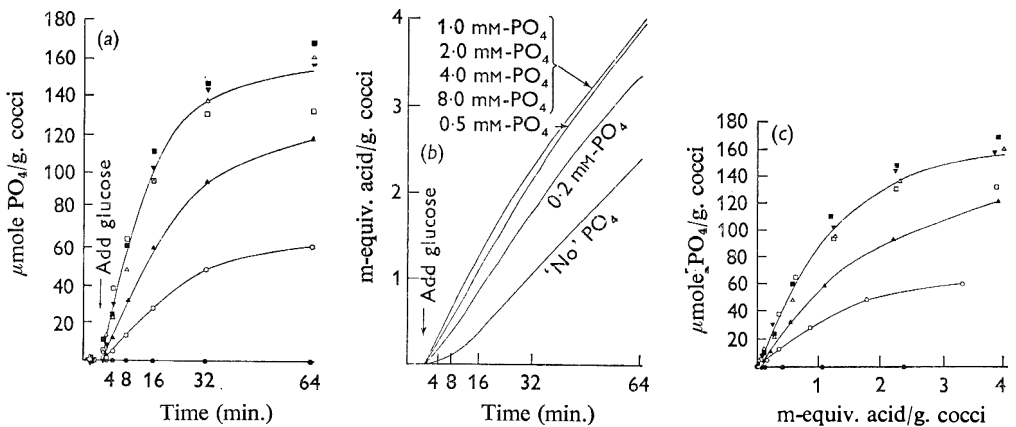
Fig. 2. Relationship of phosphate accumulation (a), acid production (b) and the stoichiometric relationship of phosphate accumulation and acid production (c) to various initial phosphate concentrations of the incubation medium. Cocci were incubated in 50 mM-KCl containing the following concentrations of phosphate (mM): 'No' phosphate (●), 0·2 (○), 0·5 (▲), 1·0 (△), 2·0 (■), 4·0 (□), 8·0 (▼). Glucose was added to the incubation medium to 0·20 % after the addition of 2·25 μC Na₂H³²PO₄.

the rate of phosphate accumulation and the rate of acid production are actually coupled, incubations were carried out with saturating levels of environmental phosphate and glucose, but with varying environmental pH, so that the rates of processes would be changed. The pH range 7·2 to 4·6 was chosen for study since this is the approximate range existing in the natural environment from which these cells were isolated (Dr G. Charlton, personal communication).

Experiments were carried out in two ways. In the first, the cocci were washed and stored in 1 mM-phosphate at the pH values at which they were subsequently incubated. In the second, the cocci were washed and stored in 1 mM-phosphate at pH 6·8 and adjusted to the required pH with N-NaOH or HCl immediately before testing.
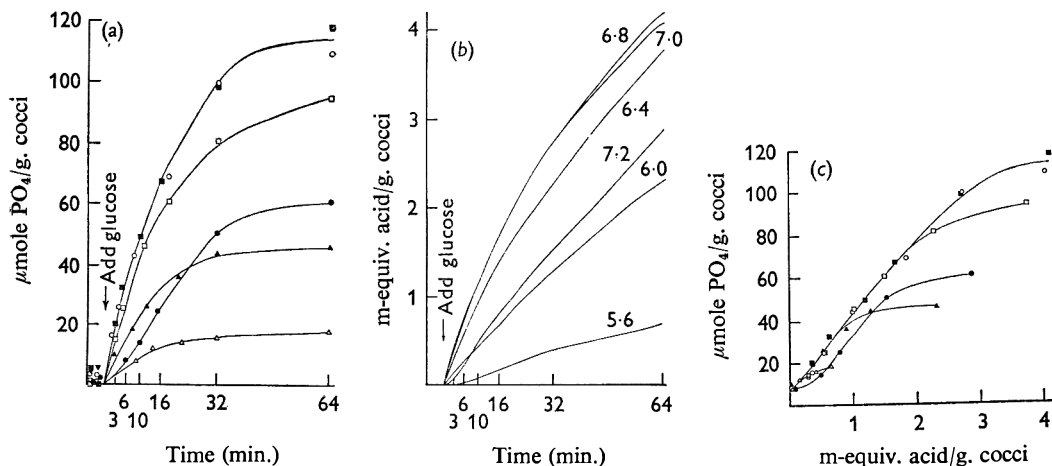
Fig. 3. Relationship of phosphate accumulation (*a*), acid production (*b*) and the stoichiometric relationship of phosphate accumulation and acid production (*c*) to the pH of the wash, storage and incubation medium. Cocci were washed, stored and incubated in 50 mM-KCl + 1 mM phosphate medium at the following pH values: 7·2 (●), 7·0 (○), 6·8 (■), 6·4 (□), 6·0 (▲) or 5·6 (△). Glucose was added to the incubation medium to 0·20 % after the addition of 2·25 μc $Na_2H^{32}PO_4$.

With cocci stored at the same pH level as the experimental incubations, the highest initial velocity of phosphate accumulation (430 μmole/g. cocci/hr) and the highest accumulative capacity were observed at pH 6·8 and 7·0 (Fig. 3*a*). The accumulation rates and capacities at pH 6·4, 6·0 and 5·6 were progressively lower. The rate and capacity at pH 7·2, after an initially slower accumulation, was intermediate between those at 6·4 and 6·0. Previous experiments had shown no phosphate uptake at pH 5·0. Thus there appeared to be a functional relationship between the rate and the capacity of phosphate accumulation under these conditions of storage and incubation.

Similarly, acid production rates (Fig. 3*b*) showed the same relative behaviour as phosphate accumulation rates: 6·8 and 7·0 > 6·4 > 7·2 > 6·0 > 5·6. The initial rates ranged from about 7·9 m-equiv./g. cocci/hr at pH 6·8 and 7·0 to 0·64 m-equiv./g. cocci/hr at pH 5·6. There was an initial delay in acid production at pH 7·2.

The stoichiometric relationship of phosphate accumulation and acid production was essentially the same at pH 7·0, 6·8, 6·4, 6·0 and 5·6 (Fig. 3*c*). At pH 7·2, the same relationship developed after a brief period of acid production unaccompanied by

phosphate uptake. At all pH values studied, a continuous decrease in the relationship of phosphate accumulated to acid produced again was evident as the limiting accumulation was approached. The environmental pH dictated the point at which the phosphate accumulation and acid production became dissociated.

By contrast, with cocci maintained at pH 6·8 during storage, the initial phosphate accumulation rates showed only slight variations in the pH range 7·2 to 5·6, with a mean of approximately 520 μmole/g. cocci/hr (Fig. 4a). At pH 5·1 and 4·6 these rates were about 255 and 90 μmole/g. cocci/hr, respectively. The change in capacity for phosphate uptake was not continuous but stepwise with respect to changing pH, the capacities being grouped as follows: pH 7·2, 7·0 and 6·8 exhibited the highest
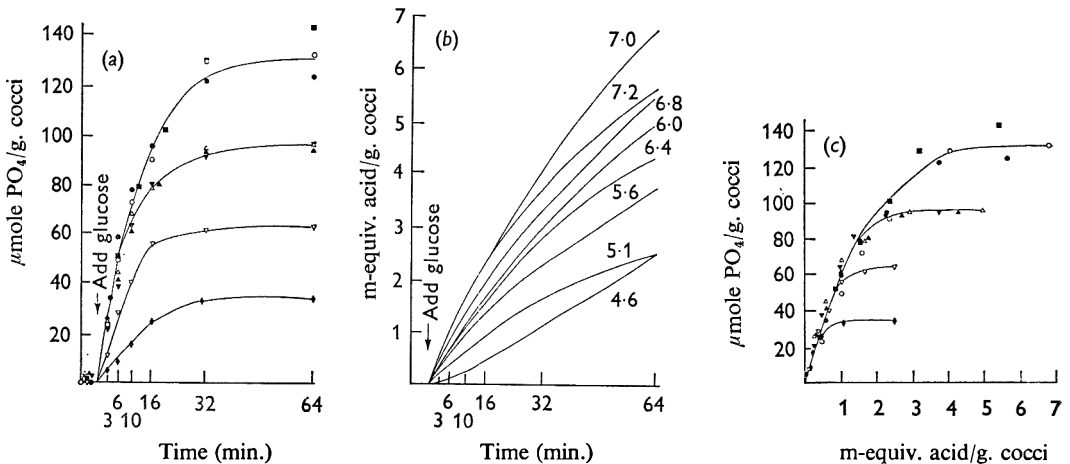
Fig. 4. Relationship of phosphate accumulation (a), acid production (b), and the stoichiometric relationship of phosphate accumulation and acid production (c) to the pH of the incubation medium. Cocci were washed and stored in 50 mM-KCl + 1 mM-phosphate medium at pH 6·8. At the time of incubation the pH was adjusted to the following values: 7·2 (●), 7·0 (○), 6·8 (■), 6·4 (▲), 6·0 (△), 5·6 (▼), 5·1 (▽) or 4·6 (◆). Glucose was added to the incubation medium to 0·20 % after the addition of 2·25 μC $Na_2H^{32}PO_4$.

value; pH 6·4, 6·0 and 5·6 gave a discretely lower value; and pH 5·1 and 4·6 yielded progressively lower total accumulations. Therefore, it appeared that the environmental pH ultimately regulated the absolute levels to which phosphate could be accumulated, but did not substantially affect the rate of that accumulation at pH 7·2 to 5·6. The initial rates of acid production are shown in Fig. 4b, they ranged from 11·0 m-equiv./g. cocci/hr to 2·6 m-equiv./g. cocci/hr (the maximal rate at pH 4·6). At all pH values studied, the stoichiometric relationship between phosphate accumulation and acid production was essentially the same until the limit of accumulation was approached for each case (Fig. 4c). Again, the environmental pH dictated the point of dissociation of these events. This dissociation occurred latest at pH 7·2, 7·0 and 6·8; earlier at pH 6·4, 6·0 and 5·6; still earlier at pH 5·1; and earliest at pH 4·6.

Thus, the rates of phosphate accumulation and acid production, although variable, remain coupled, irrespective of the pH conditions of the storage and/or incubation.

Absence of backflux of phosphate. The specific activity (c.p.m./μmole o-PO$_4$) of filtrates of the suspension was evaluated during the accumulation of labelled phosphate

to detect the possible efflux of unlabelled phosphate from cocci. The specific activity did not change detectably, since chemically analysed phosphate and radioactivity disappeared in equal proportion from the filtrate (Fig. 5). Hence, the net flux of phosphate observed in these experiments is essentially equal to influx, efflux being insignificant.
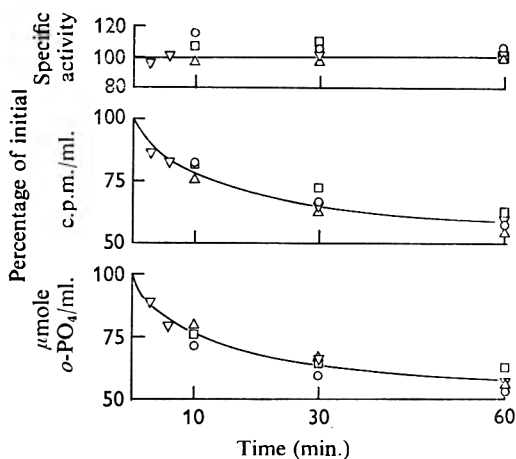
Fig. 5
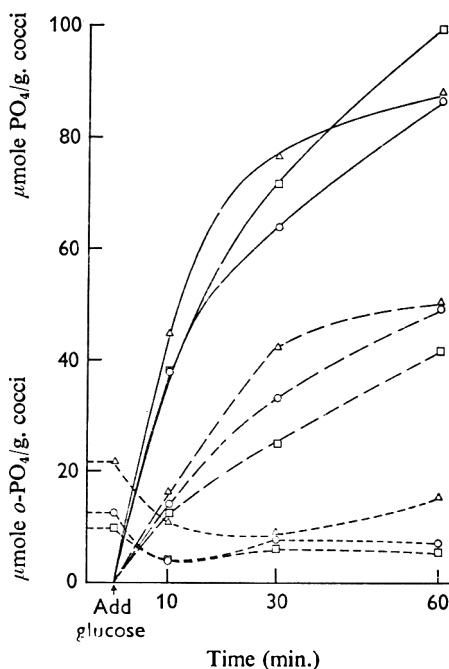
Fig. 6

Fig. 5. Change of o-PO_4 concentration, radioactivity and specific activity of the filtrates of the cell suspension during incubation. Standard conditions of incubation with glucose (0·2 %) added after the addition of 2·25 μc $Na_2H^{32}PO_4$ were used. Data are expressed as % of initial values. Four experiments are represented by different symbols. (O, □, △, ▽).

Fig. 6. Accumulation of phosphate into cold $HClO_4$-extractable and H_2SO_4-digestible fractions, and concentration of o-PO_4 in the cold $HClO_4$ extract of cocci during incubation. Standard conditions of incubation with glucose (0·2 %) added after the addition of 2·25 μc $Na_2H^{32}PO_4$ were used. Uptake of phosphate into H_2SO_4-digestible material is indicated by (———), and into cold $HClO_4$-extractable material by (– – –). The concentration of o-PO_4 in the cold $HClO_4$-extractable fraction is indicated by (- - - -). The symbols denote three experiments whose corresponding values of filtrate o-PO_4 concentration, radioactivity and specific activity are given by the same symbols in Fig. 5.

General compartmental distribution of accumulated phosphate. In view of the unidirectional flux described above, uptake into the total phosphate pool was calculated by dividing the c.p.m./g. cocci of H_2SO_4 digest by the mean c.p.m./μmole o-PO_4 of the filtrate. Uptake into the cold $HClO_4$ extractable pool was computed similarly. Figure 6 shows that approximately half of the accumulated phosphate was not extracted by cold-$HClO_4$ and presumably represents high molecular weight material. Chemical analysis of o-PO_4 in the cold $HClO_4$-extractable material showed initial values of 9·5 to 22·2 μ-mole/g. cocci. This pool contracted by over 50 % after the addition of glucose and then tended to return to the original levels.

Comparison of the cold $HClO_4$-extractable o-PO_4/g. cocci with o-PO_4/ml. of culture filtrate indicates that cellular o-PO_4 is 5·0 to 35 times more concentrated than medium o-PO_4 (these data are not corrected for the non-aqueous mass of the cocci). In other experiments with an initial concentration of 0·2 mM-o-PO_4 in the medium, 83 % of extracellular radioactivity was accumulated by the cocci in 64 min. Assuming intracellular o-PO_4 concentrations to be similar to those observed in the present experiment, one would expect cocci/medium o-PO_4 ratios in that experiment to be on the order of 25 to 130. Hence, o-$PO_4 <$ must move into the cocci against a chemical gradient even though the intracellular o-PO_4 pool is continuously being depleted.

DISCUSSION

The accumulation of phosphate by SL-1 was strictly energy-dependent, essentially unidirectional and coupled stoichiometrically to acid production. Energy-dependent and unidirectional phosphate flux has been described for yeast (Hevesy, Linderstrom-Lang & Nielsen, 1937; Goodman & Rothstein, 1957; Leggett, 1961; Borst Pauwels, 1962) and *Streptococcus faecalis* (Harold, Harold & Abrams, 1965). The stoichiometric relationship between phosphate accumulation and acid production demonstrates a coupling which has not been described by other authors.

Coupling occurred over wide ranges of extracellular glucose concentration, extracellular phosphate concentration, and extracellular pH. A point of uncoupling is, however, dictated by the environmental pH. Acid production continued longer than phosphate uptake, which appeared to have a capacity limited by extracellular pH. Phosphate accumulation did not occur in the absence of acid production; however, acid production occurred in the absence of added phosphate. The data do not indicate the closeness of the coupling nor the direct energetic cost of phosphate accumulation.

The rate of phosphate uptake was independent of the environmental glucose concentration in the range 0·01 to 0·30 % (w/v). These glucose concentrations were presumably saturating and submaximal rates of phosphate accumulation would be observed at lower glucose concentrations. The rate of phosphate accumulation showed saturation kinetics with respect to the environmental concentration of phosphate.

The rate of accumulation of phosphate by SL-1 was essentially independent of pH from 7·2–5·6 provided that the cocci studied had been harvested and stored at pH 6·8. This is similar to the behaviour of *Streptococcus faecalis* as shown by Harold *et al.* (1965), who harvested cells in salts-maleate buffer at pH 7·0 and tested phosphate accumulation immediately. By contrast, when SL-1 cocci were harvested and stored at the pH values to be tested, phosphate accumulation rates were strongly pH dependent. This finding is similar to those observed with *Staphylococcus aureus* (Mitchell, 1954) and yeast (Goodman & Rothstein, 1957). These observed variations of phosphate uptake rate with extracellular pH, at least with SL-1, may be due to disruption of the phosphate accumulative machinery incurred when energy-depleted cocci are faced with disadvantageous environmental pH for a prolonged period of storage. The hypothesis of Goodman & Rothstein (1957) that $H_2PO_4^-$ is transported in preference to HPO_4^{2-} by yeast seems inadequate to explain the experimental observations of the pH dependency of phosphate accumulation rate with SL-1 and *S. faecalis* (Harold *et al.* 1965).

The effect of the environmental pH on the rate of glycolysis is not clear from the literature. Some reports show that fermentation rates are essentially independent of

pH over a wide range. Below pH 5·0 the rate of yeast fermentation depends upon the amount of K^+ present in the incubation medium, K^+ counteracting the depressant effect of H^+ on fermentation (Rothstein, 1954). Stralfors (1950), however, showed with a number of isolates of streptococci, staphylococci, lactobacilli, Gaffkya, Neisseria and yeast that the rate of acid production from glucose depends upon the extracellular pH. No clear difference was apparent with the presence or absence of 10 mM-K^+ in the incubation fluid. The rate of acid production by SL-1 was dependent upon extracellular pH, even in the presence of 50 mM-K^+ in the medium. The effect of pH is probably not due to variation in the products of glucose fermentation since lactate is the predominant end product at pH 7·0 or below (Gunsalus & Niven, 1942; Platt & Foster, 1958). In view of the coupling of phosphate accumulation to acid production, the pH effect upon the regulation of the rate of glycolysis probably directly regulates the energy supply which in turn determines the rate of phosphate accumulation.

By contrast with the rate of phosphate accumulation, the capacity for phosphate accumulation appeared to be controlled by the extracellular pH regardless of the storage conditions. It is not clear how this effect is mediated, but it cannot be ascribed to a limitation of energy since glycolysis continued unabated long after phosphate accumulation terminated.

These experiments showed that the concentration of o-PO_4 in the intracellular pool was always greater than the extracellular concentration. They also showed that during phosphate uptake the intracellular o-PO_4 pool contracted by more than 50 %. If one assumes that o-PO_4 taken up from the extracellular fluid passes through the intracellular o-PO_4 compartment, then the incorporation of o-PO_4 into other phosphate-containing constituents must be more rapid than the transport of o-PO_4 into the coccus. On the other hand, if extracellular o-PO_4 does not initially mix with the intracellular o-PO_4 pool, it is still clear that the synthetic events attendent to phosphate accumulation take place partially at the expense of the intracellular o-PO_4 compartment. The suggestion has been made with other cell systems that o-PO_4 incorporated from extracellular fluid does not immediately pass through the intracellular o-PO_4 pool (Ginsburg, 1967; Vestergaard-Bogind, 1963).

Since approximately 50 % of the phosphate accumulated by SL-1 was not extractable with cold $HClO_4$, it would appear that a considerable synthesis of high molecular weight material occurs. Furthermore, since the pool of o-PO_4 contracted during the accumulation process, a large portion of the cold $HClO_4$-extractable accumulated phosphate must also represent synthetic products.

Phosphate accumulated into the high molecular weight material probably is not located in *de novo* synthesized pools of DNA, RNA, protein or cell-wall constituents. Two lines of reasoning lead to this conclusion. Firstly, cocci depleted of an exogenous nitrogen source are not capable of significant growth or net synthesis of DNA, RNA and protein. The absence of an increase of DNA in the experimental incubation system supported this assumption. Secondly, unpublished studies of the metabolic fate of glucose-U-^{14}C indicated complete recovery of glucose carbon in either residual sugar, carboxylic acids, small amounts of Embden-Meyerhof intermediates, or CO_2. Thus, no carbon is available for appreciable synthesis of nucleic acids, proteins or cell wall constituents. However, exchange of ^{32}P for ^{31}P in these pools is not ruled out. The presence of polyphosphate has not been described in streptococci (Harold, 1966). Although no direct evidence is now available, the data presented here are consistent

with the hypothesis that a large portion of the accumulated phosphate is polymerized as an inorganic polyphosphate.

REFERENCES

BORST PAUWELS, G. S. F. H. (1962). The uptake of radioactive phosphate by yeast. I. The uptake of phosphate by yeast compared with that by higher plants. *Biochim. biophys. Acta* **65**, 403.

BURNHAM, J. C. & HAGEAGE, G. J., JUN. (1967). Adenosine phosphate hydrolases in fractions of Vitreosilla. *J. Bact.* **93**, 19:.

BURTON, K. (1956). A study of the conditions and mechanism of the diphenylamine reaction for the colorimetric estimation of deoxyribonucleic acid. *Biochem. J.* **62**, 315.

FITZGERALD, R. J. & KEYES, P. H. (1960). Demonstration of the etiologic role of streptococci in experimental caries in the hamster. *J. Am. dent. Ass.* **61**, 9.

GINSBURG, J. M. (1967). Renal tubular transport of phosphate and ^{32}P exchange in the dog. *Physiologist* **10**, 181.

GOODMAN, J. & ROTHSTEIN, A (1957). The active transport of phosphate into the yeast cell. *J. gen. Physiol.* **40**, 915.

GORDAN, C. F. & WOLFE, A. L. (1960). Liquid scintillation counting of aqueous samples. *Analyt. Chem.* **32**, 574.

GUNSALUS, I. C. & NIVEN, C. F., JUN. (1942). The effect of pH on the lactic acid fermentation. *J. biol. Chem.* **145**, 131.

HAROLD, F. M. (1966). Inorganic polyphosphates in biology: structure, metabolism and function. *Bact. Rev.* **30**, 772.

HAROLD, F. M., HAROLD, R. L. & ABRAMS, A. (1965). A mutant of *Streptococcus faecalis* defective in phosphate uptake. *J. biol. Chem.* **240**, 3145.

HEVESY, G., LINDERSTROM-LANG, K., & NIELSEN, N. (1937). Phosphorous exchange in yeast. *Nature, Lond.* **140**, 725.

JORDAN, H. V., FITZGERALD, R. J. & BOWLER, A. E. (1960). Inhibition of experimental caries by sodium metabisulfite and its effect on the growth and metabolism of selected bacteria. *J. dent. Res.* **39**, 116.

KINARD, F. E. (1957). Liquid scintillator for the analysis of tritium in water. *Rev. scient. Instrum.* **28**, 293.

LEGGETT, J. E. (1961). Entry of phosphate into yeast cell. *Pl. Physiol.* **36**, 277.

LUOMA, H. (1964). Lability of inorganic phosphate in dental plaque and saliva. *Acta odont. scand.* **22** (Suppl. 41), 7.

MARTONOSI, A. (1960). Chromatographic separation of phosphate compounds. *Biochem. biophys. Res. Comm.* **2**, 12.

MITCHELL, P. (1954). Transport of phosphate across the osmotic barrier of *Micrococcus pyogenes*. Specificity and kinetics. *J gen. Microbiol.* **11**, 73.

OGUR, M. & ROSEN, G. (1950). The nucleic acids of plant tissues. I. The extraction and estimation of desoxypentose nucleic acid and pentose nucleic acid. *Archs. Biochem.* **25**, 262.

PLATT, T. B. & FOSTER, E. M. (1958). Products of glucose metabolism by homofermentative streptococci under anaerobic conditions. *J. Bact.* **75**, 453.

POTTER, V. R. (1947). The assay of animal tissues for respiratory enzymes. *J. biol. Chem.* **169**, 17.

ROTHSTEIN, A. (1954). Enzyme systems of the cell surface involved in the uptake of sugars by yeast. *Symp. soc. exp. Biol.* **8**, 166.

STRALFORS, A. (1950). The bacterial chemistry of dental plaques. *Odont. Tidskr.* **58**, 155.

UMBREIT, W. W., BURRIS, R. H. & STAUFFER, J. F. (1957). Methods for the analysis of phosphorylated intermediates. In *Manometric Techniques*, chap. 16, p. 268. Minneapolis: Burgess Publishing.

VESTERGAARD-BOGIND, B. (1963). The transport of phosphate ions across the human red cell membrane. II. The influence of the concentration of inorganic phosphate on the kinetics of the uptake of (^{32}P) phosphate ions. *Biochim. biophys. Acta* **66**, 93.

Regulation of Gratuitous β-Galactosidase Synthesis in *Aerobacter aerogenes* during an Adaptive Process

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SUMMARY

When cells of *Aerobacter aerogenes*, growing exponentially in either glucose + mineral salts medium, or lactose + mineral salts (being fully adapted to growth in lactose), are inoculated for the first time into maltose + mineral salts medium containing 10^{-3} M-methyl-β-D-thiogalactoside, a very large amount of β-galactosidase is synthesized gratuitously during the lag and early stages of growth in the new medium. The abnormally high enzyme levels are maintained for some time. This is a form of 'preferential synthesis', which can be ascribed to a failure of catabolite repression during exposure to an unfamiliar compound as source of carbon and energy. The extent of preferential synthesis is increased by starvation of the organisms prior to inoculation in maltose medium.

Similar experiments involving organisms partially or completely adapted to growth in maltose show a smaller degree, or complete absence, of preferential synthesis and enhanced enzyme levels. The efficiency with which maltose exerts the 'catabolite repression' effect is thus a function of the degree of adaptation of the organisms to maltose as sole carbon source.

It is suggested that this breakdown of catabolite repression, followed by its recovery during adaptation, could well be a useful general feature of the adaptive process.

INTRODUCTION

Catabolite repression has been found to be an important element in the regulation of the rate of synthesis of several microbial enzymes, both induced and constitutive. Early work (see Neidhardt & Magasanik, 1956; Neidhardt, 1960; Magasanik, 1961) led to the suggestion that the repression was caused by the accumulation of catabolic intermediates, resulting from a rate of catabolism which is excessive relative to the rate of anabolism.

Later work has tended to confirm this hypothesis, and has established a considerable amount of evidence concerning the operation of the effect. In particular, the repressor was shown to act during the stage of enzyme induction (i.e. specific messenger RNA formation) rather than that of actual protein synthesis (Nakada & Magasanik, 1964). This suggested that there might be a close link between the molecule effecting catabolite repression, and that associated with the product of the *i* (regulator) gene in normal induced enzyme synthesis. This question has not been completely resolved, but Loomis & Magasanik (1964) found that i^- mutants were subject to catabolite repression, and subsequently (Loomis & Magasanik, 1967) discovered a new gene, *CR*, a mutation in which led to insensitivity to catabolite repression in the *lac* operon.

An additional factor is the distinction between 'acute transient catabolite repres-

sion', which is overcome by the organism after varying periods of time, and a less severe permanent repression which persists (Moses & Prevost, 1966). The transient effect, as far as it affects β-galactosidase synthesis, appears to require a functional operator gene in the *lac* operon, but it is suggested that the permanent effect is non-specific, not operating via the regulatory system of the *lac* operon (Palmer & Moses, 1967).

In general it is true to say that an accumulation of catabolic intermediates, either by an increase in the rate of their formation or a restriction in the rate of their incorporation into cell macromolecules, increases the effectiveness of repression, and vice versa (see above references, and Prevost & Moses (1967) and Clark & Marr (1964)).

METHODS
Organism and growth conditions

The strains of *Aerobacter aerogenes* used in these experiments all had maximal growth rate when glucose was sole carbon source (doubling time approximately 35 min.), in a mineral salts medium. They were variously adapted to lactose and/or maltose as sole carbon source, or unadapted to either, the exact state of adaptation being given in the text. The growth medium was as follows: 3 parts of a solution containing 10 g./l. of the appropriate sugar (in the text 'G' represents glucose, 'M' maltose and 'L' lactose), 7 parts of a buffered mineral salts medium (pH = 7·12) having the composition: $Na_2HPO_4 . 12H_2O$, 10·0 g./l.; KH_2PO_4, 1·85 g./l.; $(NH_4)_2SO_4$, 1·56 g./l.; $MgSO_4$, 62 mg./l.; $FeSO_4 . 7H_2O$, 0·32 mg./l. When it was desired to induce β-galactosidase gratuitously in a culture growing in a maltose medium, a sterile solution of methyl-β-D-thiogalactoside (MTG) in mineral salts solution was added, replacing the same volume of plain mineral salts, to give a final concentration of 10^{-3} M-MTG (M + MTG medium). Cells were grown at 40° with forced aeration. For further details of culture methods see Richards & Hinshelwood (1961).

Growth rate and enzyme assay

The bacterial concentration of cultures was measured either using a Hilger 'Spekker' light absorptiometer, or at 540 mμ in a 'Unicam' SP 600 spectrophotometer, using a 1 cm. cell. The cell concentration symbol, M, is proportional to cell *mass* per unit volume, although expressed as 'number of standard cells per 10^{-6} ml.' (for a discussion of this see Richards & Hinshelwood, 1961).

The β-galactosidase activity of organisms was measured using o-nitrophenyl-β-D-galactopyranoside (ONPG) as chromogenic substrate, according to the method given in Richards & Hinshelwood (1961). The symbol α_s represents the β-galactosidase activity of washed, ultrasonically disrupted organisms (for details, see Richards & Hinshelwood, 1962), per unit mass of organisms.

α_c represents the β-galactosidase activity per unit volume of culture, i.e. $\alpha_c = \alpha_s M_c$, where M_c is the concentration of organisms in the culture from which the sample was taken.

The symbol Δ is used to indicate the change in a property (α or M) from the time of inoculation of a culture (i.e. the slope of a plot of $\Delta\alpha_c v$. ΔM is a measure of the differential rate of synthesis of β-galactosidase).

<div style="text-align:center">RESULTS</div>

<div style="text-align:center">*Transfer of non-induced organisms to M + MTG medium*</div>

Organisms fully adapted to maltose

If a strain of *Aerobacter aerogenes*, fully adapted to maltose as sole source of carbon and energy, is transferred to M + MTG medium, β-galactosidase synthesis is induced. The induction occurs without any preferential synthesis. In fact, with $[MTG] = 10^{-3}$ M the initial differential synthetic rate of β-galactosidase is lower than the equilibrium value. Figure 1 shows this: α_s increases roughly linearly with number of cell divisions (N) after addition of inducer. If the organisms were synthesizing β-galactosidase at a steady differential rate of $d\alpha_c/dM = 55$, α_s would increase with N in the manner shown by the broken line.
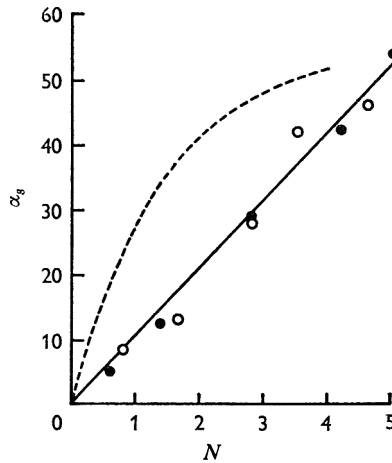
Fig. 1. Induction of β-galactosidase by 10^{-3} M-MTG in *Aerobacter aerogenes* fully adapted to maltose, and growing in maltose medium. N = number of generations after addition of inducer. ○, ●, represent separate experiments. - - -: theoretical curve for a constant differential synthetic rate of $d\alpha_c/dM = 55$.

A steady value of $d\alpha_c/dM$ has, however, been reached by $N = 5$. When organisms were transferred from maltose medium to M + MTG, grown in this for 5 generations and then transferred to fresh M + MTG medium, the plot of $\Delta\alpha_c v . \Delta M$ is subsequently a straight line of slope 51. This is in good agreement with the value of α_s maintained during serial subculture in M + MTG medium We can therefore accept $\alpha_s \approx 50$ as the equilibrium value during balanced growth.

Organisms unadapted to maltose

If an inoculum of organisms which have never been grown in maltose, nor induced with MTG, is taken from a glucose medium, washed, and added to a maltose medium, there is a lag of about 60 min. After this, growth proceeds slowly until mass has increased about 50 %, when steady exponential growth follows, with mean generation time (m.g.t.) about 60 to 80 min. MTG has no effect on growth. The mean generation time decreases steadily to a minimum over the course of several subcultures, but the bulk

of the experiments reported below refer to the induction of β-galactosidase during this early period of lag and slow growth.

If the initial transfer from glucose, after washing, is to a M + MTG medium, β-galactosidase induction follows the pattern shown in Fig. 2. Gratuitous induction of the enzyme and adaptation to growth in maltose occur simultaneously. It can be seen that β-galactosidase synthesis begins at zero time, and α_s rises linearly with respect to time, during the growth lag. This linear increase is continued beyond the point where growth begins, but is starting to level off at the end of the experiment. Accordingly, α_c rises linearly with respect to time before mass increase begins and more rapidly later. The plot of $\Delta\alpha_c$ v. ΔM necessarily has apparently infinite slope at $t = 0$ (Fig. 2c), since enzyme is synthesized without sensible mass increase. The slope of the plot falls gradually to a steady value of approximately 140 by $t = 120$ minutes. This value is some 2·8 times the steady value of $d\alpha_c/dM$ reached during induction in
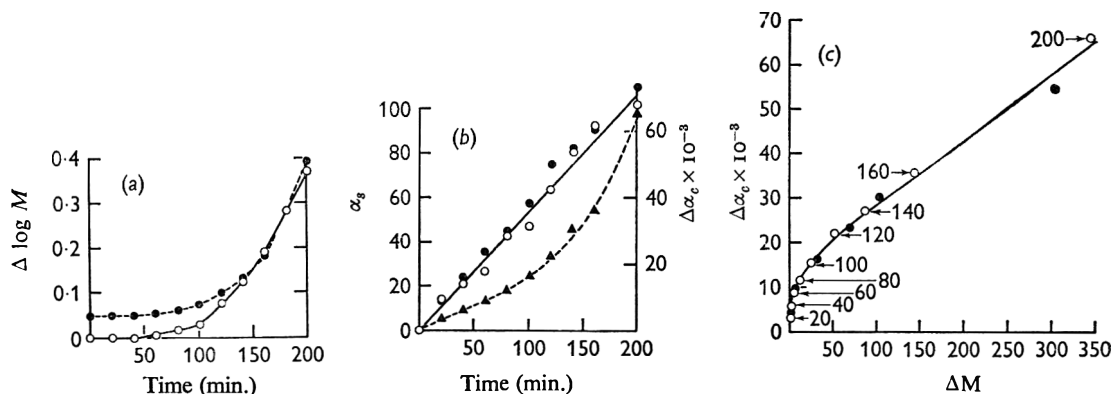
Fig. 2. Simultaneous induction of β-galactosidase and adaptation to maltose, of organisms never before exposed to either. (a) Growth, plotted as $\Delta \log M$, v. time: O—O and ● — — ● represent separate experiments (with somewhat different initial cell concentrations). Growth approaches exponential towards the end of the experiment, with m.g.t. $\approx$ 65 minutes. (b) Circles represent α_s for the corresponding culture in (a). ▲ – – – ▲ for culture indicated by closed circles. (c) Plot of $\Delta\alpha_c$ v. ΔM. Figures on curve are times after inoculation of culture O, in minutes.

maltose adapted cells. The maximum value of α_s is approximately twice the equilibrium value in adapted cells. This value is reached after 1·3 cell divisions in M + MTG medium, and the 'equilibrium' value of α_s ($\approx$ 50) is passed after 0·08 cell divisions.

This behaviour can be shown to represent a genuine preferential synthesis as follows. The alternative interpretation would be reincorporation of protein breakdown products into β-galactosidase molecules during protein turnover in the lag period, at the standard differential synthetic rate (see Mandelstam, 1957). This can be excluded because the turnover rate would have to be impossibly high to account for the large amount of enzyme synthesized. Suppose we plot the amount of enzyme synthesized per unit cell mass, $\Delta\alpha_c/M_o$, where $M_s =$ cell concentration at zero time, against time, during the lag and very early growth period (Fig. 3, curve A). The slope of this line, $(d/dt)(\Delta\alpha_c/M_o)$, subsequently referred to as ϵ, is effectively the rate of enzyme synthesis per unit mass per unit time.

Suppose that the β-galactosidase is all produced as a result of protein turnover with

reincorporation at the normal differential rate. If we define δM as the amount of mass turnover per unit total mass in unit time, ϵ must be in the same ratio to δM as is the small increment in α_c, $d\alpha_c$, to the small increment in M, dM, when a steady value of the differential rate of synthesis has been reached in actively growing organisms,

i.e. $$\frac{\epsilon}{\delta M} = \left(\frac{d\alpha_c}{dM}\right) \text{ equilibrium.}$$

One remaining difficulty lies in choosing an appropriate equilibrium value for the differential rate of enzyme synthesis in the experiments under consideration, since for most of the experiment the cells are not growing exponentially. The terminal gradient of the $\Delta\alpha_c$ v. ΔM plot in Fig. 2(c) gives the highest value which could be considered as an 'equilibrium' figure, since it is reached right at the beginning of exponential growth in maltose, about 3 hr after the inoculation into maltose medium. For different experiments it lies within the range 120–140. From Fig. 3, curve A, $\epsilon = 0.56$ enzyme units per unit mass per minute. Therefore if we write $(d\alpha_c/dM)_{\text{equilibrium}}$ $(= F_e) = 120$ to 140, it follows that $\delta M = \epsilon/F_e = 0.0040$ to 0.0046 mass units per unit mass per minute, which is equivalent to a mass turnover rate of 24 % to 28 %/hr. This would be a minimum figure, since if we took a lower value for F_e, δM would be correspondingly higher. What is more, only protein turnover is likely to lead to β-galactosidase synthesis, and since protein constitutes about 50 % of the cell mass, a minimum protein turnover of about 50 % to 60 %/hr would be required to account for all of the enzyme synthesis. This is out of the question, the results of Mandelstam (1957, 1960) and Willetts (1967) suggesting that protein turnover rates in non-growing organisms do not exceed 5 %/hr. In addition the value taken as representing F_e, 120 to 140, is much higher (by a factor of about 2·8) than the steady value in maltose adapted organisms, which itself requires explanation (it should be remembered that the m.g.t. of the organisms, during the first stages of growth in maltose, is about 2·5 times that of fully adapted organisms).

The above argument does not exclude the possibility of β-galactosidase synthesis via incorporation at a preferential (rather than a normal) rate, in contrast to *de novo* synthesis, but this would seem to be improbable on the following grounds. Protein turnover only occurs in the absence of growth (Mandelstam 1960). The hyperproduction of β-galactosidase in the present experiments continues into the period of active growth. There is no evidence of a change in mechanism at the onset of growth: α_s increases linearly from $t = 0$ to 160 min. This would seem to relegate synthesis via reincorporation to a secondary role, if it occurs at all.

Organisms partially adapted to maltose

Figure 4 gives results for organisms which had been transferred from glucose medium to maltose medium for two subcultures (ten generations) and then inoculated into M + MTG medium. β-Galactosidase synthesis was followed for about six generations after the beginning of induction. The organisms were maintained in exponential growth by transfer to fresh medium after each assay. The values of N, $\Delta\alpha_c$ and ΔM given are calculated as the total values which would have been attained if the culture could have been kept in exponential growth without transfer to fresh medium (e.g. if M in the original culture is 100, and a twofold dilution is made whenever M reaches 200 ΔM at successive transfers will be 100,300,700, ... ($100 \times 2^N - 100$)). Figure 4(b)

shows that after growth for ten generations in maltose medium there is no measurable lag before logarithmic growth, although the growth rate (m.g.t. = 48 min. at the beginning and 38 min. at the end of the experiment) is not maximal. There is no appreciable preferential synthesis of β-galactosidase, on commencing induction, and the slope of the $\Delta\alpha_c$ v. ΔM plot, 56, is very little different from that eventually reached by fully adapted cells.

Organisms stably adapted to lactose, gratuitously induced in maltose medium

The adaptation of wild-type *Aerobacter aerogenes* to lactose medium can be stabilized by prolonged subculture in that medium. The details are given in Richards & Hinshelwood (1962), but in brief such a stably adapted organism retains the ability to synthesise β-galactosidase during growth in maltose medium, and in the absence of
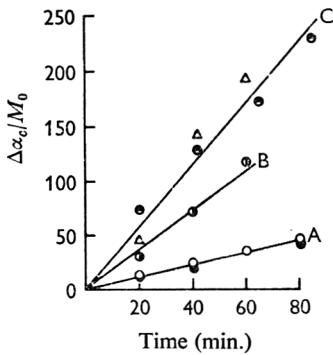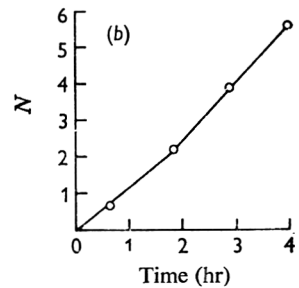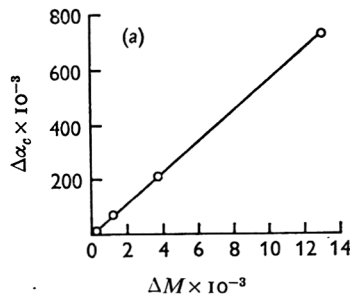
Fig. 3   Fig. 4

Fig. 3. Amount of enzyme synthesized per unit cell mass, $\Delta\,\alpha_c/M_0$, during initial lag in maltose medium + MTG. A: Organisms unadapted to lactose medium. (● and ○ represent different experiments) B and C: organisms stably adapted to lactose medium. B: washed organisms inoculated directly from lactose into maltose medium. C: organisms from lactose medium washed and incubated in medium without sugar (◒) or without ammonium sulphate (△) for 150 min. before inoculation into maltose medium.

Fig. 4. Gratuitous induction of a strain partially adapted to maltose. (a) Enzyme induction. There is an absence of preferential synthesis, and $d\alpha_c/dM$ (= 56) is very little different from that characteristic of fully adapted organisms. (b) Growth, expressed as number of generations (N) v. time, has not reached a rate characteristic of fully adapted organisms (m.g.t. at beginning of experiment = 48 min., at end, 38 min.).

inducer, to the extent of about 10–15 % of the value in induced organisms. This compares with about $\frac{1}{2}$ % in organisms not stably adapted, i.e. β-galactosidase synthesis is partially constitutive. In addition, stably adapted organisms grow immediately with maximal growth rate, on return to lactose medium after prolonged subculture in glucose or maltose medium, whereas unstably adapted strains develop lags and slow growth rates.

When such stably adapted organisms (L_s) are washed and transferred for the first time to maltose medium, growth in maltose follows the same pattern as in $G \rightarrow M$ transfers. α_s falls steadily to the value characteristic of the non-induced organisms (although enzyme synthesis continues, at a lower rate than in the induced organisms, throughout the period of dilution).

If the transfer of L_s organisms is made to M + MTG medium, there is a marked hyper-production of β-galactosidase, and α_s may rise as high as 700 (some five times as high as the normal value in L_s organisms growing exponentially in lactose, 120–150). This is shown in Fig. 5 for: (*a*) organisms which were transferred direct from lactose to M + MTG, after a single wash in buffer, (*b*) organisms which were taken from lactose, washed, and incubated at 40° in medium without the nitrogen source, ammonium sulphate, for 150 min., before inoculation into M + MTG, (*c*) organisms incubated as above, but in complete medium minus lactose.

These results would seem to exclude the possibility that any metabolic inter-
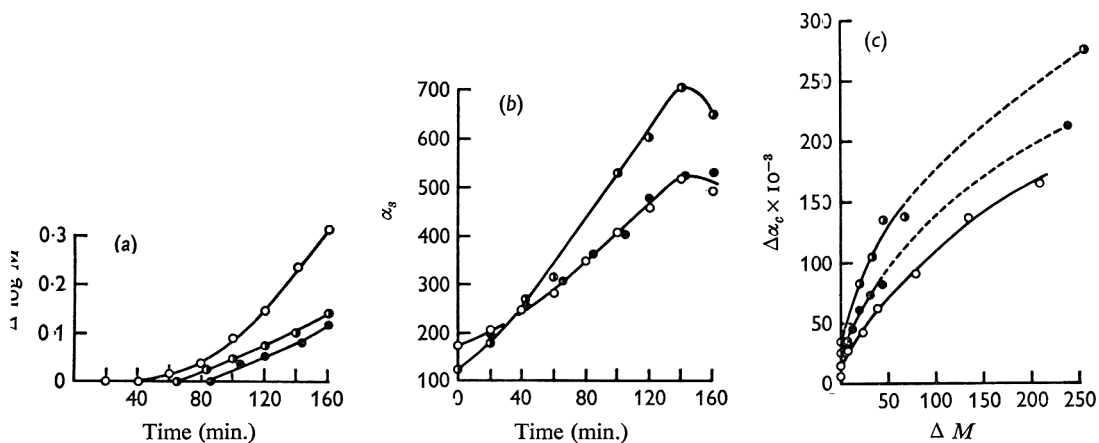
Fig. 5. A strain stably adapted to lactose (L_s), washed and transferred to maltose + MTG medium for the first time. ○, Direct transfer. ●, Organisms incubated for 150 min. in medium minus carbon source before inoculation into maltose medium. ◐, Organisms incubated for 150 min. in medium minus nitrogen source before inoculation into maltose medium. (*a*) Growth, in terms of Δ log M. (*b*) Specific enzyme activity. (*c*) New enzyme synthesis per unit volume of culture, against growth.

mediates carried over from the lactose medium might assist the preferential synthesis of β-galactosidase in M + MTG medium. In fact Fig. 3 (curves B and C) shows that enzyme synthesis during the earliest stages following transfer is most rapid (when measured in terms of enzyme units per unit mass per unit time) in the starved organisms. This suggests that, during starvation, ability to repress enzyme formation is lost, rather than ability to form enzyme on subsequent induction.

Arguments similar to those used in the previous section for G → M + MTG transfers can be applied here to show that there is a real preferential synthesis. It is harder to fix a maximum acceptable value for F_e. In none of the curves of Fig. 5 has a steady value of $d\alpha_c/dM$ been reached by the end of the experiment. α_s reaches a maximum value and begins to fall soon after the onset of exponential growth. It falls steadily during subculture in M + MTG (see later). If we assume that the maximum value of α_s corresponds roughly to the maximum reasonable value of F_e, we obtain minimum values for mass turnover rate of 22 %, 24 % and 32 %/hr for unstarved organisms, organisms starved without nitrogen source, and without carbon source, respectively. These would represent an impossibly high protein turnover rate of around 50 % per hour, if no preferential synthesis occurred.

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Relation of hyper-production of β-galactosidase, in L_s organisms, to state of adaptation to maltose

The experiments described below, involving various strains of *Aerobacter aerogenes*, suggest that the preferential synthesis, and subsequent hyper-production, of β-galactosidase, following a medium change of the type L → M + MTG, is a function of the degree of adaptation of the organisms to maltose, in much the same way as in G → M + MTG transfers.

If L_s organisms, initially unadapted to maltose, are serially subcultured in M + MTG medium, the value of α_s characteristic of mid-logarithmic phase bacteria falls steadily, as the organisms become adapted to maltose (Table 1). In all serial subcultures, inoculum size = 1/100 culture volume, so that on average each subculture is equivalent to 6·7 generations.

Table 1. *Values of specific activity of β-galactosidase (α_s) during the mid-logarithmic phase of organisms stably adapted to growth in lactose medium, being serially subcultured in maltose + MTG medium*

No. of subculture in M + MTG	α_s
1	≈ 600*
2	≈ 320
3	≈ 290
8	≈ 200
19	156
33	140
53	131
60	90
101	87

* Maximum value during exponential growth.

If the strain which is stably adapted to lactose is then subcultured in maltose until it is fully adapted (for 55 subcultures), before being grown in the presence of MTG, no preferential synthesis occurs (i.e. a plot of $\Delta\alpha_s$ v. ΔM gives a straight line through the origin), and the differential synthetic rate on gratuitous induction is approximately 150. This is much lower than the rate shortly after the change L → M + MTG with organisms unadapted to maltose, and is in fact very close to the value reached in organisms grown for the same number of subcultures in M + MTG (see Table 1).

When a strain fully adapted to maltose receives seven subcultures in lactose (conferring a partial, but not a stable, adaptation), before being washed and transferred to M + MTG medium, the graph of $\Delta\alpha_c$ v. ΔM is linear, passes through the origin, and has a slope of 50·8, characteristic of organisms adapted to maltose and growing in M + MTG with the equilibrium value of α_s.

DISCUSSION

The results confirm the existence of a genuine 'preferential gratuitous synthesis' of β-galactosidase, in which enzyme is synthesized during the growth lag following transfer to maltose medium for the first time. The amounts of enzyme synthesized during the lag are far too large to be explained in terms of a non-preferential reincorporation

of breakdown products into β-galactosidase molecules during protein turnover. This type of reincorporation was investigated by Mandelstam (1957), who suggested that it might operate in situations where other workers (e.g. Rickenberg & Lester, 1955; Richards & Hinshelwood, 1962) have reported preferential synthesis.

The preferential synthesis obtained during the present work, in the lag period, is associated with hyper-production of the enzyme during the early stages of growth in maltose: i.e. the initial differential synthetic rate is maintained at an unusually high level for some time. In fact, there seems to be no reason to distinguish between enzyme synthesis during the lag and during early growth. Both are aspects of a general hyper-production of enzyme.

The hyper-production is, in a qualitative sense, not dependent on the previous induction history of the cells. It occurs both with organisms which have never been induced for β-galactosidase, previous to their transfer to M + MTG, and also with L_s organisms transferred from lactose. It is, however, a function of the degree to which the organisms are adapted to maltose, being greatest with completely unadapted organisms, and diminishing progressively as adaptation proceeds.

An adequate and logical explanation of this behaviour seems to be possible in terms of the catabolite repression effect. The exposition of this effect given by Magasanik (1961) requires that the effectiveness of catabolite repression should be a function of the speed with which the organisms can degrade the substrate, and hence the extent to which intermediates of the degradation will accumulate in the cellular environment. In general, the more quickly a particular carbon source can be degraded by the organisms, the more effective should repression be. Several examples of this have been discovered, in particular those discussed by Mandelstam (1962). The present results are an extension to a situation where the speed of degradation of a given carbon source changes with time—to an adaptative process in fact. The factor limiting the growth rate will be the rate of degradation of the new carbon source, maltose. The original hyper-production is a result of a breakdown in catabolite repression following transfer to a slowly degraded catabolite. As adaptation proceeds, and the carbon source becomes more rapidly metabolisable, the catabolite repression reasserts itself.

In the present experiments, β-galactosidase is gratuitous and plays no part in the adaptation. It is, however, tempting to think that the phenomenon of transient catabolite de-repression may be a general feature of processes involving adaptation to a new substrate. When the organism finds itself in a situation where rapid degradation of substrate molecules is impossible, there is an initial relaxation of the control of enzyme synthesis, owing to the depletion of a repressor molecule or molecules, rendering the organism particularly sensitive to the action of any inducer, which in turn might be used as a substrate. As the efficiency of the compound as substrate increases, so does its efficiency as repressor.

REFERENCES

CLARK, D. J. & MARR, A. G. (1964). Studies on the repression of β-galactosidase in *Escherichia coli.* *Biochim. biophys. Acta* **92,** 85.

LOOMIS, W. F., JUN & MAGASANIK, B. (1964). The relation of catabolite repression to the induction system for β-galactosidase in *Escherichia coli. J. molec. Biol.* **8,** 417.

LOOMIS, W. F., JUN & MAGASANIK, B. (1967). The catabolite repression gene of the *lac* operon in *Escherichia coli. J. molec. Biol.* **23,** 487.

MAGASANIK, B. (1961). Catabolite repression. *Cold Spring Harb. Symp. quant. Biol.* **26,** 249.

MANDELSTAM, J. (1957). Turnover of protein in starved bacteria and its relationship to the induced synthesis of enzyme. *Nature, Lond.* **179,** 1179.

MANDELSTAM. J. (1960). The intracellular turnover of protein and nucleic acids and its role in bio-chemical differentiation. *Bact. Rev.* **24,** 289.

MANDELSTAM, J. (1962). The repression of constitutive β-galactosidase in *Escherichia coli* by glucose and other carbon sources. *Biochem. J.* **82,** 489.

MOSES, V. & PREVOST, C. (1966). Catabolite repression of β-galactosidase synthesis in *Escherichia coli.* *Biochem. J.* **100,** 336.

NAKADA, D. & MAGASANIK, B. (1964). The roles of inducer and catabolite repressor in the synthesis of β-galactosidase by *Escherichia coli. J. molec. Biol.* **8,** 105.

NEIDHARDT, F. C. (1960). Mutant of *Aerobacter aerogenes* lacking glucose repression. *J. Bact.* **80,** 536.

NEIDHARDT, F. C. & MAGASANIK, B. (1956). Inhibitory effect of glucose on enzyme formation. *Nature, Lond.* **178,** 801.

PALMER, J. & MOSES, V. (1967). Involvement of the *lac* regulatory genes in catabolite repression in *Escherichia coli. Biochem. J.* **103,** 358.

PREVOST, C. & MOSES, V. (1967). Pool sizes of metabolic intermediates and their relation to glucose repression of β-galactosidase synthesis in *Escherichia coli. Biochem. J.* **103,** 349.

RICHARDS, N. & HINSHELWOOD, C. (1961). Observations on the β-galactosidase activity of *Bact. lactis aerogenes* (*Aerobacter aerogenes*). *Proc. R. Soc.* B **154,** 463.

RICHARDS, N. & HINSHELWOOD, C. (1962). Gradual stabilization of adaptive β-galactosidase in *Bact. lactis aerogenes. Proc. R. Soc.* B **156,** 20.

RICKENBERG, H. V. & LESTER, G. (1955). The preferential synthesis of β-galactosidase in *Escherichia coli. J. gen. Microbiol.* **13,** 279.

WILLETTS, N. S. (1967). Intracellular protein breakdown in non-growing cells of *Escherichia coli. Biochem. J.* **103,** 453.

The Incomplete Tricarboxylic Acid Cycle in the Blue-green Alga *Anabaena variabilis*

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SUMMARY

The presence of an incomplete tricarboxylic acid cycle in *Anabaena variabilis* and *Anacystis nidulans* is described. These blue-green algae lack both α-oxoglutarate dehydrogenase and succinyl-CoA synthetase. Succinyl-CoA was formed in extracts of *A. variabilis* by 3-ketoacyl CoA-transferase using acetoacetyl-CoA as CoA donor. The activities of the other tricarboxylic cycle enzymes were measured in extracts prepared from autotrophic organisms and from those grown in the presence of acetate. No alterations in activity indicative of enzyme repression or de-repression were observed. These results are discussed in relation to possible reasons for autotrophic behaviour.

INTRODUCTION

The photosynthetic autotrophically grown blue-green algae have been shown to metabolize acetate (Hoare & Moore, 1965; Carr & Pearce, 1966). Although isocitratase and malate synthetase were detected at low activities in extracts of *Anacystis nidulans* and *Anabaena variabilis* (Pearce & Carr, 1967*a*), incorporation of [^{14}C]acetate into *A. nidulans* gave no evidence for a glycoxylate cycle operation and indicated that the tricarboxylic acid cycle did not proceed further than α-oxoglutarate (Hoare, Hoare & Moore, 1967). The absence of α-oxoglutarate dehydrogenase from extracts of *A. variabilis* was reported by Pearce & Carr (1967*b*), and Smith, London & Stanier (1967) showed that several obligate autotrophs, blue-green algae and chemosynthetic bacteria, lacked α-oxoglutarate dehydrogenase and NADH$_2$ oxidase. The latter group also examined the incorporation of [^{14}C]acetate by obligate autotrophs and showed that it was transformed into only four amino acids, which were metabolically derived from α-oxoglutarate. This restricted flow of [^{14}C]carbon from acetate into a limited number of related amino acids had also been reported in *A. nidulans* (Hoare & Moore, 1965; Hoare *et al.* 1967) and by Kelly (1967) in *Thiobacillus neapolitanus*. The present communication presents enzymic evidence for an incomplete tricarboxylic acid cycle in *A. variabilis* and *A. nidulans*, from which α-oxoglutarate dehydrogenase and succinyl-CoA synthetase are absent. The role of 3-ketoacyl CoA-transferase in succinyl-CoA formation is described and the failure to adjust enzymic activities after growth in the presence of acetate noted.

METHODS

Growth and preparation of cell-free extracts. *Anabaena variabilis* and *Anacystis nidulans* were grown as previously described (Carr & Hallaway, 1965) on a defined

mineral-salt medium which was gassed with air + carbon dioxide (95 + 5, v/v). Sodium acetate (20 mM) was added to this medium as indicated in the text. After harvesting, organisms were disrupted by ultrasonic treatment as previously described (Pearce & Carr, 1967a).

Citrate synthase (EC 4.1.3.7) assay. The disappearance of acetyl-phosphate in the presence of CoA and oxaloacetate was followed by using the procedure of Ochoa (1955). Acetyl-phosphate was estimated as the hydroxamic acid and an appropriate control permitted a correction for the small amount of acetyl-CoA metabolized by de-acylation. This assay utilized the phosphotransacetylase present in extracts of *Anabaena variabilis* (Pearce & Carr, 1967a).

Aconitase (EC 4.2.1.3) assay. The rate of reduction of NADP in the presence of citrate or cis-aconitate was followed by increase in E_{340} (Ochoa, 1948). Cuvettes contained (μmole): potassium phosphate buffer (pH 7·0), 200; $MgCl_2$, 5; NADP, 0·5; isocitrate dehydrogenase preparation, 50 μl. (activity, 9 μmole/min./ml.); cell extract containing 3 to 5 mg. protein. The reaction was started by adding substrate (10 μmole) to the experimental cuvette.

Isocitrate dehydrogenase (EC 1.1.1.42) assay. This enzyme was measured by the procedure of Ochoa (1948).

Glutamate dehydrogenase (EC 1.4.1.4) assay. The procedure used depended upon the oxidation of $NADPH_2$ in the presence of α-oxoglutarate and observation of decline in E_{340} (Bulen, 1956).

α-Oxoglutarate dehydrogenase (EC 1.2.4.2) assay. The rate of reduction of NAD or NADP in the presence of α-oxoglutarate was measured (Kaufman, Gilvarg, Cori & Ochoa, 1953). The assay mixture contained (μmole): potassium phosphate buffer (pH 7·0), 200; NAD or NADP, 0·5; cysteine, 10; CoA, 0·05; 3 to 7 mg. protein in 2·7 ml. total volume. The reaction was started by adding α-oxoglutarate (20 μ-mole) to the experimental cuvette.

Succinic dehydrogenase (EC 1.3.99.1) assay. The spectrophotometric assay of Ells (1959) was used.

Succinyl-CoA synthetase (EC 6.2.1.5) assay. Formation of succinyl hydroxamic acid in the presence of hydroxylamine was measured (Kaufman et al. 1953). The following substances (μmole) were incubated at 34° in test tubes: potassium phosphate buffer (pH 7·0), 50; $MgCl_2$, 10; cysteine, 20; CoA, 0·05; ATP, 5; freshly neutralized hydroxylamine hydrochloride, 1000; sodium succinate, 200; total volume 2·0 ml. The reaction was started by adding cell extract containing 5–10 mg. protein; after incubation the hydroxamate formed was estimated as the ferric chloride complex.

Fumarase (EC 4.2.1.2) assay. The decline in fumarate concentration, measured by E_{240}, was measured according to the procedure of Racker (1950).

Malate dehydrogenase (EC 1.1.1.37) assay. The activity of this enzyme was determined by oxidation of $NADH_2$ in the presence of oxaloacetate (Mehler, Kornberg, Grisolia & Ochoa, 1948).

Malic enzyme (EC 1.1.1.40) assay. This enzyme was measured by the procedure of Ochoa, Mehler & Kornberg, (1948).

3-Ketoacyl CoA-transferase (EC 2.8.3.5) assay. Acetoacetyl-CoA and Mg^{2+} form a complex with an absorption maximum at 303 mμ (Stern, Coon & del Campillo, 1953). Cuvettes contained (μmole): tris (pH 8·5), 200; $MgCl_2$, 10; acetoacetyl-CoA, 0·05; extract (containing 1 to 5 mg. protein); total volume of 3 ml. The control cuvette did

not contain acetoacetyl-CoA, and the reaction was started by addi:1g succinate (10 μ-mole) to each cuvette and the decline in E_{310} measured (Carr & Lascelles, 1961).

Estimation of protein. The amount of protein present in extracts was determined colorimetrically after removal of the photosynthetic pigments by hot acid ethanol as previously described (Pearce & Carr, 1967*a*).

Preparation of acetoacetyl-CoA and acetyl-CoA. Acetoacetyl-CoA was synthesized from diketene and CoA by the procedure of Lynen *et al.* (1958). Acetyl-CoA was prepared from acetic anhydride and CoA by a method based on that of Simon & Shemin (1953) for succinyl-CoA preparation.

Paper chromatography. Organic acids were identified by co-chromatography with authentic samples in *n*-butanol + acetic acid + water ($60 + 15 + 25$ by vol.), ethanol + ammonia + water ($80 + 5 + 15$) and phenol + water + formic acid ($75 + 25 + 1$). Acids were detected as yellow spots on a blue background after dipping the chromatograms in bromocresol green reagent [bromocresol green ($0\cdot1\%$) in an ethanol + acetone mixture ($20 + 81$)].

Chemicals. All chemicals were of analytical grade, or of the purest commercial grade available. ATP, ADP, AMP, CoA, ITP, NADP, NADPH$_2$, NAD and isocitrate dehydrogenase were purchased from C. F. Boehringer Ltd., Mannheim, Germany; acetyl phosphate from Sigma London Chemical Co. Ltd., Lettice Street, London, S.W.6. Diketene was kindly supplied by the Department of Organic Chemistry, University of Liverpool.

RESULTS

Cell-free extracts of *Anabaena variabilis* contained most of the tricarboxylic acid cycle enzymes at comparable degrees of activity. Succinic dehydrogenase was present but the activity was less than one tenth that of the other enzymes found. α-Oxoglutarate dehydrogenase and succinyl-CoA synthetase were not detected in any extract of *A. variabilis* or *Anacystis nidulans* (Table 1). Aconitase activity was significantly greater with cis-aconitate than with citrate as substrate. Experimental controls showed

Table 1. *Tricarboxylic acid cycle and some associated enzymes in extracts of Anabaena variabilis*

Activities are expressed as mμmole/min./mg. protein, and are the mean of several determinations. nt, not tested.

	Organism grown with	
Enzyme	CO$_2$	CO$_2$ + ace-tate (20 mM)
Citrate-condensing enzyme	$5\cdot7$	$6\cdot1$
Aconitase (citrate as substrate)	$2\cdot1$	nt
Aconitase (cis-aconitate as substrate)	$11\cdot9$	nt
Isocitrate dehydrogenase	$4\cdot7$	$5\cdot0$
α-Oxoglutarate dehydrogenase	0	0
Succinic thiokinase	0	0
Succinic dehydrogenase	$0\cdot2$	$0\cdot2$
Fumarase	$3\cdot7$	$3\cdot5$
Malate dehydrogenase	$2\cdot0$	$2\cdot3$
Glutamate dehydrogenase	$2\cdot7$	$2\cdot4$
Malic enzyme	$2\cdot0$	$2\cdot3$
3-Ketoacyl-CoA transferase	$2\cdot5$	nt

that aconitase was not present in the commercial preparation of isocitrate dehydrogenase used in the aconitase assay procedure. The isocitrate dehydrogenase in extracts of *A. variabilis* was specific for NADP, there being no activity with NAD as coenzyme. After the addition to the reaction mixture of (5 μmole) AMP or ADP no alteration in the rate with NADP was observed and NAD was still inactive. Glutamate dehydrogenase activity in these extracts was also specific for NADP, as was the conversion of malate to pyruvate and carbon dioxide by the malic enzyme.
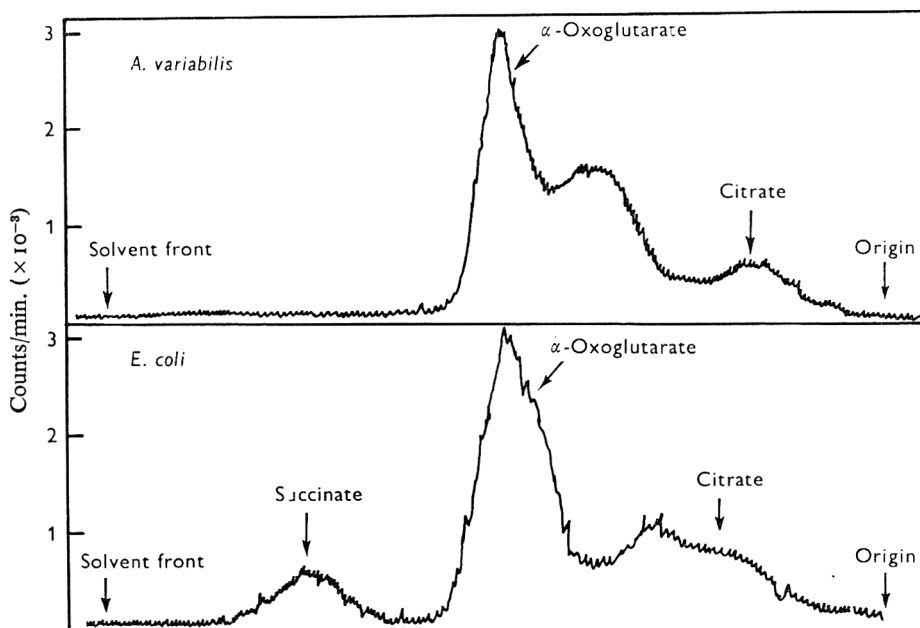
Fig. 1. Products obtained after incubation of [5-^{14}C]α-oxoglutarate (10 μmole containing 10 μc) in the presence of the *Anabaena variabilis* extract (8 mg. protein) for 20 min. and *Escherichia coli* extract (5 mg. protein) for 8 min. under the conditions used for the α-oxoglutarate dehydrogenase assay (see Methods). Products were separated by paper chromatography (butanol + acetic acid + water, 60 + 15 + 25 by vol). and radioactive compounds detected by a Nuclear-Chicago Actigraph III chromatogram scanner. Compounds were identified by co-chromatography with authentic samples (see Methods).

The slow rate of succinate oxidation by extracts of *Anabaena variabilis* was not increased by adding back pellet fraction to the cell-free extract; the activity of succinic dehydrogenase was of the same order as that of isocitrate lyase (Pearce & Carr, 1967*a*). Neither α-oxoglutarate dehydrogenase nor succinyl-CoA synthetase were detected in extracts of the two blue-green algae examined. Different pH values of the assay procedure and the addition of thiamine pyrophosphate, lipoic acid and FAD singly or together did not permit detection of the former enzyme, which was readily shown, using the identical procedure, in extracts of *Escherichia coli*. The absence of α-oxoglutarate dehydrogenase in *A. variabilis* was confirmed by examination of the products of [5-^{14}C]α-oxoglutarate incubation. The incubation mixture was that of the α-oxoglutarate dehydrogenase assay system (see Methods) with the addition of ^{14}C-labelled substrate. After anaerobic incubation at 34° the reaction was ended by the addition

of hot ethanol (20 ml.). After further extraction with ethanol the combined extracts were reduced in volume by rotary distillation under low pressure at 40° and products separated by paper chromatography (Fig. 1). Although citrate and glutamate were produced from [5-^{14}C]α-oxoglutarate, no succinate, malate or fumarate were found after varied incubation times (15 to 120 min.). This was in contrast to the results of an identical experiment with *E. coli* in which [^{14}C]succinate was readily detected.

The activation of succinate to succinyl-CoA could be not accomplished directly in extracts of *Anabaena variabilis* or *Anacystis nidulans* in spite of replacement of ATP with GTP or ITP, although enzymes necessary for acetate activation were present in such extracts (Pearce & Carr, 1967 a). Succinyl-CoA formation by a 3-ketoacyl CoA-transferase from acetoacetyl-CoA and succinate was detected; the addition of succinate, in the presence of cell free extract of *A. variabilis*, produced a marked decline in
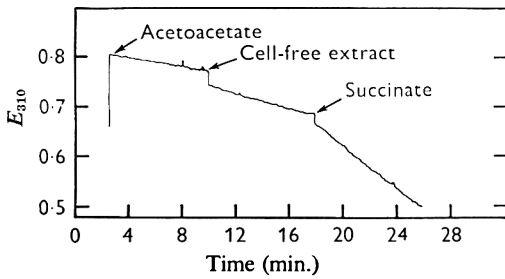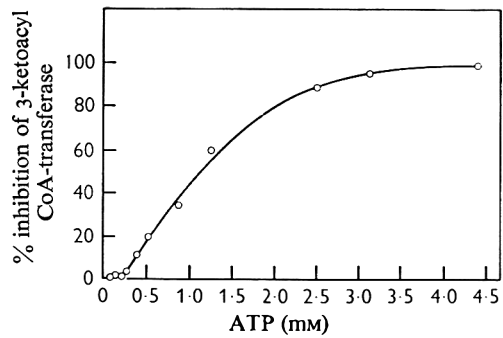
Fig. 2. 3-Ketoacyl CoA-transferase in extracts of *Anabaena variabilis* was measured by the decline in E_{310}, due to an acetoacetyl-CoA + Mg^{2+} complex, in the presence of succinate. The assay was done at room temperature (20°) in a Unicam SP 700 recording spectrophotometer (see Methods), additions to the cuvettes were as shown.

Fig. 3. The inhibition of 3-ketoacyl CoA-transferase, in extracts of *Anabaena variabilis*, by increasing amounts of ATP. Results are expressed as the % inhibition of enzyme activity, measured as in Methods except that ATP was added as shown.

the acetoacetyl-CoA + Mg^{2+} complex measured by a decrease in E_{310}, corresponding to a rate of 2·5 mμmole/min./mg. protein (Fig. 2). This enzyme was not sedimented by centrifugation for 2 hr at 75,000 g and was stable for 2 weeks at −18°. 3-Ketoacyl CoA-transferase was inhibited by ATP (Fig. 3), the sigmoidal kinetics of this inhibition being indicative of an allosteric interaction. AMP and ADP were also inhibitory but at 10-fold higher concentration and with no evidence of sigmoidal kinetics. The 3-ketoacyl CoA-transferase was specific for acetoacetyl-CoA; acetyl-CoA was only 1 % as effective as a CoA donor.

An important feature of enzyme activities concerned in tricarboxylic acid metabolism in *Anabaena variabilis* was the failure of the organism to adjust enzymic content following the inclusion of acetate (20 mM) in growth medium. There was no increase in activity of any of the tricarboxylic acid cycle enzymes, nor of the associated malic enzyme and glutamate dehydrogenase in extracts grown in the presence of acetate (Table 1). Previous work had shown that, under the conditions used, acetate was significantly incorporated and assimilated into *A. variabilis* (Pearce & Carr, 1967 a).

DISCUSSION

Enzymes present in extracts of *Anabaena variabilis* would permit the operation of an incomplete tricarboxylic acid cycle, as illustrated in Fig. 4. The absence of α-oxoglutarate dehydrogenase and succinyl-CoA synthetase activity prevents cyclic flow, and this is consistent with the entry of [^{14}C]acetate into only a limited number of amino acids, first observed by Hoare & Moore (1965). The synthesis of oxoloacetate, and hence the aspartate family of amino acids, could arise from the carboxylation of pyruvate or phosphoenol pyruvate. Some formation of succinate would be possible
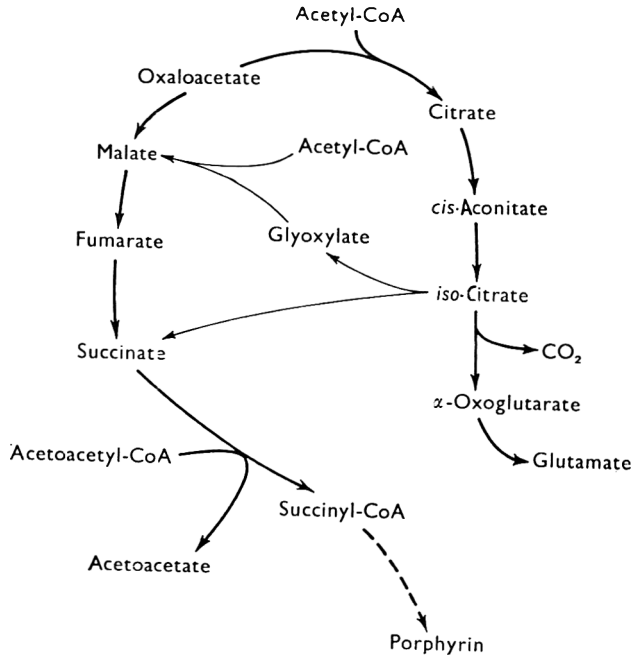
Fig. 4. The interrupted tricarboxylic acid cycle in *Anabaena variabilis*.

by the activity, detected at low levels, of the glyoxylate cycle enzymes (Pearce & Carr, 1967a). The activation of succinate to succinyl-CoA, necessary for all porphyrin formation, did not proceed directly but by a transferase enzyme from acetoacetyl-CoA, which was allosterically inhibited by ATP. Isocitrate dehydrogenase in extracts of *A. variabilis* required NADP as coenzyme and there was no evidence of activation by AMP or ADP, as is found with the NAD specific enzyme in other organisms (Kornberg & Pricer, 1951; Hathaway & Atkinson, 1963). This lack of stimulation is consistent with a biosynthetic, rather than energy-yielding, function of this enzyme. Both glutamate dehydrogenase and the malic enzyme, in *A. variabilis*, were also specific for NADP.

The production of [^{14}C]carbon dioxide from carboxyl-labelled acetate by *Anabaena variabilis* was readily shown whereas the methyl-carbon contributed only very slightly to respiratory carbon dioxide (Pearce & Carr, 1967a). The release of carbon dioxide from the [1-^{14}C] position of acetate presumably arises from the metabolism of α-oxoglutarate, via glutamate or through the operation of a glyoxylate cycle.

An incomplete tricarboxylic acid cycle lacking α-oxoglutarate has been reported in two photosynthetic bacteria, *Chloropseudomonas ethylicum* (Callely, Rigopoulos & Fuller, 1968) and Chromatium D (Fuller, Smillie, Sisler & Kornberg, 1961). The interruption of the cycle at the point of α-oxoglutarate not only deprives the organism of a site of substrate level phosphorylation, but severely curtails the formation of reduced pyridine nucleotides by oxidation of organic substrates. Smith *et al.* (1967) suggested that the absence of α-oxoglutarate dehydrogenase and of $NADH_2$ oxidase are the lesions in metabolism that cause an organism to be an obligate autotroph. The break in the tricarboxylic acid cycle could be alleviated by the exogenous supply of C_4 intermediates, and the latter workers place particular emphasis on the absence of $NADH_2$ oxidase which would prevent the organism from linking, via an electron transport chain, oxidation of organic substrates to ATP generation. However, Hempfling & Vishniac (1965) reported an $NADH_2$ oxidase in extracts of the obligate autotroph *Thiobacillus neapolitanus* and Trudinger & Kelly (1968) recorded the reduction of endogenous cytochrome *c* by $NADH_2$ in the same organism. The presence of $NADPH_2$ oxidase in extracts of several obligate autotrophic blue-green algae has also been measured (Leach & Carr, 1968).

A feature of the data presented in this communication is the absence of any alteration in enzymic activities measured after inclusion of acetate in the growth medium. Tricarboxylic acid cycle enzymes in certain other micro-organisms are markedly affected by the growth medium, inclusion of acetate increased activity generally in *Hydrogenomonas* sp. (Trüper, 1965) and *Aerobacter aerogenes* (Forget & Pichinoty, 1967). The activity of isocitrate dehydrogenase was several fold greater after growth of *Escherichia coli* and *Pseudomonas aeruginosa* on acetate rather than glucose (Pearce & Carr, unpublished). In other aspects of intermediary metabolism *Anabaena variabilis* has been shown not to exercise control over enzyme biosynthesis but only on existing enzyme molecules by end-product interaction (Carr, 1967; Pearce & Carr, 1967*a*; Pearce & Carr, 1968; Hood & Carr, 1968). The failure of *A. variabilis* and other blue-green algae to increase growth rate in the presence of substrates known to be assimilated and metabolized could be due, at least in part, to lack of control at the transcription level. We suggest that this lack of control may be a characteristic and causative feature of obligate autotrophs.

REFERENCES

BULEN, W. A. (1956). The isolation and characterization of glutamic dehydrogenase from corn leaves. *Archs Biochem. Biophys.* **62**, 173.

CALLELY, A. G., RIGOPOULOS, N. & FULLER, R. C. (1968). The assimilation of carbon by *Chloropseudomonas ethylicum. Biochem. J.* **106**, 615.

CARR, N. G. (1967). Aspects of enzymic control in blue-green algae. Abstr. *5th Meeting Fedn. Europ. Biochem. Soc.*, Universitetsforlaget, Oslo, p. 89.

CARR, N. G. & HALLAWAY, M. H. (1965). Reduction of phenolindo-2,6-dichlorophenol in dark and light by the blue-green alga, *Anabaena variabilis. J. gen. Microbiol.* **39**, 335.

CARR, N. G. & LASCELLES, J. (1961). Some enzymic reactions concerned in the metabolism of acetoacetyl-coenzyme A in Athiorhodaceae. *Biochem. J.* **80**, 70.

CARR, N. G. & PEARCE, J. (1966). Photoheterotrophism in blue-green algae. *Biochem. J.* **99**, 28 P.

ELLS, H. A. (1959). A colorimetric method for the assay of soluble succinic dehydrogenase and pyridine nucleotide-linked dehydrogenase. *Archs Biochem. Biophys.* **85**, 561.

FORGET, P. & PICHINOTY, F. (1967). Le cycle tricarboxylique chez *Aerobacter aerogenes*. *Annls Inst. Pasteur, Paris* **112**, 261.

FULLER, R. C., SMILLIE, R. M., SISLER, E. C. & KORNBERG, H. L. (1961). Carbon metabolism in Chromatium. *J. biol. Chem.* **236**, 2140.

HATHAWAY, J. A. & ATKINSON, D. E. (1963). The effect of adenylic acid on yeast nicotinamide adenine dinucleotide isocitrate dehydrogenase, a possible metabolic control mechanism. *J. biol. Chem.* **238**, 2875.

HEMPFLING, W. P. & VISHNIAC, W. (1965). Oxidative phosphorylation in extracts of Thiobacillus. *Biochem. Z.* **342**, 272.

HOARE, D. S. & MOORE, R. B. (1965). Photoassimilation of organic compounds by autotrophic blue-green algae. *Biochim. Biophys. Acta* **109**, 622.

HOARE, D. S., HOARE, S. L. & MOORE, R. B. (1967). The photoassimilation of organic compounds by autotrophic blue-green algae. *J. gen. Microbiol.* **49**, 351.

HOOD, W. & CARR, N. G. (1968). Threonine deaminase and acetolactate synthetase in *Anabaena variabilis*. *Biochem. J.* **109**, 4 P.

KAUFMAN, S., GILVARG, C., CORI, O. & OCHOA, S. (1953). Enzymatic oxidation of α-ketoglutarate and coupled phosphorylation. *J. biol. Chem.* **203**, 869.

KELLY, D. P. (1967). The incorporation of acetate by the chemoautotroph *Thiobacillus neapolitanus* strain C. *Arch. Mikrobiol.* **58**, 99.

KORNBERG, A. & PRICER, W. E. (1951). Di- and triphosphopyridine nucleotide isocitric dehydrogenase in yeast. *J. biol. Chem.* **189**, 123.

LEACH, C. K. & CARR, N. G. (1968). Reduced nicotinamide adenine dinucleotide phosphate oxidase in the autotrophic blue-green alga, *Anabaena variabilis*. *Biochem. J.* **109**, 4 P.

LYNEN, F., HENNING, U., BUBLITZ, C., SORBO, B. & KRÖPLIN-RUEFF, L. (1958). Der chemische Mechanismus der Acetessigsäurebildung in der Leber. *Biochem. Z.* **330**, 269.

MEHLER, A. H., KORNBERG, A., GRISOLIA, S. & OCHOA, S. (1948). The enzymatic mechanism of oxidation reductions between malate or isocitrate and pyruvate. *J. biol. Chem.* **174**, 961.

OCHOA, S. (1948). Biosynthesis of tricarboxylic acids by carbon dioxide fixation. *J. biol. Chem.* **174**, 133.

OCHOA, S. (1955). Crystalline condensing enzyme from pig heart. *Meth. Enzymol.* **1**, 685.

OCHOA, S., MEHLER, A. H. & KORNBERG, A. (1948). Biosynthesis of dicarboxylic acids by carbon dioxide fixation. *J. biol. Chem.* **174**, 979.

PEARCE, J. & CARR, N. G. (1967a). The metabolism of acetate by the blue-green algae, *Anabaena variabilis* and *Anacystis nidulans*. *J. gen. Microbiol.* **49**, 301.

PEARCE, J. & CARR, N. G. (1967b). An incomplete tricarboxylic acid cycle in the blue-green alga, *Anabaena variabilis*. *Biochem. J.* **105**, 45 P.

PEARCE, J. & CARR, N. G. (1968). The incorporation and metabolism of glucose by *Anabaena variabilis*. *J. gen. Microbiol.* **54**, 451.

RACKER, E. (1950). Spectrophotometric measurement of the enzymatic formation of fumaric and *cis*-aconitic acids. *Biochim. biophys. Acta* **4**, 211.

SIMON, E. J. & SHEMIN, D. (1953). The preparation of S-succinyl coenzyme A. *J. Am. chem. Soc.* **75**, 2520.

SMITH, A. J., LONDON, J. & STANIER, R. Y. (1967). Biochemical basis of obligate autotrophy in blue-green algae and thiobacilli. *J. Bact.* **94**, 972.

STERN, J. R., COON, M. J. & DEL CAMPILLO, A. (1953). Acetoacetyl coenzyme A as intermediate in the enzymatic breakdown and synthesis of acetoacetate. *J. Am. chem. Soc.* **75**, 1517.

TRUDINGER, P. A. & KELLY, D. P. (1968). Reduced nicotinamide adenine dinucleotide oxidation by *Thiobacillus neopolitanus* and *Thiobacillus* strain C. *J. Bact.* **95**, 1962.

TRÜPER, H. G. (1965). Tricarboxylic acid cycle and related enzymes in Hydrogenomonas strain H 16 G+ grown on various carbon sources. *Biochim. biophys. Acta* **111**, 565.

Studies on Negative Chemotaxis and the Survival Value of Motility in *Pseudomonas fluorescens*

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SUMMARY

Multiplication of aerobic piliated fla$^+$ and fla$^-$ *Pseudomonas fluorescens* growing in aerated mixed culture gave fla$^+$:fla$^-$ ratios approximately 1:1 over a 24 hr growth period. When fla$^+$ and fla$^-$ were cultured simultaneously in non-aerated media, fla$^+$ then outgrew the fla$^-$ mutant to a final (24 hr) ratio of 10:1 or greater, thereby lending supporting evidence to the view that motility is a survival factor in environments where 'nutrients' are limited or discontinuous.

Gravity, magnetism, and light did not influence a negative chemotactic response of fla$^+$ *P. fluorescens* to hydrogen ions. Temperature variations, pH, or viscosity alterations either inhibited or stimulated motility, thereby making impossible a determination of their specific effects. Various antibiotics, membrane-active agents, and protein or DNA synthesis inhibitors were employed in attempts to interfere with the chemotactic response, but none completely inhibited chemotaxis without also affecting motility. The cytoplasmic membrane may act as a transducer of environmental stimuli in chemotaxis.

INTRODUCTION

The positive value of motility as a survival factor for bacteria in environments where 'nutrients' or 'harmful agents' are discontinuously distributed seems fairly obvious; however, little verification of this view exists outside of a few theoretical speculations. Carlson (1962) suggested that no matter how inefficient the motility mechanism, an organism could always achieve a net gain in collection of nutrients by moving. Piliation also is reported to favour survival of aerobic bacteria by allowing surface film formation in static cultures (Brinton, 1959). The experimental results presented here were obtained from a study on a system wherein the factor favouring multiplication was oxygen, which has long been known to invoke an aerotactic response in several kinds of bacteria including Pseudomonas (Beijerinck, 1893; Baracchini & Sherris, 1959).

Tactic responses involve not only mechanisms by which bacteria move, but imply the (possible) existence of sensing and transducing devices which direct bacteria towards conditions most suitable for survival and multiplication (Doetsch & Hageage, 1968).

Little in the way of postulated mechanisms of tactic responses has been published for bacterial systems. Links (1955) maintained that any factor which can suddenly and reversibly increase the quantity or consumption of energy-supplying substance

to the motor apparatus can exercise a chemotactic effect. Clayton (1958) modified Links's hypothesis and proposed that the phototaxis in *Rhodospirillum rubrum* involved an ATP-mediated reaction closely associated with photosynthetic phosphorylation.

Recently Armstrong, Adler & Dahl (1967) isolated non-chemotactic mutants of *Escherichia coli*, but were unable to determine the reason for the lack of chemotactic activity.

Hydrogen ions, which stimulate negative chemotaxis in fla$^+$ *Pseudomonas fluorescens*, provide a means for investigation which is rapid, repeatable, and simple (Smith & Doetsch, 1968). The present paper reports experiments attempting to uncover the basis of this negative chemotactic response. In our view, 'negative chemotaxis' is characterized by an abrupt change in the direction of translational motility. This behaviour is postulated to be caused by a critical change in the membrane potential (the membrane serving as a non-specific environmental monitor) when a bacterium encounters a chemical stimulus. The energy of such stimuli is transduced into a signal causing a reversal in direction of flagellar motion. A mechanism whereby this might be accomplished has been suggested by Doetsch & Hageage (1968). All chemotactic responses by bacteria may thus be considered movements *away* from environmental conditions which induce a critical change in membrane potential.

METHODS

Bacteria studied. Pseudomonas fluorescens (University of Maryland 401), an obligately aerobic, actively motile bacterium (fla$^+$) with a single polar flagellum approximately 5 μ long was selected for this investigation.

Mutant isolation. Non-motile (fla$^-$) mutants were obtained by a method similar to that of Armstrong *et al.* (1967) by growing the fla$^+$ organisms at 30° in 10 ml. Trypticase Soy Broth (TSB) (Baltimore Biological Laboratories, Baltimore, Md., U.S.A.) overnight, inoculating one loopful of culture on to the centre of a Tryptone Semisolid Agar (TSSA) (Difco Laboratories, Detroit, Mich., U.S.A.) plate prepared with 1 % (w/v) Bacto-Tryptone and 0·35 % (w/v) Bacto-Agar. Plates were sealed with masking tape to prevent drying and incubated overnight at 30°. Growth was harvested daily with a Pasteur pipette from the centre of the large colony which developed, and washed once in 3 ml. TSB; one loopful of this suspension then was used to inoculate a fresh TSSA plate. The procedure was repeated successively for 10 days at which time the suspension was streaked on to several Trypticase Soy Agar (TSA) (Baltimore Biological Laboratories) plates for colony development and isolation. These plates were incubated 24 hr at 30°, colonies were picked and each inoculated by stabbing into tubes containing 10 ml. Sulfide Indole Motility (SIM) medium (Difco Laboratories). After overnight incubation at 30°, the tubes were examined for the presence of non-motile isolates. These were checked for: Gram-negative staining reaction (Hucker), formation of small (1 mm. diameter) colonies on TSSA, growth in phenol-red + sucrose broth (Difco), weak acidic reaction in dextrose + phenol-red broth (Difco), basic reaction in litmus milk (Difco), nitrate reduction, inability to liquefy gelatin, presence of cytochrome oxidase using Taxos N Neisseria–Pseudomonas detection discs (Baltimore Biological Laboratories), and a growth rate in TSB comparable to the parent strain. All tests requiring incubation were made at 30° for 24 hr. The mutant chosen formed

small (1 mm.), shiny, discrete colonies on TSA spread plates, whereas the parent strain formed larger, less opaque, colonies on the same medium. O antiserum prepared in rabbits against the fla$^-$ isolate was tested against the parent fla$^+$ strain and gave a positive rapid slide agglutination test reaction. When stained by Rhodes (1958) method, the mutant was non-flagellated. Both cultures had polar Type VI pili when grown 16–18 hr at 30° in TSB and stained with 1 % (w/v) sodium phosphotungstate (pH 7·0) on carbon-formvar coated copper grids and examined with an electron microscope (RCA EMU 3 f).

Selection experiments

Culture preparation. Fla$^+$ and fla$^-$ cultures were grown 16–18 hr at 25° in 100 ml. TSB contained in 150 ml. prescription bottles. Forty ml. of each culture were removed and aseptically washed twice in TSB after centrifuging at 2000 *g* for 15 min. each washing. Each suspension was adjusted with TSB to a 1·0 absorbance at 425 mμ.

Determination of survival. Fla$^+$ and fla$^-$ cultures were distributed into five 250 ml. cotton-stoppered sidearm flasks containing 100 ml. TSB in the following manner: flask 1, 2 ml. fla$^+$ suspension; flask 2, 2 ml. fla$^-$ suspension; flasks 3, 4 and 5 contained 1 ml. fla$^+$ and 1 ml. fla$^-$. Flasks 1, 2 and 3 were shaken at 20 rev./min. on an incubator-shaker at 30°, and flasks 4 and 5 were incubated without shaking. Colony counts were made from flasks 3, 4 and 5 after 8 and 24 hr by spreading 0·1 ml. of a diluted suspension on to TSA plates which had been dried overnight at 37°, then incubated at 30° for 24 hr. Multiplication of the culture also was followed by measuring absorbance of flasks 1 and 2 for 8 hr at 425 mμ. Flasks 4 and 5 were not shaken except for sampling, each being sampled only once to minimize any effect shaking would have on the results if only a single flask were used. After 24 hr incubation at 30° on TSA spread plates, fla$^+$:fla$^-$ ratios at the different time intervals tested were determined.

Chemotaxis experiments

Culture preparation. Forty ml. of a fla$^+$ *Pseudomonas fluorescens* culture grown in 100 ml. TSB in a 150 ml. prescription bottle for 16–18 hr at 30° were washed once in pH 7·0 buffer (modified from Adler, 1966*b*), and resuspended to the original volume to give an absorbance of 0·25 at 425 mμ or approximately $3·0 \times 10^8$ bacteria/ml. The buffer consisted of 10^{-2} M-mono- and di-basic K phosphates, 10^{-3} M-MgSO$_4$ and 10^{-4} M-EDTA. When chemicals were added to the buffer, this was done first and then drops of a dense suspension of bacteria (50 ml. washed as previously described and resuspended to 3 ml.) were added with a Pasteur pipette to give the above-mentioned absorbancy.

Flat glass capillary preparation. Flat glass capillaries prepared from 8 mm. internal diameter flint-glass tubing segments by the method of Wright & Colebrook (1921) were cut to 4 cm. lengths. Approximate inside dimensions were 1·2 mm. wide by 0·3 mm. deep.

Demonstration of negative chemotaxis. Capillary tubes were filled from Pasteur pipettes containing the test suspension; one end was plunged twice into plain (1·5 % w/v) 'Ionagar' (Oxoid) No. 2 (Consolidated Laboratories, Inc., Chicago, Ill.) so that a 1 cm. length agar plug was formed; the other end was plunged once into either acidic Ionagar (titrated to pH 1·0 at 55° with N-HCl) or basic Ionagar (pH 12·0 with N-NaOH), depending upon the experiment done. A bit of 'Vaseline' (Chesebrough Manuf. Co.) was placed at either end of the capillary to allow secure mounting on a glass

slide (Fig. 1). Observations were made with a Bausch & Lomb Stereozoom Variable
Power microscope, utilizing the substage concave mirror and a Nicholas Variable
Illuminator mounted in the substage lamp receptacle. The mirror was adjusted to give a
'dark-field' effect. Using an ocular micrometer, calibrated to read directly in mm. for
different magnifications, formation and migration of bacterial 'acid response bands'
(a.r.b.) or basic response bands (b.r.b.) were observed and recorded as μm. moved per
unit time. Active motility of suspensions sealed in capillary tubes for 3 hr revealed that
oxygen depletion would not be a factor affecting these experiments during the 30 min.
experimental time period. (The fla$^+$ *Pseudomonas fluorescens* used here is aerotactic
and motility ceased rapidly in very dense suspensions held under conditions of
limited aeration.)

Measurement of motility. Motility estimates made by the method of Shoesmith
(1960) were based on bright-phase observation of wet-mount preparations examined
with a Zeiss photomicroscope equipped with $\times 12 \cdot 5$ oculars and a $\times 63$ Neofluar phase
objective. The right eyepiece was fitted with a black cardboard diaphragm with a
centre opening 1 mm. in diameter. Motility of the suspensions was estimated by
counting the number of organisms swimming by the aperture during a 1 min. interval.
An average of five observations was used to estimate the 'per cent motile' for any
given sample.

Investigations of factors possibly influencing acid response bands

Temperature. Washed suspensions were divided into 3 ml. portions and incubated
30 min. at 4°, 20° and 37°. Acid response band formation and migration then were
followed for 30 min. after capillary tubes were prepared in triplicate on glass slides.
Experiments were made in 4° and 37° incubators and at room temperature ($\simeq$ 20°),
and where appropriate, a rapid visual estimation of motility was made.

Magnetic fields. Washed bacterial suspensions in the process of forming a.r.b. were
subjected to the effect of a 12,000 gauss horseshoe magnet (Alnico V) to determine the
influence of a strong non-fluctuating magnetic field on it. Capillary tubes prepared in
triplicate were placed both parallel and at right angles to the lines of force and at both
the north and south poles. Capillary tubes parallel to lines of force were positioned
with one end of the tube touching the magnetic pole, whereas tubes at right angles to the
lines of force were placed with the centre 5 mm. from one of the poles. Migration of
a.r.b. was followed for a period of 30 min. The effect of a fluctuating magnetic field was
observed by placing the capillary tube preparation on the centre of the platform of a
Mag-mix magnetic stirrer, Model 11-V-3 (Precision Scientific Co., Chicago, Ill.). The
capillaries were mounted 3 mm. above the surface of the platform to obviate any effects
of heat generated by the stirrer motor. The dissecting microscope optical system was
mounted above the platform so the a.r.b. migration could be followed, and a Nicholas
Illuminator was mounted on the adjustable lamp base. The slide preparation was
positioned so that the centre of the capillary tubes was at the centre of rotation of the
magnetic stirrer. Migration of a.r.b when viewed from above was from left to right; the
stirrer rotation was counterclockwise. Migration of a.r.b. formed was followed for
30 min. with the stirrer set at maximum output.

Gravity. Migration rates were determined in triplicate capillary tubes by mounting
the slide preparations (1) in a horizontal position, (2) with the a.r.b. ascending, and
(3) with the a.r.b. descending.

Viscosity. Buffered solutions (pH 7) of polyvinylpyrrolidone, (type NPJ-K 90:PVP) and Carbowax 4000, both 5 % (w/v), were tested for their ability to affect band formation and motility rate. Relative viscosity of each solution and the buffer was measured by noting the time necessary for the meniscus of the solution to drop a 4 cm. length in a vertically held Pasteur pipette.

Light. Capillary tubes again were prepared in triplicate. Migration rates were plotted in unfiltered light using the Nicholas Illuminator at full brightness (35 W.) and in the dark, except for brief illumination of the specimen so that readings could be made at 5 min. intervals. Wratten gelatin filters were mounted on a transparent glass
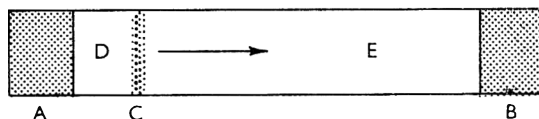
Fig. 1. Diagram of capillary tube experiment. A = acidic agar plug, B = plain Ionagar plug, C = ARB, D = zone of non-motile organisms, and E = motile organisms.
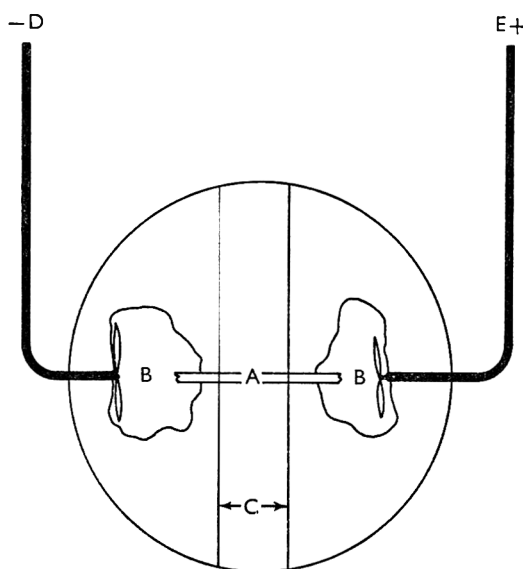
Fig. 2. 'Grid' used to measure effect of direct electric current on bacterial cells and acid response bands. A = capillary tube, B = saline agar, C = 3 cm. space cut in plastic Petri dish, D = cathode, and E = anode.

microscope stage; the capillary preparation then was placed on top of the filter. Formation and migration of a.r.b. was compared for 30 min. against the same experiment done in a darkroom. Wratten filters no. 58 (green, dominant wavelength 540·2 mμ), no. 25 (red, 615·1 mμ), no. 15 (yellow, 579·3 mμ), and no. 47 (blue, 463·7 mμ) were used.

Direct electric current. A Thomas Electrophoresis Unit, Model no. 21, was attached to a 'grid' of the design shown in Fig. 2. Capillary tubes were placed on the grid and saline agar (1 %, w/v, NaCl) was dropped on each end to ensure electrical contact with the electrodes. The voltage necessary to cause migration of heat-killed (60° for 15 min.)

bacteria was determined in cell suspensions in buffers of pH 5, 7 and 8 using Ionagar of pH 7·0 as capillary plugs. Ionagar plugs of pH 1 and 8 were used in the anode side of the capillary when the voltage necessary to disrupt any a.r.b. which formed in response to each type of plug was determined. Voltage necessary to cause migration of heat-killed bacteria with a pH 1 acid agar plug also was determined.

Influence of pH. Demonstration of the applicability of Weber's Law to negative chemotaxis was attempted by suspending washed bacteria in buffer adjusted to pH values of 5. 6, 7 and 8, and determining the reaction of each suspension to Ionagar plugs of pH 1, 2, 3 and 4. *Pseudomonas fluorescens* grown in bottles of TSB (100 ml.) at pH 6, 7 and 8 were washed and suspended in pH 7 buffer and judged for their ability to form a.r.b to agar plugs of pH 1, 2, 3 and 4.

Table 1. *Compounds tested for their effect on acid response bands and motility*

Compound	Concentration or range used*	Compound	Concentration or range used*
Dicoumarol	$1·5 \times 10^{-3}$ M	Chloramphenicol	$1·2 \times 10^{-2}$ to $1·2 \times 10^{-4}$ M
Eserine SO$_4$	1×10^{-3} M	'Triton'	1 to 10 mg./ml.
Acetylcholine Cl	1×10^{-3} M	Benzalkonium Cl	1 to 0·001 mg./ml.
Atabrine	$2·5 \times 10^{-3}$ M	*p*-Nitrophenyl glycerol	5×10^{-2} to 5×10^{-4} M
Indole acetic acid	2×10^{-3} M	(PNPG)	
Sodium dodecyl SO$_4$	$2·2 \times 10^{-3}$ M	Potassium cyanide	$2·5 \times 10^{-3}$ M
Dimethyl sulphoxide	1 to $9·8 \times 10^{-4}$ M	Polymyxin B SO$_4$	2100 units/ml.
(DMSO)		Phenethyl alcohol	1×10^{-1} to 5×10^{-4} M
Cetylpyridinium Cl	$2·6 \times 10^{-3}$ to $2·6 \times 10^{-6}$ M	*n*-Butanol	1×10^{-1} to 5×10^{-4} M
DL-*p*-Fluorophenylalanine	$5·5 \times 10^{-2}$ to $5·5 \times 10^{-3}$ M	Tyrocidine HCl	5×10^{-5} to 1×10^{-7} M
Urea	$1·67 \times 10^{-1}$ to $1·67 \times 10^{-2}$ M	Digitonin	1×10^{-6} M
Sodium barbital	$9·7 \times 10^{-3}$ to $9·7 \times 10^{-5}$ M	Serotonin creatinine SO$_4$	5×10^{-5} to 1×10^{-7} M
Morphine SO$_4$	$5·3 \times 10^{-3}$ to $2·7 \times 10^{-5}$ M	Bacitracin	5×10^{-5} to 5×10^{-7} M
Cocaine SO$_4$	$1·28 \times 10^{-2}$ to $5·9 \times 10^{-4}$ M	Strychnine SO$_4$	$4·7 \times 10^{-3}$ to $4·7 \times 10^{-5}$ M
Sanguinarine SO$_4$	$3·8 \times 10^{-3}$ to $3·8 \times 10^{-4}$ M	Pentachlorophenol	$1·5 \times 10^{-2}$ to $1·5 \times 10^{-4}$ M
Amobarbital	$1·2 \times 10^{-2}$ to $1·77 \times 10^{-4}$ M	*p*-Chloromercuribenzoate	$1·5 \times 10^{-3}$ M

* When a range of concentrations was tested, the increments were 0·5 M or 0·5 mg./ml.

Chemical agents. Various compounds reported to be inhibitors of nerve impulse transmission in 'higher' organisms, DNA- and protein synthesis inhibitors, and materials able to destroy membrane integrity were incorporated into the buffer system at various concentrations. Compounds such as dimethyl sulphoxide (DMSO) also were used if they showed some promise from reports of effects on other organisms. Concentrations are given in Table 1.

Each compound was screened for ability to inhibit a.r.b. formation, and if any was noted it was further checked to determine its effect on motility as compared with a control of plain buffer. Any compounds which showed little effect on motility and that seemed at the same time to affect a.r.b. formation were studied further.

Several compounds were tested for activity in the presence of non-inhibitory amounts of buffered (pH 7) DMSO (0·0312 M) to see whether permeability and, hence, the action of these compounds, was intensified. All compounds were prepared in buffer in as small an amount as possible on the same day they were tested. Solid compounds were weighed and dissolved in 3 ml. buffer for the desired concentration; dilutions of fluid compounds were made to the desired concentration in buffer, 3 ml. of which was

used as a test sample in the experiment. Bacteria were added only after the dissolution of the solid material, and the mixtures were allowed to incubate at room temperature (20°) in the solutions for 30 min. before any experiment was performed.

RESULTS AND DISCUSSION

Selection experiments

The ratio of fla$^+$:fla$^-$ organisms remained constant when they were grown together as mixed aerated cultures, fla$^-$ colony counts being always slightly higher than fla$^+$ counts
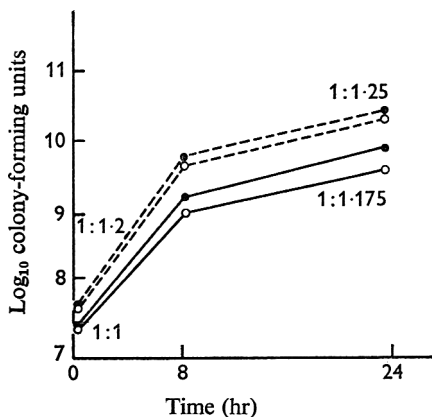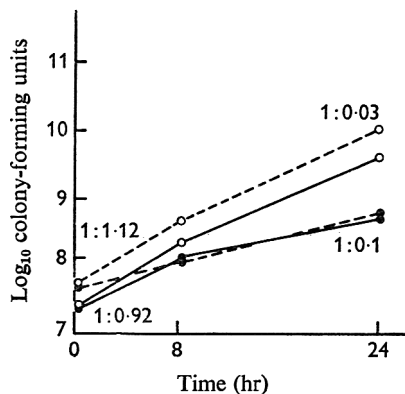
Fig. 3. Multiplication of fla$^+$ and fla$^-$ *Pseudomonas fluorescens* over a 24 hr period in two different experiments; showing fla$^+$:fla$^-$ ratios in aerated mixed cultures. ○, fla$^+$; ●, fla$^-$. —, Expt. 1; – – –, Expt. 2.

Fig. 4. Multiplication of fla$^+$ and fla$^-$ *Pseudomonas fluorescens* over a 24 hr period in two different experiments; showing fla$^+$:fla$^-$ ratios in non-aerated mixed cultures. ○, fl$^+$; ●, fl$^-$. —, Expt. 1; – – –, Expt. 2.

(Fig. 3). Fla$^+$:fla$^-$ ratios changed remarkably from a 1:1 ratio in non-aerated media: after 24 hr the fla$^+$ colony count was 10–33 times greater than the fla$^-$ count (Fig. 4).

Selective multiplication of aerobic fla$^+$ bacteria under non-aerated conditions supports the view that motility is a positive factor in survival. If a bacterium is living in an environment where favourable conditions are discontinuous or limited it is able to facilitate contact with more satisfactory conditions if it is motile. An actively motile organism could accomplish this in two ways: (1) by migrating through the medium to more favourable areas; and (2) by causing the medium to circulate in the immediate vicinity of the organism. Operation of either factor would enable the organism to increase the amount of material contacting the cell surface, thereby increasing the flux of material across the membrane, and resulting in greater availability of nutrients and dissipation of waste or growth-inhibitory substances present in the medium or secreted by the organism itself. Our data thus agree with Carlson's (1962) analysis since oxygen is a 'nutrient' in the broad sense.

The results demonstrate furthermore that motile piliated bacteria have an advantage over non-motile piliated forms in static (non-aerated) conditions. Although positive

aerotaxis and piliation admittedly both play a role in survival of aerobic forms, motility is considered the greater advantage under the particular experimental conditions used here since it allows for the expression of this response.

Chemotactic experiments

Demonstration of negative chemotaxis. Fla$^+$ *Pseudomonas fluorescens* suspensions in capillary tubes developed a single 0·1 mm. deep band in which the bacteria migrated away from the acidic Ionagar plug. The pH values of plugs tested ranged from less than 1·0 to 4·0. No band developed at the opposite end of the tube which was plugged
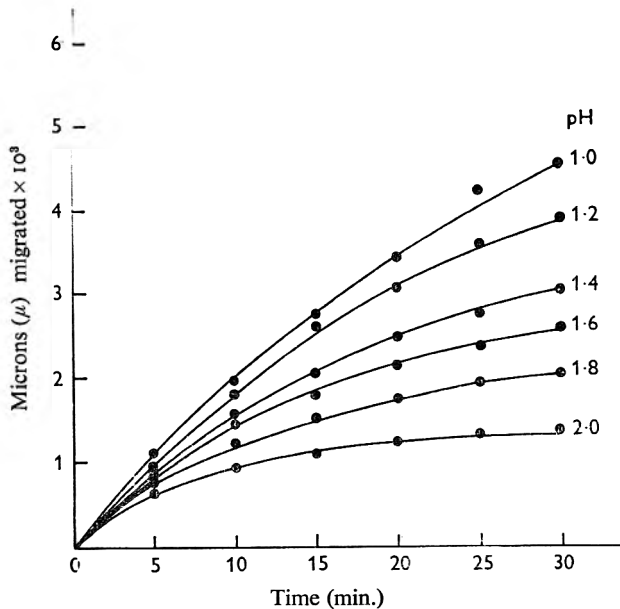
Fig. 5. Migration of acid response bands as related to pH of acidic Ionagar plug.

with plain Ionagar and served as a control. At pH 1·0 band migration was as rapid as 200 μ/min. over a period of 30 to 40 min., after which the migration rate then decreased. A sharp boundary was always observed on the side of the band directed to the origin of the stimulus, the opposite side being more diffuse (Plate 1). Phase-microscopic examination ($\times 630$) of the band showed that bacteria moving to the sharp boundary immediately reversed direction in a manner similar to the 'Schreckbewegung' described by Englemann (1883). Bacteria entering the acid zone became quickly immobilized. Basic response bands formed in reponse to the stimulus from basic agar plugs were not as sharply defined as those in response to acid and therefore were not further considered. An a.r.b. did not develop if either deflagellated (10 ml. suspension exposed for 5 min. at 16,000 rev./min. in a Sorvall Omni-Mixer) or heat-killed (60° for 15 min.) bacteria were used. Incorporation of 1 mg./ml. *m*-cresol purple and 10 mg./ml. bromthymol blue in the buffer revealed that an a.r.b. formed between pH 5·5 and 6·0, and the organisms therein moved as the acid diffused from the agar plug to always remain in this pH range, which

covered a zone approximately 2 mm. wide. Neither dye affected the motility of the organism over at least 1 hr. A mixture (1:1:1) of *Pseudomonas fluorescens*, *Spirillum serpens* and *Agrobacterium tumifaciens* suspensions gave a single a.r.b. composed of all three organisms migrating as a unit; these organisms are equally sensitive and negatively chemotactic. Microscope examination and electron micrographs revealed that bacteria remaining on the acid side of the retreating band were not motile and lacked flagella. Varying the concentrations of HCl in Ionagar plugs resulted in response curves in which migration rate was proportional to total distance traversed from the acidic agar plug (Fig. 5). If an acidic agar plug was placed in both ends of a capillary tube, two a.r.b.'s developed which migrated towards each other to disappear immediately upon contact, leaving only non-motile bacteria throughout the tube.

Studies of factors influencing acid response bands

Temperature. Bacteria incubated at 4° and then transferred to capillary tubes were non-motile, therefore no a.r.b. was formed. Acid response bands formed at 20° migrated at the same rate as controls, but subsequent tubes set up from that portion of the culture incubated for more than 1 hr at 37° failed to show a.r.b. formation, since the organisms became non-motile because of temperature effects.

Table 2. *Effect of direct electric current on heat-killed bacteria and on acid response band of living bacteria*

Stimulus	Cell condi- tion*	Buffer pH	Migration (units/min.)†	Volts to mov‹ cells‡	Volts to disperse a.r.b.§
None	Dead	5	.	10	No band
None	Dead	7	.	7	No band
None	Dead	8	.	10	No band
pH 1 agar	Dead	7	.	10	No band
pH 1 agar	Live	5	.	.	No band
pH 1 agar	Live	7	0·33	.	23
pH 1 agar	Live	8	0·23	.	32·5
pH 8 agar	Live	5	No band	.	No band
pH 8 agar	Live	7	No band	.	No band
pH 8 agar	Live	8	No band	.	No band

* Cells in buffer (adjusted to indicated pH by titration of 10^{-2} M-phosphate buffer).
† One unit is equal to 0·071 mm. (71 μ) on ocular micrometer.
‡ Indicates reading on voltmeter of Thomas Electrophoresis Unit when bacteria began migrating towards anode.
§ Indicates reading on voltmeter of Thomas Electrophoresis Unit causing dispersion of acid response band and migration towards anode.

Magnetic fields, gravity, light and age. No effect on a.r.b. was observed in the experiments on magnetic fields, gravity and light. In addition, organisms incubated in TSB for 48 hr and 1 week gave an a.r.b. similar to that of the 16–18 hr culture.

Viscosity. Comparative rates of flow of the solutions used to test for viscosity were 0·3 sec./cm. for buffer, 0·4 sec./cm. for 5% Carbowax, and 6·65 sec./cm. for 5% PVP. No band was formed with PVP in the buffer, and the band formed with Carbowax was less dense than that in the buffer control. Motility counts revealed that the reason for these differences lay in somewhat inhibited motility by the more viscous

solutions. Although motility was not completely halted (motility counts were 14 % of the buffer control in PVP and 72 % in Carbowax), the decrease in the case of PVP was sufficient to completely eliminate band formation.

Direct electric current. Voltage required to cause migration of dead cells towards the anode differed considerably from that required when living organisms were used (Table 2). Migration of dead cells occurred with an input of 10 V. whether the acid stimulus was present in the tube or not, whereas at least 23 V. were necessary to cause disruption of a.r.b. moving away from the acid stimulus. As might be expected, no a.r.b. was formed either with dead organisms with or without a stimulus, or with living

Table 3. *Effect of varying the pH of the buffer on acid response band formation*

Cell suspension pH*	Agar plug pH†	a.r.b. formation‡	Cell suspension pH*	Agar plug pH†	a.r.b. formation‡
5	1	+ −	7	1	+
5	2	+ −	7	2	+
5	2·5	+ −	7	2·5	−
5	3	−	7	3	−
5	3·5	−	7	3·5	−
5	4	−	7	4	−
6	1	+	8	1	+
6	2	+	8	2	+
6	2·5	+	8	2·5	−
6	3	+	8	3	−
6	3·5	−	8	3·5	−
6	4	−	8	4	−

* Cells in buffer (adjusted to indicated pH by titration of 10^{-2} M-phosphate buffer).
† 'Ionagar' titrated to indicated pH at 50° with N-HCl.
‡ a.r.b. formed within 15 min. after insertion of acid agar plug into capillary tube.
+ = formation of acid response band equivalent to control; + − = weak acid response band; − = no acid response band formed.

organisms when no stimulus was present. Acid response bands also were not formed with living organisms if the pH of the buffer was as low as 5, or when the Ionagar plug was pH 8·0. It is interesting to speculate upon whether the increase in voltage necessary to 'force' the living organisms into the acid causing dispersion of the a.r.b. is related in some manner to the phenomenon of negative chemotaxis.

Influence of pH. The effect of pH of the suspending medium on a.r.b. formation is depicted in Table 3. *Pseudomonas fluorescens* grown at different pH values and resuspended in buffer of pH 7 showed no difference in ability to form a.r.b. with acid agar plugs; an a.r.b. formed at any pH below 3·0.

Chemical agents. Compounds found not to inhibit motility or a.r.b. formation were: urea, indole acetic acid, digitonin, cocaine SO_4, amobarbital, eserine SO_4, 'Triton', serotonin creatinine SO_4, morphine SO_4, chloramphenicol, and dicoumarol. Compounds which inhibited motility or a.r.b. formation were: atabrine, benzalkonium Cl, sanguinarine SO_4, pentachlorophenol, polymyxin, *n*-butanol, sodium dodecyl SO_4, cetyl pyridinium Cl, sodium barbital, strychnine SO_4, *p*-chloromercuribenzoate, phenethyl alcohol, and tyrocidine HCl. All concentrations employed are given in the methods section of this paper. Those compounds which at some concentration partially inhibited motility and a.r.b. formation were: potassium cyanide, bacteria 56 %

as motile as control at $2\cdot5 \times 10^{-3}$ M; DL-*p*-fluorophenylalanine, 75 % at $5\cdot5 \times 10^{-2}$ M; PNPG, 94–59 % at $1\cdot5$ to 3×10^{-5} M; and DMSO, 90–70 % at $3\cdot12 \times 10^{-2}$ to $1\cdot25 \times 10^{-1}$ M; however, at a 10-fold greater DMSO concentration, the motility rate was only $45\cdot5$ % of the control.

Since temperature, viscosity, and pH variations all exerted inhibitory or stimulatory effects on motility of *Pseudomonas fluorescens*, any measurement, therefore, of influence on negative chemotaxis, was compromised.

The effect of variation of environmental pH on the a.r.b. initiated by varying the pH of agar plugs, did not give evidence of any conformation with Weber's Law in that fla$^+$ organisms failed to respond in a manner which indicated that the threshold response was a constant fraction of the environmental stimulus under the conditions described. It is possible that concordance under Weber's Law was not observed because: (1) motility rate is dependent upon pH (Schuetze & Doetsch, 1967) and any threshold response was masked; and (2) the response may be to pH increments finer than those chosen for measurement in this series of experiments, indeed, finer than the measuring capability of the experimental system.

Since we have found that migration rate of an a.r.b. is related to factors such as hydrogen ion concentration, degree of ionization of the acid, and ionic strength of the buffer, including that to which the chemical agents have been added, it is desirable that any compound which inhibits or stimulates the chemotactic reaction manifests itself by preventing or intensifying band formation. At the same time, such an agent ought to exhibit little effect on motility rate, particularly if motility *per se* and chemotactic responses are distinct as found by Adler (1966*a*) in the case of *Escherichia coli*.

The difficulty of 'proving' inhibition of chemotaxis lies in the technical difficulty of demonstrating that motility is not affected at the same time that chemotaxis is repressed. Measurement of motility by crude visual methods has several shortcomings, but it seems the most feasible preliminary method available. The motility rate in control organisms is not initially stable, and varies considerably, so that one must allow the suspension to stand for 10 min. after preparation; care also must be taken to wait at least one min. before counting any slide preparation, since motility rate again stabilizes after this time.

Failure to observe inhibitory effects of chemical agents on negative chemotaxis may have several explanations: (1) inhibitory agents exist but were not found; (2) both motility and chemotaxis were simultaneously affected; (3) the action of agents was not observable under the conditions imposed in the experiment; and (4) the agents did not gain access to the membrane or postulated receptors.

The idea of hydrogen ions acting as a stimulus in a negative chemotactic reaction in the organism studied is appealing when viewed in the light of possible alteration of electrical potentials along the membrane. The theoretical number of hydrogen ions 'displaced' by the volume of one *Pseudomonas fluorescens* cell ($0\cdot96 \mu^3$) at pH $5\cdot5$ is 2×10^3 or one H$^+$ for a 1 mμ thick cross-section of the organism. It is interesting to speculate as to whether this is, in fact, the 'signal' to which this organism responds.

Mechanisms which control flagellar responses of bacteria remain, as yet, unknown. Any 'neurological' system, however primitive, described in flagellated eucaryotic organisms seems completely lacking in procaryotic forms; in addition, the exact

nature of excitations (if any) elicited by stimuli also is unknown (Doetsch & Hageage, 1968).

A theory developed by us, in the course of laboratory experiences with various flagellated bacteria (Doetsch, 1966b; Doetsch, Cook & Vaituzis, 1967) suggested that the flagellum or flagellar fascicle is part of an excito-motor system developed for spacial accommodation. Stimuli are considered to be 'sensed' by the cytoplasmic membrane acting as a 'monitor' or signal receptor. The entire membrane may serve this function, or possibly there are specialized receptor areas which may or may not lie near the origin or point of insertion of the flagellum (Doetsch, 1966a). The mechanism of sensing environmental stimuli is visualized as involving an induced change in membrane potential by which means the signal is transduced and transmitted to a 'motor unit' (possibly a differentiated membrane immediately surrounding the flagellum). A gap, synapse, or an equivalent thereof, and a primitive 'muscle-membrane' or contractile element, may be coupled with, and activate, a rigid or semirigid non-contractile flagellum. The chemical events may be similar to those found in higher animals, based upon acetylcholine (Doetsch & Hageage, 1968).

Successful experiments influencing and interfering with the postulated sensing function of the membrane would add considerable support to this view of the nature of sensory processes in bacteria. The response may be, on the other hand, a result of some change, induced by the stimulus, in the general metabolic state of the organism, and controlled by a mechanism which is linked to one or several energy-yielding pathways. This possibility has yet to be ruled out in the phenomenon of negative chemotactic responses.

Studies with bacterial systems might yield valuable clues into the general problem of how the energy of stimuli is transduced into the language of the sensory code at the unicellular level. The implications of such studies are exciting to contemplate and undoubtedly would open new horizons for development of a theory of bacterial behaviour and motility.

REFERENCES

ADLER, J. (1966a). Effect of amino acids and oxygen on chemotaxis in *Escherichia coli*. *J. Bact.* **92**, 121.

ADLER, J. (1966b). Chemotaxis in bacteria. *Science, N.Y.* **153**, 708.

ARMSTRONG, J. B., ADLER, J. & DAHL, M. M. (1967). Non-chemotactic mutants of *Escherichia coli*. *J. Bact.* **93**, 390.

BARACCHINI, O. & SHERRIS, J. C. (1959). The chemotactic effect of oxygen on bacteria. *J. Path. Bact.* **77**, 565.

BEIJERINCK, M. W. (1893). Über atmungsfiguren beweglichen Bakterien. *Zentbl. Bakt. ParasitKde.* (Abt I) **14**, 827.

BRINTON, C. C. (1959). Non-flagellar appendages of bacteria. *Nature, Lond.* **183**, 782.

CARLSON, F. D. (1962). A theory of the survival value of motility. In *Spermatozoan Motility*, ed. D. W. Bishop. AAAS Pub. no. 72. Washington, D.C.

CLAYTON, R. K. (1958). On the interplay of environmental factors affecting taxis and motility in *Rhodospirillum rubrum*. *Arch. Mikrobiol.* **29**, 189.

DOETSCH, R. N. (1966a). Some speculations accounting for the movement of bacterial flagella. *J. theor. Biol.* **11**, 411.

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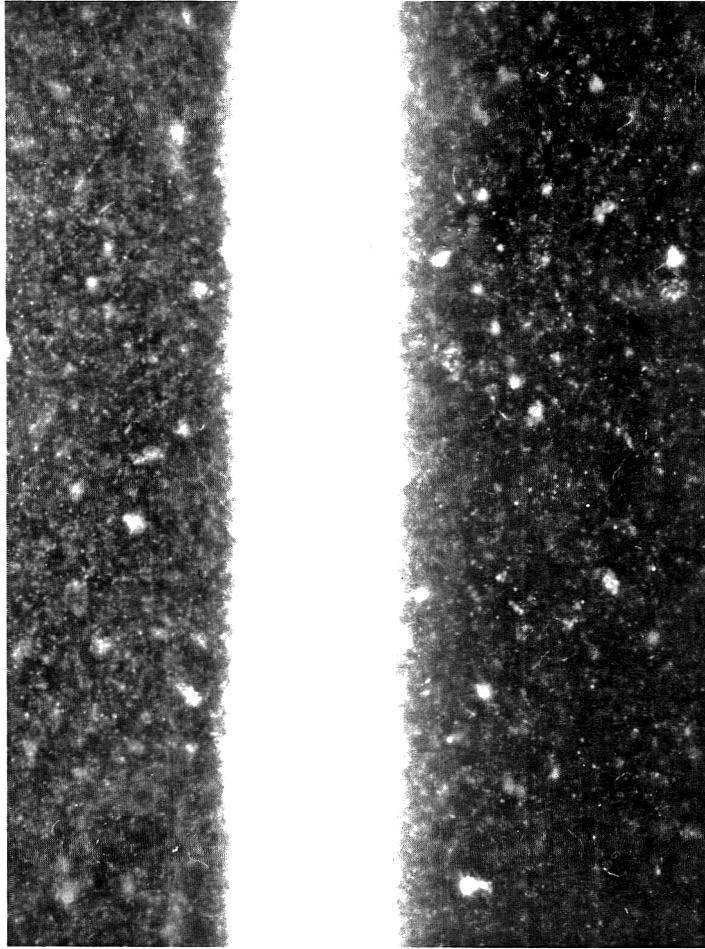
DOETSCH, R. N. (1966*b*). Notes on some structural features of *Sphaerotilus naians. Arch. Mikrobiol.* **54**, 46.

DOETSCH, R. N. & HAGEAGE, G. J. (1968). Motility in procaryotic organisms; problems, points of view, and perspectives. *Biol. Rev.* **43**, 317.

DOETSCH, R. N., COOK, T. M. & VAITUZIS, Z. (1967). On the uniqueness of the flagellum of *Thiobacillus thiooxidans. Antonie Van Leeuwenhoek.* **33**, 196.

ENGELMANN, T. W. (1883). Ein Beitrag zur vergleichen Physiologie des licht- und farbensinnes *Bacterium photometricum. Pflügers Arch. ges. Physiol.* **30**, 95.

LINKS, J. (1955). I. *Een hypothese over het mechanisme van de (phobo-) chemotaxis. II. De carotenoiden, steroiden en vetzuren van Polytoma uvella.* Dissertation, Leiden.

RHODES, M. E. (1958). The cytology of Pseudomonas as revealed by a silver-plating staining method. *J. gen. Microbiol.* **18**, 639.

SCHUETZE, P. & DOETSCH, R. N. (1967). Reversible paralysis of the flagella of certain Gram-negative bacteria. *Can. J. Microbiol.* **13**, 1114.

SHOESMITH, J. G. (1960). The measurement of bacterial motility. *J. gen. Microbiol.* **22**, 528.

SMITH, J. L. & DOETSCH, R. N. (1968). Motility in *Pseudomonas fluorescens* with special reference to survival advantage and negative chemotaxis. *Life Sci.* **7**, 875.

WRIGHT, A. E. & COLEBROOK, L. (1921). *Technique of the Teat and Capillary Tube,* p. 22. London: Constable.

EXPLANATION OF PLATE

Photomicrograph of an acid response band (× 290); stimulus is on the left

The Formation of Buds in Yeast

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SUMMARY

Vesicles accumulate at the site of bud formation in the mother cell and are also found in the growing bud during the growth of its wall. The bud is in direct communication with the mother cell until maturity when a septum which is formed across the cytoplasm grows inwards from the wall and is subsequently thickened on both sides. During the thickening process vesicles are present at both surfaces of the cross-wall which finally has two layers one of which becomes continuous with the bud cell wall. It is suggested that the vesicles carry material both into the growing bud wall and into the septum.

INTRODUCTION

The relationship between cytoplasmic organization and wall formation in yeast cells is a problem of long-standing interest and the search for organelles which are involved in the budding process has led several authors to examine thin sections of cells with the electron microscope. But the fixatives generally used—osmium tetroxide and potassium permanganate—are of limited value as they contain heavy metals and are oxidizing reagents so that they distort important structures.

To overcome fixation problems Mundkur (1960 *a*, *b* and 1961) applied a freeze-drying technique and Moor & Mühlethaler (1963) and Moor (1967) adapted the freeze-etching technique of Steere (1957) to the study of yeast. This latter technique permits the stabilization of both prolonged and transitory stages of structures that are not preserved when ordinary chemical fixation is used but the replicas obtained are difficult to interpret and therefore it is desirable to correlate the image with those obtained by other techniques.

Aldehyde fixatives, first introduced by Sabatini, Bensch & Barrnett (1963) have greatly improved our knowledge of the fine structure of animal and plant cells. Fixation occurs by cross-linkages between various chemical groups of the polymers so that there is only slight distortion of the cellular structure.

Robinow & Marak (1966) and Schmitter & Barker (1967) fixed yeast with glutaraldehyde before or after removal of the cell wall with snail gut enzyme in an attempt to obtain better intracellular resolution.

In this study the ultrastructural morphology of glutaraldehyde fixed *Saccharomyces cerevisiae* has been re-examined with special reference to the budding process.

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METHODS

Organism. A strain of *Saccharomyces cerevisiae* isolated from commercial pressed baker's yeast (Distillers Co. Ltd.) was used. This strain was maintained on Winge medium (yeast extract, 3 g.; glucose, 20 g.; water, 1 l.) in 2% agar slopes at 4°; it was cultured in Winge medium agitated in a reciprocal shaker at 28°. The yeasts were harvested at the beginning of the logarithmic phase of growth and washed twice in ice-cold distilled water.

Electron microscopy. The yeast cells were fixed in a glutaraldehyde fixative (4% glutaraldehyde in 0·02 M-phosphate buffer pH 6·8, with an added salt mixture, (Zetterquist, 1956) at 21° for 1 hr, and washed four times with the same buffer over a period of several hours. Post-fixation was done in 1% osmium tetroxide solution in veronal buffer (0·05 M, pH 7.0) for 1 hr. The pellet was embedded in 0·75% agar blocks, dehydrated with an ascending ethanol series and embedded in araldite resin.

Sections were cut with glass knives on a Sorvall Porter-Blum ultramicrotome MT 2. Gold sections were collected on carbon coated copper grids and stained with uranyl acetate (saturated solution in 50% ethanol) at 60° for 15 min. followed by lead citrate (0·09% lead; Reynolds, 1963) for 1 min. The sections were examined by a GEC-AEI, EM 6B electron microscope at 80 kV.

RESULTS

The use of glutaraldehyde as a fixative resulted in satisfactory preservation of the organelles known to be present in the yeast cell and showed an unusually complex organization of its membrane system.

The plasmalemma had small invaginations into several regions of the cytoplasm; some of these invaginations were almost circular in section but others showed much greater structural complexity (Pl. 1, fig. 1, 2, 4); they resembled packages of more or less concentric rings or aggregates of membranes (Pl. 1, fig. 1, 4). They were sometimes seen in close contact with the endoplasmic reticulum (Pl. 1, fig. 1).

The cell wall appeared on the electron micrographs as a layer of low electron density with the plasmalemma in close contact with it. The initial stage in the budding process became apparent at sites just under the plasmalemma where an accumulation of vesicles occurred (Pl. 2, fig. 5, 6, 7). The cell wall in this region bulged out and vesicles accumulated in the underlying cytoplasm (Pl. 2, fig. 5). The cell wall of the growing bud increased in area but not in thickness (Pl. 2, fig. 6, 7) and organelles other than vesicles and endoplasmic reticulum accumulated in the bud. Before the bud reached maturity a cross-wall began to be laid down at the junction between the bud and the mother cell (Pl. 1, fig. 3, Pl. 3, fig. 8, 9, 10, 11). This cross-wall which was first seen as a ring between the cell wall and the plasmalemma (Pl. 3, fig. 8), grew centripetally in a similar way to that described for bacteria and other fungi (Pl. 1, fig. 3; Pl. 3, fig. 9). During the formation of this cross-septum vesicles were seen in the cytoplasm alongside it both in the mother and in the bud cell (Pl. 1, fig. 3; Pl. 3, fig. 10, 11; Pl. 4, fig. 12). The contents of the vesicles had the same appearance as the material which made up the septum. The vesicles were similar in appearance to those involved in the formation of the bud cell wall. The cross-wall had a sinuous shape and was displaced towards the mother cell (Pl. 1, fig. 3; Pl. 3, fig. 9, 10). When the separation between the two cells was completed by the formation of a thin septum, this was

thickened to produce a double-layered cross-wall (Pl. 4, fig. 13, 14). The septum was lined on each side by plasmalemma which was continuous with that of the bud or of the mother cell. A gap appeared between the two layers but the cells remained attached around the ring formed on the mother cell wall (Pl. 4, fig. 13). The septum on the bud cell side became continuous with the wall but separate layers were clearly distinguished between the septum and the mother cell wall (Pl. 4, fig. 13, 14).

The birth scar had only one layer while the bud scar on the mother cell had at least three, the outermost layers being part of the mother cell wall, and the inner layer, part of the cross-wall (Pl. 4, fig. 15).
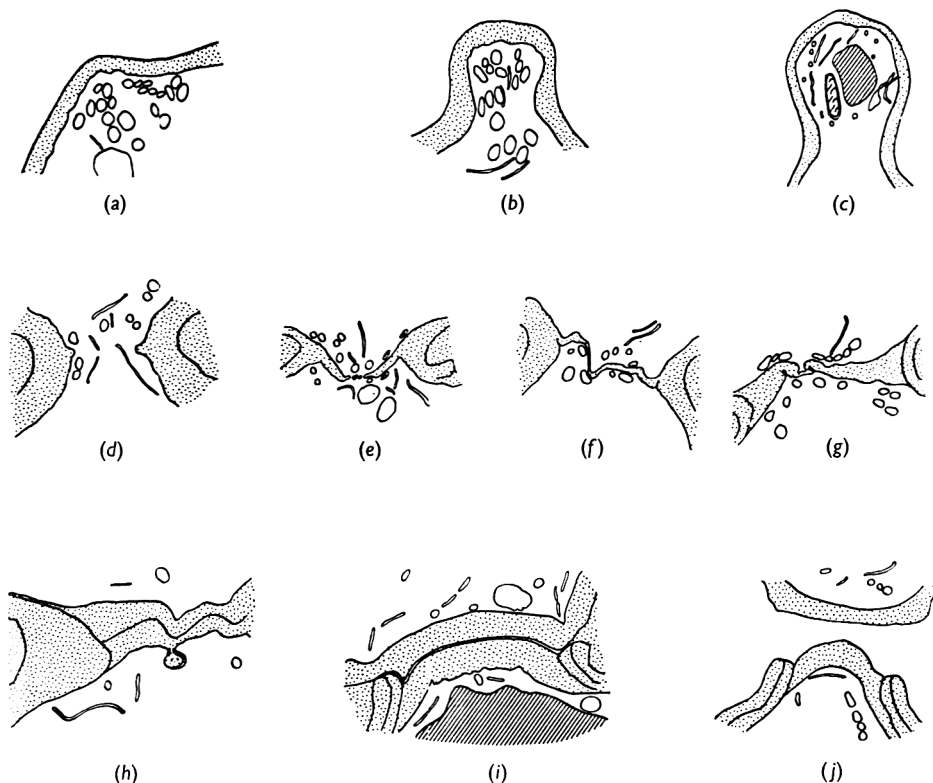
Fig. 1. Diagrams to show the sequence of bud and septum formation. The bud cell is the upper cell and the mother cell is the lower cell.

(*a*) and (*b*). Vesicles accumulate under the wall during the initial stages of bud formation. (Compare Pl. 2, fig. 5, 6 and 7.)

(*c*) Nuclear material, mitochondria and endoplasmic reticulum are passed into developing bud.

(*d*–*g*). Formation of the septum across the connexion between the bud and mother cell. The septum is formed by the incorporation of material passed to it in vesicles from both sides It grows from the wall of the cell inwards. (Compare Pl. 1, fig. 3; Pl. 3, fig. 8, 9 and 10.)

(*h*) The septum is thickened and forms two layers. (Compare Pl. 3, fig. 11; Pl. 4, fig. 12.)

(*i*) A gap appears between the layers of the septum. One layer is continuous with the wall of the bud but the other is distinct from the wall of the mother cell. (Compare Pl. 4, fig. 13 and 14.)

(*j*) The bud is separated from the mother cell. (Compare Pl. 4, fig. 15.)

DISCUSSION

The presence of invaginations of the plasmalemma into the cytoplasm of yeast cells has been clearly demonstrated by freeze etch studies (Moor & Mühlethaler, 1963; Northcote, 1968). The fixation and staining methods used in this work have enabled the membranes to be investigated and the complexity of some of the invaginations can be seen to resemble the mesosomes of Gram-positive bacteria (Ryter, 1968). When the cell walls of bacteria are digested away by lysosyme the protoplasts obtained are spherical and the mesosome is extruded from it in the form of a beaded appendage (Fitz-James, 1966; Ryter, 1968). The blebs which are formed at the surface of the protoplasts of yeast (Svihla & Schlenk, 1965) may have a similar origin.

The fine structure of the yeast cell and the budding process has been studied in thin sections by several workers; however a complete sequence of events has been impossible to describe mainly because of the difficulties of fixation, embedding and staining. By the use of glutaraldehyde as a fixative we have been able to demonstrate the initial stages of bud formation and the subsequent formation of a septum between the mother and the bud cells (Fig. 1 a–j). The formation of the septum in yeast is seen to be similar to that described for some other fungi and Gram-positive bacteria (Glauert, 1962; Hawker, 1965; Ryter, 1968).

During the initial stages of its formation the bud is extended by new cell wall material that is probably carried to the site in the vesicles which accumulate at the bud region and the material is passed across the plasmalemma by reverse pinocytosis. It has been shown by the freeze etch technique that synthesis of some of the cell wall substance probably takes place from small organized particles at the outer plasmalemma surface (Moor & Mühlethaler, 1963). Thus the cell wall is formed partly off site in vesicles which are transported to the wall and partly at the cell surface from synthetic particles (Northcote, 1968) in a way similar to that indicated for the formation of the cell walls of higher plants (Northcote & Lewis, 1968) and fungi (McClure, Park & Robinson, 1968). The vesicles seen in the sections are equivalent to the 'proteaseparticles' described by Moor (1967) from a freeze etch study, but he suggested that the vesicles carried enzymes for dissolving and weakening the wall at the point of bud formation. The origin of the vesicles is not clear but it is possible that they are derived from the endoplasmic reticulum system (Moor, 1967; Marchant & Smith, 1967).

As the wall grows out of the parent cell nuclear material, mitochondria, endoplasmic reticulum, ribosomes and other cytoplasmic inclusions are passed into the bud region. The bud is finally cut off from the parent cell by a septum which grows inwards from the wall across the intercommunicating region as a thin sinuous strand and is then thickened by the deposition of material from both sides. The thin septum is not seen very frequently in sections containing some hundreds of yeast prepared from an actively growing culture and it is likely therefore that the growth of the septum and its subsequent thickening is quite a rapid process.

During formation of the septum and its thickening, material is again probably deposited from vesicles which can be seen to accumulate at this region at both sides from the mother and from the bud cell. Although the septum increases in thickness its diameter remains fairly constant because of its attachment with the mother cell wall and during the latter stages of its formation the continuity of the material of the

septum on the bud cell side with the wall of the bud can be seen, but such a direct continuity is not clear on the mother cell side. It may be that as the bud grows a tension is developed at the junction which results in the breakage of the connexion and a release of the bud which then rounds off its wall at the birth scar. But the distinctive layered appearance of the septum at the bud scar of the mother cell remains.

REFERENCES

FITZ-JAMES, P. C. (1966). Fate of the mesosomes of *Bacillus megaterium* during protoplasting. *J. Bact.* **87**, 1483.

GLAUERT, A. M. (1962). The fine structure of bacteria. *Br. med. Bull.* **18**, 245.

HAWKER, L. E. (1965). Fine structure of fungi as revealed by electron microscopy. *Biol. Rev.* **40**, 52.

McCLURE, W. K., PARK, V. & ROBINSON, P. M. (1968). Apical organization in the somatic hyphae of fungi. *J. gen. Microbiol.* **50**, 177.

MARCHANT, R. & SMITH, D. G. (1967). Wall structure and bud formation in *Rhodotorula glutinis*. *Arch. Mikrobiol.* **58**, 248.

MOOR, H. (1967). Endoplasmic reticulum as the initiator of bud formation in yeast (*S. cerevisiae*). *Arch. Mikrobiol.* **57**, 135.

MOOR, H. & MÜHLETHALER, K. (1963). Fine structure in frozen-etched yeast cells. *J. Cell Biol.* **17**, 609.

MUNDKUR, B. (1960a). Electron microscopical studies of frozen-dried yeast. I. Localization of poly-saccharides. *Expl Cell Res.* **20**, 28.

MUNDKUR, B. (1960b). Submicroscopic morphology of frozen-dried yeast. *Expl Cell Res.* **21**, 201.

MUNDKUR, B. (1961). Electron microscopical studies of frozen-dried yeast. III. Formation of the tetrad in *Saccharomyces*. *Expl Cell Res.* **25**, 24.

NORTHCOTE, D. H. (1968). Structure and functions of plant-cell membranes. *Br. med. Bull.* **24**, 107.

NORTHCOTE, D. H. & LEWIS, D. R. (1968). Freeze-etched surfaces of membranes and organelles in the cells of pea root tips. *J. Cell Sci.* **3**, 199.

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

ROBINSON, C. F. & MARAK, J. (1966). A fiber apparatus in the nucleus of the yeast cell. *J. Cell. Biol.* **29**, 129.

RYTER, A. (1968). Association of the nucleus and the membrane of bacteria: A morphological study. *Bact. Rev.* **32**, 39.

SABATINI, D. D., BENSCH, K. & BARRNETT, R. J. (1963). Cytochemistry and electron microscopy. The preservation of cellular ultrastructure and enzymic activity by aldehyde fixation. *J. Cell Biol.* **17**, 19.

SCHMITTER, R. E. & BARKER, D. C. (1967). A new fixation method for *Schizosaccharomyces pombe*. *Expl Cell Res.* **46**, 215.

STEERE, R. L. (1957). Electron microscopy of structural detail in frozen biological specimens. *J. biophys. biochem. Cyt.* **3**, 45.

SVIHLA, G. & SCHLENK, F. (1965). The influence of culture conditions on the formation of spheroplasts from *Cardiola utilis*. In Symposium on Yeast Protoplasts. *Abh. dt. Akad. Wiss. Berl.* (Medizin) (1966), **21**, 15.

ZETTERQUIST, H. (1956). The ultrastructure organization of the columnar absorbing cells of the mouse jejunum. In *Histological Techniques for Electron Microscopy*. Ed. by D. C. Pease, 1960. London: Academic Press.

EXPLANATION OF PLATES

Fig. 1–15. Pictures of sections of yeast cells taken with an electron microscope. B, bud cell: M, mother cell: e, endoplasmic reticulum.

PLATE 1

Fig. 1. An invagination of the plasmalemma into the cytoplasm can be seen. The invagination is circular in section and is closely associated with profiles of the endoplasmic reticulum. × 87,000.

Fig. 2. Simple invaginations of the plasmalemma. These are close to profiles of the endoplasmic reticulum. × 75,000.

Fig. 3. Intermediate stage in the growth of the septum across the connexion, between the mother and bud cells. The septum is not continuous at its centre although it is well developed at its outer edge. × 19,000.

Fig. 4. A circular profile of a complex invagination of the plasmalemma into the cytoplasm of the cell is shown. × 70,000.

PLATE 2

Fig. 5. A region in the mother cell at the initial stage of bud formation. Vesicles can be seen to have accumulated in the cytoplasm of the cell just under the plasmalemma. × 67,000.

Fig. 6, 7. Initial stages in the growth of the bud. The cell wall of the bud has extended in area and vesicles can be seen to have accumulated in the cytoplasm in this region of the cell. Fig. 6 × 39,000. Fig. 7. × 52,000.

PLATE 3

Fig. 8. Initial stage in the formation of the septum between the mother and mature bud cell. A transverse section of a circular ridge formed on the wall can be seen. × 39,000.

Fig. 9. A thin septum which extends completely across the connexion between the bud and mother cell can be seen. It is lined on both sides by plasmalemma. × 54,000.

Fig. 10 and 11. Stages in the thickening of the septum. Vesicles can be seen on either side in the mother and bud cells. Fig. 10 × 30,000. Fig. 11 × 39,000.

PLATE 4

Fig. 12. A stage in the thickening of the septum. It is possible that the appearance of the septum on the mother cell side could be caused by the deposition of material from a vesicle across the plasmalemma. × 75,000.

Fig. 13 and 14. Stages in the final thickening and development of the septum. Two layers in the septum are visible. The layer on the bud cell side is continuous with the cell wall of bud. The layer on the mother cell side is distinct from the cell wall. Fig. 13. × 50,000.

Fig. 14. × 39,000.

Fig. 15. Bud scar on the mother cell. × 25,000.

Plate 1

R. SENTANDREU AND D. H. NORTHCOTE

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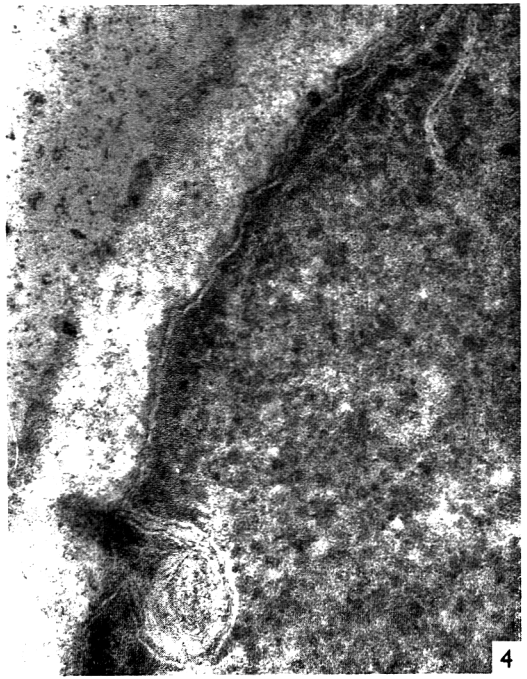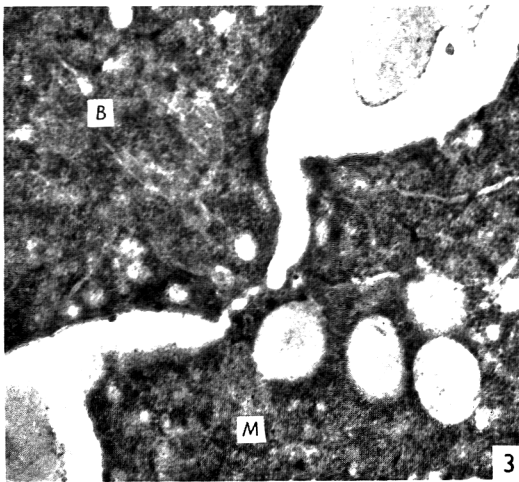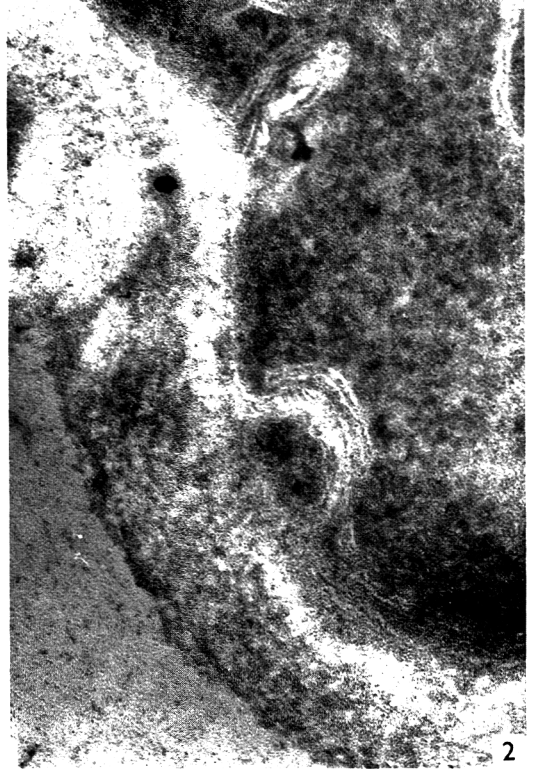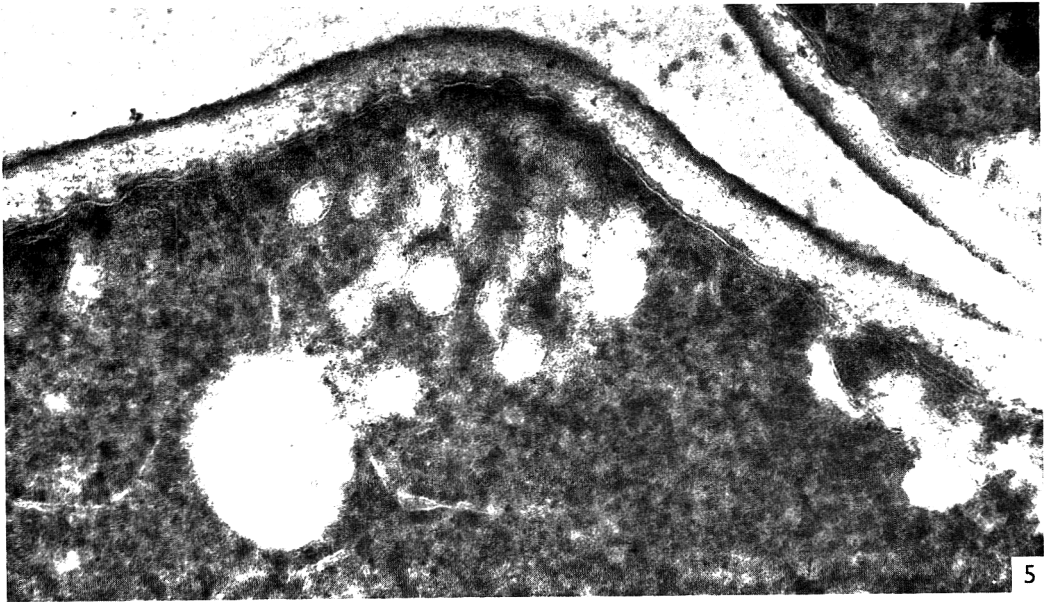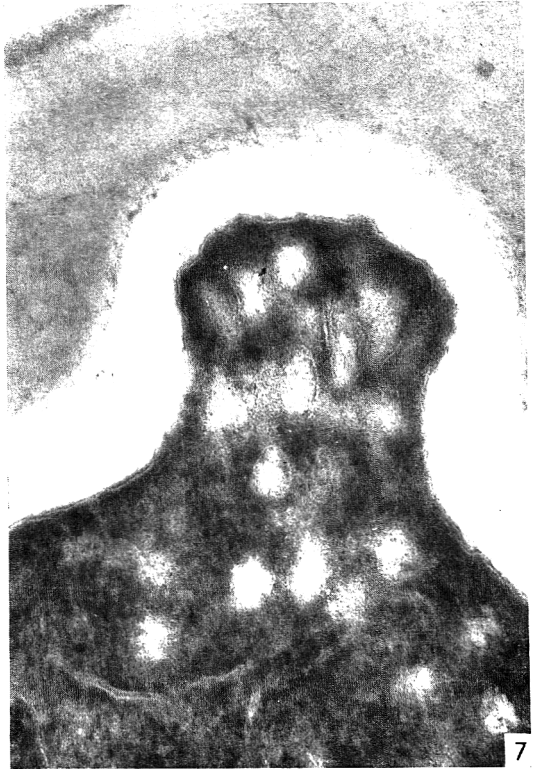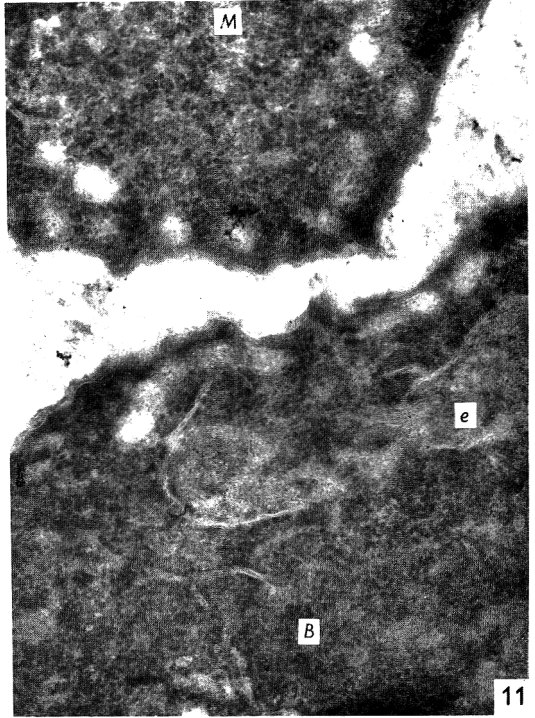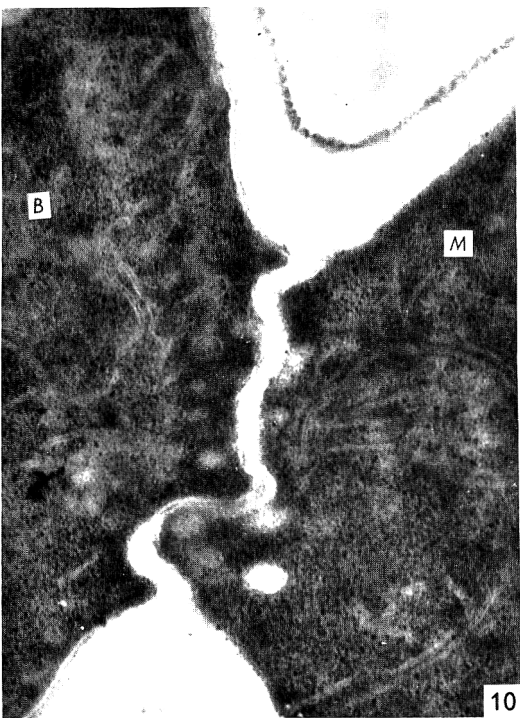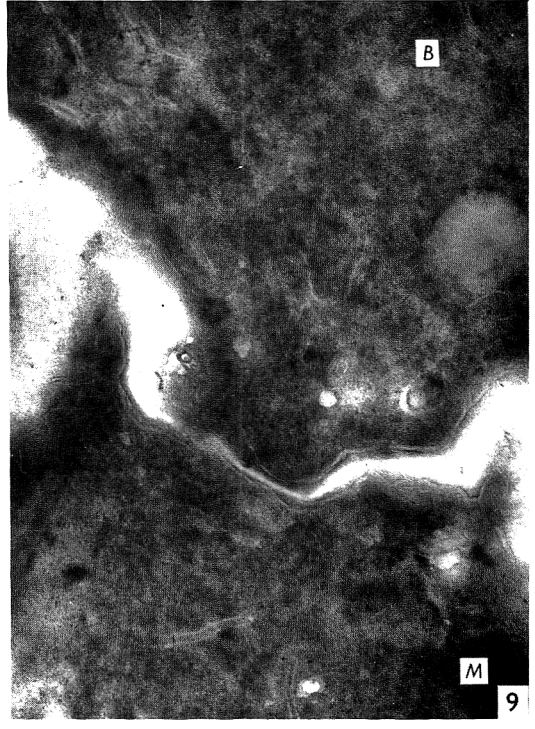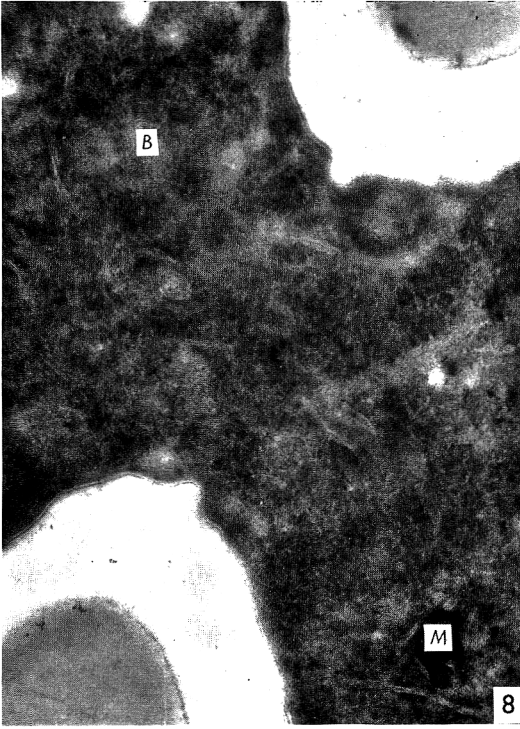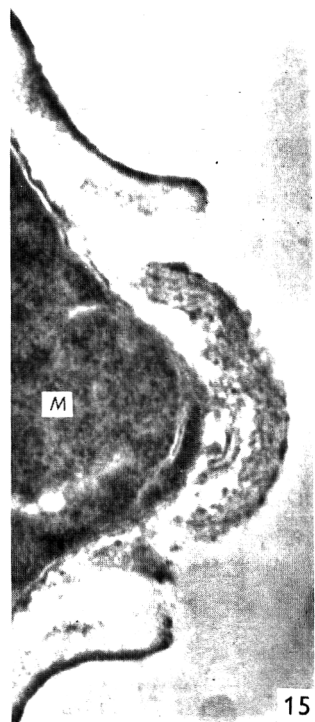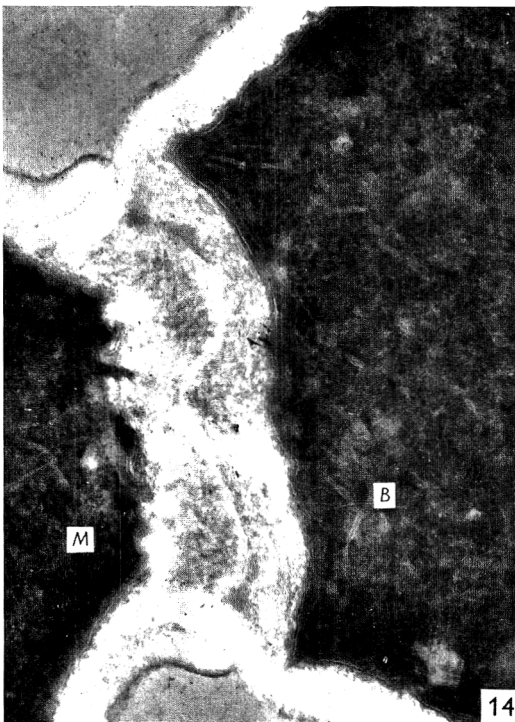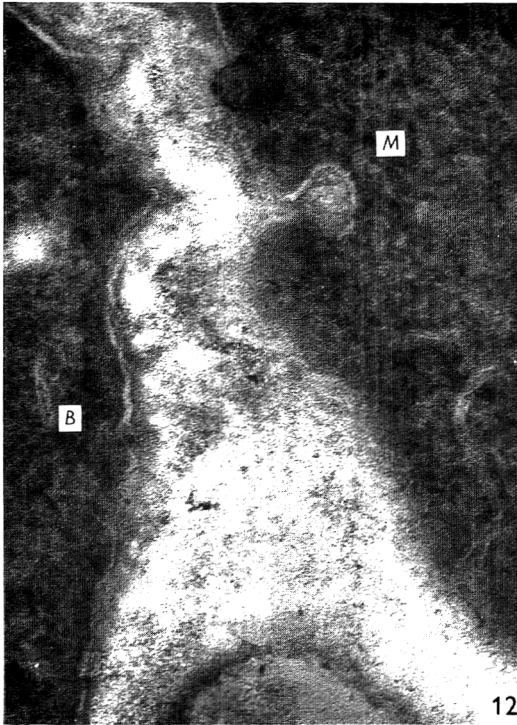
Characterization of Leptospires according to Fatty Acid Requirements

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SUMMARY

The fatty acid requirements of leptospires parasitic for mammals were compared to those of members of the 'biflexa complex' (leptospires not clearly demonstrated to be animal parasites). The parasitic leptospires grew on unsaturated fatty acids. Saturated fatty acids were not utilized unless an unsaturated fatty acid was also provided. This requirement for an unsaturated fatty acid was increased at temperatures above and below the optimal. In addition, these leptospires did not utilize fatty acids containing less than 15 to 16 carbon atoms unless a longer chain fatty acid was also present. Leptospires of the 'biflexa complex' had neither of these requirements. These leptospires grew on either long or short chain, saturated or unsaturated fatty acids. Utilizing these differences in fatty acid requirements, a means of differentiating these two groups of leptospires is provided. Members of the 'biflexa complex' but not the parasitic leptospires grew on an albumin medium containing 5×10^{-4} M-myristic acid, the 14-carbon saturated fatty acid.

INTRODUCTION

The genus *Leptospira* is at present represented by a single species, *L. interrogans* (*World Health Organization*, 1967). However, there appears to be two major 'complexes' of leptospires within the genus: The 'parasitic complex' which contains leptospires parasitic for man and animals, and the 'biflexa complex' which contains the so-called 'water' leptospires and other leptospires not clearly demonstrated to be parasitic for animals. Fatty acids are the major carbon and energy source for leptospires of both complexes. Recently, Auran & Johnson (1968) reported that long chain alcohols would also serve as a major carbon and energy source. In addition, they reported that serotype *pomona* Wickard, a member of the 'parasitic complex', required an unsaturated fatty acid for growth, whereas serotype *patoc* Patoc I, a member of the 'biflexa complex', did not. This report is concerned with the fatty acid requirements of the leptospires as related to fatty acid chain length, presence or absence of double bonds and effect of incubation temperature. A method of differentiating the two complexes based on fatty acid requirements is also described.

METHODS

Media and cultural procedures

The source of the leptospires used in this study was described by Johnson & Harris (1968). The nomenclature used for the leptospiral cultures used in this report is that recommended by a WHO Expert Group (*World Health Organization*, 1967). The cultures were maintained in a Tween 80 + albumin medium, a modification of that described by Ellinghausen & McCullough (1965), which was prepared as described by Johnson & Harris (1967). Incubation was at 30° in the dark.

The albumin medium used in the nutritional studies was prepared as follows: Separate stock solutions were made in distilled water (g./100 ml.) of NH_4Cl, 25; $ZnSO_4.7H_2O$, 0·4; $CaCl_2.2H_2O$ and $MgCl_2.6H_2O$, 1 g. each; $FeSO_4.7H_2O$, 0·5; $CuSO_4.5H_2O$, 0·3; glycerol, 10; thiamine HCl, 0·5; and vitamin B_{12}, 0·02. The pH of the stock solutions was not adjusted. The basal medium consisted of Na_2HPO_4 (1 g.), KH_2PO_4 (0·3 g.), NaCl (1 g.) and NH_4Cl, thiamine and glycerol stocks (1 ml. each) in 997 ml. of distilled water, adjusted to pH 7·4; sterilized at 121° for 20 min.

The albumin supplement was prepared by adding 20 g. of fatty acid-poor bovine albumin, Fraction V (Pentex, Inc., Kankakee, Ill.) to 100 ml. distilled water. The following stock solutions (in ml.) were added slowly to the albumin solution while it was being stirred: calcium and magnesium chloride, 2; zinc sulfate, 2; copper sulphate, 0·2; iron sulfate, 20; and vitamin B_{12}, 2·0. The pH of the albumin solution was adjusted to 7·4 and the final volume to 200 ml. The albumin supplement was sterilized by filtration. The test medium was prepared by adding 1 vol. albumin supplement to 9 vol. basal medium.

Tweens (Atlas Chemical Industries, Wilmington, Del.) were added to the basal medium and sterilized by autoclaving. The fatty acids (Hormel Institute, Austin, Minn.) were of high purity (99 %). The unsaturated fatty acids were sterilized at 121° for 20 min. in sealed vials under N_2. The sodium salts of the unsaturated acids were prepared with sterile NaOH and the fatty acid salt was added aseptically to the sterile test medium. The saturated fatty acids were dissolved in ethanol, to which NaOH was added, and added to the basal medium. Autoclaving removed the ethanol. One vol. albumin supplement was added to 9 vol. basal medium containing the saturated fatty acid.

Organisms used for the nutritional studies were from cultures in the logarithmic or early stationary phase of growth. Unless stated otherwise, a 1 % (v/v) inoculum, which yielded approximately 3×10^6 organism per ml. in the test medium, was used. Growth was measured daily with a Coleman (model 7) photonephelometer calibrated with an arbitrary standard (Roessler & Brewer, 1967). The relationship between nephelometer reading and number of organisms was verified by periodic counts with a Petroff–Hausser counting chamber.

RESULTS

Growth of leptospires on fatty acid esters at several temperatures

Preliminary experiments indicated that the ability of leptospires to grow on fatty acids varied with the fatty acid ester, incubation temperature and leptospiral serotype. Three serotypes of leptospires were selected which manifested different growth

patterns on a fatty acid-poor albumin medium containing polyoxyethylene sorbitan monolaurate (Tween 20), monopalmitate (Tween 40), monostearate (Tween 60) or monooleate (Tween 80). One of the serotypes selected, serotype *semaranga*, is a member of the 'biflexa complex'. This serotype grew on all the Tweens at 13°, 30° and 34° and to approximately the same extent on each Tween at a given temperature (Table 1). Serotypes *canicola* and *ballum*, members of the 'parasitic complex', did not grow on any of the Tweens at 13°. As the incubation temperature was increased to 17°, some growth of serotype *canicola* occurred in Tween 20 and good growth in Tween 80. However, no growth occurred in Tween 40 and 60. At 30° and 34° growth of this serotype was observed on all the Tweens (Table 1). Serotype *ballum* differed from serotype *canicola* in that Tween 80 was the only Tween that supported growth at 17°, 30° and 34°. Hence, at a near minimal growth temperature, the unsaturated fatty acid ester was the best substrate for serotype *canicola* and it was the only fatty acid ester which supported the growth of serotype *ballum* at 17°, 30° or 34°. Moreover, a difference in fatty acid requirement between members of the 'biflexa complex' and 'parasitic complex' is apparent.

Table 1. *Growth of leptospires on various Tweens at different temperatures**

		Increase in number of leptospires/ml. ($\times 10^7$)			
Serotype tested	Incubation temperature	Tween 20 (C_{12})†	Tween 40 (C_{16})†	Tween 60 (C_{18})†	Tween 80 $(C_{18:1})$†
semaranga	13°	<2	43	35	46
VELDRAT SEMARANGA	30°	<2	52	45	50
173	34°	30	24	30	28
canicola	13°	< 2	< 2	< 2	< 2
HOND UTRECHT IV	17°	23	< 2	< 2	52
	30°	48	44	37	52
	34°	34	28	27	31
ballum S 102	13°	< 2	< 2	< 2	< 2
	17°	< 2	< 2	< 2	< 2
	30°	< 2	< 2	< 2	44
	34°	< 2	< 2	< 2	30

* Tween concentration 0·1 % (v/v). Incubation time at 30° and 34° was 5–7 days, and at 13° and 17° it was 10–14 days.
† Major fatty acid component of Tween.

Growth of leptospires on fatty acids

Since the Tweens are not chemical entities but rather are mixtures of fatty acid esters with one member of the mixture being the major component, we tested media in which the Tweens were replaced by high purity fatty acids. Serotype *semaranga* grew on all the saturated and unsaturated fatty acids tested (Table 2). The growth pattern of serotype *canicola* and *ballum* on these fatty acids differed markedly from that of serotype *semaranga*. The parasitic serotypes only grew well on the long chain unsaturated fatty acids, palmitoleic ($C_{16:1}$) and oleic ($C_{18:1}$). Poor or no growth occurred on the shorter chain unsaturated fatty acid, myristoleic ($C_{14:1}$), and on the saturated fatty acids, lauric (C_{12}), myristic (C_{14}), palmitic (C_{16}) and stearic (C_{18}) (Table 2).

To determine whether the saturated fatty acids were growth inhibitory *per se* or were substrates which the members of the 'parasitic complex' could not utilize, the

growth of leptospires in the albumin medium containing 1×10^{-4} M of the unsaturated fatty acid, oleic acid, and 3×10^{-4} M of a saturated fatty acid was investigated. Serotype *semaranga* grew well on the saturated fatty acids in presence or absence of oleic acid. In contrast to serotype *semaranga*, the growth of serotypes *canicola* and *ballum* on saturated fatty acids was markedly affected by the presence of oleic acid. In the absence of oleic acid, no significant growth occurred on 3×10^{-4} M-palmitic acid. However, good growth occurred on this fatty acid in the presence of oleic acid (Table 3). The amount of growth obtained was several times that with oleic acid alone, which demonstrated that the saturated fatty acid was utilized. Similar results were obtained

Table 2. *Growth of leptospires on various fatty acids**

	Fatty acid tested (3×10^{-4} M)						
Serotype tested	Lauric (C_{12})	Myristic (C_{14})	Palmitic (C_{16})	Stearic (C_{18})	Myristoleic ($C_{14:1}$)	Palmitoleic ($C_{16:1}$)	Oleic ($C_{18.1}$)
semaranga VELDRAT SEMARANGA 173	43	38	51	48	35	55	53
canicola HOND UTRECHT IV	15	< 2	< 2	< 2	4	45	51
ballum S 102	< 2	< 2	<.2	< 2	< 2	36	41

* Growth expressed as number of leptospires $\times 10^7$/ml. Incubation was at 30° for 7–10 days. The first number in parentheses under the common name of the fatty acid indicates number of carbon atoms; the second number indicates the number of unsaturated bonds. Similar results were obtained with 2 and 4×10^{-4} M concentrations of these fatty acids.

Table 3. *Effect of an unsaturated fatty acid on the growth of leptospires on a saturated fatty acid**

	Fatty acids tested		
	Oleic ($C_{18:1}$) 1×10^{-4} M	Palmitic (C_{16}) 3×10^{-4} M	Palmitic 3×10^{-4} M +oleic 1×10^{-4} M
semaranga VELDRAT SEMARANGA 173	28	53	66
canicola HOND UTRECHT IV	27	< 2	68
ballum S 102	19	< 2	57

* Growth expressed as number of leptospires $\times 10^7$/ml. Incubation was at 30° for 7 to 10 days.

with the saturated fatty acids, lauric, myristic and stearic. This data indicated that the parasitic leptospires were unable to utilize the saturated fatty acids unless an unsaturated fatty acid was present. Serotype *semaranga*, a member of the 'biflexa complex', did not manifest this requirement for an unsaturated fatty acid.

Various unsaturated fatty acids were tested for their capacity to permit serotype *ballum* to utilize the saturated fatty acids. A concentration of 1×10^{-4} M-linolenic ($C_{18:3}$), linoleic ($C_{18:2}$), oleic (*cis*-9-18:1), palmitoleic or myristoleic acid permitted serotype *ballum* to grow on 3×10^{-4} M-palmitic acid. Thus, the polyunsaturated fatty

acids as well as unsaturated fatty acids varying in chain length from 18 to 14 carbon atoms were capable of permitting growth on palmitic acid. However, chain length became a factor when the unsaturated fatty acid was the sole carbon and energy source.

Effect of fatty acid chain length on leptospiral growth

Myristoleic acid was not a satisfactory carbon and energy source for serotypes *ballum* or *canicola*, whereas unsaturated fatty acids containing greater than 14 carbon atoms were suitable growth substrates (Table 2). These observations suggested that the parasitic leptospires required both an unsaturated fatty acid and a chain length of greater than 14 carbon atoms for growth. Serotypes *ballum* and *canicola* were therefore inoculated into media containing 3×10^{-4} M-myristoleic and 1×10^{-4} M-lauric, tridecanoic (C_{13}), myristic, pentadecanoic (C_{15}), palmitic, or heptadecanoic (C_{17}) acid. As seen in Table 4, growth of serotype *canicola* occurred in myristoleic acid only when the supplemental fatty acid contained 15 or more carbon atoms. Serotype *ballum* required a supplemental fatty acid of at least 16 carbon atoms.

Table 4. *Effect of fatty acid chain length on growth of leptospires on myristoleic acid**

Additions to test medium containing 3×10^{-4} M-myristoleic acid ($C_{14:1}$)†	Serotypes tested		
	semaranga VELDRAT SEMARANGA 173	*canicola* HOND UTRECHT IV	*ballum* S 102
None	34	3	< 2
Lauric acid (C_{12})	40	< 2	< 2
Tridecanoic acid (C_{13})	38	< 2	< 2
Myristic acid (C_{14})	41	< 2	< 2
Pentadecanoic acid (C_{15})	46	43‡	< 2
Palmitic acid (C_{16})	55	48	38
Heptadecanoic acid (C_{17})	58	54	46

* Growth expressed as number of leptospires $\times 10^7$ per ml. Incubation at 30° for 7 to 10 days.
† Concentration of saturated fatty acid tested 1×10^{-4} M.
‡ 16 day incubation time required.

Effect of temperature on fatty acid requirement

The experiment with the Tweens (Table 1) indicated that the parasitic leptospires had an increased requirement for an unsaturated fatty acid at 17°. The effect of temperature on the fatty acid requirement of serotype *canicola* was investigated. As seen in Table 5, ratios of oleic acid to myristic acid of greater than 1:4 would not support growth of serotype *canicola* at 17°, whereas at 30°, growth occurred in the 1:32 ratio. At 36°, an increase in the oleic acid requirement was also observed. Growth occurred in the 1:2 ratio but not the 1:4 ratio.

The growth of the parasitic serotypes on the Tweens also suggested that serotype *ballum* had a greater requirement for unsaturated fatty acids than serotype *canicola* (Table 1). The growth of these two serotypes on varying ratios of oleic and myristic acid at different temperatures was compared (Table 5). Serotype *ballum* was restricted to growth in media containing only oleic acid at 17° and 36°, whereas serotype *canicola* grew at these temperatures in ratios of 1:4 and 1:2 respectively. At 30° serotype *canicola* grew in a ratio of 1:32 of these fatty acids, while serotype *ballum* was restricted to ratios of 1:2 or less. Several other parasitic serotypes also were found to differ in

their requirement for unsaturated fatty acids. Serotypes *tarassovi, alexi, poi, coxi* and *celledoni* required higher proportions of oleic acid to grow on myristic acid than did serotypes *woiffi, grippotyphosa* and *butembo*. Thus, the first group of serotypes resemble serotype *ballum* and the latter, serotype *canicola*.

Table 5. *Effect of temperature on the fatty acid requirements*

Temperature of incubation	Ratio of oleic acid ($C_{18:1}$) to myristic acid (C_{14})							
	6:0	1:1	1:2	1:4	1:8	1:16	1:32	0:6
Serotype *canicola* HOND UTRECHT IV								
17°	56	70	57	31	< 2	< 2	< 2	< 2
30°	38	70	74	72	76	53	34	< 2
36°	38	33	21	< 2	< 2	< 2	< 2	< 2
Serotype *ballum* S 102								
17°	32	< 2	< 2	< 2	< 2	< 2	< 2	< 2
30°	38	33	30	< 2	< 2	< 2	< 2	< 2
36°	37	< 2	< 2	< 2	< 2	< 2	< 2	< 2

Inoculum size, 5 % (v/v). Incubation times: 17°, 18 days; 30° and 36°, 7 days. Total fatty acid concentration, 6×10^{-4} M. Growth expressed as number of leptospires $\times 10^7$ per ml.

Table 6. *Growth of 'biflexa complex' leptospires on 5×10^{-4} M-myristic acid (C_{14})*

Serogroup Serotype, strain	Number of leptospires $\times 10^7$ per ml.
Semaranga	
semaranga, VELDRAT SEMARANGA 173	48
patoc PATOC I	63
sao-paulo SAO PAULO	49
Andamana	
andamana CH 11	30
andamana CORREO	46
Biflexa	
biflexa A-284	38
biflexa CDC	33
biflexa LT 430	68
biflexa LT 965	43
biflexa WAZ	43
biflexa GENT	18

Incubation was at 30° for 7–10 days.

The parasitic leptospires, serotypes *ballum* and *canicola*, did not grow in myristic acid (Table 2) unless the medium was supplemented with an unsaturated fatty acid such as oleic acid. Serotype *semaranga*, a member of the 'biflexa complex', grew well in myristic acid in the absence of any exogenous source of unsaturated fatty acid (Table 2). These observations suggested that this difference in the requirement for an unsaturated fatty acid might serve as a taxonomic tool for differentiating leptospires of the 'parasitic complex' from those of the 'biflexa complex'. To test this possibility, media containing 5×10^{-4} M-myristic acid was tested for its ability to support the growth of 24 serotypes of the 'parasitic complex' and 11 members of the 'biflexa complex'. A 1 % (v/v) was found to be most satisfactory for this study. Serotypes *alexi* HS 616, *arboreae* ARBOREA, *australis* BALLICO, *bakeri* LT 79, *ballum* MUS 127,

bataviae SWART, *borincana* HS622, *butembo* BUTEMBO, *canicola* HOND UTRECHT IV, *castellonis* CASTELLON 3, *celledoni* CELLEDONI, *copenhageni* M20, *coxi* COX, *gatuni* LT839, *grippotyphosa* MAL1540, *icterohaemorrhagiae* W39, *javanica* VELDRAT BATAVIAE 46, *kabura* KABURA, *mankarso* MANKARSO, *pomona* POMONA, *pyrogenes* SALINEM, *rachmati* RACHMAT, *sarmin* SARMIN, *sorex-jalna* SOREX-JALNA and *wolffii* 3705, members of the 'parasitic complex', were unable to grow in 5×10^{-4} M-myristic acid. As seen in Table 6, all leptospires of the 'biflexa complex' grew in this medium.

DISCUSSION

We recently reported that leptospires of the 'biflexa complex' could be differentiated from those of the 'parasitic complex' by their ability to grow at low temperatures (Johnson & Harris, 1967). The minimal growth temperature of the parasitic leptospires was between 13° and 15°. Leptospires of the 'biflexa complex' have a minimal growth temperature between 5° and 10°, or approximately 5° below that of the pathogens. Preliminary studies indicated that differences in fatty acid requirements might be responsible in part for this difference in minimal growth temperature. As reported by Auran & Johnson (1968) and in this paper, major differences in fatty acid requirement do exist between members of the two complexes of leptospires, and temperature does influence the fatty acid requirements of the parasitic leptospires. The parasitic leptospires cannot utilize saturated fatty acids unless they are also given an unsaturated fatty acid. Moreover, the parasitic leptospires cannot utilize either saturated or unsaturated fatty acids containing fewer than 15 to 16 carbon atoms as their sole carbon source. However, a longer chain fatty acid permits these leptospires to utilize the shorter chain fatty acids. Leptospires of the 'biflexa complex' have neither the requirement for fatty acid chain length nor the requirement for unsaturated fatty acids. They grow on saturated fatty acids containing less than 15 carbon atoms, although the longer chain fatty acids are better carbon and energy sources. The growth of leptospires of the 'biflexa complex' on the Tweens at temperatures above or below their optimal did not demonstrate any temperature-relative change in their fatty acid requirements. In contrast, the parasitic leptospires, when growing on mixtures of saturated and unsaturated fatty acids, required increased proportions of unsaturated fatty acids at near minimal and maximal growth temperatures. The basis for this unsaturated fatty acid requirement of the parasitic leptospires is not known at the present time.

The fatty acid requirements of parasitic leptospires have been studied in media containing serum or serum fractions and in synthetic media. Oleic acid or compounds containing this unsaturated fatty acid supported good growth of leptospires in media containing extracted rabbit serum (Van Eseltine & Staples, 1961), extracted rabbit serum albumin (Johnson & Gary, 1963), and bovine albumin (Ellinghausen & McCullough, 1965). The inability of parasitic leptospires to utilize saturated fatty acids was not observed in our earlier work (Johnson & Gary, 1963). We reported that serotype *pomona* grew in an extracted albumin medium with either long or short chain saturated fatty acids as the major added carbon source. The most probable explanation for the discrepant results is that sufficient long chain unsaturated fatty acid remained in the albumin preparation to allow growth on the saturated fatty acids. Both saturated and unsaturated fatty acids have been reported to serve as the carbon

source for parasitic leptospires grown in synthetic media (Vogel & Hutner, 1961; Stalheim & Wilson, 1964; Van Eseltine, Adams, Prochazka & Wooley, 1967; Shenberg, 1967). It is difficult to evaluate the results of these studies since: (1) many of the strains of leptospires used were selected for their ability to grow in the media (e.g. nutritional mutants); (2) fatty acid-binding agents such as albumin were missing from these media making it difficult to differentiate between the nutritional qualities of the fatty acids and their lytic activities. In general, synthetic media containing oleate esters supported the best growth. It is of interest to note that the serotypes of parasitic leptospires that grew poorly or not at all in the synthetic medium of Shenberg (1967) are those which we found to have a high requirement for unsaturated fatty acids.

The usual habitat of the parasitic leptospires is the mammal. In this controlled environment the leptospires are exposed to a constant temperature and an abundant source of long chain saturated and unsaturated fatty acids. The leptospires of the 'biflexa complex' have not been clearly demonstrated to be animal parasites and appear to be closely associated with a soil-water environment in nature (Henry & Johnson, 1968). In this environment they are exposed to variations in available nutrients as well as variations in temperature. Because the leptospires of this group must be more adaptable, they have the enzymes necessary for utilizing either saturated or unsaturated fatty acids, the shorter chain fatty acids, and an enzymic make-up which allows them to grow in a wide range of temperatures below their optimal. In contrast, the parasitic leptospires depend upon the types of fatty acids available in the mammalian host, namely the long chain saturated and unsaturated fatty acids, and they do not grow well at the low temperatures. Serotypes such as *ballum*, which are lipase (trioleinase) negative (Johnson & Harris, 1968), having a relatively high requirement for unsaturated fatty acids and not producing a severe disease in mammals, are probably older, well-adapted parasites. Serotypes such as *canicola*, which possess lipase activity, having a lesser requirement for unsaturated fatty acids and producing a more severe disease, may be parasites of more recent origin.

REFERENCES

AURAN, T. B. & JOHNSON, R. C. (1968). Nutrition and growth of leptospires. *Bact. Proc.* p. 27.
ELLINGHAUSEN, H. C., JUN. & McCULLOUGH, W. G. (1965). Nutrition of *Leptospira pomona* and growth of 13 other serotypes: Fractionation of oleic albumin complex and a medium of bovine albumin and polysorbate. *Am. J. vet. Res.* **26**, 45.
HENRY, R. A. & JOHNSON, R. C. (1968). Ecological studies on leptospires of the 'biflexa complex'. *Bact. Proc.* 1968, p. 33.
JOHNSON, R. C. & GARY, N. C. (1963). Nutrition of *Leptospira pomona*. II. Fatty acid requirements. *J. Bact.* **85**, 976.
JOHNSON, R. C. & HARRIS, V. G. (1967). Differentiation of pathogenic and saprophytic leptospires. I. Growth at low temperatures. *J. Bact.* **94**, 27.
JOHNSON, R. C. & HARRIS, V. G. (1968). Purine analogue and lipase activity of leptospires. *Appl. Microbiol.* **16**, 1584.
ROESSLER, W. G. & BREWER, C. R. (1967). Permanent turbidity standards. *Appl. Microbiol.* **15**, 1114.

SHENBERG, E. (1967). Growth of pathogenic Leptospira in chemically defined media. *J. Bact.* **93**, 1598.

STALHEIM, O. H. V. & WILSON, J. B. (1964). Cultivation of leptospirae. II. Growth and lysis in synthetic medium. *J. Bact.* **88**, 55.

VAN ESELTINE, W. P. & STAPLES, S. A. (1961). Nutritional requirements of leptospirae. I. Studies on oleic acid as a growth factor for a strain of *Leptospira pomona*. *J. infect. Dis.* **108**, 262.

VAN ESELTINE, W. P., ADAMS, M. A., PROCHAZKA, J. K. & WOOLEY, R. E. (1967). Nutritional requirements of leptospirae. II. A chemically defined medium supporting growth of *Leptospira pomona*. *Bull. Georgia Acad. Sci.* **25**, 222.

VOGEL, H. & HUTNER, S. H. (1961). Growth of Leptospira in defined media. *J. gen. Microbiol.* **26**, 223.

WORLD HEALTH ORGANIZATION. (1967). *Current problems in leptospirosis research. Wld Hlth Org. techn. Rep. Ser.* **380**.

Lysogenic Mycobacteria: Phage Variations and Changes in Host Cells

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SUMMARY

Lysogenic conversion of mycobacteria by phage Roy results in reciprocal changes in the phase and in the host cells. The temperate phage released after lysogenization differs from the converting phage in its host range. The lysogenic derivatives show marked changes in their colony morphology and in their sensitivity to phagolysis. Some, but not all, of the lysogenic bacteria have suffered losses in enzymic activity. An attempt is made to distinguish between host-determined and phage-determined variations.

INTRODUCTION

Phage-determined changes in colony morphology, enzymic activity and other properties of lysogenized mycobacteria were described in 1961 (Mankiewicz, 1961). White, Foster & Lyon (1962) reported alterations of colony morphology in strain ATCC607 after lysogenization with phages R1 and D32. Russell, Jann & Froman (1963), however, did not detect differences between stock cultures of strain ATCC607 and derivatives of this strain produced by lysogenization with one or other of five phages. Vandra & Takats (1965) lysogenized *Mycobacterium fribergensis* and *M. rabinowitsch* with lytic phages. The staining properties, colony morphology, amidase and catalase activity and synthesis of niacin were similar in both the phage-sensitive parent strains and their lysogenic derivatives. Juhasz & Boenicke (1966) reported 'changes' in colony morphology, and decrease in the growth rate of *M. phlei* F89 after its lysogenization with phage B2. Recently, Jones & White (1968) described the conversion of colony morphology, nitrate reductase activity and Tween hydrolysis of strain ATCC607 associated with its lysogenization by phages D29 and B4.

Changes in bacterial properties obtained with some phage-host systems but not with others could be due to the selective effect of the phage on mycobacterial mutants that pre-existed within the particular bacterial population.

The following experiments were undertaken to demonstrate that, within some pure and homogeneous mycobacterial strains, lysogenization determines host- and phage-variants, while in other phage-sensitive strains no such reciprocal changes occur. This indicates that lysogenization may involve, or determine, different genetic processes in different mycobacterial strains.

METHODS

Mycobacteriophage Roy was isolated in our laboratories from the sputum of a patient with thoracic sarcoidosis. Repeated cultures of sputum from this patient had been negative for mycobacteria. Since its isolation in liquid medium enriched with strain ATCC607 (R) this phage had been propagated on this strain only, and its host range and lytic activity have remained the same. Phage Roy produces 0·8–1·0 mm. clear plaques with regular borders on strains ATCC607 and on TMC1515. At a phage: bacterium ratio of 1 : 10, its lytic cycle in ATCC607 consists of an absorption period of 30 min. (65 % absorption), a latent period of 70 min., a rise period of 30 min. The lytic cycle is completed in approximately 2½ hr. The average burst size is 92. Electron-microscopic studies show this phage to be formed by a head hexagonal in structure measuring 92–94 mμ in length and 53–54 mμ in width. The length of the tail is approximately 250 mμ.

Media for phage study. The RVB liquid medium for the preparation of phage filtrates and the RVA agar medium for phage typing and phage titrations (in r.t.d. and p.f.u.) were prepared and used according to the recommendations of Redmond & Ward (1966).

Table 1. *Mycobacterial strains used in this study*

Species and group	Strain or source
M. tuberculosis, var. *hominis*	H37RV, Steenken, Trudeau
M. tuberculosis, var. *bovis*	BCG, Université de Montréal
Atypical Mycobacteria:	
Runyon group I	TMC1201
	TMC1203
	TMC1204
	1518, Royal Edward Chest Hospital
Runyon group II	TMC1302
	TMC1306
	18685, Royal Edward Chest Hospital
Runyon group III	TMC1403
	TMC1404
M. fortuitum	TMC1512
M. smegmatis	ATCC607
	TMC1515
M. fribergensis	E. Vandra, Budapest
M. phlei	E. Vandra, Budapest
M. rabinowitsch	E. Vandra, Budapest
M. stercoides	E. Vandra, Budapest
M. lacticola	ATCC9626
Mycobacterium sp.	ATCC9033
Mycobacterium sp.	ATCC4243

TMC = Trudeau Mycobacterial Collection.
ATCC = American Type Culture Collection.

Bacteria. The twenty-one mycobacterial strains included in this study are tabulated in Table 1. Before use, they were plated on RVA agar medium and one colony was selected, suspended in nutrient broth (Difco) and replated on solid medium. This process of colony selection was repeated twice for each strain. Three colonies were selected from the last plating and propagated separately. The three subcultures of each strain were submitted to the tests indicated below, in order to compare their properties and to ascertain the homogeneity and purity of each strain.

The following characters were studied: growth rate in liquid and on solid medium at 37° and at 22°; colony morphology and the conditions for their pigmentation; cord formation and acid fastness in liquid medium; utilization of paraffin as the sole carbon source in Czapek's mineral medium (without sucrose); catalase and peroxydase activity (Tirunaranay & Fischer, 1957), the amidase reactions (Boenicke, 1958), synthesis of niacin (Konno, 1957) and, for bacilli from group IV, the transformation of ammonium ferrocitrate (Tison, Tacquet & Devulder, 1964). Sensitivity to strepto-mycin, isoniazid and to p-aminosalicylic acid was determined as well as the sensitivity of each strain to phagolysis by phages DS6, G4E, AG1 of Redmond's phage-typing scheme (Redmond, 1963). The presence or absence of an inducible prophage was studied in filtrates of cultures that had been irradiated with u.v. for 30 sec. at 10 cm. distance (15 watt G.E. germicidal tube) or had been exposed to hydrogen peroxide (10 %) or Actinomycin-C (10 μg./ml.). Lysis of the mycobacterial strains by phage Roy was determined in the following way: the bacteria were plated on solid medium and incubated for three hours. Droplets of a phage suspension that just produced an area of clear lysis on ATCC607 were deposited on the bacterial lawns. The plates were re-incubated and read after intervals of 48 hr to 10 days. Only complete clearing of the 'spots' was considered as true lysis.

Lysogenization of the mycobacterial strains. Five cultures of each bacterial strain in RVB medium were infected with phage Roy in a phage/cell ratio of 5:1. The infection took place during the exponential growth period of the mycobacteria: the fast growing bacteria were infected after three hr incubation; the slow growing organisms, after 18 hr. The cultures were re-incubated for 3 hr and one ml. samples of the cultures were added to equal volumes of phage antiserum that neutralized 98 % of free phage particles. After three hr incubation with the antiserum, the cultures were centrifuged and the sediments were suspended, each in 0·2 ml. of RVB medium. These suspensions were then plated on RVA agar. Colonies produced by bacteria that survived the phage infection were isolated and propagated in RVB medium to which Tween 80 at a concentration of 0·1 % was added in order to avoid re-infection by free phage particles. Subcultures on RVA medium and on Loewenstein medium were then submitted to a study of their bacterial properties by means of the above-mentioned tests.

As proposed by Russell, Jann & Froman (1963), temperate phages released by lysogenic mycobacteria are designated by the name of the phage, followed in paren-theses by the name of the mycobacterium in which lysogeny was established; lysogenic derivatives are designated by the name of the parent strain, followed in parentheses by the name of the converting phage.

RESULTS

Phage variations. Phage Roy (607) and phage Roy (1515) produce clear spots on mycobacterial strains 607 and 1515. Under experimental conditions for lysogenization, they also produce phage-resistant lysogenic bacteria within these two strains. These release phage particles of the same lytic pattern and produce the lysogenic conversion of the same bacterial strains as did the virulent phage. By its lytic activity, phage Roy is a phage specific for *Mycobacterium smegmatis*.

The nineteen remaining strains resisted phagolysis. Seven strains, however, produced lysogenic colonies after incubation with phage Roy. The number of colonies under-

going lysis after u.v.-induction of the phage-infected population varied from 65 to 90 %. The phage particles released by the lysogenic bacteria showed marked changes in lytic pattern. With one exception, the lytic spectrum had expanded and included, in every instance, the respective host strain. The strains susceptible to lysogenization by temperate phages were the same that were susceptible to the parent phage; efficiency to establish the lysogenic state was, however, much greater for the temperate phages. The lytic spectrum of phage Roy and of the phage variants is summarized in Table 2.

Table 2. *Lytic activity of phage Roy and its host-determinant variants*

Indicator	Phage Roy (607)	(1518)	(1302)	(1306)	(1512)	(Phlei)	(Ster-coides)	(Lacti-cola)
Strain 607	+	+	+	+	+	−	+	+
1515	+	+	+	+	+	−	+	+
1518	−	+	+	+	−	−	−	−
1302	−	−	+	+	−	−	−	−
1306	−	−	+	+	−	−	−	−
1512	−	−	−	−	+	−	−	−
M. phlei	−	−	−	−	−	+	+	−
M. stercoides	−	−	−	−	+	+	+	+
M. lacticola	−	−	−	−	−	−	−	+

Table 3. *Changes in growth rate, colony morphology, pigmentation of colonies and in the microscopic aspect in mycobacteria lysogenized with phage Roy*

Lysogenic derivative of	Growth rate	Colony	Pigmentation	Bacterial morphology
607	Delay	R. S nippled	Earlier	Swollen cytoplasma which lost acid fastness. Heavy beading
1515	Delay	R, S to S	No change	Partial loss of acid fastness
1518	Delay	S	No change	Partial loss of acid fastness
1302	Delay	R, S	Dissociation into pig-mented and non-pigmented umbilicated colonies	Partial loss of acid fastness
1306	Delay	R to S	No change	No change
1512	Delay	S	No change	Partial loss of acid fastness
M. phlei	Delay	S	No change	Relative loss of acid fast-ness
M. stercoides	Delay	S, nippled colonies	No change	Heavy beading, partial loss of acid fastness
M. lacticola	Delay	S	Increase in pigmen-tation	Heavy beading, partial loss of acid fastness

R = Rough.   S = Smooth.

Changes in bacterial properties associated with lysogenization. Ten mycobacterial variants were isolated from the nine strains lysogenized by phage Roy: two from strain 1302; one from each of the following: 607, 1515, 1518, 1306, 1512, *Myco-bacterium phlei*, *M. stercoides* and *M. lacticola*. The lysogenic derivatives shared the ability to produce phage particles, the lytic activity of which has been described previously. They all showed decrease in their growth rate during the first two or three

subcultures. Instead of the rough or wrinkled colonies of most of the parent strains, lysogenic strains produced smooth colonies, some of them heaped or nippled. Microscopically, bacteria from these colonies showed a relative loss in acid fastness that affected mainly the cytoplasma; Much's granula were purple and caused heavy 'beading' of the bacteria. The lysogenic derivatives were resistant to phagolysis by the converting phage and by the phage variants. The colonies of 607 (Roy) and 1515

Table 4. *Comparison between strain* ATCC607 *and strain* ATCC607 *(Roy) isolated 8 days after phage infection*

Character on test	Parent strain	Lysogenic derivative
Colony morphology on RVA agar	Small, wrinkled or rough	Large, smooth, nippled
Pigmentation	Slight, increases with age of culture	Slight, appears immediately
Bacterial morphology and acid fastness	Long granulated rods, 95 % acid fast	Long and short (coccoid) bacilli. Loss of cytoplasma which retains less dye. Large acid fast granules (heavy 'beading')
Growth rate in RVB medium	24 hr at 37° 48 hr at 22°	4–5 days at 37° 9 days at 22°
Paraffin utilization	+ + +	—
Cord formation	—	—
Amidase reactions:		
Urease	+ +	+ +
Pyrazinamidase	+ +	+ +
Nicotinamidase	+ +	+ +
Acetamidase	±	—
Benzamidase	+ +	+ +
Succinamidase	+	+
Niacin synthesis	—	—
Catalase activity	+ + +	±
Peroxydase	—	—
Nitrate reduction	+	—
Transformation of ammonium ferrocitrate	+ +	—
Sensitivity to:		
Streptomycin (10–100 mcg./ml.)	Sensitive	Sensitive
Isoniazid (1–10 mcg./ml.)	Resistant	Resistant
PAS (1–100 mcg./ml.)	Resistant	Resistant
Lysis by phage:		
DS 6A	—	—
G 4E	—	—
AG 1	+	—
Roy	+	—
Induction by:		
u.v. (30 sec., 10 cm. distance)	—	+ +
Actinomycin-C (10 mcg./ml.)	—	+ +
H_2O_2 (10 %)	—	+ +

(Roy) and of *M. lacticola* (Roy) showed earlier and more pronounced pigmentation than did the respective parent strains. In contrast, strain 1302 (Roy) produced two kinds of colonies: one, pigmented as the parent strain; the other, nonpigmented and umbilicated. The above observations are summarized in Table 3.

The time of appearance of lysogenic derivatives was studied with fast- and slow-growing bacteria. The smooth colonies of 607 (Roy) appeared first in cultures plated 24 hr after infection. They required nine days to attain maturity in these cultures. The slow-growing 1302 (Roy) colonies appeared 10 days after phage infection. Their lag period was 5–6 days. After repeated subcultures, the lag periods were reduced considerably.

Comparison between strain 607 and its lysogenic derivative is summarized in Table 4. In addition to differences in colony morphology (Pl. 1, fig. 1), the microscopic aspect of the bacilli (Pl. 1, fig. 2) and the changes in phagolytic pattern with resistance to phage Roy as well as to phage AG 1, there was a loss of enzymic activity associated with lysogenization. These bacilli were not able to utilize paraffin as their sole carbon source. Their catalase reaction and nitrate reduction tests were negative; ammonium ferrocitrate was no longer transformed. The acetamidase reaction which already was weak in the parent strain, had completely disappeared. There was no change in the sensitivity to antimycobacterial drugs.

The lysogenic derivative of strain 1515 (Pl. 1, fig. 3) showed almost the same changes as strain 607 (Roy): it produced smooth nippled colonies, it had acquired resistance to phagolysis by phage Roy and phage AG 1 and it had lost its nitrate reductase activity. Strain 1518 (Roy) also showed resistance to phagolysis by phage Roy and phage AG 1. In this case the lysogenic conversion was associated with the loss of nicotinamidase and urease activity. The non-pigmented umbilicated colonies of strain 1302 (Roy) (Pl. 1, fig. 4) showed loss of their nicotinamidase, succinamidase and their nitrate reductase activity. Both the parent strain and the lysogenic variant are resistant to phagolysis. No changes in enzymic activity could be detected between the lysogenic strains of 1512, *Mycobacterium phlei*, *M. stercoides*, *M. lacticola* or the pigmented strain of 1302.

DISCUSSION

Variations and 'adaptations' of mycobacteriophages have been reported many times. Among thirty or more phages isolated, in Redmond's laboratories only one had been free of some variations (Redmond, 1963). Russell, Jann & Froman (1963) have shown alterations in host range between lytic phages and their temperate derivatives. These authors suggested that 'some of these alterations may be due to host-controlled modification, while others may be due to the activation of a previously undetected prophage'. The latter mechanism, or recombination, was suggested by observations recently reported on the lytic phenomena of phage Leo propagated on ATCC607 or on strain $H_{37}RV$ as alternate hosts (Mankiewicz & Redmond, 1968).

Failure to demonstrate a prophage in the bacterial parent cells may be due to lack of appropriate techniques or indicator strains, or to the particular state of the prophage. The prophage may be defective or inhibited in the expression of its genes by the action of a repressor present in the bacterial chromosome. Superinfection with phage Roy and recombination, however, may have caused the prophage to become detached from the chromosome.

Phage Roy does not lyse or lysogenize strains of *Mycobacterium tuberculosis* or representative strains from atypical mycobacteria group III. Only one of four strains of photochromogenic mycobacteria was susceptible to lysogenization by this phage. Strain 1518 has recently been isolated in our laboratories and has been classified as a

photochromogen on the basis of photo-induced pigmentation, growth rate, amidase activity, phage-typing and the other above-mentioned tests. Scotochromogenic bacteria, *M. fortuitum* and fast-growing saprophytic mycobacteria appeared to be most susceptible to phage Roy infection.

In their recent study of the conversion of colony morphology and certain enzyme systems of ATCC607 lysogenized with phage D29 or B4, Jones & White (1968) described the appearance of phage-resistant smooth colonies which were lysogenic. Their morphology appears to be quite similar to the colonies of 607 (Roy) and of 1515 (Roy). In contrast to the observations of these authors, strains lysogenized by phage Roy did not revert to the non-lysogenic state nor did they form rough colonies following subculture in media preventing phage readsorption. The high frequency of the loss of the lysogenic state together with the reversion to the formation of R colonies by the 'cured' strain of 607 suggested to Jones and White that the genetic material that controls these changes is found in the extrachromosomal plasmids. This does not seem to apply to 607 (Roy) where the described changes were hereditary and permanent.

Jones & White (1968) reported that the smooth colonies of 607 (D29 or B4) showed increased nitrate reductase activity and a higher degree of hydrolysis of Tween 80. In this study the lysogenic derivative 607 (Roy) showed a reduction in the activity of several enzyme systems including that of the nitrate reductase.

These differences—provided they are not due to technical reasons—may indicate which of the bacterial enzyme systems affected by lysogenization are phage controlled and which are host determined. Similarities in colony conversion suggest that they are independent of the specific phage, while the different effects on the enzymic activity may be determined by the converting or recombinant phage. Further studies along these lines may help to clarify the process of lysogenization and the nature of certain mycobacterial variants.

REFERENCES

BOENICKE, R. (1958). Classification des mycobactéries à l'aide des épreuves chimiques. *Bull. Un. int. Tuberc.* **28**, 162.

JONES, W. & WHITE, A. (1968). Lysogeny in mycobacteria. I. Conversion of colony morphology, nitrate reductase activity, and Tween hydrolysis of *Mycobacterium* sp. ATCC607 associated with lysogeny. *Can. J. Microbiol.* **14**, 551.

JUHASZ, S. E. & BOENICKE, R. (1966). Reciprocal genetic changes in mycobacterial host-virus systems: effect of lysogeny on the phage. *Nature, Lond.* **210**, 1186.

KONNO, K. (1957). The metabolism of nicotinic acid in mycobacteria: A method for differentiating tubercle bacilli of human origin from other mycobacteria. *Am. Rev. Tuberc.* **75**, 529.

MANKIEWICZ, E. (1961). Morphological and biological properties of a strain of chromogenic acid-fast bacteria and of its phage-immune variant. *J. gen. Microbiol.* **24**, 63.

MANKIEWICZ, E. & REDMOND, W. B. (1968). Lytic phenomena of phage Leo isolated from a sarcoid lesion. *Am. Rev. resp. Dis.* **98**, 41.

REDMOND, W. B. (1963). Bacteriophages of the mycobacteria. A review. *Adv. Tuberc. Res.* **12**, 191.

REDMOND, W. B. & WARD, D. M. (1966). Media and methods for phage-typing mycobacteria. *Bull. Wld. Hlth Org.* **35**, 563.

RUSSELL, R. L., JANN, G. J. & FROMAN, S. (1963). Lysogeny in mycobacteria. II. Some phage-host relationships of lysogenic mycobacteria. *Am. Rev. resp. Dis.* **88**, 528.

TIRUNARANAY, M. & FISCHER, W. (1957). Relationship of isoniazid to the metabolism of mycobacteria. *Am. Rev. Tuberc.* **75**, 62.

TISON, F., TACQUET, A. & DEVULDER, B. (1964). Un test simple d'étude des mycobactéries: La transformation du citrate de fer ammoniacal. *Annls Inst. Pasteur, Paris* **106**, 797.

VANDRA, E. & TAKATS, A. (1965). Induced lysogenesis of mycobacteria. *Acta microbiol. hung.* **12**, 29.

WHITE, A., FOSTER, F. & LYON, L. (1962). Alteration of colony morphology of mycobacteria associated with lysogeny. *J. Bact.* **84**, 815.

EXPLANATION OF PLATE

Fig. 1. Conversion from rough to smooth nippled colonies of strain 607 and its lysogenic derivative 607 (Roy). (*a*) Wrinkled dry colonies; strain 607. (*b*) Smooth nippled colonies after lysogenization; strain 607 (Roy).

Fig. 2. Microscopic aspect of 607 (Roy) showing large granules and loss of acid fastness of cytoplasma. ($\times$ 1400).

Fig. 3. Conversion from rough to smooth nippled colonies of strain 1515 and 1515 (Roy). (*a*) Rough dry colonies; strain 1515. (*b*) Smooth nippled colonies after lysogenization; strain 1515 (Roy).

Fig. 4. Dissociated into smooth pigmented and nonpigmented umbilicated colonies of strain 1302 and 1302 (Roy). (*a*) Round regular colonies; strain 1302. (*b*) After lysogenization, two types of colonies distinct from those of the parent strain: smooth pigmented and umbilicated non-pigmented colonies; strain 1302 (Roy).
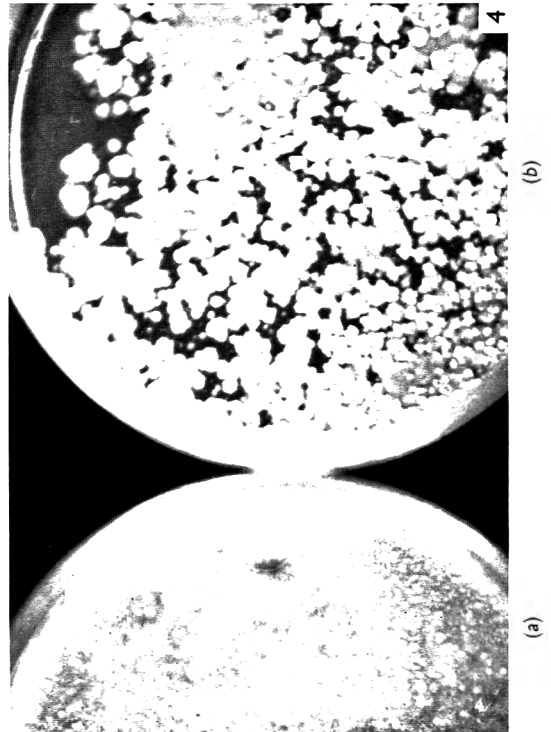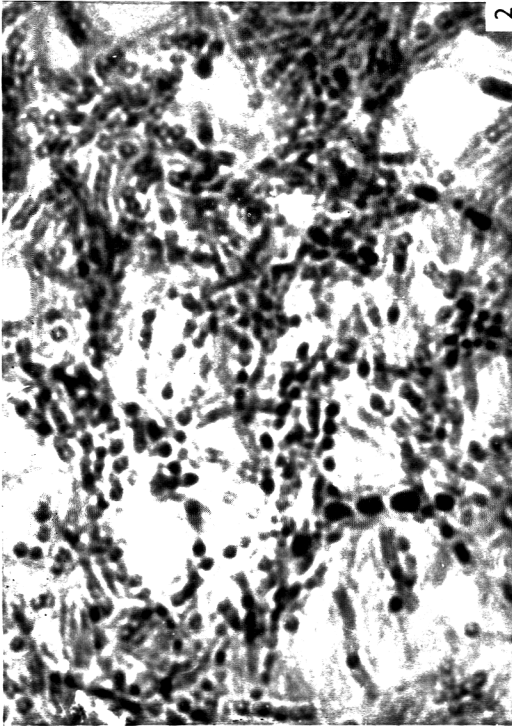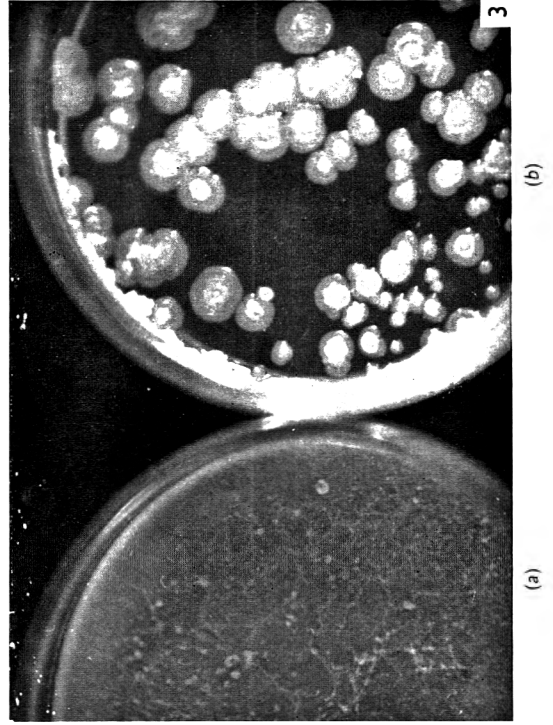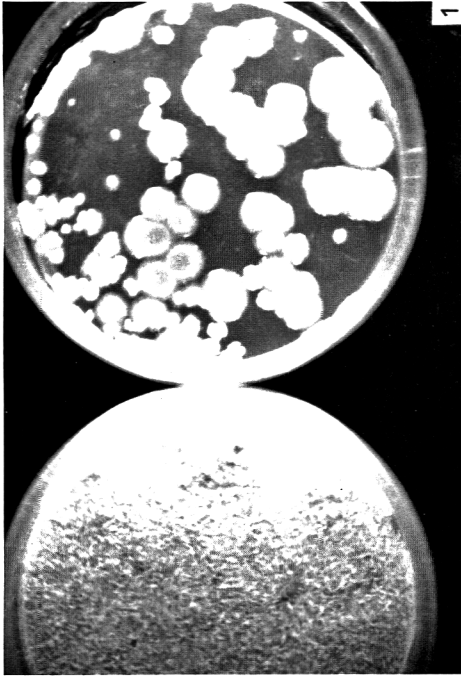
Proteins Immunologically Related to Neurospora Histidinol Dehydrogenase

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SUMMARY

Antibody to pure Neurospora histidinol dehydrogenase has been prepared from rabbits. Extracts of Neurospora strains mutant at the *his*-3 locus have been examined by the double diffusion technique to ascertain whether or not they produce proteins which cross-react with this antibody. A total of 129 mutants, including 52 non-complementing ones, gave a positive reaction; no mutants have yet been observed which gave a negative result. In all cases two major precipitin bands were produced and it was shown that these corresponded to the 4S and the 7S forms of the enzyme. Dehydrogenation of histidinol by purified wild-type enzyme was completely inhibited by the antibody prepared against pure histidinol dehydrogenase and by antibody prepared against pure HD from the mutants K445 and K959, which produce a modified HD enzyme. HD activity was also inhibited by antibody prepared against partially purified homologous protein from the two non-complementing mutants K492 and K474. It was possible to reduce the efficiency of neutralisation of HD by antibody if this was treated with extracts of mutants K492 and K474.

INTRODUCTION

In *Neurospora crassa* the *his-3* gene is responsible for the synthesis of a protein which has three enzymic functions; the most easily measured one is the dehydrogenation of histidine. This enzyme has been purified and partially characterized by Creaser, Bennett & Drysdale (1967), and it became of interest to study the effect of amino acid substitutions due to mutation on its structure and activity. Certain mutants produce proteins which have activity and these enzymes can be purified and their properties investigated (Creaser, Bennett & Drysdale, 1965, 1967, and Bennett & Creaser, 1967), but the majority of mutations at this locus do not have histidinol dehydrogenase (HD) activity. It is likely that these represent more drastic changes in the protein structure which abolish enzymic activity. One way in which these inactive proteins can be purified and analysed is if they react with antiserum prepared against pure wild type HD. This paper reports an immunological survey of the proteins produced by *his-3* mutants of *Neurospora crassa*.

Immunological studies have been performed on many enzymes (Cinader 1963) and two well studied Neurospora enzymes have been examined in this way. These are tryptophan synthetase (Suskind, 1957; Garrick & Suskind, 1963) and glutamic dehydrogenase (Roberts & Pateman, 1964 and Roberts, 1966, 1967) and in both cases proteins were detected which were not enzymically active but reacted with antisera to purified enzyme (CRM).

METHODS

Organisms. Neurospora crassa strain EMERSON A and all the mutants prefixed K used in this work were provided by Professor D. G. Catcheside. Mutants prefixed M were obtained from Dr Mary Case of Yale. All mutants, except K874 which was isolated after x irradiation, had been obtained by u.v. radiation and selected by the filtration enrichment procedure of Catcheside (1954). Mutants were grown on Vogel minimal medium (Vogel, 1955) containing 2% sucrose and 30 mg. L-histidine per litre with forced aeration. They were maintained in the same medium to which 1·5% Ionagar had been added. The wild-type organism was grown on this medium without histidine.

Antibody production. 3–10 mg. of pure enzyme made according to the procedure of Creaser *et al.* (1967), normally in 0·05 M-phosphate buffer resulting from the last stage in purification, was emulsified with an equal volume of Freund complete adjuvant and injected intramuscularly into rabbits. When necessary subsequent injections were given by the same technique but using incomplete instead of complete adjuvant.

After 2 weeks the rabbits were bled by removing 20–30 ml. from the ear vein. This blood was allowed to clot in a test tube and the clot released from the side of the tube which was then left overnight in the refrigerator for the clot to contract. The serum was decanted and centrifuged to remove blood cells and stored frozen.

Double diffusion technique. Plates were poured with 0·01 M-phosphate buffer (pH 6·8) containing 1% Ionagar. A circular mould was used 2 in. diameter and after the agar had set holes were cut with a cork borer to the patterns shown in the figures. For use the centre well was normally filled with antibody and the orbital wells with the material under test. The plates were then stored at 5° for 48 hr to permit precipitin bands to form.

Enzyme assays. Histidinol dehydrogenase activity was assayed by reduction of NAD at 340 mμ using a Shimatzu recording spectrophotometer. To 0·1–0·5 ml. of enzyme preparation was added 0·2 ml. (2 μmole) of NAD solution and tris/HCl buffer, 0·05 M (pH 9·1) containing 1 mg./ml. of 2-mercaptoethanol. After incubation of the spectrophotometer cuvette for 2 min. at 34° to permit temperature equilibration, 0·2 ml. (0·4 μmole) of histidinol solution was added and the increase in extinction at 340 mμ was recorded.

RESULTS

Preparation of antigens and mutant extracts. Pure Neurospora histidinol dehydrogenase (NHD) was prepared according to the previously published procedure (Creaser *et al.* 1967). Pure enzyme was also prepared from mutants K445 and K959 by this procedure—differing in that fractionation with ammonium sulphate was achieved with different percent saturations from those used in purifying the wild-type enzyme and the elution positions on the final chromatogram were slightly different. Less highly purified proteins were prepared from mutants K492 and K474 by following the published procedure, again modifying the ammonium sulphate fractionation step, to a point where NHD would be purified about 100-fold.

Crude extracts were prepared from the mutants by the following procedure. Mutants were grown in 8 l. batches in 10 l. bottles for 3 days at 25° with aeration; about 15 g. dry wt/organism being produced per bottle. The Neurospora was harvested

by filtration, lyophilized and stored at $-10°$ until needed. The mutant powder was moistened with 0·05 M-tris buffer (pH 9·1) and ground with glass in a cooled mortar. After grinding the preparation was extracted with the same buffer and centrifuged at 20,000g for 20 min. to remove glass particles and mycelial debris. The supernatant was decanted and used without further treatment. It was found in earlier work that the yield of active NHD was enhanced by the inclusion of 1 mg./ml. 2-mercaptoethanol in the extraction buffer. However, when the ground mycelium was extracted with pH 9·1 buffer no differences were observed in the precipitin reactions whether mercaptoethanol had been present in the buffer or not.

Survey of antigen-antibody reactions in his-3 mutants. It was found that the serum taken from rabbits 2 to 10 weeks after they had been injected with NHD would precipitate and enzymically inactivate this antigen. Accordingly a survey was made of the reaction between this antiserum and crude extracts of the available *his-3* mutants by the double diffusion technique as described. This survey was performed twice with two different batches of antiserum elicited by separate HD purifications. The results were the same in all cases in that all mutant extracts gave precipitin bands

Table 1. *Neurospora histidine-3 mutants producing CRM*

A. Non-complementing mutants

K 109, K 249, K 282, K 424, K 430, K 457, K 459, K 462, K 464, K 466,
K 479, K 485, K 487, K 491, K 492, K 494, K 502, K 503, K 504, K 57,
K 284, K 431, K 435, K 438, K 474, K 475, K 478, K 486, K 499, K 506,
K 872, K 879, K 1005, K 1077, K 1102, K 1120, K 1181, K 1201, K 1218,
K 1223, K 1224, K 1229, K 1250, K 1268, K 1271, K 1332, K 1381,
K 1488, K 1320, K 1528, K 1566, M 111, K 1079.

B. Complementing mutants

K 959, K 70, K 241, K 442, K 436, K 467, K 471, K 439, K 500, C 140,
K 1350, K 1352, K 477, K 1314, K 445, K 458, K 498, K 501, K 508,
K 441, K 1276, K 1511, M 16, K 26, K 438, K 434, K 461, K 483, K 469,
K 470, K 472, K 482, K 495, K 1179, K 1210, K 1269, K 1319, K 1440,
K 1466, K 1531, K 1151, K 509, K 511, T 1710, K 480, K 488, K 1006,
K 1142, K 1447, K 1530, K 1551, K 1023, K 737, K 874, K 1164, K 1178,
K 1208, K 1261, K 1457, K 1504, K 446, K 127, K 53, K 232, K 1069,
K 1135, K 935, K 476, K 727, M 261, M 585, M 579, M 565, M 496.

with antiserum to NHD. Table 1 lists these mutants and it can be seen that, of a total of 129 tested, 52 were non-complementing mutants. Figure 1 shows typical reactions between pure wild type HD and five mutant extracts. In all experiments in the survey each plate had one well with either crude extract of wild type or purified HD. Purified HD can exist either in the single molecular species or as a mixture of forms (see below). In Fig. 1 the pure HD used was the multiple species type. All mutants in this experiment give two bands, continuous with the two bands of the pure wild type HD and this result was obtained with all mutants listed in Table 1.

Multiplicity of Precipitin bands. It is known that NHD can exist in several polymeric forms—the two major forms being characterised by approximate sedimentation constants of 4S and 7S respectively (Creaser *et al.* 1967). Creaser *et al.* also showed that it was possible to prepare pure NHD in the single species 7S form and as the mixture of 4S+7S. Such preparations were made and tested against antiserum to pure NHD. The pure 7S form was used as the antigen but the mixed 4S+7S gave identical results. Pure 7S enzyme gave one major band, pure 4S+7S enzyme gave two

major bands which correspond to those found in crude extracts of mutants and wild type (Fig. 2). Older preparations of 7S NHD contained progressively more of the 4S form as they age.

A different type of multiple banding occurred when samples of NHD lose enzyme activity on standing at 5°. As the sample became inactivated the single 7S band split into several very close bands, possibly due to molecules in different states of oxidation.

Fig. 1   Fig. 2
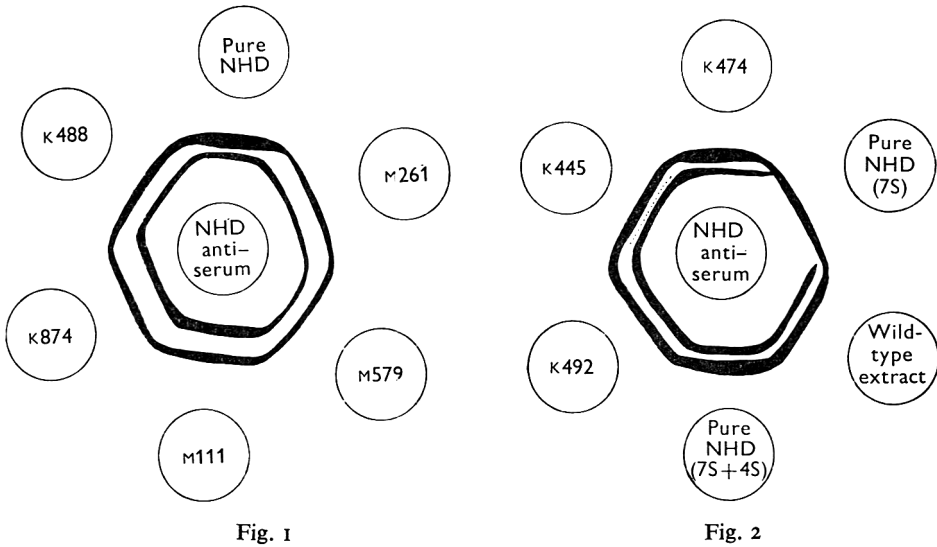
Fig. 1. Reaction between antiserum to wild type Neurospora histidinol dehydrogenase (NHD) (centre well), various crude extracts of *his-3* mutants and pure NHD (peripheral wells).

Fig. 2. Reaction between anti NHD in centre well with pure NHD in the 7S form and the 7S+4S form, crude extracts of wild type, two non-complementing mutants (K434, K492) and one complementing one (K445).
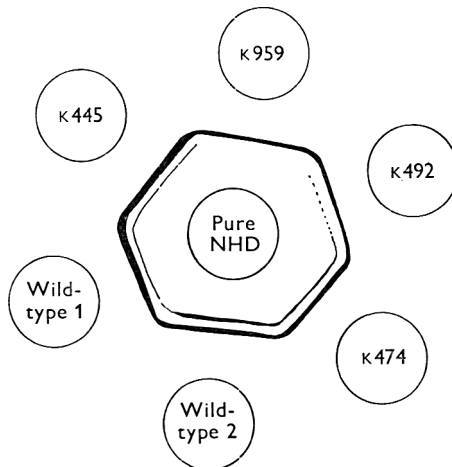
Fig. 3. Reactions between NHD and various antisera. Pure NHD in centre well with six antisera in peripheral wells. Antisera to pure NHD: two separate preparations of wild type, and preparations of K445, K595: antisera to partially purified K492 and K474.

If an enzyme preparation, either the 7S form or the mixed 4S−7S, was converted to its monomeric condition all antigenic activity was lost. Enzyme sub-units can be prepared either by oxidation with performic acid (Moore 1963) or by reduction and carboxymethylation (Moore, Cole, Gundlach & Stein 1958) but neither preparation gave precipitin bands when tested against anti-NHD serum.
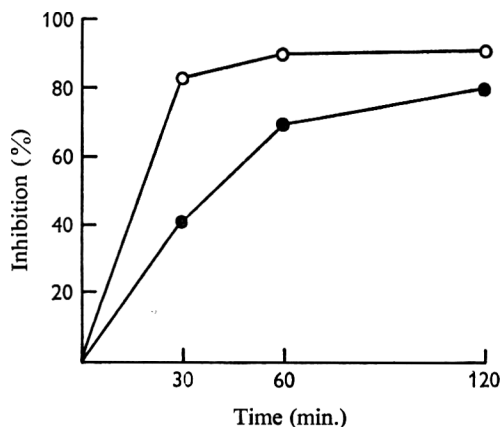
Fig. 4. Inhibition of NHD activity by 1·0 ml. antiserum O—O 0·1 ml. antiserum ●—●.
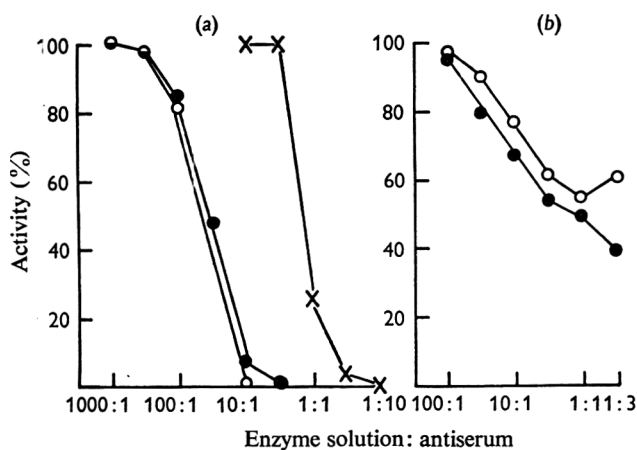
Fig. 5
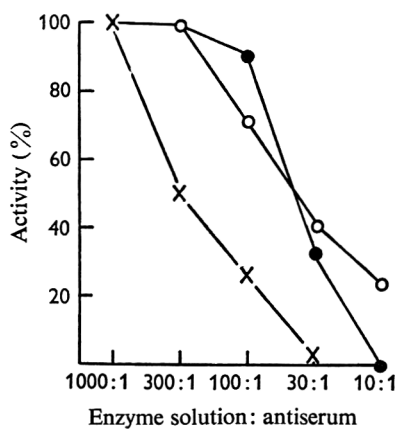
Fig. 6

Fig. 5. Inhibition of NHD activity by (*a*) Antiserum to pure enzymes; ●—● wild type, O—O K445, ×—× K959. (*b*) Antiserum to partially purified O—O K492 and ●—● K474 enzymes.

Fig. 6. Protection by two non-complementing mutant extracts O—O K492 and ●—● K474 of inhibition of NHD activity by antisera to pure NHD ×—×.

Reactions of other antisera. Antisera were prepared to pure NHD from mutants K445 and K959 originally to investigate their reactions with mutant extracts which did not react with anti wild type NHD. In the event their production was superfluous but it was confirmed that both these antisera would react with wild type enzyme and also with a random selection of mutant extracts. In addition antisera were prepared

against partly purified extracts of mutants K492, K474. These antisera although weaker than the antisera produced against pure enzymes would give precipitin bands. The reactions of various antisera against pure NHD are shown in Fig. 3.

Inhibition of enzyme activity by antisera. It was found that, as would be expected from the double diffusion experiments, histidinol dehydrogenase activity of either crude or pure NHD was inhibited by antiserum prepared against this enzyme. Preliminary experiments established the range for which inhibition occurred and Fig. 4 shows that the reaction was quite slow when the lower concentration of antibody was used. Normally, therefore, the reaction was allowed to proceed for 3 hr at 37°. For this experiment o·1 ml. of enzyme, normally about 25 μg, and with an activity of o·05 i.u./o·1 ml. was incubated with o·1 ml. of antibody of various dilutions. After incubation the sample was assayed for histidinol dehydrogenase activity as described above and the results were expressed as percentage of the activity of a similar sample incubated in the absence of antiserum. Figures 5a and 5b show the inhibition by five antisera. The antisera produced to the purified enzymes (a) are more effective than those produced in response to injections of the partially purified homologous proteins (b). Control sera taken from these rabbits before injecting the antigens did not inhibit HD activity.

Figure 6 shows that incorporation of extracts of K474 and K492 in the incubation mixture had a considerable effect in antagonizing the effect of anti NHD on the dehydrogenation reaction.

DISCUSSION

In view of the surprising result that all mutants tested, including the non-complementing ones, produced material which would cross react with anti-NHD serum it was thought advisable to check this observation by using antiserum to several mutants, especially non-complementing ones. The supporting evidence resulting, together with experiments on the effects of various antisera on dehydrogenation of histidinol by NHD and the protective effect of K474 and K492 extracts, makes it reasonably certain that all of the *his-3* mutants tested produced a protein which was sufficiently similar to NHD to give a cross-reaction.

These proteins must have some tertiary structure because monomeric units would not cross react; mutations giving rise to single amino acid changes—missense mutants—could produce such proteins. If one assumes that a considerable portion of the HD polypeptide is needed for polymerisation to occur then chain termination and frame shift mutations could only give rise to protein which is antigenically recognizable when the mutation was close to that end of the gene which was translated last. In this event all such mutations could not be distinguished antigenically from simple missense mutations.

REFERENCES

BENNETT, D. J. & CREASER, E. H. (1967). Amino acid substitutions in mutant forms of histidinol dehydrogenase from *Neurospora crassa*. *Biochem. J.* **105**, 39.

CATCHESIDE, D. G. (1954). Isolation of nutritional mutants of *Neurospora crassa* by filtration enrichment. *J. gen. Microbiol.* **11**, 34.

CINADER, B. (1963). Introduction: Immunochemistry of enzymes. *Ann. N.Y. Acad. Sci.* **103**, 495.

CREASER, E. H., BENNETT, D. J. & DRYSDALE, R. B. (1965). Mutant forms of histidinol dehydrogenase from *Neurospora crassa. Can. J. Biochem.* **43**, 993.

CREASER, E. H., BENNETT, D. J. & DRYSDALE, R. B. (1967). The purification and properties of histidinol dehydrogenase from *Neurospora crassa. Biochem. J.* **103**, 36.

GARRICK, M. D. & SUSKIND, S. R. (1963). Antigenically active fragments of tryptophane synthetase. *Ann. N.Y. Acad. Sci.* **103**, 793.

MOORE, S. (1963). On the determination of cystine as cysteic acid. *J. biol. Chem.* **238**, 235.

MOORE, S., COLE, D. R., GUNDLACH, H. G. & STEIN, W. H. (1958). On the cleavage of disulfide bonds in proteins by reduction. *Proc. IV. int. Congr. Biochem.* **8**, 52.

ROBERTS, D. B. (1966). Immunological and enzymatic studies on glutamic dehydrogenase and a related protein from *Neurospora crassa. J. Bact.* **91**, 1888.

ROBERTS, D. B. (1967). Immunological studies on glutamic dehydrogenase and two mutant forms of the protein. *J. Bact.* **94**, 958.

ROBERTS, D. B. & PATEMAN, J. A. (1964). Immunological studies of amination deficient strains of *Neurospora crassa. J. gen. Microbiol.* **34**, 295.

SUSKIND, S. R. (1957). Properties of a protein antigenically related to tryptophane synthetase in *Neurospora crassa. J. Bact.* **74**, 308.

VOGEL, A. J. (1955). A convenient growth medium for Neurospora. *Microb. Genet. Bull.* **13**, 42.

Spore Concentrations in the Atmosphere at Ahmadi, a New Town in Kuwait

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SUMMARY

The fungal air spora and the microfungi in house dust are described. Pollens from indigenous weeds of the Chenopodiaceae which were deliberately planted in the area were most numerous, that from the introduced trees of *Prosopis spicigera* (an important allergen) was trapped only sporadically at roof-top level, but found in high concentration beneath the trees. Low Cladosporium counts in the hot season are related to degree-days above 29°. The origins of the spora and the possibility of dust being colonized by fungi in air-cooled houses is discussed. Since inhalant allergy is likely to increase among the population as planting programmes extend the areas of vegetative cover in this hot arid region, future surveys on the air spora are desirable.

INTRODUCTION

The air spora in areas where vegetative cover is sparce or entirely absent has been studied by a number of workers. Pady & Kapica (1953) and Polunin (1955) have examined air samples from Arctic regions. The content of the air at Tucson, in the shrub and cactus-covered landscape of the Sonoran desert of Arizona has been briefly described by Dworin (1967). Wilkinson (1964) has produced a pollen calendar for Ahmadi which lies in the area described by Blatter (1919) as the extra-tropical desert of Arabia. Dworin reported a two-peak curve for the occurrence of airborne pollen, one in the spring and one in the autumn, and Wilkinson reported a similar curve for the incidence of hay fever. The sampling technique used by Dworin was not described but that used to study airborne pollen by Polunin and by Wilkinson was the horizontally exposed sticky slide which Gregory & Stedman (1953) have shown to have serious defects as a quantitative method for sampling such particles outdoors. Nevertheless, pollen grains in the air of the Arctic and hot desert areas have been demonstrated. The present study describes observations on the concentrations in which pollens and fungal spores occur in the air at Ahmadi and discusses their origins and relevance to the development of inhalant allergy in the population.

The establishment of a town at Ahmadi on the Persian Gulf, some 36 km. south of the state capital of Kuwait, commenced in 1946. Since then the town has been developed into a suburb-like district of tree-lined streets and houses with lawns and gardens. Wilkinson (1964) reported that among the trees planted in an area of 6 km.[2] there were 10,000 *Prosopis spicigera* Linn., 10,000 Tamarisk, 6000 Eucalyptus, 2500 Parkinsonia, 1500 Albizzia and many thousands of shrubs such as Oleander and flowering currant. An even more extensive planting programme has been adopted in the state capital, Kuwait.

METHODS

A spore trap (Hirst, 1952), which is volumetric and continuously recording, was set up on the flat roof of the company store in central Ahmadi. The methods used were as described by Davies, Denny & Newton (1963) except that silicone grease, dissolved in chloroform for ease of application, although optically inferior to petroleum jelly, replaced petroleum jelly because of the very high summer temperatures. The greased slides, prepared in this laboratory and sent to Kuwait in polythene staining jars with screw caps, were exposed for 24 hr every 2 or 3 days between August 1962 and March 1963, and, after exposure, returned to London and mounted in glycerol jelly. They were scanned as previously described (Davies *et al.* 1963) and the mean concentration for the pollen and spores trapped was calculated. The amount of sand trapped made scanning difficult and the deposits obtained during sandstorms were useless: sand tends to obscure small spores already trapped and, when airborne in high concentrations, quickly decreases the area of adhesive surface and consequently the retentivity of the slide. The concentrations of pollens and spores described therefore tend to be underestimates.

RESULTS AND DISCUSSION

Pollen. During the period studied pollen of the Chenopodiaceae was predominant, forming 66 % of the total pollen catch (Fig. 1). The maximum concentration recorded on any one day was 140 grains/m.³ on 6 October. Catches could not be correlated with any particular wind direction.

Grass pollen accounted for 3 % of the pollen trapped, it occurred sporadically in the air between August and the end of October and the highest concentration recorded was 10 grains/m.³

The pollen of Prosopis was trapped on four occasions only: 10 grains/m.³ on 10 September and 24 November and 5 grains m³ on 28 November and 4 December. Between the beginning of August and the end of October pollens similar to those of Artemisia, Betula, Platanus, the Rosaceae and Umbelliferae were occasionally trapped, and they together comprised 3 % of the entire pollen catch. Artemisia was the most common and reached a maximum concentration of 20 grains/m.³

About 28 % of the pollens in the deposits were not identified, partly through unfamiliarity with the local plants, but mainly because many of the grains were damaged, some showing conspicuous holes. Damaged pollen grains have not been a conspicuous feature in deposits obtained from the air of European cities and the damage may be attributed to the effect of attrition by wind-blown sand.

Fungal spores. The most common fungal spores were those of the genus *Cladosporium*; they occurred throughout the period sampled and their maximum daily concentration was 1725 spores/m.³ on 28 November. They formed 66 % of the total fungal spores (Fig. 2). The highest concentrations of Cladosporium and total fungal spores were obtained when the wind was from the N.W. sector.

Ustilago spores were next in frequency; average monthly concentrations with those for some of the other spore types commonly trapped are given in Table 1.

Among the more rarely encountered spores were: Aspergillaceae, Botrytis, Chaetomium, Curvularia, Epicoccum, Erysiphe, Ganoderma, Leptosphaeria, Monotospora,

Mucoraceae, Pleospora, Polythrinicium, Pullularia, Sordaria, rust uredospores and teleutospores, Tilletia, Trichothecium and brown ascospores morphologically similar to Venturia. There were also dematiaceous spores which, through unfamiliarity and because they could not be removed from the slides and grown in culture, were not identified.
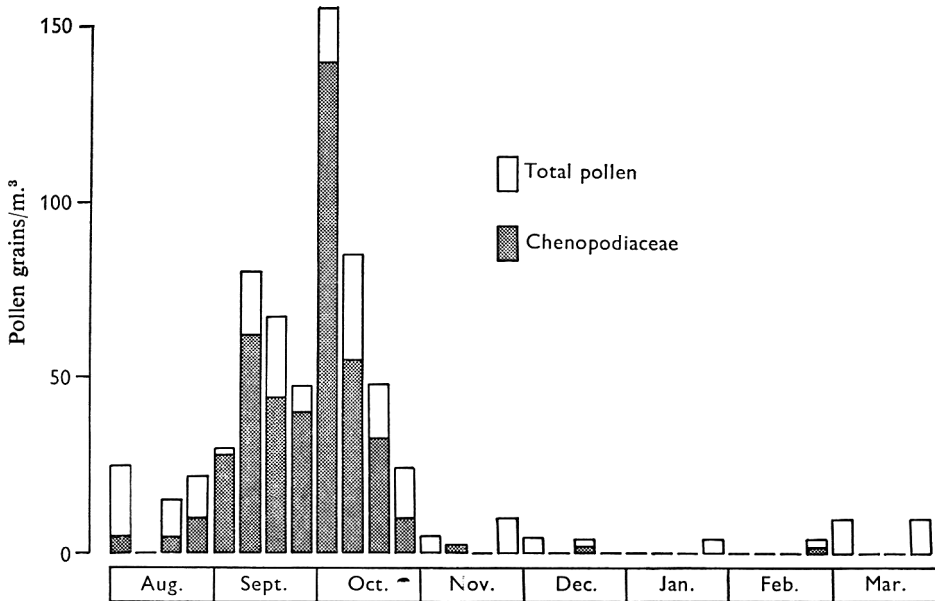
Fig. 1. Weekly average concentration for pollens of the Chenopodiaceae in relation to the total pollen catch.

Table 1. *Airborne spore concentrations at Ahmadi*

Spore	% of total spores	Average numbers/m.³/month							
		Aug.	Sept.	Oct.	Nov.	Dec.	Jan.	Feb.	Mar.
Ustilago	8	95	106	41	31	25	14	11	17
Alternaria	4	6	13	11	14	4	24	14	9
Helmintho-sporium	3	10	18	14	51	3	6	6	4
Ascospores	3	24	18	10	6	24	14	12	13
Basidiospores	4	12	22	11	57	26	12	4	6
Hyaline indeterminate spores	0·2	2	1	3	2	1	0	0	0
Mycelial fragments	6	26	49	42	30	32	19	19	23

On one of the exposed slides the conidiophore of *Aspergillus terreus* Thom with over 50 conidia attached was specifically identified and on another the whole sporangium of a Mucor packed with endospores.

Effect of climate on the air spora. The tropical desert of Arabia is hot in summer. Shade temperatures greater than 49° were recorded and in August 1962 at Ahmadi the average shade temperatures were 44.4° maximum and 29·2° minimum. Most fungi

have an optimum temperature for development between 20° and 24° and do not grow above 29°. In temperate climates, where winter temperatures are too low for the growth of higher green plants, the values of 'Accumulated Temperature' above a base temperature of 5°, the minimum temperature at which plant development is sustained, has been of use to the ecologist and agricultural meteorologist. (It denotes the combined amount and duration of the excess or deficit of air temperature above or below
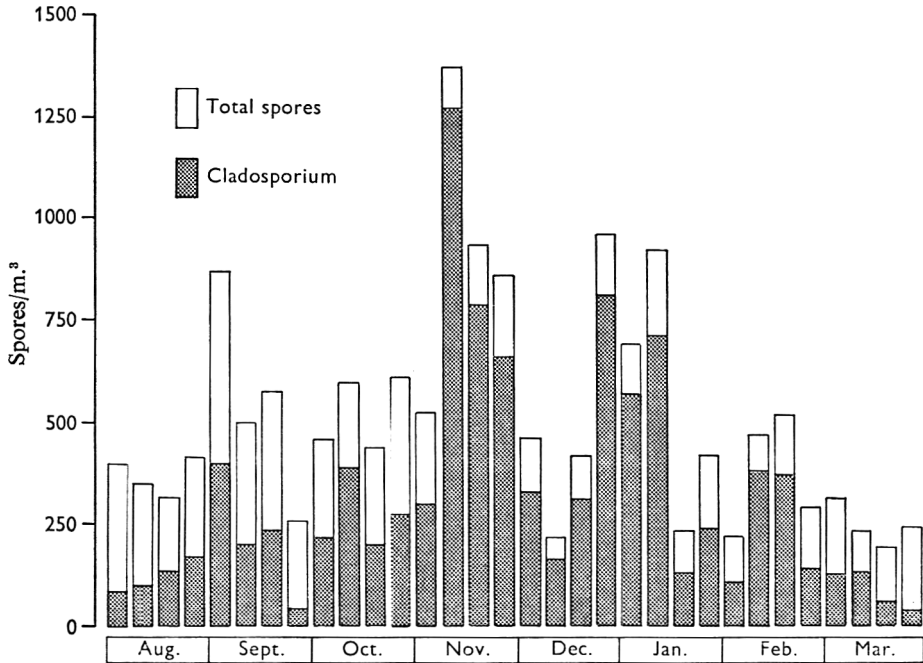
Fig. 2. Weekly average concentrations of Cladosporium in relation
to the total spores trapped.

Table 2. *Mean concentrations of chenopod pollen and Cladosporium spores with monthly values of accumulated temperature in degree-days below 29 and 20°*

Month	Aug.	Sept.	Oct.	Nov.	Dec.	Jan.
Accumulated temp. below 29°	6	30	76	235	367	436
Accumulated temp. below 20°	0	0	0	61	105	167
Chenopod pollen	5	44	60	1	1	0
Cladosporium spores	126	220	269	758	405	404

a base temperature. It is expressed in units of degree-days so that amounts for individual days can be added together to obtain the amount for all the days together.) Since shade temperatures in Kuwait during summer appeared too high for develop-

ment of most fungi, the values of accumulated temperature below 29° and below 20° for each month during the period studied are given together with the mean concentrations for Cladosporium and chenopod pollen in Table 2.

Between August and November, as the monthly accumulated temperature below 29° rose, the concentration of Cladosporium increased to its seasonal maximum in November. From November onwards, the accumulated temperatures below 20° also increased, and the concentrations of Cladosporium then decreased, as does the rate of growth in artificial culture at incubation temperatures below 20°. The development of Cladosporium is not only limited by temperature but also by the availability of moisture and moribund vegetation upon which it grows. Although during the period studied the relative humidity of the atmosphere increased from a mean of 49 % in August to 61 % in December, no rain fell before 8 November when the monthly total of 5 mm. was precipitated on that day. The total rainfall for December was 4.4 mm.; 3·7 mm. was recorded on the 11th and on only three other days was rain recorded.

Among the Cladosporium spores trapped in November and December were large clumps containing as many as 40 conidia, indicating production from a local source. Much of the Cladosporium trapped in August and September most probably had an origin in spore clouds developed to the N.W. of Kuwait in more temperate regions where cereals and grasses are grown. Evidence of spore transport into the area is also given by Ustilago; its monthly concentrations (Table 1) were highest in August and September and then declined.

Chenopod pollen occurred from 1 August onwards and by the 25th reached 25 grains/m.³; the plants producing this pollen were clearly less inhibited by high temperature than Cladosporium and other fungi.

Viable moulds in house dust. The concentrations in which fungi occur in the indoor atmosphere in Kuwait are unknown but, when house dust was collected during the routine vaccum cleaning of two houses in Ahmadi, the following were isolated: *Aspergillus niger*, van Tieghem, *A. flavus*, Link, *Neurospora sitophila*, Shear and Dodge, *Rhizopus arrhizus*, Fischer, *R. stolonifer* (Ehrenb. ex Sar.) Lind., *Ulocladium atrum* Preuss. and Penicillium, Cladosporium, Helminthosporium and Stemphylium species.

Aspergillus niger was the most common mould in the samples examined and colony counts were from 3000 to 40,000/g. dust. I also found *A. niger* to be the most common mould in dust from houses and the desert in Egypt.

Origins of the air spora at Ahmadi. When the development of the township was commenced the seeds of two indigenous plants, the salt bush *Salsola baryosma* (Roem. et Schult) Dandy and a thorny shrub *Cornulaca leucacantha* Charif et Aellen were sown in the area to bind the sand. Dickson (1955) reported Cornulaca as common near the sea shore and flowering in October, and Salsola as common in parts of the desert and flowering in May and September. Both these members of the Chenopodiaceae have spread in profusion (Wilkinson, 1964) although at present Salsola is the common weed in the uncultivated spaces and is the most likely source of the chenopod pollen.

The tree *Prosopis spicigera* Linn is a new introduction to the area and although it is reported to be an important source of airborne pollen by Wilkinson (1964), little was encountered in the Hirst trap deposits when it was flowering in November and December. These trees also flower in the spring, and when air was sampled with a rotorod (Perkins, 1957) beneath trees in April clumps of grains were trapped and a

local concentration of 60 grains/m.³ was recorded. Since large clumps of pollen grains settle quickly to the ground it seems likely that Prosopis pollen occurs in high concentration only in the vicinity of the trees.

The source of the grass pollen lies in the lawns of *Cynodon dactylon* (Linn.) Pers. It is the only grass which will withstand the heat and it occurs naturally in the area. None of the other pollens trapped could be related to introduced plants and they seem to be of indigenous origin.

Ustilago and *Leptosphaeria eustoma* (Fries) Sacc. of the fungal spores appear to be borne into the area by the prevailing winds from more temperate cereal-growing areas (e.g. in Iraq to the N.W.). That there are local sources of spores is also evident. Dickson (1955) reported that toadstools, puff balls and the truffles Tirmania and Terfezia species occur in the desert and highest basidiospore concentrations were obtained in November when temperatures were optimal for fungal growth. The concentrations of Cladosporium rose as ambient temperatures declined from the extreme heat of the summer.

The mean concentration of Cladosporium in August and September of 178 spores/m.³ is similar to that of 132/m.³ reported by Pady & Kapica (1953) during the summer sampling at Churchill in the Arctic. At Churchill the tundra contributes to the air spora, in Kuwait, even when temperatures are inimical to the growth of Cladosporium, its spores may become airborne from fragments of vegetation and even whole plants uprooted and blown about the desert by the wind. Davies (1959) reported that Cladosporium conidia are detached from conidiophores by the impact of minute water droplets (< 0·2 mm. diam.) in a moving mist or cloud, and Hirst & Stedman (1963) reported that the collision of glass beads with deposits of Lycopodium spores disseminates the spores into the air. The effect of windblown sand will be similar and probably accounts for long lengths of hypha, conidiophores and the sporangium in the Ahmadi deposits. Damage to plant tissue by windblown sand is described by Rotem (1965).

Under mesophytic conditions such as prevail in Britain, hyaline conidia, hyaline ascospores, and Sporobolomyces and Tilletiopsis spores can become airborne in high concentration. Neither Sporobolomyces nor Tilletiopsis was trapped in Kuwait. It is usually assumed that hyaline spores are less adapted to withstand ultraviolet irradiation than dematiaceous ones and that the brown pigment confers protection. The sparcity of a hyaline spora in Kuwait is probably unrelated to ultraviolet irradiation since Sporobolomyces and Tilletiopsis grow and sporulate on the transpiring surfaces of leaves (Last, 1955), on dorsiventral leaves growth is therefore confined to the shaded surfaces, and apart from the plants introduced and grown under irrigation in Kuwait, the flora is xerophytic with minimal transpiration.

Indoors at Ahmadi, where the air is cooled, moulds may colonize dust collected in the air ducts. In August 1962 the mean temperature outdoors was 37° and the average relative humidity 49 %; if no water was removed in the air-conditioning equipment, lowering the temperature 10° to 27° would increase the relative humidity to 90 % and cooling to 25° would produce dew point. Davies (1960) reported that moulds such as *Aspergillus repens* develop in house dust at relative humidities as low as 75 %, many common moulds such as other Aspergillus species and the penicillia colonize house dust at relative humidities of 85 % and Absidia and Mucor species develop when the humidity reaches 93·7 %.

The air spora in relation to inhalant allergy. The pollen of *Prosopis spicigera* is the most important cause of pollinosis in Kuwait (Wilkinson, 1964; A. W. Frankland, personal communication). As mentioned earlier, over 10,000 trees of this species have been introduced into the area of Ahmadi and many more planted in Kuwait town. The houses are surrounded by the trees which were planted for shade; pollen clumps are shed in the vicinity and settle quickly but high concentrations occur beneath the trees. In future planting programmes the importance of this tree as a cause of inhalant allergy should be noted, and until such time as they can be successively replaced without loss of amenity, allergic people should be housed to the N.W. of the planted area and instructed to avoid the trees as much as possible in the April/May and November/ December flowering periods.

In Wilkinson's (1964) study, the 'gravity slide' appears to have overestimated the numerical importance of grass and other pollens relative to those of the Chenopodiaceae, a family not only common in desert floras but well adapted to anemophily. Dr A. W. Frankland (personal communication) finds that atopic patients normally resident in Arabia usually show skin sensitivity to extracts of pollen from *Chenopodium album* Linn. Hence the chenopod pollen to which these patients are commonly exposed is antigenically similar to the English grown *C. album*. At Ahmadi, chenopod pollen concentrations averaged over 50 grains/m.^{3} for 3 weeks in September and October; such concentrations are comparable with those for grass pollen in England in June and July, and experience has shown that concentrations of grass pollen of 50 grains/m.^{3} in Central London almost invariably produced hay fever in patients clinically sensitive to grass pollen (Frankland and Davies, unpublished observations). Chenopod concentrations in Central London are rarely as high as 10 grains/m.^{3} and it is an uncommon cause of pollinosis in England. Wilkinson (1964) reported that many hay fever subjects at Ahmadi were hypersensitive to the Chenopodiaceae and Bermuda grass in addition to Prosopis. Extracts of pollen from *Salsola baryosma* and other chenopods indigenous to the area, such as *Anabasis articulata* (Forsk.) Moq. and *Haloxylon salicornicum* (Moq.) Boiss, which are common in the westerly parts of Kuwait (Dickson, 1955), have not yet been obtained. From the aerobiological evidence, grass pollen is unlikely to be an important cause of pollinosis in Kuwait since it was only occasionally trapped. The maximum concentration recorded was only 10 grains/m.^{3} and in London where 0·5 % of the population experience grass pollinosis in the June/July season, daily concentrations of hundreds of grains/m.^{3} are recorded, and average concentrations during the seasons from 1961 to 1968 ranged between 50 and 130 grains/m.^{3}

Hypersensitivity to fungal spores does not yet appear to have become a serious problem among the population at Ahmadi although Dr. D. W. Frankland (personal communication) reported that positive skin (prick) reactions to *Aspergillus niger* are not uncommon in allergic patients from this area, and Wilkinson (1964) reported that many patients complained that their symptoms were worse at night indoors. The possibility of mould development in the dust that accumulates in air cooling ducts has been mentioned.

Outdoors the concentration of Cladosporium spores reached a maximum weekly average of 1250/m.^{3} and the highest daily concentration was 1750. Frankland & Davies (1965) report that, in patients with hypersensitivity to this spore, symptoms are precipitated when mean daily concentrations rise to 3000 or more/m.^{3} From this

evidence it needs but a twofold increase in the concentrations of Cladosporium in the air of Kuwait for it to become clinically significant in the atopic population.

Since this survey was undertaken in 1962–3, the planting programmes have been continued, especially in the vicinity of the state capital, Kuwait. As the areas of vegetative cover are extended, the fungal flora will increase and inhalant allergy due to fungal spores will inevitably become more common. Periodic surveys of the air spora would appear to be desirable so that in the light of the knowledge obtained, the planned ecological succession may be from time to time modified in the interest of the people prone to allergic diseases.

REFERENCES

BLATTER, E. (1919). *Records of the Botanical Survey of India*, 8, *Flora Arabica* i. Calcutta: Superintendent of Government printing.

DAVIES, R. R. (1959). Detachment of conidia by cloud droplets. *Nature, Lond.* **183**, 1695.

DAVIES, R. R. (1960). Viable moulds in house dust. *Trans. Br. mycol. Soc.* **43**, 617.

DAVIES, R. R., DENNY, M. J. & NEWTON, L. M. (1953). A comparison between the summer and autumn air-sporas at London and Liverpool. *Acta allerg.* **18**, 131.

DICKSON, V. (1955). *The Wild Flowers of Kuwait and Bahrain*. London: Allen and Unwin.

DWORIN, M. (1967). Allergy in the Sonoran desert. *Int. Congr. Allerg., Excerpta Medica* **144**, 41.

FRANKLAND, A. W. & DAVIES, R. R. (1965). Allergie aux spores de moisissures en Angleterre. *Le Poumon et le Coeur* **1**, 11.

GREGORY, P. H. & STEDMAN, O. J. (1953). Deposition of airborne *Lycopodium* spores on plane surfaces. *Ann. appl. Biol.* **40**, 651.

HIRST, J. M. (1952). An automatic volumetric spore trap. *Ann. appl. Biol.* **39**, 257.

HIRST, J. M. & STEDMAN, O. J. (1963). Dry liberation of fungus spores by raindrops. *J. gen. Microbiol.* **33**, 335.

LAST, F. T. (1955). The seasonal incidence of *Sporobolomyces* on cereal leaves. *Trans. Br. mycol. Soc.* **38**, 221.

PADY, S. M. & KAPICA, L. (1953). Air-borne fungi in the Arctic and other parts of Canada. *Can. J. Bot.* **31**, 309.

PERKINS, W. A. (1957). The rotorod sampler. *Semi-ann. Rep. Aerosol Lab. Stanford Univ. CML.* **186**, 1; quoted by Gregory, P. H. & Lacey, M. E. (1964). *Trans. Br. mycol. Soc.* **47**, 25.

POLUNIN, N. (1955). Arctic aeropalynology. Spora observed on sticky slides exposed in various regions in 1950. *Can. J. Bot.* **33**, 401.

ROTEM, J. (1965). Sand and dust storms as factors leading to Alternaria blight epidemics on potatoes and tomatoes. *Agric. Meteorology* **2**, 281.

WILKINSON, W. M. (1964). Development of allergy in the desert. *J. trop. Med. Hyg.* **67**, 16.

Characterization of Human Cutaneous Lipophilic Diphtheroids

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SUMMARY

Sixty lipophilic diphtheroids (LD) isolated from human skin were characterized by a variety of morphological, biochemical, physiological and nutritional tests. The LD strains were tentatively placed into six fermentative groups and one non-fermentative group. Most LD strains required amino nitrogen, others required vitamins plus amino acids for growth. Nine LD isolates initiated growth with ammonia as a sole nitrogen source. Urea and nitrate were not utilized. A total of 149 cutaneous bacteria were compared for esterase and lipase action. Esterase activity was common but few LD strains appear capable of obtaining their required lipids by their own lipase action. The grouping scheme derived from studies with the 60 LD strains was tested as a screening procedure to recognize and categorize other LD strains. A second series of 115 cultures from seven cutaneous sites were isolated. Six of the original seven groups were identified and one additional subgroup was formed. The screening method was partially effective as a means of studying the location and types of LD strains on skin. There were 31 strains which by determinative features could be grouped as *Corynebacterium xerosis*-like species. Human skin appears to have an unrecognized diversity of lipophilic corynebacteria yet to be classified into species.

INTRODUCTION

Normal human skin contains a relatively stable microflora although the numbers of bacteria present on skin may vary considerably depending on the site sampled (Marples, 1965). The predominant bacteria are the Gram-positive, coagulase-negative staphylococci or micrococci, aerobic diphtheroids and *Corynebacterium acnes* which is anaerobic and resides primarily in pilosebaceous units. There are also yeasts usually classified as Pityrosporum species (Pillsbury & Rebell, 1952). A small percentage of people may be carriers of coliforms or bacteria of the tribe Mimeae (Taplin, Rebell & Zaias, 1963).

Few studies have been conducted on the ecological associations among the autochthonous skin bacteria. Some resident skin bacteria may provide certain factors which act as defence against invasion by other non-cutaneous organisms (Marples, 1965).

A large group of bacteria commonly isolated from normal skin are designated lipophilic diphtheroids (LD) because they require certain lipids for growth *in vitro* (Pollock, Wainwright & Mansion, 1949). This LD group has been relatively obscure taxonomically although some authors consider LD skin species identical to *Corynebacterium xerosis* (Pillsbury & Kligman, 1954). Evans (1968) recently attempted to classify cutaneous aerobic diphtheroids. The LD groups was not specifically sought

but Evans was able to identify some of her isolates as *C. xerosis* or other recognized corynebacteria species.

This study was conducted to characterize LD strains from normal human skin and to compare them with several known species of the genus *Corynebacterium* and other closely related bacteria, to determine if LD strains can be classified as *C. xerosis* or its variants, and to compare their lipolytic activity and nutritional requirements to other groups of cutaneous bacteria. An attempt was made to measure the cutaneous distribution of LD strains by using a selected number of minimal screening tests.

The characterization of LD strains was considered a necessary preliminary to more satisfactory ecological investigations of the cutaneous diphtheroids and other skin bacteria.

METHODS

Reference strains. Cultures (Table 1) were maintained on slopes of Todd–Hewitt agar (BBL; Baltimore Biological Laboratories, Baltimore, Md., U.S.A.) except for *Propionibacterium acnes*, which was kept in thioglycollate, and strains of *Corynebacterium bovis*, which were maintained on trypticase soy (TS) agar without glucose (BBL) containing 0·1 % Tween 80. Stock cultures were stored at 4°.

Table 1. *Designations and origin of reference strains*

Strain	Source*
Brevibacterium linens	ATCC 9172
Microbacterium lacticum	ATCC 8180
Listeria monocytogenes	ATCC 15313
Mima polymorpha	ATCC 14291
Kurthia zopfii	ATCC 6900
Propionibacterium acnes	ATCC 11828
Corynebacterium diphtheriae	—
C. striatum	ATCC 6940
C. xerosis	ATCC 373, 7064
	MWCS 29
C. pseudodiphtheriticum	MWCS 28
C. minutissimum	ATCC 23346, 23347, 23348, 23349
C. bovis	ATCC 7715, NIRD 65,
	77, 120, 122, 125, 151, 29

* ATCC, American Type Culture Collection, Rockville, Maryland, U.S.A. MWCS, Midwest Culture Service, Terra Haute, Indiana, U.S.A. NIRD, National Institute for Research in Dairying, Reading, United Kingdom. *C. diphtheriae* was obtained from H. M. Cobe, *C. minutissimum* strains from D. Taplin, and the NIRD strains from D. J. Jayne-Williams. All other strains were obtained directly from the culture service indicated.

Isolation of cutaneous strains. Various sites on the skin of 62 students and six staff members were rubbed with cotton-wool swabs in Triton phosphate buffer (Williamson & Kligman, 1965). Swabs were directly streaked on media or first mixed in 5 ml. of buffer to express the bacteria from the swabs. Loopfuls of diluent were then streaked on plates. Gram-positive cocci were isolated on mannitol salt agar (BBL) and classified by the methods of Baird-Parker (1963). mimeae-like bacteria were isolated on MacConkey agar (BBL) and identified by the methods of Taplin *et al.* (1963). The LD strains were isolated on 5 % (v/v) human blood agar by the method of Smith & Willett (1968). They were maintained on the same slopes as were the *Corynebacterium bovis* strains.

Lipid growth requirements. The LD strains were separated from non-lipid-requiring strains by inoculating TS broth as a base medium and TS broth containing 0·1 % Tween 80. Isolates which grew in the latter medium only were considered LD strains. To confirm the lipid requirement, nutrient broth (Difco) was extracted twice with equal volumes of chloroform to remove lipids and dispensed in glassware washed with chloroform and methanol. Strains which grew in this broth only after the addition of 0·5 % Tween 80 or 10 μg. sodium oleate/ml. were considered obligate LD strains.

Characterization tests. Tween 80 (0·05 %) was added to all media used in this group of tests.

Growth studies. Forty-eight hr TS broth cultures were streaked on TS agar and incubated at 37 and 22°, in candle jars (10 % CO_2) and in Brewer jars for anaerobic growth. Phenethyl alcohol agar (PEA) (BBL) was incubated aerobically at 37°. Filter sterilized sodium azide and potassium tellurite were added to TS broth. One loopful of each 48 hr culture was inoculated into each of two TS broths. One acted as a control and the other was heated in a 60° water bath for 30 min. The tubes were removed, rapidly cooled and incubated at 37°. Nutrient broth was adjusted to pH 5·0, 6·0, 7·0 and 8·0 electrometrically to determine ability to initiate growth in these broths.

Biochemical tests. Action on egg yolk was tested by the method of Esselmann & Liu (1961). Methyl red and Voges-Proskauer tests were determined with MR-VP broth (BBL). Nitrate reduction was determined with nitrate broth (BBL) and Bacto-Peptone broth (Difco) was used for indole production. Extracellular nuclease activity was measured by the methods of Smith & Bodily (1968). Phosphatase activity was measured by the method of Baird-Parker (1963). Kligler iron agar and s.i.m. (sulphide–indole–motility) agar (BBL) were used to detect hydrogen sulphide production. Haemolysis was observed on 5·0 % (v/v) whole unwashed human blood agar. The remaining tests were conducted by the methods of Jayne-Williams & Skerman (1966). Reagents were prepared according to standard methods (*Manual of Microbiological Methods*, 1957). Cultures were observed and tested in media from 1 to 8 days except litmus milk, which was incubated for 15 days.

Carbohydrate fermentations. Carbohydrates were incorporated into phenol red broth base (BBL) in final concentrations of 0·5 %. Media were autoclaved at 10 lb/in.² (115°) for 10 min. and rapidly cooled to 25° in a water bath.

Esterase and lipase activity. Substrates used in this section were obtained from the Nutritional Biochemical Corp., Cleveland, Ohio. Esterase action was determined using polyoxyethylene sorbitan derivatives of several fatty acids. Compounds were incorporated in TS agar without glucose containing 0·01 % $CaCl_2$. Preliminary tests revealed that some Tween compounds were inhibitory to the LD strains at 1·0 % concentrations. Growth was judged as retarded when poor growth or no growth occurred on agar with substrate concentrations from 0·1 to 1·0 %. Inhibition was considered to have occurred when growth developed at lower but not higher substrate concentrations. Each Tween was tested to find the concentration permitting growth of all LD strains. Esterase action was noted by the formation of cloudy zones around and beneath the colonies resulting from calcium soap formation. Triacetin hydrolysis was measured by the method of Davis & Ewing (1964). Lipase action of emulsified substrates was observed on Spirit blue agar (Difco) using the methods of Smith & Willett (1968).

Nutritional studies. A medium used as a minimal salts medium had the following

composition per litre: anhydrous K_2HPO_4, 700 mg.; KH_2PO_4, 300 mg.; $MgCl_2$, 100 mg.; NaCl, 100 mg.; $MnCl_2,4H_2O$, 10 mg.; $FeCl_3$, 10 mg.; $CaCl_2$, 1 mg. This medium was supplemented with combinations of NH_4Cl, 1 g. (w/v); glucose, 10 g. (w/v); vitamin-free Casamino acids (Difco), 10 g. (w/v); urea (filter sterilized) or KNO_3, 1 g. (w/v); and Tween 80, 0·05–0·10 %. All batches of media were adjusted to pH 7·2. Vitamins sterilized by filtration were added alone or in combination in final concentrations of 10–100 μg./ml. These included riboflavine, niacin, nicotinic acid, thiamine and sodium pantothenate. Sodium oleate (1–10 μg./ml.) was used as a substitute for Tween 80. In some experiments carbon assimilation was compared with 0·06M-glycerol, glucose and the sodium salts of acetate, citrate and lactate. Growth measurements were made in a Bausch and Lomb Spectronic-20 colorimeter.

Screening tests. A second series of LD strains was isolated from various cutaneous sites. Each strain was examined for its Tween 80 growth requirement, Tween 80 hydrolysis, growth on and hydrolysis of tributyrin and triolein, growth in 9·0 % NaCl broth, nitrate reduction and the fermentation of glucose, maltose, fructose, galactose, sucrose, lactose and salicin. The isolates were grouped on the basis of test results from the initial group of LD strains.

RESULTS

General characterization tests. Sixteen of the reference bacteria were compared with 60 LD strains (Table 2). *Listeria monocytogenes* was the only species which was motile, produced haemolysis and hydrolysed aesculin. An opaque lecithinase type reaction was produced on egg yolk agar. *Brevibacterium linens* produced a distinct orange pigment on solid media and produced a lecithinase reaction on egg-yolk agar surrounded by an outer clear proteolytic zone. The three *Corynebacterium xerosis* strains produced a pale yellow to tan pigment; *C. xerosis* ATCC 373 formed rough dry colonies and the other two strains were smooth.

Morphologically, the 60 LD strains were non-acid fast, asporogenous and pleomorphic. Generally, there was a lack of metachromatic granules in the organisms but always a tendency to form palisades (Pl. 1, fig. 1–4). Strains varied from small coccobacilli to longer bacillary forms both straight and club-shaped. None of the LD strains produced pigments as did the *Corynebacterium xerosis* strains. Colonies were smooth, entire, raised and butyrous in consistency. There were no rough colony types formed. During the course of 1 yr. in which the isolates were maintained and repeatedly subcultured, only four (6·6 %) of the strains lost their obligate *in vitro* requirement for oleate or Tween 80. The 60 strains were catalase positive and initiated growth in 10 % CO_2 in air and at pH 6·0, 7·0 or 8·0. The group was uniformly negative in the following characters: anaerobic growth, gas production from carbohydrate fermentation, motility, growth in 0·1 % sodium azide and potassium tellurite, urease, gelatinase, haemolysis and action on egg yolk agar. There were no changes detected in litmus milk after 15 days incubation. Hydrogen sulphide and indole were not produced by any test organisms. None of the strains hydrolysed aesculin or fermented lactate, glycerol and mannitol. The LD strains were variable in the remaining tests. They were arranged into seven subgroups by their fermentation reactions. Groups I–VI were fermentative and group VII was non-fermentative. Group I differed from the other fermentative groups by fermenting lactose, trehalose and salicin, and produced an acid butt and slant on Kligler agar, as did *Listeria monocytogenes*. The remaining LD

Table 2. Some variable characteristics of LD strains and other bacilli

Reaction tested	B. linens (1)	M. lacticum (1)	L. monocytogenes (1)	C. diphtheriae (1)	C. striatum (1)	C. pseudodiphtheriticum (1)	C. minutissimum (4)	C. bovis (3)	C. xerosis (3)	LD groups I (2)	II (3)	III (11)	IV (10)	V (10)	VI (10)	VII (14)
Growth 22°	+	+	+	+	+	+	+	+	+	−	+	7	4	2	2	3
Survive 60°	−	+	−	+	+	−	2	2	−	−	+	6	4	7	5	5
DNase	−	+	−	+	+	+	+	2	−	−	−	2	2	2	3	9
RNase	+	+	−	−	−	−	+	−	−	−	−	1	1	1	1	8
Nitrate red	−	−	−	+	+	+	−	−	+	−	−	2	1	3	3	−
Growth PEA	−	−	−	+	+	−	+	+	+	−	+	+	+	9	+	10
Urease	−	−	−	−	−	+	+	+	−	−	−	9	+	9	+	9
Growth 9% NaCl	+	−	−	+	+	−	+	+	+	−	+	9	+	9	+	−
Methyl red	−	−	+	+	−	−	2	−	−	1	2	7	4	3	1	−
Voges-Proskauer	−	−	+	−	−	−	−	+	−	−	−	2	−	7	2	−
Phosphatase	+	−	+	−	+	−	+	−	2	−	−	9	+	+	+	11
Acid from:																
glucose	−	+	+	+	+	−	+	+	+	+	+	+	+	+	+	−
maltose	−	+	+	+	−	−	+	1	+	+	+	3	5	2	−	−
fructose	−	−	+	+	+	−	+	+	+	+	+	+	+	−	−	−
galactose	−	+	+	−	+	−	−	+	+	+	+	9	2	6	−	−
sucrose	−	+	+	−	+	−	2	−	+	+	+	10	3	2	−	−
lactose	−	+	+	−	−	−	−	−	−	+	−	−	−	−	−	−
trehalose	−	+	+	−	−	−	−	−	1	+	−	−	−	−	−	−
salicin	−	+	+	−	−	−	−	1	−	+	−	−	−	−	−	−
glycerol	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−
Terminal pH																
7·2–7·4	.	.	.	.	.	+	.	.	.	.	.	.	.	.	.	+
6·0–6·4	+	+	.	.	.	.	.	2	1	.	.	.	.	2	2	.
5·4–5·8	.	.	.	.	+	.	.	1	.	1	1	2	3	5	2	.
5·2 or less	.	.	+	+	.	.	+	−	2	1	1	9	7	3	6	.

Strains listed with number tested in parentheses.
+, −, Strains all positive or negative.
Number given for reaction indicates positive strains of total tested.

PEA-growth on phenethyl alcohol agar.
C. bovis strains were ATCC 7715, NIRD 65 and 77.
Terminal pH measured in glucose broth after 8 days incubation.

groups produced a weakly positive acid butt and alkaline slant or were negative. Group II fermented glucose, maltose, fructose, galactose and sucrose, as did the C. xerosis strains. Groups III–V were separated on the basis of the number and combinations of four sugars (maltose, galactose, fructose and sucrose) which were fermented. Group III fermented three of four sugars. Group IV fermented two sugars and group V only one sugar. Within each group no strain differed from any other by more than one reaction among the four sugar tests. Group VI fermented only glucose. Twenty-two strains grew at 22° and 32 survived 60°. Nuclease activity was variable and produced mostly by group VII. Forty-eight strains grew in 9 % NaCl broth.

Table 3. *Comparative esterase and lipase activity of various human skin bacteria and others*

Organisms	No. of strains tested	Hydrolysis of								
		Tweens					Tri-butyrin	Tri-olein	Methyl oleate	Cotton-seed oil
		20	40	60	80	85				
Gram-positive cocci	46	42	32	30	23	20	32	42	33	32
M. polymorpha	1	+	+	+	+	−	+	−	−	−
Mima-like strains	20	+	+	+	+	−	+	−	−	−
P. acnes	1	−	+	+	−	−	+	−	−	−
K. zopfii	1	−	−	−	−	−	−	−	−	−
B. linens	1	+	+	+	+	+	+	+	+	+
M. lacticum	1	−	−	−	−	−	−	−	−	−
L. monocytogenes	1	+	+	+	−	−	−	−	−	−
C. diphtheriae	1	−	−	−	−	−	+	−	−	−
C. striatum	1	+	+	+	+	+	+	−	−	−
C. pseudodiph-theriticum	1	+	+	+	+	−	+	−	−	−
C. minutissimum	3	+	+	+	+	+	+	−	−	−
C. xerosis	3	+	−	+	1	−	1	−	−	−
C. bovis	8	5	1	N	−	7	N	−	−	−
LD strains:										
Group I	2	1	1	1	−	−	−	−	−	−
II	3	3	3	3	3	−	−	−	−	−
III	11	+	+	10	+	7	1	−	−	−
IV	10	+	+	9	9	8	−	−	−	−
V	10	9	9	4	8	5	3	−	−	−
VI	10	+	+	8	8	3	2	−	−	−
VII	14	10	10	6	7	3	1	−	−	−
Total no. LD strains active on each substrate		54	54	41	46	26	7	0	0	0

Final test concentration for Tweens 20, 40, 60 and 85 was 0·5 %; Tween 80 was 1·0 %.
Emulsified lipids were used in final concentrations of 0·5 %.
+, −, Strain or strains tested all positive or negative.
Numbers indicate strains positive if more than one tested.
N, No growth on media.

Esterase and lipase activity. The bacteria were compared with cutaneous isolates of Gram-positive cocci and Gram-variable coccobacilli of the Mima-like group (Table 3). None of the 149 cultures tested hydrolysed 0·1 % Tween 65 or degraded squalene. The test for triacetin hydrolysis was unreliable because incubation of the medium beyond

3 days caused non-specific changes in the agar. The strains were compared for their action on soluble Tween substrates, emulsified substrates and one oil. The results indicated that only Gram-positive cocci and *Brevibacterium linens* produced extra-cellular lipase capable of acting on long chain lipids. *Mima polymorpha* and the other Mima-like strains hydrolysed certain Tweens and tributyrin. They also had lecithinase activity when tested on egg agar and in egg-yolk broth, producing a ring of fatty curd in the latter medium. Tributyrin was hydrolysed by most of the non-lipophilic diph-theroids but this property was not always related to the strains' ability to hydrolyse Tweens or split emulsified lipid substances. Tweens 20, 40, 60 and 80 and tributyrin did not support the growth of some *Corynebacterium bovis* strains. With the exception of certain group VII strains, the LD bacteria grew well on all Tween media. Seven strains hydrolysed tributyrin, 22 strains grew poorly on this medium and did not hydrolyse the substrate. There were 31 other LD strains which grew normally on tributyrin agar but did not produce a lipase. The Gram-positive cocci, non-lipophilic bacilli and the Mima group grew on squalene media but the LD strains and *C. bovis* cultures did not grow.

Table 4. *Nutritional patterns of LD strains and other bacilli*

Organisms	No. of strains tested	Nitrogen source		
		Ammonia	Amino acids required	Amino acids and vitamins required
B. linens	1	+	−	−
M. lacticum	1	−	−	+
L. monocytogenes	1	−	+	−
C. diphtheriae	1	−	+	−
C. striatum	1	−	+	−
C. minutissimum	2	−	+	−
C. pseudodiphtheriticum	1	−	+	−
C. xerosis	2	−	+	−
C. bovis	6	+	−	−
LD strains:				
Group I	2	−	1	1
II	3	−	2	1
III	11	−	6	5
IV	9	1	4	4
V	7	6	−	1
VI	8	1	5	2
VII	12	1	10	1

Basal medium contained NH_4Cl, glucose and salts. Cultures were inoculated in TS Tween 80 broth as a growth control.
Number indicates strains positive of total tested.

Nutritional studies. *Brevibacterium linens* and *Corynebacterium bovis* utilized ammonia as a sole nitrogen source (Table 4). The other corynebacteria, including *C. xerosis*, required amino acids. *Microbacterium lacticum* did not grow without added vitamins. Urea as sole nitrogen source was utilized only by the *C. bovis* strains. None of the bacilli tested including the LD strains utilized nitrate as sole nitrogen source. The majority of the LD strains required nitrogen as NH_2 in the form of casamino acids, others had an additional requirement for vitamins. The use of vitamins with NH_4Cl did not replace casamino acids for those strains which required nitrogen as NH_2.

Group V had the largest number of ammonia-utilizing strains. The substitution of glucose in the basic medium by citrate, glycerol, lactate or acetate was not stimulatory to the majority of the strains. Only three LD strains were stimulated partially by acetate and glycerol.

Screening tests for distribution of LD strains on skin. A second series of 115 strains was isolated from seven cutaneous sites to test the usefulness of the initial grouping scheme as a preliminary means of classifying these bacteria (Table 5). None of the isolates in this series fermented lactose or salicin, thus eliminating group I. All the strains grew in 9% NaCl broth. There were 34 isolates mostly from the face, nose and toes which did not hydrolyse Tween 80. Thirty strains grew on tributyrin agar but only 12 hydrolysed the substrate.

Table 5. *Distribution and characteristics of LD strains from human skin*

Site sampled	No. of strains isolated	Tween 80 not hydro-lysed	Tributyrin* Growth	Tributyrin* Hydro-lysis	I	II	III	IV	IV a	V	VI	VII
Scalp	26	5	5	1	0	0	4	12	7	1	2	0
Face	8	5	3	0	0	0	0	4	0	4	0	0
Nose	14	12	0	0	0	0	0	2	12	0	0	0
Axilla	10	0	8	0	0	1	5	3	0	0	1	0
Sternum	21	1	1	0	0	0	3	10	3	2	2	1
Groin	24	2	1	7	0	1	0	22	1	0	0	0
Toeweb	12	9	0	4	0	3	0	9	0	0	0	0
Totals	115	34	18	12	0	5	12	62	23	7	5	1

* Growth on tributyrin agar was compared to growth on TS Tween 80 agar.
Strains which hydrolysed tributyrin grew normally.
Group IV a reduced nitrate.

One or more isolates from the remaining six groups were recognized. The most commonly occurring type was groups IV (53% of total). The groin area yielded the largest number of this group consisting of strains fermenting glucose, sucrose and fructose but not maltose or galactose. One additional group was recognized (IV a) as being identical to group IV but reducing nitrate. Nearly all of the strains in group IV a were from the nasal area. Strains from the other groups did not reduce nitrate.

The strains in this series were examined for growth in media containing Tween 40, tripropionin, triolein, methyl oleate, methyl ricinolate and methyl palmitate. Tween 40 was split by all strains. Growth on tripropionin and methyl palmitate was poor or absent. Nine strains with apparent lipase activity from the face, scalp and nose were found. All acted on methyl ricinolate, three hydrolysed triolein and four split methyl oleate.

In the process of isolating LD strains 13 strains of contaminating Gram-positive cocci were found. This occurred because of their apparent requirement for Tween 80. This was shown by inoculating mannitol salt agar with and without Tween 80. Growth developed eugonically on the latter medium only and they were distinguished from the diphtheroids by Gram staining the cultures several times under different growth conditions.

DISCUSSION

Determinative features of the reference group of bacteria were in general agreement with the recognized properties of these species (*Bergey's Manual*, 1957). These investigations also confirm the descriptions for *Corynebacterium minutissimum* and *C. bovis* as reported by Sarkany, Taplin & Blank (1962) and Jayne-Williams & Skerman (1966).

Diagnostically the LD strains fell into the family Corynebacteriaceae as they were Gram-positive, non-acid fast, non-motile, non-sporeforming, catalase-positive, aerobic, pleomorphic bacilli (Skerman, 1967). Human cutaneous diphtheroids have always been regarded as corynebacteria (Rosebury, 1962; Marples, 1965) and the data obtained in this study provide no evidence against this assumption. A precise distinction between the Brevibacterium and the Corynebacteriaceae does not exist but brevibacteria are not considered indigenous to man (Rosebury, 1962) and this group is usually not pleomorphic (Skerman, 1967). Harrington (1966) showed the similarity of nocardias, corynebacteria and mycobacteria. The familial status of human indigenous diphtheroids may be more difficult to resolve than recognition at the species or generic level. Pleomorphism offered little as an aid to species recognition or grouping of the LD strains.

The assumption that LD strains are identical with *Corynebacterium xerosis* is erroneous. *Corynebacterium xerosis* ferments glucose, maltose, fructose, galactose and sucrose and does not reduce nitrate or split urea (*Bergey's Manual*, 1957). These characters are used to separate *C. xerosis* from other closely related human species such as *C. striatum*, which has the same characteristics but usually ferments other sugars (Winford & Haberman, 1966). *Corynebacterium xerosis* has no other known distinguishing diagnostic features. The three reference cultures of *C. xerosis* reduced nitrate. Evans (1968) identified as *C. xerosis* 23 non-lipophilic cutaneous diphtheroids which reduced nitrate, fermented glucose, sucrose and weakly fermented maltose. She identified as *C. striatum* nitrate negative strains which fermented the three sugars. The *C. striatum* strain tested here reduced nitrate but did not ferment maltose. Assuming that *C. xerosis* reduces nitrate and ferments glucose, fructose, maltose, galactose and sucrose then none of the 60 initial LD strains were identical to *C. xerosis*. If *C. xerosis* is considered variable in nitrate reduction and in the fermentation of one of the above sugars except glucose then 31 LD strains (groups II and III) from both series of 175 strains could be tentatively identified as *C. xerosis*. The 31 strains mentioned would be overlooked, however, or considered biochemically inert if cultivated in media without Tweens or other lipids. Obligate lipid requirements are the important difference between LD strains and *C. xerosis*. Apart from those strains which resemble *C. xerosis*, a far greater number exist as unrecognized, unnamed corynebacteria on human skin. Few studies have been attempted to describe them (McBride, Freeman & Knox, 1968). None of the LD strains resembled *C. tenuis*, which has no lipid requirements (Crissey, Rebell & Laskas, 1952), nor are they closely related to *C. bovis* or *C. pyogenes*, which do require lipids (Roberts, 1968).

The fermentation scheme used to group the LD strains was not intended as a classification but was a workable means to categorize the bacteria. Evans (1968) placed non-lipophilic cutaneous diphtheroids into seven groups by using nitrate and urease tests and the fermentation of glucose, sucrose and maltose. She found that most nitrate reducers, as in this study, were from the nasal area. Evans found another nitrate-positive

group from the groin area that did not ferment sucrose. A similar group was not recognized in the present study.

The most commonly occurring LD strains fermented glucose, fructose and sucrose (group IV) and a second group (IV a) was identical to group IV except for nitrate reduction. These two groups may represent closely related common cutaneous bacteria. Within the groups differentiated, however, there were some strains which differed in their utilization of ammonia or need for amino acids or vitamins. Nutritional differences plus lipid requirements should have some priority in a natural classification for the LD strains.

Variations in growth and activity on lipid substrates by LD strains indicate the heterogenous nature of the lipid requirements and lipolytic activity of the groups. Apparently few strains can derive their required lipids by their own lipase action. Esterase action was a common feature of the groups. It appears that some ecological association exists between LD species which require lipids and the lipolytic Gram-positive cocci of skin. On the other hand, cutaneous cocci are ureolytic (Auletta & Kennedy, 1966) and the LD strains are not. Cocci may produce ammonia from urea which could be utilized by some skin diphtheroids.

Growth stimulation by various Tweens was not always related to hydrolysis of the compounds. Eugonic and sometimes retarded growth occurred on Tween media. If each Tween contained some free fatty acids or slowly released them for utilization by the bacteria then several fatty acids could support or inhibit growth. These include laurate (Tween 20), palmitate (Tween 40), stearate (Tween 60), and oleate (Tweens 80 and 85). The strains tested for Tween 40 hydrolysis were all positive. Palmitate is the most common fatty acid found in human surface lipids (Wheatley, 1963) but other fatty acids produced by skin or liberated from triglycerides by cocci may be available for the diphtheroids. A detailed study of the lipid requirements for each designated group of LD strains is in progress and is required before further consideration can be given to each group as a species.

The method of Evans (1968) and the screening and grouping scheme reported here can be useful for presumptive studies of the aerobic cutaneous diphtheroids.

REFERENCES

AULETTA, A. E. & KENNEDY, E. R. (1966). Deoxyribonucleic acid base composition of some members of the Micrococcaceae. *J. Bact.* **92**, 28.

BAIRD-PARKER, A. C. (1963). A classification of micrococci and staphylococci based on physiological and biochemical tests. *J. gen. Microbiol.* **30**, 409.

Bergey's Manual of Determinative Bacteriology. (1957). 7th ed. Ed. by R. S. Breed, E. G. D. Murray and N. R. Smith. Baltimore: Williams and Wilkins.

CRISSEY, J. T., REBELL, G. M. & LASKAS, J. (1952). Studies on the causative organism of *Trichomycosis axillaris. J. invest. Dermatol.* **19**, 187.

DAVIS, B. R. & EWING, W. H. (1964). Lipolytic, pectinolytic and alginolytic activities of *Enterobacteriaceae. J. Bact.* **88**, 16.

ESSELMANN, M. T. & LIU, P. V. (1961). Lecithinase production by Gram-negative bacteria. *J. Bact.* **81**, 939.

EVANS, N. M. (1968). The classification of aerobic diphtheroids from human skin. *Br. J. Dermatol.* **80**, 81.

HARRINGTON, B. J. (1966). A numerical taxonomical study of some corynebacteria and related organisms. *J. gen. Microbiol.* **45**, 31.

JAYNE-WILLIAMS, D. J. & SKERMAN, T. M. (1966). Comparative studies on coryneform bacteria from milk and dairy sources. *J. appl. Bacteriol.* **29**, 72.
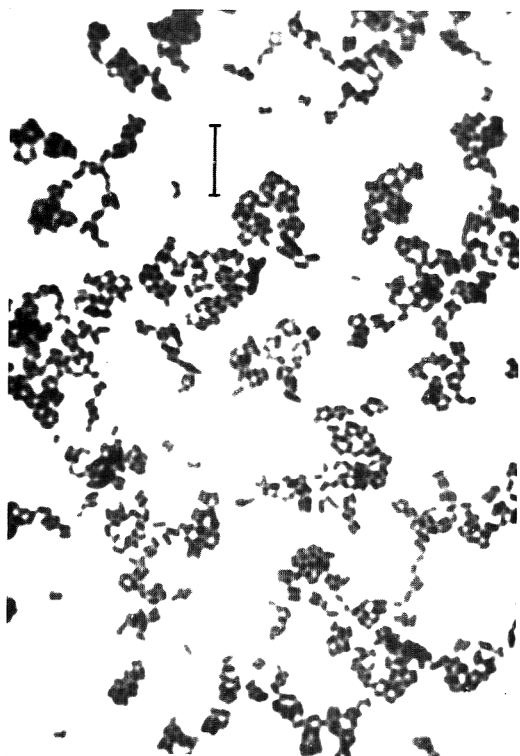
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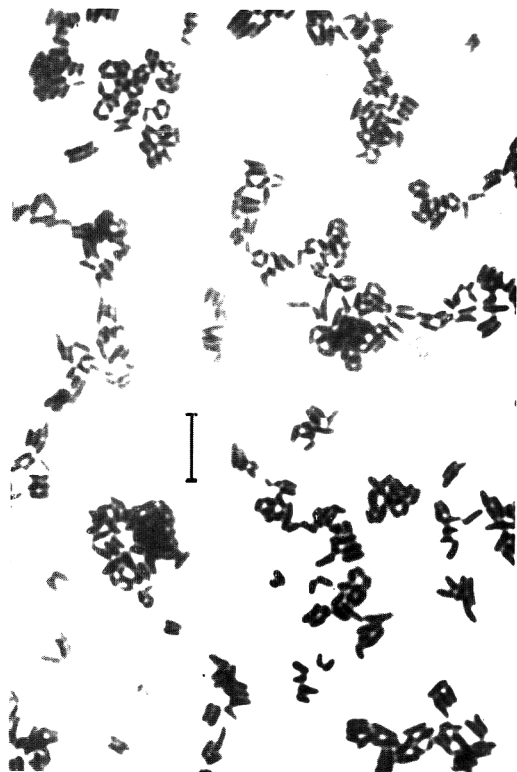
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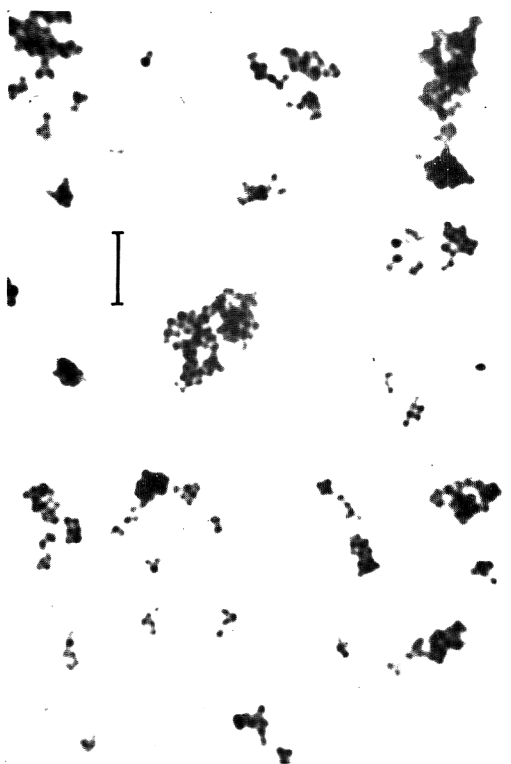
Fig. 1
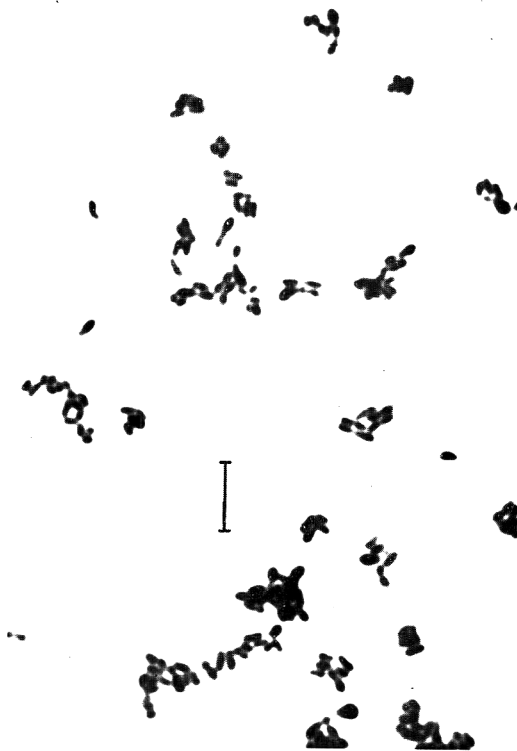
Fig. 3

Manual of Microbiological Methods. (1957). *Society of American Bacteriologists.* New York: McGraw-Hill.

MARPLES, M. S. (1965). *The Ecology of Human Skin.* Springfield, Ill.: C. C. Thomas.

MCBRIDE, M. E., FREEMAN, R. G. & KNOX, J. M. (1968). Characterization of keratinophilic corynebacteria. *Bact. Proc.* p. 78.

PILLSBURY, D. M. & KLIGMAN, A. M. (1954). Some current problems in cutaneous bacteriology. In *Modern Trends in Dermatology.* Ed. by R. M. B. MacKenna. New York: P. Hoeber, Inc.

PILLSBURY, D. M. & REBELL, G. (1952). The bacterial flora of the skin. *J. invest. Dermatol.* **18**, 173.

POLLOCK, M. R., WAINWRIGHT, S. D. & MANSION, E. D. (1949). The presence of oleic acid requiring diphtheroids on human skin. *J. Path. Bact.* **61**, 274.

ROBERTS, R. J. (1968). Biochemical reactions of *Corynebacterium pyogenes. J. Path. Bact.* **95**, 127.

ROSEBURY, T. (1962). *Microorganisms Indigenous to Man.* New York: McGraw-Hill.

SARKANY, D., TAPLIN, D. & BLANK, H. (1962). Erythrasma, a bacterial disease. *Excerpta Medica Int. Congress.* Series **55**, 1010.

SKERMAN, V. B. D. (1967). *A Guide to the Identification of the Genera of Bacteria.* Baltimore: Williams and Wilkins.

SMITH, R. F. & BODILY, H. L. (1968). Characterization of anaerobic diphtheroids. *Hlth. Lab. Sci.* **5**, 95.

SMITH, R. F. & WILLETT, N. P. (1968). Lipolytic activity of human cutaneous bacteria. *J. gen. Microbiol.* **52**, 441.

TAPLIN, D., REBELL, G. & ZAIAS, N. (1963). The human skin as a source of Mima-Herellea infections. *J. Am. med. Ass.* **186**, 952.

WHEATLEY, V. R. (1963). Problems in the analysis of sebum. In *Advances in the Biology of Skin,* **4**, 135. New York: MacMillan Co.

WILLIAMSON, P. & KLIGMAN, M. (1965). A new method for the quantitative investigation of cutaneous bacteria. *J. invest. Dermatol.* **45**, 498.

WINFORD, T. E. & HABERMAN, S. (1966). Isolation of aerobic Gram-positive filamentous rods from diseased gingivae. *J. dent. Res.* **45**, 1159.

EXPLANATION OF PLATE

Morphology of representative LD strains and *Corynebacterium xerosis.* Gram stain of 48 hr cultures grown on TS Tween 80 agar. Magnification, ×970. Bar in photographs represents 5 μ.

Fig. 1. *Corynebacterium xerosis* ATCC 373.

Fig. 2. LD strain 54, small coccobacilli.

Fig. 3. LD strain 51, intermediate size oval-shaped cells with one swollen end.

Fig. 4. LD strain 40, largest type with more even bacillary shape.

Cellulolytic Bacteria Occurring in the Rumen of Sheep Conditioned to Low-protein Teff Hay

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SUMMARY

From a large number of cultures of cellulolytic bacteria isolated from rumen contents of sheep conditioned to low-protein teff hay through the use of both a selective medium containing finely ground cellulose as energy source and a less selective medium containing cellobiose, xylan and starch, thirty isolates, representing three morphological types, were selected for detailed characterization. Nineteen isolates of Gram-negative curved rods belonged to the genus *Butyrivibrio*. Of these one was identical with and ten closely related to *B. fibrisolvens*. The remaining eight Butyrivibrio cultures differed in several respects from the only other defined species within this genus, *B. alactacidigens*. Four coccal isolates were identified as *Ruminococcus albus* and five as *R. flavefaciens*. Two strains of sporeformers belonged to the genus *Clostridium* but could not be identified with any of the cellulolytic species of this genus listed by *Bergey's Manual*.

INTRODUCTION

In a study of the carbohydrate and lactate-fermenting bacteria of the rumen of sheep conditioned to low-protein teff hay, Gilchrist & Kistner (1962) found that the most abundant cellulose-digesting bacteria were generally Gram-negative curved rods which usually produced narrow zones of cellulolysis in thin cellulose agar films. Gram-negative and Gram-positive cocci were also present in small numbers in cultures inoculated with high dilutions of the samples.

The present paper gives a fuller description of the cellulolytic bacteria occurring in sheep conditioned to this diet and relates them to previously described species. The majority of the cultures studied were isolated from agar media which contained acid-treated, ball-milled cotton wool cellulose (Hungate, 1950) as sole energy source. However, in view of the report of Halliwell & Bryant (1963) that this form of cellulose is an unsuitable substrate for some types of cellulolytic bacteria from the rumen, isolations were also made on a less selective medium containing cellobiose, xylan and starch. Of those isolates from this medium which were found to be cellulolytic, representative strains were included in the study.

METHODS

The methods used in the characterization of the isolates were similar to those described by Kistner (1960) and Kistner & Gouws (1964). In the following, details will, therefore. only be given of amendments to the original procedures.

Media

Cellobiose-xylan-starch medium. A medium of the same basal composition as the cellulose and cellobiose agar media, but containing 0·3 % (w/v) of each of cellobiose, xylan and starch (CXS-agar) was prepared. This medium was used for counts, isolations and maintenance of isolates of a section of the bacterial population of the rumen which would include all the cellulolytic species but exclude non-cellulolytic, glucose-fermenting species.

Media for fermentation tests. The clarified rumen fluid content of the basal medium was decreased from 30 to 20 % (v/v), the bicarbonate concentration was reduced to one-tenth of that used originally and the medium equilibrated with a gas mixture containing 10 % CO_2. In this way the buffering capacity was drastically lowered so that even slight growth gave rise to measurable pH changes. The concentration of cotton wool and filter paper cellulose was diminished to 0·2 % (w/v), while the concentrations of the remaining carbon sources were retained at 0·5 % (w/v).

Medium for end-product determination. Only the cellobiose medium was used and this was dispensed in 50 ml. volumes into rubber-stoppered, CO_2-purged, 6 oz. bottles.

Media for other tests. The media for the determination of temperature tolerance, carbon dioxide requirement, nitrate reduction, Voges–Proskauer reaction and gelatin. liquefaction were similar in composition to the counting medium, but contained 0·5 % (w/v) cellobiose. Bicarbonate was omitted from the carbon dioxide requirement test medium and it was equilibrated with oxygen-free nitrogen. The pH value was adjusted to 6·7 before sterilization. Potassium nitrate was added to the nitrate reduction test medium to give a final concentration of 0·1 % (w/v); the gelatin liquefaction test medium contained 0·4 % (w/v) gelatin and 1·5 % (w/v) agar. The medium for determining H_2S production contained 0·1 % cellobiose and 0·05 % (w/v) ferric ammonium citrate.

Anaerobic phosphate buffer. This was modified to contain 0·025 % instead of 0·05 % (w/v) cysteine $HCl.H_2O$.

Bacteriological procedures

Isolations from CXS-agar. Isolations were also made from CXS-agar film cultures inoculated with the 10^{-7} and 10^{-8} dilutions of samples of strained rumen contents. Colonies from the primary cultures were immediately transferred to slopes of the same medium. Subsequently, the cultures were tested for cellulolytic activity and only those which possessed such activity were further purified, where necessary.

Determination of cell morphology, motility and flagellation. The water of syneresis of 17–24 hr old cultures on rumen fluid agar slants containing 0·1 % (w/v) glucose or cellobiose was used for these tests, as recommended by Bryant & Small (1956). Fresh wet mounts of this material were examined without delay for motility and morphology. Gram reaction and cell dimensions were determined in Gram-stained smears of this material, while type of flagellation was determined by examination of smears stained according to Leifson (1951).

Further tests. The tests for production of acetylmethylcarbinol, indole and H_2S and the tests for nitrate reduction and gelatin liquefaction were carried out according to standard methods (*Society of American Bacteriologists*, 1957).

Determination of fermentation acids

The acids were extracted from the deproteinized culture liquid by the method of Neish (1952), with the exception that excess of alkali was added to the receiver containing ether to prevent loss of the lower acids by evaporation. The excess of alkali was back-titrated and water evaporated off. The acids were liberated from their sodium salts and separated by column chromatography on silicic acid by the method of Ramsey (1963), but using, successively, benzene, chloroform, 5 and 8 % (v/v) tertiary butyl alcohol in chloroform as eluants. The effluent from the column was fractionated into 5 ml. fractions, each of which was titrated with 0·01 N-alcoholic KOH solution.

RESULTS

Isolation

Seventy-eight cultures were isolated non-selectively from cellulose agar films inoculated with the 10^{-6} and 10^{-7} dilutions of samples of strained rumen contents obtained on different dates from six Merino wethers which had been fed low-protein teff hay for at least 4 weeks. Of these, 27 were lost on serial transfer in cellulose agar as the result of poor growth. A further 16 of the 98 cultures isolated from CXS-agar films inoculated with the 10^{-7} or 10^{-8} dilutions of samples from the same sheep were found to be cellulolytic according to the test of Bryant & Burkey (1953a). Five of these were also lost on serial transfer in cellulose agar. The surviving cultures were examined for cell morphology, Gram reaction, motility, type of flagellation, the appearance of the colonies in cellobiose and cellulose agar films and the nature of the zones of cellulolysis surrounding the colonies in the latter medium.

Characteristics of the isolates

Three morphological types of cellulolytic bacteria were observed. In agreement with earlier findings (Gilchrist & Kistner, 1962; Gouws & Kistner, 1965), Gram-negative, curved rods were the most abundant cellulose-digesting bacteria in strained rumen fluid from sheep on this diet. The cells were 0·4–0·5 μ in diameter by 1·0–1·8 μ long, with tapered ends. One isolate was exceptional in that the mean dimensions of the cells were 0·7 by 2·7 μ. The cells occurred singly, in pairs and in short to long chains. With one exception, the isolates were found to be motile, though even in 17 hr cultures the proportion of motile cells varied from test to test and was sometimes very low, especially in the chain-forming strains. The motile strains generally had single polar to subpolar flagella. However, in the case of those isolates which occurred mainly in long chains, it was difficult to determine the number of flagella per organism and the site of attachment. Surface colonies on cellobiose agar were generally tan-coloured, smooth, entire and flat to convex, while deep colonies in the same medium were spindle-shaped or Y-shaped. The zones of cellulolysis in cellulose agar films were narrow and often indistinct.

Second in abundance were Gram-negative, Gram-variable and Gram-positive cocci, the diameters of which varied between 0·6 and 0·9 μ. The cells occurred singly, in pairs and, in the case of some isolates, in chains of variable length. Most of the isolates produced capsules. Colonies of the cocci on cellobiose agar were circular, spindle-shaped or punctiform, smooth, opaque, butyrous, with entire margins and

flat to convex in elevation. The colonies in cellulose agar films mostly remained small, even after extended incubation, and the zones of cellulolysis were narrow and indistinct. However, two isolates gave rise to rhizoid colonies surrounded by fairly wide, distinct clearings. Two isolates produced yellow pigment when grown in cellulose agar.

Gram-negative, motile rods 2·4–3·0 μ long by 0·6–0·7 μ in diameter, with peritrichous arrangement of flagella, occurred in high numbers in rumen contents of two of the sheep on two sampling days. Old cells of these rods showed single, terminal to subterminal oval spores 1·3–1·6 μ long by 0·5–0·8 μ in diameter, in swollen sporangia. Surface colonies on cellobiose agar films were punctiform to circular, sometimes rhizoid, smooth, opaque, butyrous, flat to convex, with entire margins. The colonies in cellulose agar films were small and irregularly shaped, surrounded by large transparent zones which were sharply demarcated from the background of undigested cellulose.

On the results of the preliminary tests, twenty-five newly isolated cultures were selected for more detailed characterization. In the case of the curved rods and the cocci, the isolates were selected to include a wide range of combinations of cell dimensions, Gram-reaction, chain formation, pigment production and appearance of the zones of cellulolysis. Since the isolates of sporeformers were very uniform in their morphological characteristics, only two cultures were included in the selection. To the newly isolated cultures were added four isolates of cellulolytic curved rods and one of a pigment-producing coccus obtained 1–2 years previously from four sheep which had been fed a similar batch of low-protein teff hay. Details of the origin of the selected isolates are shown in Table 1. The morphological, cultural and biochemical characteristics determined for the curved rods and sporeformers are presented in Table 2 and those of the cocci in Table 3.

Curved rods. The 19 isolates studied were generally similar in Gram reaction, morphology, motility, type of flagellation and in the size of the zones of clearing in cellulose agar. Few strains grew at 22°, most at 30° and 45° and all at 37°. Ten strains grew without CO_2, none produced acetylmethylcarbinol or indole, none reduced nitrate and only one produced H_2S. Unlike the non-cellulolytic Butyrivibrio species isolated on a casein-containing medium by Blackburn & Hobson (1962), most of our cultures of curved rods did not liquefy gelatin. The carbon sources L-arabinose, galactose, sucrose, cellobiose, pectin, starch and xylan were fermented by all the isolates of curved rods, while none fermented L-rhamnose, glycerol, mannitol, inositol or sodium lactate. The isolates differed in the utilization of the remaining carbon sources listed in Table 2. Although all cultures had produced zones of clearing in cellulose agar films, only four produced visually detectable solubilization of cotton wool and filter paper cellulose in liquid medium. All 19 isolates produced formate, butyrate, hydrogen and at least traces of ethanol in the fermentation of cellobiose in rumen fluid-containing medium. One group produced appreciable amounts of lactate and removed acetate from the medium during cellobiose fermentation, while a second group produced acetate but little or no lactate. These differences in fermentation patterns correlated well with differences in the nutritional requirements of the isolates, the acetate-producing cultures being more fastidious (Shane, 1966). It therefore seemed justifiable to subdivide the curved rods ino groups 1 and 2, as shown in Table 2. The characteristics of isolates CE 52 and CE 74 were on the borderline between groups 1 and 2, both neither producing nor utilizing appreciable amounts of acetate,

though the fairly large concentrations of lactate produced seemed to indicate that they were more closely related to group 1 strains. However, isolate CE 74 was later moved to group 2 when its nutritional requirements were found to fit in more closely with this group.

Table 1. *Origins of isolates of cellulolytic rods and cocci*

Isolate*	Sheep no.	Days on diet	Sample dilution
Curved rods, Group 1			
CE 36	K 10	41	10^{-7}
CE 46	K 10	61	10^{-6}
CE 47	K 10	61	10^{-6}
CE 51	K 3	68	10^{-6}
CE 52	K 3	68	10^{-6}
CE 53	K 3	68	10^{-6}
CE 56	K 3	68	10^{-6}
CE 58	K 10	68	10^{-6}
CXS 13	K 10	40	10^{-7}
CXS 18	K 10	40	10^{-7}
7	R 3	33	10^{-7}
Curved rods, Group 2			
CE 64	K 33	28	10^{-7}
CE 65	K 33	55	10^{-7}
CE 66	K 33	55	10^{-6}
CE 74	K 23	55	10^{-7}
CE 78	K 23	55	10^{-6}
6 B	K 10	28	10^{-6}
6 D	K 20	49	10^{-6}
7 A	K 13	21	10^{-7}
Sporeformers			
CE 3	K 2	41	10^{-7}
CE 30	K 3	41	10^{-7}
Cocci, Group 1			
CE 54	K 3	68	10^{-6}
CE 63	K 23	28	10^{-7}
CXS 60	K 23	55	10^{-8}
CXS 65	K 23	55	10^{-8}
Cocci, Group 2			
CE 70	K 33	55	10^{-6}
CE 77	K 23	55	10^{-6}
CXS 36	K 3	46	10^{-8}
CXS 67	K 23	55	10^{-8}
4 A	R 3	29	10^{-4}

* Code numbers with prefix 'CXS' indicate isolates from cellobiose–xylan–starch agar films. The remaining isolates were obtained from cellulose agar.

Isolates 7, 6 D, 4 A were isolated in 1961: 6 B, 7 A in 1962: all the rest in 1963.

Cocci. The group of coccal isolates chosen for further characterization was comprised of Gram-negative, Gram-variable and Gram-positive cultures. It included cultures in which the cells occurred in short or long chains. The nine isolates had the following characteristics in common: growth at 37°, no growth at 22°; no growth in the absence of CO_2; no production of H_2S or indole; nitrate not reduced, gelatin not liquefied; cellobiose and xylan fermented and either cotton wool or filter paper cellulose visibly solubilized; L-arabinose, L-rhamnose, D-xylose, trehalose, glycerol, mannitol, inositol, salicin, aesculin, sodium lactate, inulin and starch not utilized;

Table 2. *Characters of Gram-negative cellulolytic rods from the rumen of sheep fed low-protein teff hay*

Characters	Curved rods, Group 1										
	CE 36	CE 46	CE 47	CE 51	CE 52	CE 53	CE 56	CE 58	CXS 13	CXS 18	7
Cells from water of syneresis*											
Dimensions (μ)	1·2×0·5	1·3×0·4	1·2×0·4	1·2×0·4	1·3×0·4	1·2×0·4	1·4×0·5	1·3×0·5	1·2×0·4	1·2×0·4	1·0×0·4
Aggregates†	−	−	−	−	−	−	−	sc	−	−	−
Motility	+	+	+	−	+	+	+	+	+	+	+
Flagella‡	p, m	p, m	p, m	−	p, m	p, m	p, m	p, m	sp, m	sp, m	p, m
Colonies in cellulose agar											
Zone of cellulolysis§	s, v	s, c	s, c	s, c	s, c	s, c	s, c	s, c	s, c	s, c	s, c
Cultural growth at											
22°	−	−	−	−	−	−	+	+	−	−	−
30°	+	+	+	+	+	+	+	+	+	+	+
37°	+	+	+	+	+	+	+	+	+	+	+
45°	+	+	−	−	+	+	+	+	+	+	+
Growth without CO₂	−	−	−	−	−	−	−	−	−	−	−
H₂S production	−	−	−	−	−	−	+	−	−	−	−
Nitrate reduction	−	−	−	−	−	−	+	−	−	−	−
Gelatin liquefaction	+	−	−	+	−	−	−	+	−	−	−
Carbon sources utilized‖											
L-Rhamnose	−	−	−	−	−	−	−	−	−	−	−
D-Xylose	+	+	+	+	+	+	+	+	+	+	+
D-Glucose	+	−	+	+	+	+	+	+	+	+	−
Fructose	+	+	+	+	+	+	+	+	+	+	+
D-Mannose	+	+	+	+	+	+	+	+	+	+	+
Lactose	+	+	+	+	+	+	+	+	+	+	+
Maltose	−	+	−	−	+	+	−	−	−	+	+
Trehalose	+	+	+	+	+	+	+	+	+	+	+
Cellobiose	+	+	+	+	+	+	+	+	+	+	+
Raffinose	−	+	−	−	+	+	−	−	−	+	+
Glycerol	−	−	−	−	−	−	−	−	−	−	−
Mannitol	+	−	+	+	+	+	+	+	+	+	+
Salicin	−	+	+	+	+	+	+	+	+	+	+
Aesculin	+	+	−	−	+	+	+	−	−	−	−
Inulin	+	+	−	+	−	−	+	−	+	+	+
Cellulose (cotton wool)	−	−	−	+	−	−	+	−	−	−	−
Cellulose (filter paper)	−	−	−	+	−	−	+	−	−	−	−

Table 2 (cont.)

Curved rods, Group 1

Characters	CE 36	CE 46	CE 47	CE 51	CE 52	CE 53	CE 56	CE 58	CXS 13	CXS 18	7
Fermentation end products¶											
Formate	0·31	0·38	0·47	0·56	0·27	0·22	0·51	0·35	0·45	0·28	0·43
Acetate	−0·49	−0·79	−0·62	−0·60	−0·02	−0·14	−0·50	−0·83	−0·47	−0·61	−0·36
Butyrate	1·13	1·17	1·10	1·09	1·74	1·15	1·34	1·12	1·06	1·21	0·71
Lactate	1·08	0·97	1·11	0·87	0·46	1·55	1·04	0·96	1·09	1·25	1·62
Succinate	0·00	0·00	0·00	0·00	0·00	0·00	0·00	0·00	0·00	0·00	0·00
Ethanol	0·04	0·05	0·03	0·06	0·05	0·04	0·19	0·04	0·10	0·01	0·03
Hydrogen	0·85	0·94	0·78	0·93	0·86	0·82	0·87	1·07	0·77	0·89	0·41

Curved rods, Group 2 / Sporeformers

Characters	CE 64	CE 65	CE 66	CE 74	CE 78	6 B	6 D	7 A	CE 3	CE 30
Cells from water of syneresis*										
Dimensions (μ)	2·7×0·7	1·5×0·5	1·5×0·4	1·1×0·4	1·7×0·5	1·4×0·4	1·6×0·4	1·7×0·4	2·9×0·7	2·4×0·6
Aggregates†	p, lc	p, lc	p, lc	+	sc	sc	sc	+	+	−
Motility	+	+	+	+	+	+	+	+	+	+
Flagella‡	sp, m	sp, m	sp, m	sp, m	p, m	p, m	p, m	p, m	per	per
Colonies in cellulose agar										
Zone of cellulolysis§	s, v	s, c	s	s, v	s	s, c	s, c	s, c	s, c	s, c
Cultural growth at										
22°	−	−	−	+	−	−	−	−	+	−
30°	−	−	−	+	+	+	−	+	+	+
37°	+	+	+	+	+	+	+	+	+	+
45°	−	−	+	+	+	+	+	+	+	+
Growth without CO_2	−	−	−	−	−	−	−	−	−	−
H_2S production	−	−	−	−	−	−	−	−	+	+
Nitrate reduction	−	−	−	−	+	−	−	−	+	±
Gelatin liquefaction	−	−	−	−	−	−	−	−	−	−

Table 2 (cont.)

Characters	Curved rods, Group 2								Sporeformers	
	CE 64	CE 65	CE 66	CE 74	CE 78	6 B	6 D	7 A	CE 3	CE 30
Carbon sources utilised‖										
L-Rhamnose	–	–	–	–	–	–	–	–	+	+
D-Xylose	–	+	+	–	–	+	+	–	+	+
D-Glucose	+	+	+	–	+	+	+	+	+	+
Fructose	+	+	–	–	–	+	–	+	+	+
D-Mannose	–	+	–	–	–	–	–	–	+	+
Lactose	+	–	+	+	+	+	+	+	+	+
Maltose	+	+	+	+	+	+	+	+	+	+
Trehalose	+	–	+	+	+	+	+	+	+	+
Cellobiose	+	+	+	+	+	+	+	+	+	+
Raffinose	–	+	–	+	–	+	+	+	+	+
Glycerol	–	+	–	–	–	–	–	–	+	+
Mannitol	–	–	–	–	–	–	–	–	+	+
Salicin	–	+	+	+	+	+	+	+	+	+
Aesculin	–	–	–	–	–	–	–	–	+	+
Inulin	–	+	+	–	+	+	+	+	+	+
Cellulose (cotton wool)	–	–	–	–	–	–	+	+	–	–
Cellulose (filter paper)	–	–	–	–	–	–	+	+	–	–
Fermentation end products¶										
Formate	1·61	0·98	1·71	1·84	0·56	1·46	1·15	1·73	2·21	1·54
Acetate	0·27	0·72	0·60	0·01	0·51	0·34	0·81	0·62	1·60	1·42
Butyrate	0·38	0·61	0·96	0·84	0·73	0·87	1·07	0·91	–0·06	–0·05
Lactate	0·00	0·09	0·00	0·45	0·00	0·11	0·05	0·00	0·00	0·00
Succinate	0·00	0·00	0·00	0·00	0·00	0·00	0·00	0·00	0·00	0·00
Ethanol	0·12	0·04	0·15	0·08	1·14	0·04	0·10	0·06	1·44	2·15
Hydrogen	0·44	0·38	0·43	0·14	0·87	0·45	0·73	0·67	0·31	0·14

* All isolates were Gram-negative; Voges–Proskauer and Indole production were always negative. † lc, long chains; sc, short chains. ‡ p, polar; sp, subpolar; m, monotrichous; per, peritrichous. § s, small; c, clear; v, vague; ‖ L-arabinose, galactose, sucrose, pectin, starch, and xylan were utilized as carbon sources by all isolates; inositol and sodium lactate were not utilized by any isolate. ¶ concentrations expressed in mmole/100 ml. medium, corrected for concentrations present in un-inoculated medium; – sign indicates uptake.

Table 3. *Characters of cellulolytic cocci from the rumen of sheep fed low-protein teff hay*

Characters	Group 1				Group 2				
	CE 54	CE 63	CXS 60	CXS 65	CE 70	CE 77	CXS 36	CXS 67	4A
Cells from water of syneresis									
Gram reaction*	−	−	±	−	−	±	−	+	+
Diameter (μ)	0·8	0·8	0·8	0·8	0·8	0·9	0·6	0·8	0·8
Aggregates†	p, sc	−	p	p	p	sc	lc	c	lc
Colonies in cellulose agar									
Zone of cellulolysis§	s, c	v	c	c	v	s, v	s, v	v	c
Yellow pigment	−	−	−	−	+	−	−	+	+
Cultural growth at									
22°	−	−	−	−	−	−	−	−	−
30°	−	−	+	+	−	+	+	+	+
37°	+	+	+	+	+	+	+	+	+
45°	−	−	+	−	−	+	−	−	−
Growth without CO₂	−	−	−	−	−	−	−	−	−
Voges–Proskauer test	−	−	−	−	−	+	−	−	−
H₂S production	−	−	−	−	−	±	−	−	−
Indole production	−	−	−	+	−	±	−	−	−
Nitrate reduction	−	−	−	−	−	−	−	−	−
Gelatin liquefaction	−	−	−	−	−	−	−	−	−
Carbon sources utilized‖									
D-Glucose	−	−	−	−	−	−	−	−	−
Fructose	+	+	−	−	−	+	−	−	−
Galactose	−	−	−	−	−	+	−	−	−
D-Mannose	+	+	−	+	−	+	−	−	−
Lactose	+	+	+	−	−	−	−	−	−
Sucrose	−	−	−	+	−	+	−	−	−
Maltose	+	+	+	+	−	+	−	−	+
Cellobiose	+	+	+	+	+	+	+	+	+
Pectin	−	−	−	−	+	+	+	+	−
Raffinose	−	−	−	+	−	+	−	+	+
Xylan	+	+	+	−	+	+	+	+	−
Cellulose (cotton wool)	+	+	+	+	+	+	+	+	+
Cellulose (filter paper)	+	+	+	+	−	−	+	+	+
Fermentation end products¶									
Formate	0·58	0·94	0·97	0·45	0·14	0·80	0·15	0·22	0·58
Acetate	0·66	0·62	1·15	0·77	0·60	0·68	0·79	1·06	0·85
Butyrate	−0·09	−0·10	−0·11	0·10	−0·07	−0·11	−0·04	0·02	−0·11
Lactate	1·07	0·00	0·00	0·14	1·49	0·58	1·78	0·51	0·00
Succinate	0·00	0·00	0·00	0·00	1·17	1·30	0·99	1·33	0·62
Ethanol	1·00	1·18	1·30	1·43	0·12	0·10	0·03	0·09	0·24
Hydrogen	0·10	0·39	0·34	0·24	0·10	0·14	0·16	0·16	0·09

* −, Gram-negative; +, Gram-positive; ±, Gram-variable. † p, pairs; c, chains; lc, long chains; sc, short chains. § s, small; c, clear; v, vague. ‖ L-Arabinose, L-rhamnose, D-xylotrese, chalose, glycerol, mannitol, inositol, salicin, aesculin, sodium lactate, inulin, and starch were not utilized by any isolate in group 1 or 2. ¶ concentrations, expressed in mmole/100 ml. medium, corrected for concentrations present in uninoculated medium; − sign indicates uptake.

large amounts of formate and acetate produced in the fermentation of cellobiose. A good correlation was found between production of large amounts of ethanol and lack of production of succinate by isolates CE 54, CE 63, CXS 60 and CXS 65. With the exception of isolate CE 54, cells of these cultures did not occur in chains. In the chain-forming isolates CE 70, CE 77, CXS 36, CXS 67 and 4 A the production of large amounts of succinate correlated well with the production of only traces of ethanol. Accordingly, the coccal isolates were divided into groups 1 and 2 in Table 3.

Sporeformers. The two cultures of sporeformers, CE 3 and CE 30, which were isolated from different sheep, were similar in all characteristics, except growth at 22°. Both grew at temperatures between 30° and 45° and required no CO_2 for growth. Neither produced acetylmethylcarbinol, H_2S or indole, reduced nitrate or liquefied gelatine. Of the carbon sources tested, only inositol and sodium lactate were not utilized. In spite of the fact that the sporeformers had produced extensive clearings in cellulose agar films, neither cotton wool nor filter paper cellulose were visibly solubilized in a liquid medium. The sporeformers produced large quantities of formate, acetate and ethanol and lesser amounts of hydrogen in the fermentation of cellobiose. Butyrate, lactate and succinate were not produced.

DISCUSSION

In so far as they are anaerobic, non-sporeforming, monotrichous, Gram-negative, curved rods that ferment carbohydrates with the production of large amounts of butyric acid, 18 of the 19 isolates of curved rods studied here doubtlessly belong to the genus *Butyrivibrio* (Bryant & Small, 1956). In the case of isolate CE 51, neither motility nor occurrence of flagella were demonstrated. Nevertheless, in view of general similarity of this isolate to other members of Group 1, it seems justifiable to regard CE 51 as a member of the genus. A similar view was taken by Margherita & Hungate (1963) of a culture for which motility and presence of flagella could not be demonstrated, but which otherwise agreed with the description of the species *B. fibrisolvens*. With the exception of culture CE 64, the cells of our isolates are rather smaller than those of the strains described by Bryant & Small (1956), but similar in size to those described by Blackburn & Hobson (1962) and Van Gylswyk (1968).

Within the genus *Butyrivibrio*, Bryant & Small defined only a single species, viz. *B. fibrisolvens*. The characteristics of 17 of the 48 isolates studied in some detail by these workers did not fit the description of *B. fibrisolvens* and the isolates differed so much amongst themselves, that no further species were defined. More recently, Hungate (1966) has proposed a second species, *B. alactacidigens*, to accommodate those strains which do not produce lactic acid in the fermentation of carbohydrate.

Of the lactate-producing isolates listed in group 1 of Table 2, the characteristics of culture CE 53, only, fall within the definitions of *Butyrivibrio fibrisolvens* (Bryant & Small, 1956), provided the differences in cell dimensions are ignored. The other members of our Group 1 may be regarded as closely related variants of this species. The characteristics in which they differ from *B. fibrisolvens* are: possible absence of flagella and lack of motility (strain CE 51); growth at 22° (strains CE 56 and CE 58); no growth at 45° (CE 47); failure to grow in the absence of CO_2 (CE 36, CE 46, CE 47, CE 51, CE 52, CE 58, CXS 13); inability to utilize glucose (CE 46, 7); inability to utilize salicin (CE 46); and inability to utilize aesculin (CE 36, CE 47, CE 51, CE 58, CXS 13, CXS 18, 7).

The acetate-producing isolates listed under Group 2 in Table 2 differ more from one another than the acetate-utilizing isolates of group 1. None of the former could be identified with *Butyrivibrio alactacidigens* (Hungate, 1966). Isolate 6 B was most closely related to this species, differing from it in fermenting D-xylose, solubilizing cellulose (though not visibly in liquid medium) and not fermenting L-rhamnose. The other members of group 2 differed from *B. alactacidigens* in six to eight characters.

The results presented in Table 2 seem to suggest that the good correlations between acetate utilization, 'high lactate' and 'low formate' on the one hand, and between acetate production, 'low lactate' and 'high formate' on the other hand would possibly provide a better basis for dividing the genus *Butyrivibrio* into species than the scheme based mainly on production or lack of production of lactic acid. A detailed study, with standardized methods, of a large number of Butyrivibrio isolates obtained from different habitats and different geographic locations, followed by estimation of the degree of similarity between the isolates, could help much to resolve the present unsatisfactory taxonomic state of the genus *Butyrivibrio*. An investigation along these lines, albeit of a more limited scope, for Ruminococcus isolates from the rumen of sheep on three different diets was described by Jarvis & Annison (1967).

The coccal isolates listed in Table 3 all belong to the genus *Ruminococcus* (Sijpesteijn, 1951), as redefined by Hungate (1957), Bryant, Small, Bouma & Robinson (1958) and Hungate (1966). The characteristics of the four strains classed under Group 1 fall within the definitions of the species *R. albus* (Hungate, 1957), as amended by Bryant *et al.* (1958) and Hungate (1966), with the minor exception that strains CXS 60 and CXS 65 showed growth at 45°. Isolates CE 70, CXS 36, CXS 67 and 4 belong to the species *R. flavefaciens*, as redefined by Hungate (1966). Isolate CE 77 is closely related to this species but differs from it in being able to ferment a larger variety of carbohydrates. This also holds for one of the strains studied by Dehority (1962).

By virtue of their morphological characteristics and requirement for anaerobic conditions for growth, the sporeformers belong to the genus *Clostridium*. However, they differ in many respects from the cellulolytic sporeformers *C. cellobioparus* (Hungate, 1944), *C. lochheadii* and *C. longisporum* (Hungate, 1957) previously isolated from the rumen. Moreover, they could not be identified with any of the cellulolytic Clostridium species listed in *Bergey's Manual* (1957). In view of their sporadic occurrence in high dilutions of rumen contents from only two sheep on low-protein teff hay, they are probably of minor significance in cellulose digestion in the rumen. *Clostridium lochheadii* and *C. longisporum*, likewise, occurred only sporadically in high numbers in rumen contents of cows fed different combinations of timothy hay, salt and concentrates (Hungate, 1957).

It should be noted that the isolates obtained from CXS-agar films belonged to the same groups as those obtained from cellulose agar films. *Bacteroides succinogenes* which has been reported as one of the more abundant cellulolytic bacteria in rumen contents of cattle on different diets, including low-protein wheat straw (Hungate, 1950; Bryant & Burkey, 1953 *b*) was not found among the isolates. Even though the cotton wool cellulose used in our media for direct cellulolytic counts and isolations could have been a physically unsuitable substrate for growth of this organism, it should have been able to utilize the cellobiose in the CXS-agar and therefore have shown up among the isolates from this medium, had it been present in high numbers in the samples.

Though Table 1 presents information on the origin of only those isolates which were studied in detail, it is evident that morphologically and physiologically similar strains of butyrivibrios and ruminococci were isolated from the same sheep on different sampling dates and also simultaneously from different sheep. On the other hand, different species of the same genus occurred in the same sample of rumen contents. The four Butyrivibrio cultures and one *R. flavefaciens* strain which had been isolated 1–2 years previously from sheep conditioned to low-protein teff hay, did not differ more from the newly isolated members of their respective groups than these amongst themselves. Thus, although a particular strain of a functional rumen micro-organism may disappear from the rumen in a matter of months, as is suggested by the serological studies of Margherita & Hungate (1963), it is probably replaced by physiologically similar strains.

The fact that a greater diversity of cellulose-digesting bacterial species normally occurs in the rumen contents from sheep conditioned to low-protein teff hay than in rumen contents of sheep conditioned to lucerne hay, suggests that in the former habitat environmental conditions do not favour the selection of a particular species to the same extent as in the latter. The degree to which the nutritional requirements of the various species are met by the environment probably plays a major rôle in the establishment of a balance between competing species. As a preliminary step in examining such relationships, a subsequent paper will deal with studies of some nutritional requirements of different species of cellulolytic bacteria.

This paper forms Part V of a series on 'Bacteria of the ovine rumen'; for parts I, II and IV see *J. agric. Sci.* **59**, 77, 85 (1962) and **64**, 51 (1965); for Part III see *J. gen. Microbiol.* **34**, 447 (1964).

REFERENCES

BLACKBURN, T. H. & HOBSON, P. N. (1962). Further studies on the isolation of proteolytic bacteria from the sheep rumen. *J. gen. Microbiol.* **29**, 69.

Bergey's Manual of Determinative Bacteriology, 7th ed. Ed. by R. S. Breed, E. G. D. Murray & N. R. Smith, (1957). London: Baillière, Tindal and Cox Ltd.

BRYANT, M. P. & BURKEY, L. A. (1953a). Cultural methods and some characteristics of some of the more numerous groups of bacteria in the bovine rumen. *J. Dairy Sci.* **36**, 205.

BRYANT, M. P. & BURKEY, L. A. (1953b). Numbers and some predominant groups of bacteria in the rumen of cows fed different rations. *J. Dairy Sci.* **36**, 218.

BRYANT, M. P. & SMALL, N. (1956). The anaerobic monotrichous butyric acid-producing curved rod-shaped bacteria of the rumen. *J. Bact.* **72**, 16.

BRYANT, M. P., SMALL, N., BOUMA, C. & ROBINSON, I. M. (1958). Characteristics of ruminal anaerobic cellulolytic cocci and *Cillobacterium cellulosolvens* n.sp. *J. Bact.* **76**, 529.

DEHORITY, B. A. (1962). Isolation and characterization of several cellulolytic bacteria from *in vitro* rumen fermentations. *J. Dairy Sci.* **46**, 217.

GILCHRIST, F. M. C. & KISTNER, A. (1962). Bacteria of the ovine rumen I. The composition of the population on a diet of poor teff hay. *J. agric. Sci.* **59**, 77.

GOUWS, L. & KISTNER, A. (1965). Bacteria of the ovine rumen IV. Effect of change of diet on the predominant type of cellulose-digesting bacteria. *J. agric. Sci.* **64**, 51.

HALLIWELL, G. & BRYANT, M. P. (1963). The cellulolytic activity of pure strains of bacteria from the rumen of cattle. *J. gen. Microbiol.* **32**, 441.

HUNGATE, R. E. (1944). Studies on cellulose fermentation. I. The culture and physiology of an anaerobic cellulose-digesting bacterium. *J. Bact.* **48**, 489.

HUNGATE, R. E. (1950). The anaerobic mesophilic cellulolytic bacteria. *Bact. Rev.* **14**, 1.

HUNGATE, R. E. (1957). Micro-organisms in the rumen of cattle fed a constant ration. *Can. J. Microbiol.* **3**, 289.

HUNGATE, R. E. (1966). *The Rumen and its Microbes*, 1st ed. New York and London: Academic Press.

JARVIS, B. D. W. & ANNISON, E. F. (1967). Isolation, classification and nutritional requirements of cellulolytic cocci in the sheep rumen. *J. gen. Microbiol.* **47**, 295.

KISTNER, A. (1960). An improved method for viable counts of bacteria of the ovine rumen which ferment carbohydrates. *J. gen. Microbiol.* **23**, 565.

KISTNER, A. & GOUWS, L. (1964). Cellulolytic cocci occurring in the rumen of sheep conditioned to lucerne hay. *J. gen. Microbiol.* **34**, 447.

LEIFSON, E. (1951). Staining, shape and arrangement of bacterial flagella. *J. Bact.* **62**, 377.

MARGHERITA, S. S. & HUNGATE, R. E. (1963). Serological analysis of *Butyrivibrio* from the bovine rumen. *J. Bact.* **86**, 855.

NEISH, A. C. (1952). *Analytical methods for bacterial fermentations*, 2nd revision. Saskatoon, Nat. Research Council of Canada, Report No. 46-8-3.

RAMSEY, H. A. (1963). Separation of organic acids in blood by partition chromatography. *J. Dairy Sci.* **46**, 480.

SHANE, B. S. (1966). *The predominant cellulolytic rumen bacteria of sheep fed low-protein teff hay.* Ph.D. thesis, University of Witwatersrand.

SIJPESTEIJN, A. K. (1951). On *Ruminococcus flavefaciens*, a cellulose-decomposing bacterium from the rumen of sheep and cattle. *J. gen. Microbiol.* **5**, 869.

SOCIETY OF AMERICAN BACTERIOLOGISTS (1957). *Manual of Microbiological Methods.* New York, Toronto, London: McGraw-Hill Book Company, Inc.

VAN GYLSWYK, N. O. (1968). *The cellulolytic rumen bacteria of sheep fed supplemented hay diets.* M.Sc. thesis, University of Pretoria.

Extent of Solubilization
of α-Cellulose and Hemicellulose of Low-protein Teff Hay by Pure Cultures of Cellulolytic Rumen Bacteria

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SUMMARY

Ten isolates belonging to the genus *Butyri vibrio* and two each of *Ruminococcus albus*, *R. flavefaciens* and an unidentified *Clostridium* sp. were isolated from high dilutions of rumen fluid from sheep conditioned to low-protein teff hay. The butyrivibrios solubilized between 10 and 37 % of the α-cellulose of the hay (average 21 %). The two isolates of *Ruminococcus albus* degraded 43 and 56 % of the α-cellulose and the two of *R. flavefaciens* 39 and 66 %, while the two *Clostridium* cultures achieved only 10 % degradation. Hemicellulose degradation by the Butyrivibrio isolates was between 25 and 67 % (mean 48 %) and that by the four Ruminococcus cultures between 47 and 65 %. One of the Clostridium cultures solubilized 21 % and the other 51 % of the hemicellulose. The butyrivibrios appear capable of contributing a greater share towards cellulose digestion in ruminants on low-protein hay than has been inferred from qualitative *in vitro* tests for cellulolysis, using refined cellulose preparations; they are probably less active than the ruminococci.

INTRODUCTION

Gram-negative curved rods were repeatedly found to be either the most abundant or among the most abundant cellulolytic bacteria in the rumen of sheep conditioned to low-protein teff (*Eragrostis tef*) hay (Gilchrist & Kistner, 1962; Gouws & Kistner, 1965; Van Gylswyk, 1968). These rods were identified as belonging to the genus *Butyrivibrio* (Bryant & Small, 1956) and were subdivided into two groups, one of which was closely related to *B. fibrisolvens* while the other was somewhat more heterogeneous and differed in several respects from the only other species of the genus, *B. alactacidigens*, proposed by Hungate (1966) (Shane, Gouws & Kistner, 1968). Although the isolates of both groups originated from colonies in primary cultures in cellulose agar films which had produced zones of cellulolysis, the majority did not give rise to a visually detectable disappearance of either cotton-wool or filter paper cellulose in liquid media containing 0·2 % (w/v) of these substrates. This appeared to be at variance with their supposed role as important cellulose digesters in sheep conditioned to low-protein teff hay. It seemed feasible that these refined forms of cellulose were less suitable substrates for the butyrivibrios than the forage fed to the sheep from which they were isolated. A selection of isolates, representing the two groups of Butyrivibrio mentioned above, were therefore tested for their ability to solubilize α-cellulose

and hemicellulose in the form present in finely ground teff hay. Hemicellulose was included in the study since all the isolates had been found to ferment corn cob xylan. Two strains of an unidentified *Clostridium* sp. which had also produced zones of cellulolysis in the primary cellulose agar cultures but shown little cellulolytic activity in subsequent tests in liquid medium, and two strains each of *Ruminococcus albus* and *R. flavefaciens* which had produced visually detectable solubilization of either filter paper or cotton-wool cellulose, were tested in parallel with the Butyrivibrio isolates.

METHODS

Substrate. A sample of the same batch of teff hay as was fed to the sheep from which the bacterial cultures were isolated was used as substrate. It contained 36.2% α-cellulose and 40.5% hemicellulose. The hay was ground in a Wiley mill to pass a 44-mesh sieve. Lipid was removed by extraction with ethanol–benzene (2:1, v/v) for 90 min. in a Soxhlet extractor.

Test medium. One g. portions of well-mixed, air-dried hay were placed in 600 ml. bottles, 200 ml. portions of basal minerals–rumen fluid medium, as used by Kistner & Gouws (1964) for fermentation tests, were added, the bottles were purged with oxygen-free CO_2, sealed with rubber stoppers and sterilized at $121°$ for 25 min.

Organisms. The ten cultures of *Butyrivibrio* spp., and two each of *Ruminococcus albus*, *R. flavefaciens* and the unidentified *Clostridium* sp. used in this study, have been described by Shane *et al.* (1968). The cultures were maintained on minerals-rumen fluid-cellobiose agar. The surface growth of an 18 hr slant culture, suspended in 0·5 ml. anaerobic phosphate buffer (Kistner & Gouws, 1964), served as inoculum for a test in duplicate. The inoculated test media were incubated at $38°$ for 3 weeks, to ensure that incubation time did not limit the extent of solubilization of α-cellulose and hemicellulose. Uninoculated controls were similarly treated.

Analytical procedures. On completion of the incubation period, the cultures were quantitatively transferred to plastic centrifuge bottles and centrifuged at $1200\,g$ for 25 min. The supernatant fluid was discarded, the sediment suspended in 96% (v/v) ethanol, transferred to 100 ml. plastic centrifuge tubes and recentrifuged. The sediment was treated once more with ethanol to dehydrate bacterial slime which tended to clog the sintered glass filter crucibles in subsequent procedures. Alpha-cellulose and hemicellulose were determined by the procedure described by Wise, Murphy & D'Addieco (1946) for the isolation of holocellulose and α-cellulose from wood. However, in view of the small weight of sample remaining after incubation and the lower proportion of lignin, the residual hay was suspended in 128 ml. of deionized water and treated twice, instead of three to four times, with one-third of the quantities of glacial acetic acid and sodium chlorite used by these workers. The holocellulose was dried at $110°$ for 4 hr before weighing. For the present purpose, hemicellulose fractions A and B were not determined separately, nor were the hemicelluloses isolated. Instead, hemicellulose content of the hay was calculated from the loss in weight on treating the isolated holocellulose with 24% (w/v) KOH solution at $20°$.

The extent of solubilization of α-cellulose and hemicellulose was calculated with reference to the analytical values obtained on uninoculated control media after incubation.

RESULTS AND DISCUSSION

The means of duplicate determinations of the extent of solubilization of α-cellulose and hemicellulose by the 16 cultures are shown in Table 1. Under the conditions of the *in vitro* test, the Butyrivibrio isolates solubilized between 10 and 37% of the α-cellulose of the hay, with an average value of 21%. There was no marked difference in the cellulolytic potential between the two groups. It would therefore appear that the butyrivibrios can play a significant part in the digestion of cellulose in the rumen of sheep under conditions where they are the predominant cellulolytic bacteria isolated. The extent of cellulose digestion by the pure cultures used in the present study is, however, considerably less than that found *in vivo* in four sheep conditioned to low-protein teff hay, namely a mean of 57% (van Gylswyk, 1968). It may be that the activity of these organisms is increased *in vivo* by synergism with non-cellulolytic

Table 1. *Extent of solubilization of α-cellulose and hemicellulose of low-protein teff hay by pure cultures of cellulolytic rumen bacteria isolated from sheep conditioned to such hay*

	Extent of solubilization (%)	
Cultures	α-Cellulose	Hemicellulose
Butyrivibrio, group 1		
CE 47	20·5	49·5
CE 51	27·1	66·8
CE 52	17·9	54·8
CXS 18	22·8	44·8
Butyrivibrio, group 2		
CE 64	10·0	25·3
CE 65	37·1	61·1
CE 66	21·7	49·6
CE 78	14·7	31·5
6B	17·4	40·7
7A	24·9	53·C
Clostridium sp.		
CE 3	10·1	20·6
CE 30	10·7	51·2
Ruminococcus albus		
CE 54	43·4	47·9
CXS 60	56·0	56·3
R. flavefaciens		
CE 77	66·2	64·6
4A	38·9	47·2

species. Dehority & Scott (1967) have shown such a synergistic effect on degradation of cellulose in different forages *in vitro* by combining a strain of the hemicellulolytic, but non-cellulolytic organism *Bacteroides ruminocola* with any of two strains of *Ruminococcus flavefaciens*, one of *R. albus* and one of *B. succinogenes*. However, in the teff hay-fed sheep studied by van Gylswyk (1968), the higher cellulose digestion could have been due to large numbers of ruminococci which were also present and which, the present results show, are more active cellulolytically than the butyrivibrios. Unfortunately, no figures for percentage degradation of cellulose are available for the sheep examined by Gilchrist & Kistner (1962) in which butyrivibrios were the predominant cellulose-digesting bacteria.

The two isolates of *Ruminococcus albus* degraded 43 and 56% of the α-cellulose

present in the hay samples and the two strains of *R. flavefaciens* 39 and 66%. That ruminococci are more active than butyrivibrios *in vivo* as well as *in vitro* is indicated by the results of van Gylswyk (1968), who found that the percentage digestion of cellulose of low-protein teff hay increased as the percentage proportion of ruminococci among the cellulolytic bacteria increased as a result of supplementation. The highest percentage of cellulose solubilized by a Ruminococcus isolate was 66·2% by strain CE 77. This is very similar to the highest value reported by Dehority & Scott (1967) for their most active strain of *R. flavefaciens*, when grown on timothy hay cut at boot stage. It is also very close to the mean percentage of teff hay α-cellulose digested *in vivo* (72%) when the hay was supplemented with urea and the potassium salts of isobutyric, 2-methylbutyric, isovaleric and *n*-valeric acids. In this case, about 90% of the cellulolytic population of the rumen consisted of ruminococci (van Gylswyk, 1968). Thus, for this species, the *in vitro* results are a fair reflexion of their capacity to digest cellulose *in vivo*.

The clostridia appear to be of little importance in the digestion of cellulose in sheep on teff hay rations, since not only do they occur only sporadically in high numbers, but their ability to attack native cellulose is limited.

The extent of hemicellulose degradation by the Butyrivibrio isolates was between 25 and 67% (mean 48%) and that by the four Ruminococcus cultures between 47 and 65%. One of the Clostridium cultures solubilized 21% and the other 51% of the hemicellulose present in the samples. The fact that the butyrivibrios and ruminococci have similar activity in degrading teff hay hemicellulose means that the increase in digestibility of this component in sheep on supplemented teff hay diets cannot be explained by the increase in the preponderance of ruminococci in the rumen (van Gylswyk, 1968). Other organisms which degrade hemicellulose, but not cellulose, may be involved.

This paper forms part 6 of a series on 'Bacteria of the ovine rumen'.

REFERENCES

BRYANT, M. P. & SMALL, N. (1956). The anaerobic monotrichous butyric acid-producing curved rod-shaped bacteria of the rumen. *J. Bact.* **72**, 16.

DEHORITY, B. A. & SCOTT, H. W. (1967). Extent of cellulose and hemicellulose digestion in various forages by pure cultures of rumen bacteria. *J. Dairy Sci.* **50**, 1136.

GILCHRIST, F. M. C. & KISTNER, A. (1962). Bacteria of the ovine rumen. I. The composition of the population on a diet of poor teff hay. *J. agric. Sci., Camb.* **59**, 77.

GOUWS, L. & KISTNER, A. (1965). Bacteria of the ovine rumen. IV. Effect of change of diet on the predominant type of cellulose-digesting bacteria. *J. agric. Sci., Camb.* **64**, 51.

HUNGATE, R. E. (1966). *The Rumen and its Microbes*, 1st ed. New York and London: Academic Press.

KISTNER, A. & GOUWS, L. (1964). Cellulolytic cocci occurring in the rumen of sheep conditioned to lucerne hay. *J. gen. Microbiol.* **34**, 447.

SHANE, B. S., GOUWS, L. & KISTNER, A. (1968). Cellulolytic bacteria occurring in the rumen of sheep conditioned to low-protein teff hay. (In the Press.)

VAN GYLSWYK, N. O. (1968). *The Cellulolytic Rumen Bacteria of Sheep Fed Supplemented Hay Diets*. M.Sc. thesis, University of Pretoria.

WISE, L. E., MURPHY, M. & D'ADDIECO, A. A. (1946). Chlorite holocellulose, its fractionation and bearing on summative wood analysis and on studies on the hemicelluloses. *Paper Trade J.* **122**, 35.

Books Received

Annual Review of Microbiology, Volume 22, 1968. Edited by CHARLES E. CLIFTON, SIDNEY RAFFEL and MORTIMER P. STARR. Published by Annual Reviews, Inc., 4139 El Camino Way, Palo Alto, California 94306, U.S.A. 596 pp. Price $9.00.

Antimicrobial Agents and Chemotherapy—1967. Proceedings of the Seventh Interscience Conference on Antimicrobial Agents and Chemotherapy, Chicago, Illinois, 25–27 October 1967. Edited by GLADYS L. HOBBY. Published by The Williams and Wilkins Company, 428 East Preston Street, Baltimore, Maryland 21202, U.S.A. English agents: E. and S. Livingstone Limited, 15–17 Teviot Place, Edinburgh. 779 pp. Price £7.

Caldas Médico. No. 8, September 1967. Published by Universidad de Caldas, Manizales, Apartado Aéreo 275, Colombia. 78 pp.

Metabolism. Compiled and edited by PHILIP L. ALTMAN and DOROTHY S. DITTMER. Published by the Federation of American Societies for Experimental Biology, 9650 Rockville Pike, Bethesda, Maryland 20014, U.S.A. 737 pp. Price $20.00.

Modern Biology. By HANS JOACHIM BOGEN. Published by Weidenfeld and Nicolson, 5 Winsley Street, Oxford Circus, London W. 1. 336 pp. Price £2. 5s.

Nucleic Acids in Immunology. Edited by OTTO J. PLESCIA and WERNER BRAUN. Proceedings of the Symposium on Nucleic Acids in Immunology held at the Institute of Microbiology, Rutgers, The State University, New Brunswick, New Jersey, 16–18 October 1967. Published by Springer-Verlag New York Inc., 175 Fifth Avenue, New York, New York 10010, U.S.A. 724 pp. Price $22.00.

Vistas in Connective Tissue Diseases. Price incorrectly given in Volume 54 as $10.50. This has been changed to $19.75.

THE SOCIETY FOR GENERAL MICROBIOLOGY

The Society for General Microbiology held its fifty-third General Meeting at the College of Agriculture, University of Edinburgh, in conjunction with the first Meeting of the North West European Microbiological Group on 16, 17, 18 *September* 1968. *The following communications were made:*

SYMPOSIUM: EXTRACHROMOSOMAL GENETIC ELEMENTS IN BACTERIA

Introduction. By W. HAYES (*Department of Molecular Biology, University of Edinburgh*)

Episomes were originally defined as genetic elements which are additional to the normal genome of the cell, are transmissible by infection and, in particular, may be propagated in one or the other of two alternative states—either independently in the cytoplasm or, following insertion, as an integral part of their host's chromosome. This definition emerged from a comparison of the properties and behaviour of the temperate phage λ, and the prototype sex factor F of *Escherichia coli* K 12. The word 'episome', like the word 'cistron', subsequently proved of great value in provoking new ideas and experiments. More recently, however, the word has engendered more confusion than enlightenment by being linked to the properties of infectivity and, particularly, of chromosomal integration and raises the question, 'When is an episome not an episome?' For instance, does the sex factor F cease to be an episome when transferred to *Shigella* in which it is propagated but shows no overt signs of chromosomal integration? Again, many independent genetic elements have now been recognized which display a spectrum of properties ranging from those of highly transmissible sex factors which promote chromosome transfer with an efficiency comparable to that of F-prime factors, to those of factors which are quite devoid of independent transmissibility.

All these factors clearly fall into the same general category as the sex factor F but, strangely enough, F remains the only element, apart from temperate phages, for which a relatively stable, chromosomally integrated state has been demonstrated. One class of sex factors, the fi^+ transfer factors, have close phylogenetic relationships with F, producing a cytoplasmic repressor which switches off synthesis of sex fimbriae (pili) by F as well as by themselves. Such factors, therefore, are naturally repressed and show a very low normal frequency of conjugation, but derepressed mutants have been isolated which display the same high frequency of conjugation as F, and whose fimbriae infection permit by F-specific RNA phages. The sex factor F thus turns out to be an exceptional, derepressed fi^+ transfer factor. A number of other factors, which do not show these functional interactions with F, produce sex fimbriae similar to those determined by the colicinogeny factor I, and constitute a second phylogenetically related class.

Another criterion of relatedness is 'incompatibility'; i.e. the inability of two factors to coexist stably in the same cell. While F-like and I-like elements appear always to be compatible, elements within each class, although often compatible, may display varying degrees of incompatibility. Because of these relationships between many independently isolated and otherwise distinguishable elements, and since such important attributes as transmissibility can be gained or lost by mutation, it is suggested that the term 'episome' can be abandoned on account of its specific connotations and replaced by the term 'plasmid'. This word, introduced by Lederberg in 1952, was originally defined as an extra-chromosomal element prior to the concept of episomes. However, as this symposium will reveal, there is now good evidence that genetic interactions, which may result in the integration or expulsion of genetic material,

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occur as an innate property, between different plasmids in the same cell, between plasmids and the bacterial chromosome, and even between different non-allelic regions of the bacterial chromosome itself. One cannot therefore distinguish between cytoplasmic genetic elements on a clear-cut basis of whether they can or cannot be inserted, but only by the usual frequency of insertion. A more meaningful operational distinction is that between transmissible plasmids, or sex factors, and non-transmissible plasmids whose infectivity depends on the concomitant presence of a sex factor or on transduction.

The Physical Demonstration and Characterization of Extrachromosomal Elements. By STANLEY
FALKOW (*Department of Microbiology, Schools of Medicine and Dentistry, Georgetown University, Washington, D.C.* 20007)

The transfer of extrachromosomal elements from their 'usual' host *E. coli* (50 % G + C; density 1·710 g./cm.$^{-3}$) to hosts like *P. mirabilis* (39 % G + C; density 1·698 g./cm.$^{-3}$) has provided a method of biological fractionation. The physical separation of the DNA of the extrachromosomal element from that of the host cell in caesium chloride density gradients has revealed that F-merogenotes as well as F_0-*lac*$^+$ and P-*lac*$^+$ consist of double-stranded DNA with an average base composition of 50 % G + C. In contrast, most multiple-drug-resistance elements (R-factors) of the *fi*$^+$ type have been found to consist of two DNA sub-fractions with over-all base compositions of 50 % and 56 % G + C. In Proteus, F-merogenotes, F_0-*lac*$^+$, and probably P-*lac*$^+$, are present as only a single copy per chromosome. R-factors, however, replicate from 3 to 7 times during each generation of bacterial growth. The higher replication rate was achieved just as the Proteus cells entered the stationary phase of growth and was accompanied by a 'burst' in the synthesis of the R-specific enzymes, chloramphenicol acetyl-transferase and β-lactamase. In some cases the R-factor multiplication was also correlated with lysis of about 25 % of the Proteus cells. The R-factor DNA synthesized during growth of the Proteus cell accounts for 20–30 % of the total DNA. D. K. Haapala of our laboratory has examined the renaturation kinetics of DNA extracted from Proteus with multiple R-factor copies. The entire R-factor specific DNA complex reassociates about 50 times more rapidly than chromosomal DNA because of its smaller size and the presence of the multiple copies. The renaturation kinetics permit us to estimate that the information length of the combined 50 % and 56 % G + C components averages $20 \pm 6 \times 10^6$ daltons and that from 40–60 copies of the R-factor complex is present in a stationary phase Proteus cell. The 50 % G + C component of the R-factor was often found as a closed (circular) form; the 56 % G + C component was also found as a circular molecule as was the F-merogenote, F-*lac*$^+$. Initially we suspected that the 50 % and 56 % G + C components of R-factors were the result of the breakage of a single large molecule. The different molecular states of the two components as well as their independent behaviour depending upon drug selection suggest, however, that we are dealing with more than one distinct replicon.

It is also possible to isolate and characterize extrachromosomal elements directly from *E. coli*. R. P. Silver of our laboratory has employed a system which permits the detection of an extrachromosomal element after transfer to a recipient. In this system, F-*lac*$^+$ was identified as a single component in sucrose gradients sedimenting about 1·7 times faster than linear monomers of λ phage DNA. The derepressed R-factor, R_1, presented a more complicated situation. Twenty to forty minutes after transfer, R-factor species were identified which sedimented at the same distance, less than half the distance and roughly twice the distance as λ DNA. Sixty minutes after transfer, the vast majority of R-factor DNA sediments as two peaks approximately 3 and 4 times faster than λ. Both the 50 % and 56 % G + C components were present in both rapidly sedimenting peaks. Preliminary indications are that these rapidly sedimenting peaks are not composed, in large part, of circular molecules. Other preliminary experiments suggest that the two molecular species of R-factors are capable of alternating between a state of independence and a state in which the two molecular species are united. These data may be useful for explaining some of the conflicting genetic observations observed with R-factors and serve as well as a basis for a model explaining the accretion of additional drug resistance genes by an R-factor.

Staphylococcal Plasmids. By R. P. NOVICK (*The Public Health Research Institute of the City of New York, New York* 10016, *U.S.A.*)

Most penicillin-resistant strains of *S. aureus* harbour extrachromosomal elements responsible for the synthesis of penicillinase (Novick, R. P. (1963), *J. gen. Microbiol.* **33**, 121). These extrachromosomal elements, the penicillinase plasmids, are in general similar to nontransmissible R-factors of enteric bacteria and are autonomous replicons as shown by the isolation of thermosensitive mutations affecting specifically plasmid replication at high temperature (Novick, R. P. (1967), *Proc. 5th Int. Cong. Chemother. Vienna*, pp. 269–73). In addition to structural and control loci for penicillinase synthesis, the plasmids carry some or all of a group of genes responsible for resistance to a series of inorganic ions, including arsenate, arsenite, bismuth, lead and cadmium (Novick, R. P. (1967), *Fedn Proc.* **26**, 29; Novick, R. P. & Roth, C. (1968), *J. Bact.* **95**, 1335), mercury (Richmond, M. H. & John, M. (1964), *Nature, Lond.* **202**, 1360), and sometimes to erythromycin (Hashimoto. H., Kono, K. & Mitsuhashi, S. (1964), *J. Bact.* **88**, 261). There is also a localized region, the *mc* region, in which reside determinants of functions required for autononous replication and a determinant of plasmid compatibility (Novick, R. P. (1967). *Fedn Proc.* **26**, 29).

As with the transmissible R-factors (Watanabe, T., Nishida, H., Ogata, C., Arai, T. & Sato, S. (1964), *J. Bact.* **88**, 716), there are two incompatibility sets of staphylococcal penicillinase plasmids, mc_I and mc_{II} (Novick, R. P. & Richmond, M. H. (1965), *J. Bact.* **90**, 467); incompatibility between two plasmids is evidently the result of competition for a single host maintenance site—compatible plasmids are apparently attuned to different host maintenance sites (Novick, R. P. (1967), *Fedn Proc.* **26**, 29). In a recent study of 25 naturally occurring plasmids which were transduced from wild strains into a standard laboratory strain by selection for cadmium resistance (Peyru, G., Fooner, L. & Novick, R. P., manuscript in preparation), a variety of different patterns of the above-mentioned markers was found; each of the plasmids could be assigned unequivocally to one of the incompatibility sets; all of the elements of set mc_{II} carried a gene that increased the *sensitivity* of the host to bismuth ion; the corresponding allele (?) of the mc_I plasmids either increased bismuth *resistance* or had no effect. Among the elements of set mc_{II}, three lacked the penicillinase gene, although the wild-type strains from which these three plasmids were obtained were in each case pencillinase-producing. Whether the penicillinase gene in these three strains is chromosomal or is borne by a separate cadmium-sensitive plasmid remains to be seen. Occasional strains carry penicillinase determinants that are apparently chromosomal (Poston, D. (1966), *Nature, Lond.* **210**, 802; Asheshov, E. (1966), *Nature, Lond.* **210**, 804), but all strains thus far examined that have an extrachromosomal penicillinase determinant carry cadmium resistance linked to it.

The penicillinase plasmids do not appear to be sex factors, nor are they appreciably sensitive to curing by acridine dyes. They are transmitted by transduction via the commonly occurring transducing phages that are resident as prophages in many staphylococcal strains; transmission betwen strains by transduction *in vivo* has been demonstrated (Novick, R. P. & Morse, S. I. (1967), *J. exp. Med.* **125**, 45) and may be in some measure responsible for the spread of plasmids in the natural environment. Although genetics of resistance to other drugs has been studied less thoroughly, there is good evidence that tetracycline resistance is, in some strains at least, extrachromosomal (May, J. W., Houghton, R. H. & Perret, C. J. (1964), *J. gen. Microbiol.* **37**, 157) and there is very preliminary evidence that resistance to kanamycin and to chloramphenicol may also be extrachromosomal. The latter three are evidently unlinked to one another and to the penicillinase determinant.

It thus seems that extrachromosomal drug resistance may be as prevalent in staphylococcus as it is in the enteric bacteria; since inter-strain transfer by spontaneous transduction is rather less frequent than is transfer by conjugation, it is not certain that the primary evolutionary significance of extrachromosomal resistance factors in staphylococcus is their transferability.

Genetic Analysis of ColB Factors and the Identification of Composite Circular Molecules of R-Factors. (R. C. Clowes, Celma Hausmann, T. Nisioka & Michiko Mitani, *Department of Biology, Southwest Center for Advanced Studies, Dallas, Texas*)

Certain genetic elements elaborating a B-type colicin have previously been reported to show sex-factor activity. We have investigated four of these factors, carried by the Frédericq wild-type *E. coli* strains CA 38, K 77, K 166 and K 98, referred to as ColB1, ColB2. ColB3 and ColB4 respectively. ColB3 is present in strain K 166 together with a fertility factor (termed Fβ), from which it can be separated by either acridine-orange treatment or P 1 transduction. Fβ produces no colicin and is a sex factor with derepressed-fertility, co-existing with F. The separated ColB3 factor behaves as does ColB1, ColB2 and ColB4 as a sex factor of the repressed-fertility type, i.e. normal transfer of the factors is low (LFC), but transfer from newly infected cells is greatly increased (HFC); F-piliation as judged by male specific phage (μ 2)—adsorption and multiplication is not seen under LFC conditions but can be demonstrated under HFC conditions, with the exception of HFC cultures of ColB1. All four factors co-exist stably with the F sex factor, either in its autonomous state (as F′-lac) or in the integrated (Hfr) state. Under these conditions, normal F activity (μ 2 adsorption, chromosome and F′-lac transfer) is seen in the presence of ColB3, but in the presence of ColB1 these activities are severely repressed. In the case of ColB2 and ColB4, F-fertility repression is also seen, but to a less marked degree.

All four ColB factors co-exist with R-factors of the fi^+ or fi^- type, except that ColB2 leads to the elimination of Rfi^-. Fertility-derepressed (FDR) mutants have been isolated from ColB1 and ColB2. ColB1 (FDR) is transferred efficiently, does not repress F fertility and, in contrast to phenotypically fertility-derepressed cultures (HFC) of ColB1, gives rise to male-specific phage sensitivity and to efficient F-piliation. Its fertility is repressed when present together with an Rfi^+ factor in the same cell. Several ColB2 (FDR) mutants have been isolated, none of which are repressed by Rfi^+. Moreover, when Rfi^+ is present in the intermediate strain of an HFC culture, there is no limitation in development of the HFC state of ColB1. The results will be discussed in terms of the interaction of genes controlling piliation and its regulation, and others controlling replication of extrachromosomal elements.

By density-gradient analysis of DNA isolated from *Proteus mirabilis* strains (density 1·698 g./cm.$^{-3}$), infected with one of a series of R-factors, the molecular nature of several R-factors has been identified as DNA; in some instances with a density of 1·708 g./cm.$^{-3}$ and in others including DNA with a density of 1·716 g./cm.$^{-3}$ (Falkow *et al.* (1966), *J. molec. Biol.* **17**, 102; Rownd *et al.* (1966), *J. molec. Biol.* **17**, 3376). The work discussed involves two R-factors, one (*R15*) carrying resistance to streptomycin and sulphonamide (SM^r SU^r) and another (*222/R3*) to streptomycin, sulphonamide and chloramphenicol (SM^{r}SU^{r}CM^r). These factors have been transferred to a *P. mirabilis* strain. The DNA was extracted and subjected to density-gradient centrifugation followed by electron microscopy of isolated fractions.

Analytical centrifugation of *R15*-DNA showed a single satellite band at a density of 1·709 g./cm.$^{-3}$ (with reference to *Bacillus subtilis* phage SP01-DNA taken as 1·742 g./cm.$^{-3}$). Electron microscopy of samples from the corresponding preparative CsCl gradient peak showed circular molecules with a contour length of 18 μ (35–10^6 daltons). In contrast, *222/R3*-DNA formed a satellite band with three peaks at densities 1·708, 1·711 and 1·717 g./cm.$^{-3}$. Electron micrographs showed circular molecular structures in each band with respective contour lengths of 29 μ (54 × 10^6 daltons), 36 μ (68 × 10^6 daltons), and 6 μ (12 × 10^6 daltons). Supertwisted forms of several molecular species were found. It is suggested that *222–R3* DNA may be comprised of either a single 36 μ molecule or of two individual molecules of 29 μ and 6 μ in length, and that this may reflect the evolutionary development of R-factors.

Resistance Transfer (R) Factors. Relationship to F (fertility factor). By Elinor Meynell (*Department of Microbiology, Lister Institute of Preventive Medicine, London, S.W.1*)

Fortunately for the understanding of transmissible drug resistance, the discovery of F preceded its appearance by many years. F was discovered as the result of experiments demonstrating that genetic recombination could occur between one bacterium and another (Leder-

berg, J. & Tatum, E. L. (1946), *Cold Spring Harb. Symp. quant. Bio!.* **11**, 113), which was soon shown to come about through the agency of a novel kind of genetic element (Hayes, W. (1953), *J. gen. Microbiol.* **8**, 72), originally described as having some properties of a virus, since one cell could be infected from another, and some of a supernumerary bacterial chromosome, since it converted the bacterium to a genetic donor. The work which followed (Jacob, F. & Wollman, E. L. (1961), *Sexuality and the Genetics of Bacteria*) provided an understanding of the nature and behaviour of F before the phenonemon of transmissible antibiotic resistance, by its practical importance, came to the attention of medical and veterinary bacteriologists (see Watanabe, T. (1963), *Bact. Rev.* **27**, 87; (1967) *Fedn Proc.* **26**, 23). R factors could therefore be examined, knowing how F is perpetuated and transferred with other genetic material in the donor cell. In addition, certain colicin determinants, notably *colI*, were also found to be carried on extrachromosomal elements, or 'plasmids', which shared with F the ability to bring about conjugation (Fredericq, P. (1954), *C. r. Séanc. Soc. Biol., Paris* **148**, 399; Ozeki, H., Stocker, B. A. D. & Smith, S. (1962), *J. gen. Microbiol.* **28**, 671).

In an F^+ bacterium, the ability to conjugate is correlated with the presence of specialized hair-like appendages, or 'sex pili', recognizable by their morphology by electron microscopy (Brinton, C. C., Gemski, P. & Carnahan, J. (1964), *Proc. natn. Acad. Sci. U.S.A.* **52**, 776), by their serological properties (Ørskov, I. & Ørskov, F. (1960), *Acta path. microbiol. scand.* **48**, 37) and by their acting as receptors for F-specific phages. Conjugation leads to transfer of F itself and, in an occasional bacterium, to transfer of segments of chromosome and the production of a genetic recombinant. Many, or all, of these are a consequence of integration of F with the bacterial chromosome to form a continuous structure which is then transferred, just as F alone is transferred in the autonomous state. Once integrated, F can also be released, but if the cross-over leading to release does not occur exactly at the junction of F and chromosome, the result is a composite F factor, or F′, that has incorporated one or more bacterial genes, which thereafter constitute part of the plasmid. In effect, R factors generally resemble F′ factors in being composed of genes for conjugation (the 'sex factor') linked to genes modifying the behaviour of the bacterium; although resistance genes are occasionally found which depend on unlinked sex factor genes for their transfer in the way that ColE2 depends for its transmissibility on ColI. R factors are about as efficient as F in promoting chromosome transfer, but it has yet to be shown that the resistance genes were acquired from a bacterium in the same way as the chromosomal genes of an F′.

Although an R factor endows the bacterium with ability to conjugate, no pili such as F determines can normally be seen. Moreover, about 50 % of naturally occurring R factors actually inhibit the production of F pili by an F^+ bacterium (the fi^+ character: Watanabe, T. (1963), *Bact. Rev.* **27**, 87). The absence of pili is due, not to absence of pilus genes, but to a repressor which regulates the activity of these genes. When mutant factors without repressor are obtained, pili are produced by the majority of bacteria, and the frequency of conjugation and resistance transfer are correspondingly increased. The fi^+ class of R factors, which suppress the activity of F, determine a pilus closely resembling the F pilus in morphology, antigenic structure and ability to absorb F phage; and it is presumably the activity of their own repressor acting upon homologous pilus genes in F which is responsible for the fi^+ effect. Those factors which are naturally fi^- determine a different pilus and a repressor inactive against F: they prove to be related to ColI (Meynell, E., Meynell, G. G. & Datta, N. (1968), *Bact. Rev.* **32**, 55).

A transmissible plasmid shows its identity in two ways: by the nature of the pilus and, like a temperate phage, by specificity of superinfection immunity. Fi^+ R factors are related to F by the first criterion, but are not identical to the classical F factor since both can co-exist in the same bacterium, and thus fail to show cross-immunity towards each other.

Some aspects of Replication of R factors. By TSUTOMU WATANABE (*Department of Microbiology, Keio University School of Medicine, Tokyo, Japan*)

R factors are extrachromosomal, infectious agents conferring resistance to several drugs upon host bacteria (Watanabe, T. (1963). *Bact. Rev.* **27**, 87) and can be classified into two groups, fi^+ and fi^-, depending on the presence and absence of 'fertility inhibition' (Watanabe,

T. *et al.* (1964), *J. Bact.* **88**, 716). R factors are thought to ordinarily replicate in cytoplasm in synchrony with host chromosomes. There must be a stage, however, in which R factors replicate in new hosts with a pace faster than the replication of host chromosomes, because in a conjugal event usually only one copy of R factor is transferred to a recipient cell. Otherwise the newly transferred R factor should be unable to attain the number equal to host chromosomes in multinucleate bacteria. The recipient cells which have newly received R factors show not only high donor competency but also increased levels of drug resistance (Watanabe, T. (1963), *Genetics Today* **1**, 42). This phenomenon may have some connexion to the mode of replication of the newly transferred R factors. On the other hand, spontaneous segregation of R factors may occur although at low frequencies. The spontaneous segregation indicates that the replication of R factors may be reduced to a level slower than that of host chromosomes for some unknown reasons. The 'elimination' of R factors with acridine dyes has been ascribed to the suppression of replication of R factors at concentrations of acridines which still allow the replication of host chromosomes (Watanabe, T. & Fukasawa, T. (1961), *J. Bact.* **81**, 679). Yoshikawa (personal communication) has recently found, however, that R factors give to host bacteria increased sensitivity to acridines and that the spontaneous segregants which lost R factors become accumulated in the media containing acridine dyes. The genetic stability of R factors may differ from host to host and from R factor to R factor. We have previously reported that fi^+ R factors segregated their drug resistance markers at high frequencies in *Salmonella typhimurium*, whereas they were stable in *Escherichia coli*. There was a tendency that sulphanilamide, streptomycin, chloramphenicol and kanamycin-neomycin markers are frequently lost as a unit in *S. typhimurium*, while the tetracycline marker was quite stable in the same host. The drug resistance markers remaining retained their conjugal transferability. The genetic stability of a number of R factors, fi^+ and fi^-, have recently been investigated in *E. coli* and in *S. typhimurium*. All of the R factors, fi^+ and fi^-, were quite stable in *E. coli*. In contrast, fi^+ R factors were unstable and fi^- factors quite stable in *S. typhimurium*. A drug-sensitive segregant obtained from *S. typhimurium* carrying an fi^+ R factor still retained 'resistance transfer factor' (RTF), an episomal portion of R factor (Watanabe, T. (1963), *Bact. Rev.* **27**, 87). Two different R factors, fi^+ and fi^-, can coexist in a cell without causing mutual exclusion and recombination, which easily occur between two homologous R factors. Thus we can study the genetic stability of two R factors in a single host strain. It was found that the genetic stability of each R factor is not affected by the coexisting heterologous R factor. The mechanism of spontaneous segregation of the drug resistance markers of fi^+ R factors seems most likely to be due to deletion assuming from the similarity in the patterns of their segregation which occurs spontaneously and by transduction. The reasons for their genetic instability and the genetic stability of the drug resistance markers of fi^- R factors in *S. typhimurium* are still unknown.

Practical Significance of Extrachromosomal Elements. By E. S. ANDERSON (*Enteric Reference Laboratory, Public Health Laboratory Service, Colindale Avenue, London, N.W. 9*)

The field in which the practical importance of extrachromosomal genetic elements (plasmids) has been most clearly established is that of drug resistance. Penicillin resistance in staphylococci may be plasmid in origin (Novick, R. P. (1963), *J. gen. Microbiol.* **33**, 121) and the importance of penicillin-resistant staphylococci is well recognized, especially in hospitals. Plasmids may also govern staphylococcal resistance to tetracyclines, chloramphenicol and erythromycin.

If an antibiotic is used on a large-scale, drug-resistant organisms will almost certainly appear. And if conditions favour the dissemination of these organisms, in hospital wards or animal herds for example, they will spread to most or all of the population at risk. If such an organism happens to be a highly communicable pathogen, the disease concerned will spread rapidly through the population and, because of the presence of the drug resistance, the respective antibiotics will be ineffective in treatment. When resistance is transferable, as in the enterobacteria, pathogens can acquire resistance by contact in the intestine of the host with a non-pathogen carrying an extrachromosomal transferable resistance factor (R factor). Thus, non-pathogenic enterobacteria may act as a reservoir of R factors which can be trans-

ferred to pathogens. Some of these R factors transfer a range of resistances as a single linkage group, so that the recipient pathogen may acquire resistance to several drugs simultaneously. Alternatively, a drug-sensitive pathogen may become progressively resistant to a series of drugs by consecutive transfer of resistance determinants. This happened with phage type 29 of *Salmonella typhimurium*, which caused a protracted outbreak of infectious diarrhoea in intensively reared calves between 1963 and 1966. All cultures of type 29 received before the second quarter of 1963 were drug-sensitive. In that quarter, streptomycin and sulphonamide resistance appeared in the type, and it was followed in turn by resistances to tetracyclines, ampicillin, furazolidone, neomycin and chloramphenicol. By early 1965, most type 29 cultures were resistant to four or more drugs. The resistances appeared shortly after the respective drugs were used in efforts to control the widespread *S. typhimurium* infection in calves (Anderson, E. S. (1967), *Ann. Inst. Pasteur, Paris* **112**, 547, (1968), *Brit. Med. J.* **2**, 333).

Most drug-sensitive strains of type 29 isolated before the onset of these outbreaks carried a transfer factor. Transfer factors can migrate from drug-sensitive strains into strains carrying mobilizable resistance determinants with which they recombine to form R factors. These R factors can then be transferred to the strain which provided the transfer factor, so that the originally sensitive donor strain becomes drug-resistant. (Anderson, E. S. (1965), *Brit. med. J.* **2**, 1289). It is assumed that the progressive expansion of the drug resistance of type 29 was produced in this way, and that the determinants concerned came from non-pathogenic enterobacteria in the intestine of the host animals. The selective pressure exerted by the use of the relevant antibiotics made the transfer of resistance more certain by ensuring that resistant organisms predominated in the intestine. Naturally, the appearance and expansion of resistance abolished the efficacy of the respective drugs against the infection. Drug-resistant phage type 29 was transmitted to man, and the presence of the resistance invalidated drugs that might have been used to treat human infections.

Other transferable determinants of potential practical significance are those for haemolysin synthesis and enterotoxin synthesis, which have recently been reported in *Escherichia coli* (Smith, H. W. & Halls, S. (1967), *J. gen. Microbiol.* **47**, 154; (1968), **52**, 319). These characters may be associated with virulence of the carrier organisms. If determinants associated with virulence were closely linked to transfer factors that also carried drug resistance determinants, the dissemination of the resistance by the selective pressure of antibiotics might also promote the spread of the virulence determinants.

Comparison of Bacterial and Other Extrachromosomal Genetic Elements. By G. H. BEALE
(*Department of Animal Genetics, Edinburgh University*)

Although there are many examples of non-Mendelian heredity in eukaryotic organisms ranging from protozoa and algae to insects and flowering plants, the exact location and nature of the extrachromosomal genetic elements concerned are usually unknown. Three types of cell organelle, namely mitochondria, plastids and basal granules (or centrioles), are considered to contain genetic elements, and the evidence for this will be briefly reviewed. All three organelles are thought to contain DNA, and in regard to mitochondria and plastids a more-or-less complete protein-synthesizing system. Another type of structure falling into the category of extrachromosomal elements is the kappa particle of *Paramecium*, and although this particle is now considered to be an endosymbiont rather than a cell organelle having genetic continuity, there are a number of reasons for believing that study of the kappa particles is still relevant to the subject of extranuclear heredity. Many different types of kappa-like particles are now known in ciliates, mainly in the cytoplasm, but some in the macronucleus. Some of the particles contain virus-like structures.

A characteristic of all these organelles and symbionts is their dependence to a greater or lesser degree on genes in the nucleus of the cell. Many nuclear genes are known to be concerned with the synthetic processes occurring in plastids. With regard to mitochondria it is possible that only the inner membranes are under control of the autonomous mitochondrial genetic system, the external membranes and soluble proteins being under gene control. However, this is only a tenatative view. In the case of the *Paramecium* symbionts, many are known to require the presence of specific *Paramecium* genes for maintenance of the particles.

When one compares these various extrachromosomal elements of eukaryotes with bacterial episomes and plasmids, a number of differences are immediately apparent. In regard to size, such structures as mitochondria, plastids and kappa particles are more comparable to whole bacteria than to plasmids. Secondly, the eukaryotic elements usually, if not always, comprise not merely genetic material (presumably DNA), but elaborate structures such as the mitochondrial membranes, the plastid lamellae, fibrillar systems of basal bodies, etc. This leads to the view, which has recently become again fashionable, that these organelles are derived by an ancient symbiosis of prokaryotic organisms (bacteria, blue-green algae etc.) with some primeval eukaryotic ancestors.

It is also worth pointing out that there is no definite proof of an integration of cytoplasmic elements of eukaryotes in the chromosomes like that occurring with bacterial episomes, though there has been discussion of this in regard to the CO_2-resistance factor (sigma) of *Drosophila*.

Probably the closest analogies between extra-chromosomal elements of higher organisms and bacterial plasmids, are to be found amongst those particles in higher organisms resembling viruses.

ORIGINAL PAPERS

SESSION A

Chloramphenicol Acetylation by Resistant *Staphylococcus aureus*. By W. V. SHAW (*Department of Medicine, Columbia University, New York, U.S.A.*)

An extra-chromosomal ('plasmid') locus has been proposed for the genetic determinant of chloramphenicol resistance in *Staphylococcus aureus* (Chabbert, Y. A., Baudeus, J. G. & Gerbaud, G. R. (1964). *Ann. Inst. Pasteur, Paris* (Suppl.), **107**, 678; Mitsuhashi, S. (1967), *Jap. J. Microbiol.* **11**, 49). The biochemical mechanism underlying chloramphenicol resistance has been shown to involve O-acetylation of the antibiotic by the enzyme chloramphenicol acetyltransferase in the presence of acetyl-Coenzyne A (Shaw, W. V. & Brodsky, R. F. (1968), *J. Bact.* **95**, 28). Synthesis of the enzyme was shown to be induced by chloramphenicol and certain of its analogues possessing identical stereochemistry and similar structure. Further analysis of the system was complicated by the initial inhibitory effect of chloramphenicol on protein synthesis and by the rapid conversion of the compound to the acetyl derivative which is not an inducer.

A detailed analysis of the induction of chloramphenicol acetyltransferase became feasible following the observation that the 3-deoxy analogue of D-threochloramphenicol is (a) ineffective as an antiobitic, (b) a potent inducer of the acetylating enzyme, and (c) not a substrate by virtue of the absence of a C_3 hydroxyl substituent. The availability of such a 'gratuitous inducer' has permitted an analysis of the kinetics and optimum conditions for the induced synthesis of the acetylating enzyme. When grown in Penassay broth (Difco) at 37° on a roatary shaker the presence of enzyme can be detected approximately 8 min. after the addition of the analogue to a culture in the early logarithmic phase of growth. After induction the absolute quantity of enzyme activity is proportional to the net increase in total protein of the culture throughout the logarithmic phase of growth. Enzyme induction was maximal at pH 7·0 (potassium phosphate buffer). The concentration of the 3-deoxy analogue required to achieve 50 % of the maximal rate of differential synthesis of the acetylating enzyme was 8×10^{-7} M. No evidence implicating the presence of a specific chloramphenicol 'permease' has been obtained.

Effects of Narcotics on *Staphylococcus aureus*. By E. F. GALE (*Sub-Department of Chemical Microbiology, Department of Biochemistry, University of Cambridge*)

Simon, E. J. & Van Praag, D. (1964, *Proc. natn. Acad. Sci., U.S.A.* **51**, 877, 1151) reported that levorphanol inhibits the synthesis of protein and nucleic acid in *Escherichia coli*. Ribosomal RNA appeared to be the most sensitive of the macromolecular synthesis studied but the

drug had no significant effect on RNA synthesis *in vitro*. Greene, R. & Magasanik, B. (1967, *J. molec. Pharm.* **3**, 463) confirmed that levorphanol and levallorphan inhibited protein and nucleic acid synthesis but showed that the inhibition arose from an effect on an earlier common process which they suggested was ATP breakdown although no proof of this was obtained.

Heroin, levorphanol and levallorphan inhibit protein and nucleic acid synthesis in intact Staphylococcal cells but are without significant effect on protein synthesis in *in vitro* systems obtained from the cells. Heroin increases the rate of transport into the cells of some amino acids, such as aspartate and alanine, and inhibits that of others, such as lysine and proline. The inhibition of protein synthesis in intact cells can be correlated with the inhibition of lysine transport. These results suggest an alteration in the properties of the membrane and the three narcotics have been found to produce changes in the phospholipid fraction of *Staphylococcus aureus* cells. Heroin markedly increases the rate of incorporation of glycerol into the lipid fraction and also the rate of turnover of phosphatidylglycerol. Chromatographic examination of the phospholipid (Gale, E. F. & Folkes, J. P. (1967), *Biochim. biophys. Acta* **44**, 452) shows the presence of a new component. If cells treated with heroin are washed and re-incubated in the absence of heroin, the new component disappears and is replaced by phosphatidylglycerol. Qualitatively similar results have been obtained with levorphanol and levallorphan but the new components obtained differ in chromatographic properties from that obtained in the presence of heroin. No cytosine can be detected in these new components; the ratio of phosphate to glycerol incorporated into these substances is the same as for the phosphatidylglycerol fraction.

The Incidence of Bacteriocinogeny in Some Bacillus species. By D. J. STICKLER. (*Department of Applied Biology, University of Wales Institute of Science and Technology, Cathays Park, Cardiff*)

Thirty strains of bacteria from three *Bacillus* species have been examined for bacteriocinogeny using the conventional screening techniques (Holland, I. B. (1967), in *Antibiotics*, vol. 1, p. 684, ed. by D. Gottlieb and P. D. Shaw, Springer-Verlag, Berlin). Five of six strains of *B. sphaericus*, twelve of seventeen strains of *B. cereus* and five of seven strains of *B. pumilis* were revealed as bacteriocinogenic (B⁺). Seven strains of *B. subtilis* whose intra-species killing abilities have been shown to be a function of their production of defective bacteriophage (Stickler, D. J., Tucker, R. G. & Kay, D. (1965), *Virology* **26**, 142) are also detected as B⁺ using these techniques, and Bradley (1967, *Bact. Rev.* **31**, 230) has classified these phages, together with other virus-like particles, as bacteriocins. In view of this the thirty strains of bacilli were examined for their ability to produce phage. Early log. phase broth cultures of the organisms were treated with the inducing agent hydrogen peroxide (0·44 mM) and incubation at 37° with aeration was continued. The cultures generally lysed 1–2 hr after induction and the cellular material was removed by centrifugation at 3000 *g* for 20 min. Any pellet then produced by centrifugation of the supernatant fluid at 30,000 *g* for 1 hr. was resuspended in phosphate buffer (Hayes, W. J. (1957), *J. gen. Microbiol.* **16**, 96) and examined with the electron microscope after negative staining with uranyl acetate. All six strains of *B. sphaericus*, three of the (B⁺) strains of *B. cereus* and three of the (B⁺) strains of *B. pumilis* produced phage particles. When the lysates containing phage particles were plated against sensitive bacteria they produced inhibition of growth but not plaque formation. It has yet to be established whether these apparently defective phage are responsible for the bacteriocin-like activity.

Phenotypic Masking and Drug Dependence in Streptomycin-dependent and Paromomycin-dependent strains of Escherichia coli. By L. B. QUESNEL and P. YORK (*Department of Bacteriology and Virology, University of Manchester, England*).

Recently, Gorini, L., Rosset, R. & Zimmermann, R. A. (1967, *Science, N.Y.* **157**, 1314) described a new phenomenon which they called 'phenotypic masking', showing that their streptomycin-dependent mutants of *E. coli* were actually 'drug-dependent' because their requirement could be satisfied by several drugs. We investigated a streptomycin-dependent

strain SD (originally a hospital isolate) and a paromomycin-dependent strain PD (isolated from a sensitive strain BQ by selection on nutrient agar containing 200 μg. paromomycin/ml. The MIC of streptomycin (Sm) for strain BQ was 2·0 μg./ml., and of paromomycin (Pm), 10 μg./ml. of nutrient broth. Strain SD grew optimally at a streptomycin concentration ([Sm]) of 100–400 μg./ml. nutrient broth or agar; the optimum for strain PD was 50–100 μg. Pm/ml. medium.

Strains SD and PD each grew normally in the absence of Sm or Pm if the medium contained 3 % alcohol (EtOH). The alcohol-grown strains (Drug^{D}SD and Drug^{D}PD) could subsequently be grown on either Sm or Pm media, but not on a mixture of the two, which was lethal. Either Drug^D strain would also grow in mixtures of EtOH + Sm, EtOH + Pm, or kanamycin (Km) + Pm; but not in mixtures of EtOH + Km, Sm + Km, or Km alone. Strain PD, after growth on Pm, would grow on EtOH + Pm, EtOH + Km, or Pm + Km; but in no media containing Sm. Similarly strain SD, after growth on Sm, would grow on EtOH + Sm, but not on any mixture containing Pm or Km separately or together.

The lethality of Sm + Pm for Drug^{D}PD strains was investigated by holding [Sm] constant at 500 μg./ml. and varying [Pm] from 0 to 20 μg./ml.: the mixture was lethal at all [Pm] of 10 μg./ml. or above, i.e. $\geqslant$ MIC for the parent strain BQ. Similarly, holding [Pm] constant at 200 μg./ml. and varying [Sm], the mixture was lethal at [Sm] of 2·0 μg./ml. or greater, again the MIC of strain BQ.

The strain BQ grew equally well in drug-free synthetic media at [Mg^{2+}] from 0·01 M to 0·1 M; it did not grow in the presence of 10 μg. Sm/ml. at [Mg^{2+}] of 0·02 M or less, but there was a direct relationship between growth and [Mg^{2+}] from 0·02 M to 0·09 M. Strain SD in presence of 25 μg. Sm/ml. gave a similar direct relationship from 0·01 M to 0·04 M Mg^{2+}, but was unaffected by higher concentrations of Mg^{2+}.

Variable Gene Linkage and the Phased Appearance of Transformants in *Bacillus licheniformis*. By D. J. SHERRATT and J. F. COLLINS (*Department of Molecular Biology, University of Edinburgh, Edinburgh*)

The genetic transformation system in *Bacillus licheniformis* strain ATCC 9945A (Thorne, C. B. & Stull, H. B. (1966), *J. Bact.* **91**, 1012) has been used to transform the structural and regulatory genes for penicillinase, as well as nutritional and antibiotic resistance markers. Competent cultures were obtained by diluting a stationary-state overnight broth culture into a minimal medium. Experiments designed to show the development of competence in the minimal medium revealed that maximal competence for different markers in a multiple auxotroph occurred at different times.

For example, using a strain requiring both adenine (ade$^-$) and isoleucine and valine (ilv$^-$), the ability to produce ade$^+$ transformants was greatest immediately after the dilution, but decreased by 90 % during the next hour. The ability to produce ilv$^+$ transformants reached its maximum only after 3½ hr. incubation in the minimal medium. Both the structural and regulatory genes for penicillinase could be co-transformed with this ilv$^+$ gene, but it was observed that the degree of linkage shown by the penicillinase genes to the ilv$^+$ gene varied from > 50 % at the time of maximum production of ilv$^+$ transformants, to < 10 % at other times.

The experimental evidence points to a phased behaviour of the culture leading to the sequential appearance of transformants, the transforming DNA repairing a unique position on the replicating genome at any given time. Further, DNA added to the culture at the time of maximal competence is integrated in larger pieces than DNA added at a time of poor competence; the latter DNA appears to be degraded into small pieces and largely rendered ineffective for the production of transformants during the time that elapses before the cells can repair the marker under study.

The Influence of Environment on the Synthesis of Magnesium-binding Sites in *Bacillus subtilis* var. *niger* Cell Walls. By J. L. MEERS and D. W. TEMPEST (*Microbiological Research Establishment, Porton Down, nr. Salisbury, Wilts.*)

Compared with *Aerobacter aerogenes*, *Bacillus subtilis* var. *niger* organisms had a greater capacity but lower affinity for magnesium adsorption. However, the nature of the growth

environment markedly affected the cation adsorption properties of *B. subtilis* cell walls. In particular, when a constraint to the adsorption of magnesium was applied (that is, either by growing the organisms in a magnesium-limited environment, or in one that contained a high concentration of a competing ion), cell walls were synthesized that had an improved affinity for magnesium adsorption. An explanation of this finding was sought by studying the physico-chemical nature of the magnesium-binding sites.

Potentiometric titrations were carried out on cell-wall preparations from various *B. subtilis* chemostat cultures. Two prominent pK values (at about pH 2·2 and pH 4) were observed which probably were due to phosphate and carbyoxyl groups, respectively. The buffering capacity between pH 2·0 and pH 2·5 of isolated cell walls varied considerably with the bacterial growth conditions, and in a manner that closely paralleled changes in their magnesium-binding affinity. Thus, the walls of phosphate-limited organisms showed almost no buffering in this region and bound magnesium poorly. But the walls of similarly limited *B. subtilis* cells grown in the presence of 4 % NaCl bound magnesium more avidly and had substantial buffering capacity in the pH 2·0–2·5 region. Treatment of the latter walls with alkaline solutions decreased their buffering capacity and magnesium-binding capacity proportionately. It is suggested that magnesium is bound strongly to *B. subtilis* cell walls by phosphate groups which increase in number when the organisms are grown under conditions where magnesium adsorption is impeded.

Loss of Sensitivity to EDTA by *Pseudomonas aeruginosa* Grown Under Conditions of Mg Limitation. By M. R. W. BROWN and J. MELLING (*Pharmaceutical Microbiology Group, School of Pharmacy, Bath University of Technology, Bath, Somerset*)

The effect of magnesium on sensitivity of *Pseudomonas aeruginosa* to EDTA was tested using cells grown in batch culture in simple salts, glucose medium containing varying concentrations of Mg. The growth rate was initially the same in all cultures but became progressively reduced due to Mg limitation. Eventually cell division ceased in all cultures owing to depletion of glucose at an E_{470} of about 0·18 (3·5 × 10^8 cells/ml.). Unwashed cultures in this condition were used for all studies with EDTA and were taken for treatment when the E_{470} had remained constant for 1 hr. Sensitivity to EDTA was measured in terms of lysis (E_{470}) and drop in colony count. The activity of several EDTA concentrations was measured against cells grown in several Mg concentrations. As the EDTA concentration was increased, the rate of lysis increased up to a maximum for cells grown in any one Mg concentration. Sensitivity to EDTA decreased with decrease in Mg concentration and there was a linear relationship between the maximum rate of lysis and the concentration of Mg in the growth medium below about 0·3 μg./ml. The EDTA concentration required to produce a maximum effect was greater the higher the Mg concentration in the growth medium, and was in agreement with earlier work (Brown, M. R. W. & Richards, R. M. E. (1965), *Nature, Lond.* 207, 1391), which showed that cations in excess of growth requirements reduced the activity of EDTA against nutrient broth grown cells. Colony counts followed a similar pattern to that for lysis. Loss of viability occurred more rapidly than lysis and suggested that lysis was not the primary lesion.

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

Recombination of R Factors with Col Factors and Chromosomal Genes in *Escherichia coli*. By P. FREDERICQ and E. DELHALLE (*University of Liège, Belgium*)

When a colicinogenic factor is transferred by conjugation to a strain already carrying another episome or plasmid, such as an R factor, there is, as a rule, coexistence of both or, eventually, mutual exclusion. On the other hand, recombination may occur if the Col factor is transferred by transduction. The difficulty of selecting for transfer of the Col marker was circumvented by using a donor carrying a ColB factor, linked to genes of the tryptophane region in an F′ episome, and selecting for transfer of these *trp*$^+$ genes. In many instances, where the recipients were already carrying an episome such as F, Fd, F′-*lac* or another Col factor, a number of Try$^+$ transductants were shown to carry a recombinant episome, in-

heriting markers from both parental episomes. Recombination was also obtained with R factors but only when the recipient also carried a ColV factor in addition to the R factor. Recombination was evidenced by the fact that: (1) all markers from the presumably hybrid episome were transferred *en bloc* by conjugation; (2) markers originating from both parental episomes were cotransducible and could be arranged on a circular linkage map; (3) all markers were generally lost together by spontaneous segregation.

In this way a variety of recombinant episomes was obtained, associating in a single unit genes governing resistance to Su, SM, CM and TC, production of colicines V and B, biosynthesis of cysteine and tryptophane, susceptibility to phage T_1, and fertility, in many different combinations. When such hybrid episomes were further transduced to recipients carrying F'-*lac*, recombination of the donor episome with the *lac* genes of the recipient episome was also observed.

Failure of Colicin Factor I to Integrate in the Bacterial Chromosome. By G. G. MEYNELL and
    SUSAN EDWARDS (*Guinness–Lister Research Unit, Lister Institute, London, S.W.* 1)

Chromosomal recombination mediated by colicin factor I (ColI) may not depend on preliminary integration of the plasmid with the host chromosome since recombination frequencies are unaltered if the donor strain is recombination-deficient (*rec⁻*: Clowes, R. C. and Moody, E. E. M. (1966), *Genetics*, **53**, 717). This interpretation is supported by two new findings reported here.

(1) With the de-repressed I-like sex factor, I-16*drd*, derived from strains carrying ColEia (Edwards, S. & Meynell, G. G. (1968), *Nature, Lond.* (in the Press); Meynell, G. G. & Lawn, A. M. (1967), *Proc. Soc. gen. Microbiol.* (in the Press)), chromosomal recombination occurs in *Salmonella typhimurium* LT 2 at a frequency of 10^{-9}. When ColEi-30 is also carried by the donor, the frequency is 10^{-8}. In fluctuation tests, the numbers of recombinants produced by independent clones of the donor strain do not differ significantly. Thus, recombination does not depend on the random appearance of stable variants during growth of the donor clones; e.g. on integration of the sex factor and chromosome as demonstrated for F (Jacob, F. & Wollman, E. (1961), *Sexuality and the Genetics of Bacteria*, p. 157, New York, Academic Press).

(2) When a wild type ColIa− Ib+ E2+ donor is mated with a ColIa+ Ib− E2− recipient, no recipients ($< 10^{-6}$) express *colib*, the determinants of colicin Ib, although conjugation occurs as shown by transfer of ColE2. In an F-*lac* × F+ cross, *lac* is only stably expressed by the recipient if it is *rec⁺* because expression results from integration of the donated *lac⁺* gene in the recipient chromosome (Dubnau, E. & Maas, W. K. (1968), *J. Bact.* **95**, 531). By analogy, the failure of the determinants of colicin Ib to be expressed in Ib+ × Ia+ crosses suggests that integration cannot occur. The chromosomal transfer accompanying ColI transfer may therefore take place because the donor chromosome has a small probability of entering the I sex pilus and passing to the recipient.

The Transfer of a Factor which Determines Glutamate Secretion from *Citrobacter intermedium* C-3 **to** *Paracolobactrum intermedium* (ATCC 1166). By A. RAMOS, J. GUINEA
    and R. PARÉS-FARRÁS (*Department of Microbiology, Faculty of Sciences, University of Barcelona, Spain*)

According to previous investigations (Pares, R. & Clotet, R. (1963), *VII Jornadas Bioquimicas Latinas*, Genova; (Pares, R., Guinea, J. & Clotet, R. (1965), *III Reunión Bioquimicos Españoles*, Oviedo), *Citrobacter intermedium* C-3 is a bacterial strain which has an extrachromosomal genetic factor (S factor) that in its integrated state produces a failure on the regulation of amino acid synthesis, by showing extracellular glutamate accumulation.

The episomal nature of S factor is principally based on the studies of transfer within the same *C. intermedium* C-3 and on the effects of A.O. and agitation.

The present work shows the experiments that have made it possible to transfer S factor from *C. intermedium* C-3 to *Paracolobactrum intermedium* (ATCC 1166) which had never shown glutamate secretion.

SESSION B

Sensitivity of Bifidobacteria to Oxygen. By W. de VRIES and A. H. STOUTHAMER (*Microbiology Department, Botanical Laboratory, Free University, Amsterdam, The Netherlands*).

Recently, most groups of investigators dealing with the question of anaerobiosis of bifido-bacteria (*Lactobacillus bifidus*) hold the view that they are strictly anaerobic (e.g. Dehnert, J. (1957), *Zentbl. Bakt. Parasitenk* (Abt. I, Orig.), **169**, 66: Sebald, M., Gasser, F. & Werner, H. (1965), *Ann. Inst. Pasteur, Paris* **109**, 251).

In the present work, it is shown that the size of the inhibition zone obtained when bifido-bacteria were grown in deep agar cultures under air varied from 2 to 21 mm for different strains.

Two strains having small inhibition zones in agar (2–5 mm) remained growing in liquid medium when shifted from anaerobic to aerobic shaking conditions. During active growth, no H_2O_2 accumulated in the medium. Extracts of cells grown aerobically displayed the same characteristic enzyme pattern as found previously (de Vries, W. & Stouthamer, A. H. (1967), *J. Bact.* **93**, 574). Growth of one of these strains ceased about 2 hr after the shift. Concomitant production of H_2O_2 was observed. At this stage, fructose-6-phosphate phosphoketolase appeared to be inactivated.

For three other strains the zone of inhibition in agar medium was large (16–22 mm.). These strains did not grow after the shift from anaerobic to aerobic conditions. The high sensitivity of one of these strains to oxygen could be explained by accumulation of H_2O_2 in the culture medium. However, in the culture medium of the other strains no H_2O_2 could be detected.

Except for one strain, cell suspensions of all strains studied formed H_2O_2 when shaken in a 37° water bath in the presence of 0·1 M glucose (0·4–4·1 μmole/ml. within 1 hr). The specific activity of $NADH_2$ oxidase, expressed as μmole of $NADH_2$ oxidized/mg. of protein/hr, varied from 0·4 to 2·8 for different strains. $H_2O_2/NADH_2$ ratios were between 0·12 and 0·35. Catalase and $NADH_2$ peroxidase were absent.

The results show that the degree of anaerobiosis of bifidobacteria differs from strain to strain.

Mode of Action of a Bacteriocin Produced by *Enterobacter cloacae*. By F. K. de GRAAF and A. H. STOUTHAMER (*Microbiology Department, Botanical Laboratory, Free University, Amsterdam, The Netherlands*)

Enterobacter cloacae strain DF 13 has been found to produce a bacteriocin which is a protein with a molecular weight of about 61,000 (Stouthamer, A. H. & Tieze, G. A. (1966), *Antonie van Leeuwenhoek* **32**, 171; de Graaf, F. K. *et al.* (1968), *J. Bact.* **95**, 631). The killing action of the bacteriocin has been demonstrated on *Klebsiella edwardsii* by mixing various dilutions of a bacteriocin preparation with log.-phase cells from the indicator strain and plotting the log proportion of survivors against time. The slopes of the survivor curves exhibit single-hit kinetics. The response to bacteriocin treatment, however, depends on the medium used. When grown in Brain Heart Infusion, addition of bacteriocin in different con-centrations still permits bacterial growth for some generations although there is an immediate strongly reduced colony-forming ability. In broth and minimal medium this effect is much less but still significant. Trypsin is known to reverse the inactivation of colony-forming ability by bacteriocins due to digestion of the bacteriocin which remains on the bacterial surface (Nomura, M. (1964), *Proc. natn. Acad. Sci. U.S.A.* **52**, 1514). When trypsin is added to a culture in Brain Heart Infusion 1 hr after bacteriocin treatment, the cells just continue to grow in a normal way. When grown in broth or minimal medium, trypsin restores colony-forming ability in about 1 hr, while there is a lag-time of 4–6 hr before growth started again after only 15 min. of bacteriocin treatment.

The bacteriocin has no effect on respiration and fermentation of growing cultures or cell suspensions. Influences on macromolecular synthesis has been measured by the incorporation of ^{14}C-cysteine, ^{14}C-uracil and ^{14}C-thymine in bacteriocin-treated cultures. The bacteriocin

strongly inhibits protein synthesis, while RNA and DNA are synthesized at a normal rate. RNA-synthesis in *Klebsiella edwardsii* is of the 'stringent' type and thus bacteriocin activity on this strain seems to uncouple RNA and protein synthesis.

Regulation of Reductase Formation in *Proteus mirabilis*. By A. H. STOUTHAMER and G. N. de GROOT (*Microbiology Department, Botanical Laboratory, Free University, Amsterdam, The Netherlands*)

Proteus mirabilis can form three reductases under anaerobic conditions. Thiosulphate reductase, which reduces thiosulphate to sulphite and sulphide, is formed constitutively. Nitrate reductase, which reduces nitrate to nitrite, is induced by growth with nitrate. Furthermore a specific chlorate reductase is formed constitutively. This enzyme differs from nitrate reductase, which also reduces chlorate, by being insensitive to inhibition to azide. A similar enzyme was detected before in a *Hafnia* sp. (Pichinoty, F (1968), *Ann. Inst. Pasteur, Paris* **144**, 79).

In a mutant, which is blocked in the formation of δ-aminolaevulinate, nitrate, thiosulphate and chlorate are not reduced during growth in the absence of this factor, although the reductases were present. These results indicate that cytochromes are involved in the reduction of these anions and that these reductases are no haemoproteins. Under these conditions nitrate reductase is formed constitutively and a considerable repression of thiosulphate reductase is observed. During growth in the presence of δ-aminolaevulinate the mutant behaves in the same way as wild-type *P. mirabilis*. Under aerobic conditions no reductases are ever formed by the wild type or the mutant. Thus three factors seem to control nitrate reductase formation: oxygen, an operative respiratory chain, and the presence of nitrate.

Inoculation of cells of wild-type *P. mirabilis*, with high thiosulphate reductase activity, in fresh medium results in a rapid inactivation of this reductase. No sulphide is produced before synthesis starts again. Both inactivation and resynthesis are blocked by chloramphenicol. Addition of nitrate to a culture growing with thiosulphate results in complete repression of thiosulphate and chlorate reductase formation. Both enzymes are rapidly inactivated under these conditions. Aeration has a similar effect. These effects are typical examples of 'inactivation repression' (Ferguson, J. J., Boll, B. & Holzer, H. (1967), *Eur. J. Biochem.* **1**, 21).

Localization and Solubilization of Reductases of *Proteus mirabilis*. By G. N. DE GROOT and A. H. STOUTHAMER (*Microbiology Department, Botanical Laboratory, Free University, Amsterdam, The Netherlands*)

Proteus mirabilis can form nitrate reductase, thiosulphate reductase and chlorate reductase under anaerobic conditions. These enzymes appear to be bound to the protoplasmic membrane, which also contains the enzymes of the respiratory chain, e.g. cytochromes b_1, a_1, a_2 and o. Both NADH and formate function as electron donors for the reduction of nitrate, thiosulphate or chlorate. In a haeme-deficient mutant these reductases do not function. Reduced cytochrome b_1 is reoxidized by nitrate or thiosulphate (with membranes of nitrate- or thiosulphate-grown cells respectively), indicating that this cytochrome is an intermediate in electron transfer from NADH or formate to these reductases.

On treatment of the membrane fraction with a K_2CO_3-solution about 25 % of the membrane proteins are solubilized. By this treatment nearly all nitrate reductase and thiosulphate reductase activity are solubilized whereas cytochromes and chlorate reductase remain insoluble. On centrifugation of this solubilized preparation on a sucrose gradient, thiosulphate reductase moves as a single protein peak with a sedimentation constant of about 8 *S*. Nitrate reductase activity is recovered after sucrose gradient centrifugation as two peaks. One sharp peak, containing most probably the solubilized enzyme, moves with a sedimentation constant of about 10–11 *S*. Most of the activity is present, however, in a broad peak with a higher sedimentation constant (15–20 *S*). Most probably this fraction contains nitrate reductase in an enzyme complex. By the K_2CO_3 solubilization method the specific activity of nitrate reductase

increases by a factor 3·5 and that of thiosulphate reductase by a factor 1·5. Thiosulphate reductase can be further purified by gelfiltration over Sephadex G-150, which results in an increment of the specific activity by a factor 3. The complete purification scheme results in an increase of the specific activity of both reductases by a factor 25 over the crude cell-free extract.

Methane as a Minor Product of Pyruvic Phosphoroclasm in Desulfovibrio. By J. R. POSTGATE (*A.R.C. Unit of Nitrogen Fixation, University of Sussex, Brighton, Sussex*)

Methane has been detected over some cultures of sulphate-reducing bacteria (Sisler, F. D. & ZoBell, C. E. (1951), *J. Bact.* **62**, 117) but not others (Sorokin, Y. (1956), *Proc. Bajkal Limnol Stn* **15**, 397). *D. desulfuricans* var. *azotovorans*, strain 'Berre Sol' (NCIB 8388) formed traces of methane, detected by gas chromatography, in continuous nitrogen-fixing culture; when washed and disrupted by freezing in liquid nitrogen, cells showed ATP-activated pyruvic phosphoroclastic activity (Yates, M. G. (1967), *Biochem. J.* **103**, 32c), yielding CO_2, H_2 and CH_4. Methane formation from such preparations (4–8 mg. bacterial N/2 ml. 0·025 M 'tris', pH 7·5, under argon) required sodium pyruvate (10–100 mg.), adenine nucleotide (2–10 μmole), acetyl phosphate (1 mg.), co-enzyme A (1 mg.), thiamine pyrophosphate (0·1 mg.). Methyl viologen (0·5 μmole) inhibited CH_4 but not hydrogen and CO_2 evolution; ferredoxin, rubredoxin or cytochrome c_3 extracted from 'Berre Sol' (about 100 μg. each) had no effect; cyanocobalamin (100 μg.) stimulated methane formation and became bleached during the reaction. CO_2 or $NaHCO_3$ decreased CH_4 formation by 60–75 %; an atmosphere of H_2 decreased it by 55 %. ADP, AMP and ATP stimulated CH_4 formation in order of effectiveness 4·5:2·95:1, with optimal concentrations of all co-factors, preparations formed 20–30 nl. CH_4/mg. bacterial protein/hr, amounting to below 1 % of the gaseous products of pyruvic phosphoroclasm; intact cells formed less than 0·23 nl./mg./hr. The strain formed methane when grown with fixed nitrogen; it showed no sign of contamination by methane bacteria. Four out of six other strains of *Desulfovibrio* also showed methane production. The system in sulphate-reducing bacteria thus resembles that of the methane bacteria (Stadtman, 1967, *A. Rev. Microbiol.* **21**, 122) and its presence suggests an evolutionary interrelationship.

Nitrogen Fixation by Cultures and Cell-free Extracts of *Mycobacterium flavum* 301. By D. R. BIGGINS and J. R. POSTGATE (*A.R.C. Unit of Nitrogen Fixation, University of Sussex, Brighton, Sussex*)

Mycobacterium flavum 301 (NCIB 10,071) is a nitrogen-fixing organism isolated from Russian turf podzol soils by M. V. Federov & T. A. Kalininskaya (1961, *Dokl. mosk. sel'.-khoz. Akad. K. A. Trimiryazeva* **70**, 145).

Growth with sodium lactate, pyruvate, gluconate or succinate reached 0·42–0·56 mg. dry wt/ml. compared with 0·29 mg. dry wt/ml. ethanol, a recommended substrate. Nitrogen fixation and acetylene reduction were correspondingly augmented. Yeast extract could be replaced with $(NH_4)_2SO_4$; in continuous culture a source of fixed nitrogen could be omitted altogether. When fixing nitrogen, *M. flavum* tended to be microaerophilic: at P_{O_2} = 0·1, 12-day shake cultures contained 1·37 mg. dry wt. and 60·0 μg. fixed N/ml. at 30°, cf. 0·63 mg. dry wt and 29·6 μg. fixed N/ml. at P_{O_2} = 0·2. Acetylene reduction by samples from a continuous culture increased from < 0·01 μmole/mg. dry wt/hr at P_{O_2} = 0·2 to 15·5 μmole/mg. dry wt/hr at P_{O_2} = 0·05. Wholly anaerobic conditions did not support growth.

Sonication or extrusion from the French press, followed by centrifugation at 38,000 *g* for 30 min., gave extracts which, when provided with an ATP-generating system (2·5 mM ATP), and Mg^{2+} (5 mM) + $Na_2S_2O_4$ (20 mM), performed the following reductions: N_2 to NH_3, H^+ to H_2, C_2H_2 to C_2H_4, KCN to CH_4, CH_3NC to $CH_4 + C_2H_4 + C_2H_6$. Anaerobic conditions during preparation and assay were required. Pyruvate, α-ketoglutarate, succinate, glucose and glucose-6-phosphate did not replace dithionite for acetylene reduction. ATP at high concentrations (> 8 mM), ADP (2 mM) or AMP (2 mM) inhibited acetylene reduction. Activity was associated with a particle which sedimented at 145,000 *g* over 3½ hr.

The nitrogenase from *M. flavum* 301 thus resembles the particulate system of *Azotobacter* (Bulen, W. A., Burns, R. C. & LeComte, J. R. (1964), *Biochem. biophys, Res. Comm.* **17**, 265) rather than the soluble system of *Clostridium pasteurianum* (Carnahan, J. E., Mortenson, L. E., Mower, H. F. & Castle, J. E. (1960). *Biochim. biophys. Acta* **44**, 520).

Formaldehyde Oxidation by the Methanol Dehydrogenase of Pseudomonas PP. By A. LADNER and L. J. ZATMAN (*Department of Microbiology, University of Reading, Reading, Berks.*)

Pseudomonas PP is a pink organism obtained from river mud using vitamin-fortified media with monomethylamine hydrochloride as carbon + energy source. It resembles *Pseudomonas* sp. M 27 and *Pseudomonas* AM 1, but growth of *Pseudomonas* PP is enhanced by either pantothenate or β-alanine. This enhancement is most obvious with alcohol substrates but pantothenate has not been implicated specifically in alcohol metabolism.

A methanol (alcohol) dehydrogenase as described for *Pseudomonas* sp. M 27 by Anthony & Zatman (1964, *Biochem. J.* **92**, 614) was detected in crude cell-free extracts of *Pseudomonas* PP. No evidence of nicotinamide nucleotide-linked methanol or alcohol dehydrogenase activity was obtained. These extracts contained three formaldehyde-oxidizing enzymes; two resembled previously described enzymes but the third (PP enzyme) was apparently new. These three activities were separated by ammonium sulphate fractionation; the two known enzymes occurred together in one fraction whilst the PP-enzyme was found with the methanol dehydrogenase in a later fraction. As the assay conditions for the PP-enzyme (except for the substrate) were identical with those required for the methanol dehydrogenase, it was suspected that both activities might be due to a single enzyme. Data from thermal denaturation and gel-electrophoresis experiments supported this notion. Attempts to separate these two activities by ion-exchange (DEAE-cellulose) and gel-filtration (Sephadex G-150) treatments were unsuccessful, again suggesting that a single protein was involved. Further data obtained using purified enzyme preparations support the conclusion that the PP-enzyme and the methanol dehydrogenase from *Pseudomonas* sp. M 27 are identical.

Substrate specificity studies showed that whilst the PP-enzyme oxidized many alcohols, only formaldehyde was oxidized from the wide range of aldehydes tested. Thus, this enzyme (and the M 27-enzyme) may be regarded as having dual substrate specificity, i.e. catalysing formaldehyde and alcohol oxidation.

After this abstract has been submitted, it was learnt that the 'Analar' grade formaldehyde used as the substrate in the above experiments contains 11 to 14 % (w/w) methanol added as a stabilizing agent; this makes many of the results difficult to interpret. Although some of the data support the conclusion that formaldehyde is oxidized, further experiments are required before this conclusion can be considered unequivocal.

A Mutant of Pseudomonas AM 1 which Lacks Methanol Dehydrogenase Activity. By J. HEPTINSTALL and J. R. QUAYLE (*Department of Microbiology, University of Sheffield, S 10 2 TN*).

Extracts of methanol-grown *Pseudomonas* AM 1 contain three enzymes which together can effect the oxidation of methanol to carbon dioxide (Johnson, P. A. & Quayle, J. R. (1964), *Biochem. J.* **93**, 281): (*a*) a phenazine methosulphate (PMS)-linked methanol dehydrogenase similar to that described in *Pseudomonas* M 27 (Anthony, C. & Zatman, L. J. (1964), *Biochem. J.* **92**, 614); (*b*) a 2,6-dichlorophenolindophenol (DCPIP)-linked aldehyde dehydrogenase; (*c*) an NAD-dependent formate dehydrogenase. Methylamine-grown *Pseudomonas* AM 1 has been shown to contain a PMS-linked methylamine dehydrogenase which converts methylamine to formaldehyde and which is distinct from methanol dehydrogenase (Eady, R. R. & Large, P. J. (1968), *Biochem. J.* **106**, 245). These authors concluded that methylamine was oxidized directly to formaldehyde, rather than via methanol as an intermediary metabolite.

A mutant of *Pseudomonas* AM 1, designated M-15A, has now been isolated which is capable of growth on methylamine and formate but not on methanol. Washed cell suspensions were found to be incapable of oxidizing methanol. Methanol dehydrogenase activity was present in extracts of methylamine-grown wild-type cells, but was absent in similar

extracts of mutant M-15A grown on methylamine. This shows that although methanol dehydrogenase is normally present during growth on methylamine it is not necessary for growth on this substrate.

Ladner & Zatman (preceding abstract) have found that the purified methanol dehydrogenase from *Pseudomonas* M 27 will also oxidize formaldehyde. Crude extracts of methylamine-grown *Pseudomonas* AM 1 will oxidize both methanol and formaldehyde at similar rates at pH 9·0 using PMS as primary electron acceptor. Similar extracts of mutant M-15A showed no activity with either substrate, thus confirming that the oxidation of both methanol and formaldehyde which is observed under the above experimental conditions in extracts of wild-type cells is catalysed by one enzyme rather than two.

The methylamine dehydrogenase of *Pseudomonas* AM 1 does not oxidize formaldehyde (Eady, R. R. & Large, P. J. (1966), *Biochem. J.* **100**, 57*P*), and since mutant 15-A contains the DCPIP-linked aldehyde dehydrogenase in comparable activity to wild type, the latter enzyme is implicated in the oxidation of formaldehyde *in vivo*.

Addendum. Subsequent to the submission of this abstract it was found that the A.R. formaldehyde used in these experiments contained 11 to 14% methanol added as stabilizer. It is now found that formaldehyde, prepared for paraformaldehyde, is oxidized by crude extracts of *Pseudomonas* AM 1 at pH 9 in the presence of NH_3, at 26% of the rate at which methanol is oxidized under the same conditions (instead of the same rate as reported in the abstract).

Kinetic Parameters of Mesophilic and Thermophilic Isocitrate Lyase. By T. K. SUNDARAM and H. L. KORNBERG (*Department of Biochemistry, University of Leicester*)

It has been suggested (Brock, T. D. (1968), *Science, N.Y.* **158**, 1012) that thermophilic micro-organisms, which are found in high-temperature habitats, are relics of primordial forms of life and that, in the course of evolution to the mesophilic forms, the macromolecules of the thermophiles sacrificed thermostability in favour of a more flexible conformation. On the basis of this theory, significant differences might be observed between thermophilic and mesophilic enzymes in their kinetic and allosteric properties.

We wish to report some experiments on the kinetic parameters of the enzyme, isocitrate lyase (a key enzyme of the glyoxylate cycle (Kornberg, H. L., *Essays in Biochemistry* (1966), **2**, 1)) derived from the mesophiles, *Escherichia coli*, K 12, and *Bacillus megaterium*, and from an obligate thermophile, *Bacillus coagulans* (subspecies) (kindly donated by Professor N. Grossowicz, Jerusalem).

No significant differences were observed in the temperature coefficients (activation energies 14·5–18·5 kcal./mole), or in the affinity for isocitrate (as indicated by K_m), of the three species of isocitrate lyase. Moreover, the thermophilic enzyme was as powerfully inhibited by the allosteric effector phospho*enol*pyruvate as was the *E. coli* enzyme (Ashworth, J. M. & Kornberg, H. L. (1963), *Biochim. biophys, Acta* **73**, 519). The only major difference between the three types of isocitrate lyase detected is their stability to heat. For example, when kept at 45° C. in a buffer at pH 7·4 containing 20 mM-phosphate, 1 mM-ethylenediaminetetraacetate and 10 mM-$MgCl_2$, the enzymes from the mesophiles *B. megaterium* and *E. coli* lost half of their activity in 7·5 min. and 35 min. respectively, whereas the isocitrate lyase of the thermophile maintained over 90 % of its activity even after 45 min. under these conditions.

These findings show that, although an enzyme from a thermophilic organism is considerably more thermostable than similar enzymes from mesophiles, this difference need not be accompanied by differences in kinetic or regulatory properties.

Step-wise Resistance to Polymyxin and Other Agents by *Pseudomonas aeruginosa*. By M. R. W. BROWN, W. M. WATKINS and J. H. SCOTT FOSTER (*Pharmaceutical Microbiology Group, School of Pharmacy, Bath University of Technology, Bath, Somerset*)

Preliminary training experiments with *Pseudomonas aeruginosa* NCTC strains 6750, 7244, 8203 and OSU 64 in broth culture showed a step-wise increase in resistance with each strain to

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benzalkonium chloride, chlorhexidine diacetate, chlorocresol and polymyxin B sulphate. Resistance to chlorbutanol and EDTA did not occur. Further studies were confined to polymyxin and *P. aeruginosa* NCTC 6750. With inocula of 10^5 cells or more, training to polymyxin resistance occurred exponentially by a series of steps differing 2- to 3-fold in resistance until virtual infinite resistance. Inocula of 10^2 cells consistently failed to train to resistance. The filtered extract from smashed resistant cells failed to inactivate polymycin on incubation. Further, a filtered culture of resistant cells retained activity against fresh inocula. These experiments suggested that resistance was due to mutation at a rate of about 1 in 10^5 or less cells and polymyxin was not inactivated enzymically. Little loss of resistance occurred after one subculture in nutrient broth (about seven generations). Subsequent subcultures each reduced resistance by about half, regardless of level of resistance. Uptake of polymyxin was less for resistant cells than for sensitive. Training by replica plating with velvet or wire stab did not give results as reproducible as with broth culture. A step-wise mutant grown in 5000 units/ml. polymyxin was harvested by washing in plain broth, grown through a subculture in plain broth and the cell walls isolated (Gray, G. W. & Wilkinson, S. G. (1965), *J. appl. Bact.* 28, 153). Lipid (Hugo, W. B. & Stretton, R. J. (1965), *J. gen. Microbiol.* 42, 133) and phosphorus (Allen, R. J. L. (1940), *Biochem. J.* 34, 858) were both found to be about double that in sensitive cells, suggesting a possible role of phospholipid in resistance.

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

Studies on Methane-oxidizing Bacteria: Isolation and Description. By R. WHITTENBURY, S. L. DAVIES, A. G. McLEE, K. C. PHILLIPS AND J. F. WILKINSON (*Department of General Microbiology, University of Edinburgh*)

A wide variety of methane-oxidizing bacteria have been isolated by enrichment procedures on media containing methane and inorganic salts. They are all obligate methane or methanol utilizers. Some of the organisms are similar to those that have been already described but most are types not previously isolated and some are unique in their shape and growth characteristics amongst bacteria as a whole.

Studies on Methane-oxidizing Bacteria: Structure. R. WHITTENBURY, S. L. DAVIES, A. G. McLEE, K. C. PHILLIPS and J. F. WILKINSON (*Department of General Microbiology, University of Edinburgh*)

The fine structure of some of the bacteria outlined previously will be described. All the organisms have a complex internal membrane system which fall into two broad categories according to orientation and shape of the convolutions. These are both shown to differ radically from the membranes of bacteria oxidizing ethane and higher hydrocarbons.

SESSION C

Spore Germination Mutants of *Bacillus cereus*. By S. C. WARREN (*Unilever Research Laboratory, Colworth House, Sharnbrook, Bedford*)

Germination of spores of *Bacillus cereus* strain T can be initiated either by 0·1–10 mM L-alanine or by a mixture of 0·005–0·1 mM inosine and an equimolar concentration of L-alanine (Warren, S. C. & Gould, G. W. (1968), in press). We have isolated some mutants of *B. cereus* T whose spores show an altered response to either or both of these germinants. Two of the mutants germinate only very slowly in L-alanine alone, but do so at a normal rate in inosine + alanine. Eight mutants which germinate normally in L-alanine do so only at a slow rate in inosine + alanine. Six mutants germinate very slowly in either alanine alone or inosine + alanine. Four of these six also show reduced rates of germination *n*-dodecylamine (Rode, L. J. & Foster, J. W. (1961), *J. Bact.* 81, 768) but all six germinate normally in 30 mM calcium 2,6-dipicolinate (Riemann, M. & Ordal, Z. J. (1961), *Science, N.Y.* 133, 1703) and also, after treatment with thioglycolic acid and urea, in lysozyme and in a sporelytic enzyme (Gould, G. W., Hitchins, A. D. & King, W. L. (1966), *J. gen. Microbiol.* 44, 293).

Some tentative conclusions may be drawn from these results. At least one reaction involved in germination by L-alanine alone is not essential for germination in inosine + alanine and at least one reaction involved in germination in inosine + alanine is not essential for germination in alanine alone. At least two reactions are essential for germination in both alanine and inosine + alanine, but only one of these is essential for germination in *n*-dodecylamine. None of these reactions is essential for germination in calcium dipicolinate, lysozyme, or spore-lytic enzyme. Biochemical studies of the mutants may throw some light on the nature of the germination reactions, and possibly also on the nature of the 'superdormant fraction' of spores which invariably remain after the majority of a population have germinated (Gould, G. W., Jones, A. & Wrighton, C. (1968), *J. appl. Bact.* **31**, in the Press).

Immunity to Streptococcal Infection in Pigs with Special Reference to the Bactericidal Test.
By K. K. AGARWAL (*Department of Animal Pathology, School of Veterinary Medicine, University of Cambridge*)

During the first few weeks of life, piglets are susceptible to infection with group D strepto-cocci, while the adult pigs are resistant (Field, H. I., Buntain, D. & Done, J. T. (1954), *Vet. Rec.* **66**, 453; Elliott, S. D., Alexander, T. J. L. & Thomas, J. H. (1966). *J. Hyg., Camb.* **64**, 213). The causative agent is an encapsulated streptococcus of specific serological type belonging to *Streptococcus suis*, a new subgroup in Lancefield's group D (Elliott, S. D. (1966), *J. Hyg., Camb.* **64**, 205). The disease can be produced in susceptible piglets by spraying broth culture of *S. suis* into the nasopharynx, but such infection can be prevented by prior admini-stration of serum from normal adult pigs (Agarwal, K. K. (1968), Ph.D. thesis, University of Cambridge). The experiments reported here were carried out to demonstrate the age suscepti-bility of piglets, and to characterize the immune factor present in adult pig serum.

The results of the experiments are briefly as follows. (1) Blood from piglets weaned within 48 hr of birth had no bactericidal effect against *S. suis*. Blood from conventionally reared piglets often had no bactericidal effect during the first 4 weeks of life. Blood from adult pigs was always found to be bactericidal against *S. suis*. (2) Pig blood cells or pig blood plasma had no bactericidal effect against *S. suis*. (3) Addition of serum from normal sows or pigs over 8 weeks of age conferred bactericidal property on piglet blood. (4) Sow blood polymorpho-nuclear (PMN) leucocytes suspended in sow blood plasma phagocytized *S. suis*. Piglet blood PMN leucocytes suspended in piglet plasma did not phagocytize *S. suis*. Addition of sow serum to piglet blood PMN leucocytes, suspensed in piglet plasma, conferred the property of phagocytosis on PMN leucocytes. (5) The bactericidal factor present in sow serum was heat-stable, it did not deteriorate on storage at + 4° but was inactivated by the addition of purified *S. suis* capsular polysaccharide. In normal sow serum the bactericidal factor was present in too small amounts to be detected by conventional serological methods such as precipitation, agglutination, indirect antiglobulin or complement-fixation tests, but was demonstrable in bactericidal tests. (6) Fractionation and chromatographical studies indicate that the bacteri-cidal activity of the sow serum was due to macro-globulins. (7) It is suggested that the resis-tance in adult pigs is probably due to subclinical infection with *S. suis* during the first few weeks of life.

Continuous Flow Culture of Mixtures of Faecal Organisms. By P. COLLARD and JENNIFER GOSSLING (*Department of Bacteriology and Virology, University of Manchester*)

When organisms typical of human faecal isolates are grown together in continuous flow culture, using a complex medium (casein hydrolysate, sugar and yeast extract), their numbers are in proportions similar to those found in the faeces of healthy humans: the eubiotic flora described by Haenel and others (Haenel, H. (1965), *Ernährungsforschung* **10**, 289).

One isolate of each group, each from a different source, is used for each culture. So far no variation has been found which was attributable to the use of different isolates of the same species.

By plating serial dilutions of the culture on selective media, the dominant organisms are bifidobacteria (small, Gram-positive, non-sporeforming, obligately anaerobic, pleomorphic

bacilli), usually $10^{8.5}$–$10^{9.5}$ per ml., and Gram-negative anaerobes—bacteroides and veillonellae—10^8–10^9. *Escherichia coli, Streptococcus faecalis* and *Lactobacillus acidophilus* vary in the range $10^{4.5}$–$10^{8.5}$, *Clostridium perfringens*, 10^6–$10^{7.5}$, *Staphylococcus albus*, 10^2–10^6, and *Candida albicans*, 10^1–$10^{4.5}$.

Marked variation in numbers results from changing the sugar concentration in relation to the casein hydrolysate concentration. The effects of such changes on cultures containing most of the organisms has been reported previously (Collard, P. J. & Gossling, J. (1968), *J. gen. Microbiol.* **50**, iv). Most of these changes have been shown to be due to the change in pH which accompanies the change in sugar concentration.

Addition of deoxycholate to the medium reduces the counts of clostridia, streptococci and staphylococci ($\times 10^{-4}$ to $\times 10^{-5}$). Addition of mucin temporarily reduces the counts of staphylococci ($\times 10^{-1}$). Mucin and deoxycholate together result in less reduction of the counts than deoxycholate alone. Addition of oleate results in increased counts of staphylococci $\times 10^2$) and slightly increased counts of bifidobacteria ($\times 10^{0.5}$).

Correlation of Sensitivity to Ionizing Radiation and Mild Heating in Bacteria. By B. A. BRIDGES, M. J. ASHWOOD-SMITH and R. J. MUNSON (*M.R.C. Radiobiological Research Unit, Harwell, Berks.*)

The sensitivity of various strains of *E. coli* to incubation at 52° parallels their sensitivity to gamma radiation. DNA from bacteria heated at 52° shows a similar sedimentation pattern in alkaline sucrose gradients to DNA from irradiated bacteria, both treatments producing single-strand breaks or alkali-labile sites. It is concluded that heat-sensitive strains of *E. coli* are inactivated at least in part by a similar sort of DNA damage to that which ionizing radiation produces. It is suggested that enzymes capable of repairing ionizing radiation damage might have as their normal function the repair of apurinic sites and single strand breaks in DNA which arise spontaneously even at normal growth temperature (e.g. 37°) and which are considerably increased in number at 52°.

Molecular Bases of Obligate Psychrophily. By N. L. MALCOLM (*Sub-Department of Chemical Microbiology, Department of Biochemistry, University of Cambridge*)

An inquiry into the biochemical reasons for the temperature-sensitivity of an obligate psychrophile, *Micrococcus cryophilus*, has revealed that at growth-restricting temperatures (above 25°) macromolecular syntheses are inhibited (Malcolm, N. L. (1968), *J. Bact.* **95**, 1388). The primary, temperature-induced lesion lies in the mechanism of protein synthesis and has been further located in the activation and attachment of certain species of amino acid to transfer ribonucleic acid (tRNA) (Malcolm, N. L. (1968), *Biochim. biophys. Acta* **157**, 493).

In this communication further investigation into the effect of temperature on these charging reactions is described. It is shown that this impairment of amino acid-tRNA attachment is a cumulative effect of temperature on both the aminoacyl-tRNA synthetases and their cognate species of tRNA. The postulated conformational change occurring during the heating of tRNA affects only the charging function of the molecule, not its ability to bind to ribosomes in the presence of an appropriate codon. The enzyme fraction from the psychrophile will catalyse the charging reaction in a heterogeneous system using tRNA preparations from a mesophile, *Escherichia coli*, and a thermophile, *Bacillus stearothermophilus*, only if no prior heating is involved. Psychrophile tRNA, whether pre-incubated at 30° or not, will accept amino acids activated by both mesophile and thermophile aminoacyl-tRNA synthetases.

T-Mycoplasmas from Various Animal Species. By D. TAYLOR-ROBINSON, J. P. ADDEY (*Clinical Research Centre, Harvard Hospital, Salisbury*), M. H. WILLIAMS (*Department of Rheumatology, Middlesex Hospital, London*) and D. A. HAIG (*Institute for Research on Animal Diseases, Compton, Berkshire*)

T-mycoplasmas were first isolated from the human male urogenital tract (Shepard, M. C. (1954), *Am. J. Syph. Gonor. vener. Dis.* **38**, 113). They were so named because they produced very small colonies (T = tiny) on solid medium. Their ability to metabolize urea with

ammonia production and to produce an alkaline colour change in medium containing urea and phenol red (Purcell, R. H. *et al.* (1966), *J. Bact.* 92, 6) has facilitated their study. We have isolated them from man, cattle, monkeys and dogs by means of this technique. Apart from T-mycoplasmas of human origin, those of cattle have been investigated most extensively. They have been recovered from the vagina, urethra and bladder of slaughtered cows and from the penile sheath and semen of bulls. Semen contains a growth inhibitor. In various physical and biological properties T-mycoplasmas of animals do not differ from those isolated from man. In addition, electron microscopy of bovine and human T-mycoplasmas has revealed cells which vary in size from 80 to 420 mμ and which are bounded by a triple-layered membrane— features similar to those of known classical mycoplasmas which produce large colonies.

Staining of *Escherichia coli* k 88 Antigen and Other Fimbrial Antigens for Light Microscopy. By F. ØRSKOV and IDA ØRSKOV (*W.H.O. International Escherichia Centre, Statens Seruminstitut, Copenhagen, Denmark*)

The first description of fimbriae or pili was based on electron microscopy and all later investigators have agreed that because of the small width of the fimbrial threads light microscopy could not be used.

The *E. coli* k 88 antigen is a typical L antigen; it is genetically determined by an episome (Ørskov, I. & Ørskov, F. (1966), *J. Bact.* 91, 69), it consist of protein (Stirm, S., Ørskov, F., Ørskov, I. & Mansa, B. (1967), *J. Bact.* 93, 731), and it shows its fimbrial nature in electron micrographs (Stirm, S., Ørskov, F., Ørskov, I. & Birch-Andersen, A. (1967), *J. Bact.* 93, 740).

This antigen can be stained for examination in the light microscope by prolonged staining according to Leifson's flagellar technique. However, because of the inhibitory influence of even very small amounts of material eluted from the bacteria, this method is not very reliable for staining of k 88 fimbriae.

Much better preparations and more reproducible results were obtained when the bacterial spreads were prepared in a slide centrifuge developed for spreads of blood cells (Doré, C. F. & Balfour, B. (1965), *Immunology* 9, 403). The staining time necessary was considerably longer than that employed for staining of flagella in the same preparation.

Some other fimbrial antigens could also be stained by this method.

SYMPOSIUM: THE SPECIAL PHYSIOLOGY
OF EXOTIC BACTERIA

Introduction. By H. VELDKAMP (*Microbiological Laboratory, State University, Groningen, The Netherlands.*)

The isolation of a large variety of 'exotic microbes' became possible after introduction of the enrichment culture technique. Closed culture systems have nearly exclusively been used for this purpose. Generally the type of organism to be enriched is offered an excess of nutrients, allowing growth at maximal rate. The aim is to create conditions in which this rate is higher than that of competing microbes. In this type of enrichment the result of selection is thus determined by maximum specific growth rates. Some of the 'exotics' thus enriched might well be truly exotic in that they do not play a role of any ecological importance.

It is too little realized that under natural conditions microbes often must compete for growth-limiting substrates which occur at very low concentration. And under these conditions it is another growth characteristic which determines the result of microbial competition. The organism which shows the highest growth rate at these low concentrations of a growth-limiting substrate will become dominant. It has been shown that bacteria which have a relatively low maximal growth rate at substrate saturation, often grow comparatively fast at the very low concentrations (Jannasch, H. W. (1967), *Limnol. Oceanogr.* 12, 264; *Arch. Mikrobiol.* 59, 165).

Microbial competition at different concentrations of growth-limiting substrates can only be studied in a homo-continuous culture system (chemostat), and application of this system has revealed the existence of hitherto unknown 'exotics'. Nothing is known about the physiological basis of fast growth at low concentrations of nutrients.

When studying microbial competition in relation to other factors affecting growth rates (e.g. temperature) the above-mentioned substrate affinity may also play an important role. An obligately psychrophilic *Pseudomonas* spec. showed a higher maximum specific growth rate than a facultatively psychrophilic *Spirillum* spec. at 4°, when grown in a mineral medium with excess lactate. However, the *Spirillum* spec. appeared to outgrow the *Pseudomonas* spec. at 4° in a chemostat at very low concentrations of growth-limiting lactate (Harder, W. (1968), unpublished).

The survival of organisms exposed to the continually changing conditions in nature is not only determined by growth rates under conditions which favour growth. The ability to survive periods during which growth is impossible is equally important. It has been shown that the 'dormant cell' needs a certain amount of energy per unit time for maintenance purposes. In this respect the dark metabolism of phototrophs is of interest. It was shown (van Gemerden, H. (1968), *Arch. Mikrobiol.*, in the Press) that *Chromatium* synthesizes storage carbohydrate in the light when grown with H_2S as electron donor. During dark periods this reserve material was converted to poly-β-hydroxybutyrate. Under these conditions intracellular elemental sulphur served as electron acceptor, and H_2S as well as CO_2 were released. It is conceivable that this dark process is energy generating and thus explains motility of *Chromatium* during prolonged periods of darkness.

Reserve material has also been shown of importance in the dark-synthesis of bacterio-chlorophyll by *Rhodospirillum* (Schön, G. & Drews, G. (1966), *Arch. Mikrobiol.* 54, 199). Cells grown aerobically in the dark contain hardly any chlorophyll, but immediately start to synthesize this pigment at a linear rate when exposed to anaerobic conditions. The period of synthesis was proportional to the amount of reserve material initially present. Thus, under anaerobic conditions in the dark *Rhodospirillum* produces the pigment without which anaerobic growth would be impossible; even though it would be useless if the dark period were to last for ever.

Extremely Halophilic Bacteria. By H. LARSEN (*Departmental of Biochemistry, The Technical University of Norway, Trondheim*)

Organisms which require for growth sodium chloride at a minimum concentration of 10–15 % and an optimum concentration of 20–30 % are of two distinct kinds, 'the halobacteria' and 'the halococci'. Save the extreme sodium chloride requirement, these organisms have the common bacteriological characteristics of the ordinary pseudomonads and micrococci respectively. They are ubiquitously distributed in waters of very high salinity, and utilize most readily amino acids as carbon source.

These organisms are also occasionally referred to as 'the red halophiles' because they contain carotenoids. A function of the carotenoids is to protect the cells against detrimental effects of the sunlight, which is usually quite intense over natural salines.

The salt concentration inside the cells is at least as high as that in their environment. The dominating intracellular salt components are Na^+, K^+ and Cl^-. It is especially notable that K^+, which is normally a minor component of the environment, is concentrated inside the cells to such an extent that the solubility limit for KCl is reached.

The metabolic apparatus is adapted to function at the high salt concentrations inside the cells and even requires the salt for normal functions. The individual enzymes are extremely salt-tolerant and most of them strongly stimulated by salt; KCl normally stimulates better than NaCl. Upon removal of salt from the enzymes these tend to become irreversibly denatured.

In contrast to the halococci, which contain a thick polysaccharide cell wall and are unaffected in their structure upon dilution of the saline environment with water, the halobacteria contain a fragile cell envelope and lyse at salt concentrations below 8–10 %. The lysis phenomenon is primarily due to special properties of the cell envelope. In the halobacteria the cell envelope is composed of a cell membrane and a proteinaceous layer coating the outside of the membrane.

The polysaccharide content of the cell envelope is very low. No muramic acid has been found, but small amounts of other amino sugars. Upon lowering the salt concentration in the environment of the cells the protein of the outer layer of the cell envelope goes in solution, and the membrane to which amino polysaccharide adheres, tends to split open. Upon complete removal of the salt the amino polysaccharide is released from the cell membrane and the latter disintegrates to smaller flakes still having the appearance of 'unit membranes'. These membrane flakes are mainly composed of protein and lipid in about equal amounts; they contain carotenoids and cytochromes. The lysis of the cells in hypotonic salt solutions seems, at least in part, to be due to the special chemical nature of the outer layer of the cell envelope. The protein of this layer is quite acidic and thus possesses an excess of negative charges which tend to disperse the protein. Only in the presence of high concentrations of salt seem the negative charges effectively neutralized by the cations of the salt so that the protein units are held together to make the proper surface layer. Mg^{2+} seems to play a role in the binding of protein to the lipid of the membrane.

A similar behaviour is shown for the ribosomes of the halobacteria. When exposed to hypotonic solutions the ribosomes disintegrate; protein with quite acidic properties goes in solution. The sub-units of the ribosomes are held together only in the presence of high concentrations of KCl and $MgCl_2$.

The acidic nature of the proteins is possibly a unique feature of the extremely halophilic bacteria and a dominating factor in impressing upon these organisms their extremely halophilic character.

Bacteria Resistant to Radiation Damage. By B. E. B. MOSELEY (*Department of General Microbiology, University of Edinburgh*)

Vegetative bacteria show large variations in their response to the lethal action of ultraviolet (u.v.) and ionizing radiation. For example, the dose of u.v. radiation required to inactivate 90 % of a suspension of *Escherichia coli* B_{s-1} is less than 1 erg/mm.^{2} (Hill, R. F. & Simson, E. (1961), *J. gen. Microbiol.* **24**, 1) while a dose of 7000 ergs/mm.^{2} is needed to achieve the same effect with *Micrococcus radiodurans* (Setlow, J. K. & Duggan, D. E. (1964), *Biochim. biophys. Acta* **86**, 664). A similar situation exists with ionizing radiation. Thus a 90 % inactivating dose for various *Pseudomonas* spp. is less than 2 krad (Kaplan, H. S. & Zavarine, R. (1962), *Biochem. biophys. Res. Commun.* **8**, 432) while for *M. radiodurans* it is 750 krad (Moseley, B. E. B. & Schein, A. H. (1964), *Nature, Lond.* **206**, 373). These differences can now be largely explained in terms of the absence or presence in the bacteria of DNA repair mechanisms, and if present, of their efficiency.

When vegetative bacteria are exposed to either u.v. or ionizing radiation, the potentially lethal damage, which does not vary quantitatively very much from one bacterial species to another, is that in the DNA. However, the actual lesions created by the two forms of radiation are quite different. Thus during u.v. irradiation cyclobutane-type pyrimidine dimers are formed between adjacent pyrimidine bases on the same DNA strand which act as blocks to DNA replication. Ionizing radiation, on the other hand, exerts its main lethal effect through the formation of single- or double-strand breaks in the DNA. Repair mechanisms exist for both kinds of damage.

Two mechanisms have been described for the removal of u.v.-induced pyrimidine dimers. Bacteria which possess a photoreactivating enzyme can monomerize the dimers *in situ* in the presence of light. The fraction of potentially lethal u.v. damage which can be reversed by photoreactivation (the photoreactivable sector) is between 0·5 and 0·8 in *E. coli*. Photoreactivation does not operate for ionizing radiation damage. The second type of repair which may occur and which is potentially much more useful to a bacterium is the so-called dark repair (Setlow, R. B. & Carrier, W. L. (1964), *Proc. natn. Acad. Sci. U.S.A.* **51**, 226; Boyce, R. P. & Howard-Flanders, P. (1964), *Proc. natn. Acad. Sci., U.S.A.* **51**, 293). In this case the pyrimidine dimer is recognized as a lesion in the DNA and is excised together with some adjacent bases. The resulting gap, which is now a single-strand break, is repaired by enzymes which repolymerize bases in the region of the excision, using information on the complementary DNA strand, and which finally rejoin the broken ends. Vegetative bacteria which lack the

ability to excise pyrimidine dimers are much more sensitive to u.v. radiation than those which possess it, although among bacteria in the latter group there is a wide range of resistance to u.v. radiation.

Since ionizing radiation causes single-strand breaks in DNA, and this is effectively a stage in the repair of u.v.-induced damage, it is not surprising that the dark repair mechanism operates also for the repair of ionizing radiation damage. Thus bacteria which are unusually resistant to u.v. radiation are also resistant to ionizing radiation. *M. radiodurans*, for example, is the most resistant bacterium, yet investigated, to both u.v. and ionizing radiation. Its high resistance to u.v. radiation is not merely a result of the possession of an excision mechanism but rather of its high rate of excision of pyrimidine dimers coupled with a high rate of strand mending. For example, it can excise approximately 1200 dimers from its DNA in about 15 min. and yet no more than two single-strand breaks can be demonstrated at any time during the repair (personal observation).

It is this close co-ordination of excision and strand mending which apparently makes for a radiation-resistant bacterium.

Chemolithotrophic Bacteria. By R. WHITTENBURY (*Department of General Microbiology, University of Edinburgh*)

Chemolithotrophic bacteria are characterized by their ability to use CO_2 as their only carbon source and inorganic compounds as their source of energy and reducing power by a cytochrome-mediated respiratory system, in the dark. Most chemolithotrophs are unable to use organic compounds as sole energy and carbon sources and are termed obligate chemolithotrophs. Hydrogen oxidizers are a general exception in that none are obligate.

Four groups of inorganic compounds serve as energy and reducing power sources to chemolithotrophs, reduced sulphur compounds, reduced nitrogen compounds, hydrogen gas and ferrous iron. Organisms using these substrates will be described and emphasis placed upon what appear to be the more unusual and less well known organisms. From this discussion, the hydrogen oxidizing organisms are shown to be the most difficult of the groups to classify in that many organisms able to use hydrogen are unable to use CO_2 as their sole carbon source. Added to these variations on the chemolithotrophic theme are the mechanisms by which oxidisers of 1-C compounds obtain carbon and energy. Some of these organisms, particularly the formate and methane oxidisers, appear to have a similar metabolism to chemolithotrophs with the exception that an organic compound replaces the inorganic compound as the energy and reducing power source. Cell carbon appears to be fixed chemolithotrophically with CO_2 or from formaldehyde by a pathway with similarities to the ribulose diphosphate pathway of CO_2 fixation.

The possibilities that some strict anaerobes might be termed chemolithotrophs is considered, specifically the methane producers and the clostridium species forming acetic acid. Both use hydrogen as an energy source and CO_2 as a reductant.

The varying patterns of metabolism related to chemolithotrophy raise the question of what is really unique about obligate chemolithotrophic organisms. Is it their ability to use inorganic compounds as energy and reducing power sources, their ability to use only CO_2 as a carbon source, or their inability to use organic compounds either as a source of carbon or reducing power? These questions will be discussed in the light of recent evidence on the nutrition of these organisms, particularly that relating to the absence of pyridine nucleotide oxidases and α-ketoglutarate dehydrogenase in certain obligate autotrophs (Smith, A. J., London J. & Stanier, R. Y. (1968), *J. Bact.* **94,** 972).

Electron micrographs of thin sections of some chemolithotrophs and similar organisms reveal the presence of various types of complex membraneous systems which are similar to some seen in photosynthetic bacteria and heterotrophic budding bacteria. Possible reasons for the presence of these membranes in the chemolithotrophs will be discussed and the effect that the presence of certain types of membraneous systems seem to impose on the mode of cell growth and multiplication.

Unusual Characteristics of the Phototrophic Bacteria. By NORBERT PFENNIG (*Institut für Mikrobiologie, Universitat Gottingen, Germany*)

The purple non-sulphur bacteria and the purple and green sulphur bacteria are anaerobic aquatic organisms inhabiting both marine and freshwater habitats. Enrichment cultures of Athiorhodaceae are achieved in media containing simple organic substrates and growth factors; purple and green sulphur bacteria are enriched in sulphide-containing mineral media (vitamin B_{12} is required by some species). Sulphide inhibits the growth of the Athiorhodaceae.

The different bacteriochlorophylls of the purple (bchl. a and b) and green (bchl. c and d) sulphur bacteria (Jensen, A., Aasmundrud, O. & Eimhjellen, K. E. (1964), *Biochim. biophys. Acta* 88, 466) allow a selective enrichment of either group by using filters with transmittance below or above 800 mm. Bacteriochlorophyll-b-containing purple bacteria are enriched at wavelengths above 1000 nm.

Two physiological subgroups are recognized in the purple and green sulphur bacteria with respect to their selective enrichment under high or low extremes of light intensity, sulphide concentration and temperature. Genera without gas vacuoles compete successfully at the positive extreme, while those with gas vacuoles have a selective advantage at low extremes.

The cellular arrangement and motility in the purple sulphur bacteria are controlled by sulphide concentration and light intensity. When both factors are high, all flagellated motile forms become immotile and grow as aggregates embedded in slime. The genera differ with respect to the amounts of both factors at which the cells become motile.

The formation of three-dimensional networks is characteristic for *Thiodictyon* and *Pelodictyon*. In the former the network is irregular while that of the latter organisms arises by true branching and ternary division. Whittenbury & McLee (*Arch. Mikrobiol* (1967), **57**, 324) demonstrated that in addition to *Rhodomicrobium* both *Rhodopseudomonas palustris* and *viridis* multiply by budding. Common to these forms is a photosynthetic membrane system of peripheral lamellae parallel to the long axis of the cells.

The following types of membrane extensions (continuous with the cytoplasmic membrane) were recognized in those species multiplying by binary division: (1) vesicularly intruded membrane systems: *Rhodopseudomonas rubrum*, *R. spheroides*, most Thiorhodaceae; (2) tubular membrane systems: *Thiococcus*; (3) stacks of short lamellae in the form of disc-shaped infoldings: brown *Rhodospirilla* and *Ectothiorhodospira*. The pigment-bearing elements of the green bacteria are structurally unique; the large oblong vesicular bodies are bounded by a thin membrane and attached to the cytoplasmic membrane.

Peculiar structures of blue-green alga, halobacteria and phototrophic sulphur bacteria are the gas vacuoles. Thin sections revealed groups of cylindrical vesicles with conical ends. The experimental data presently available indicate that N_2 is the vacuole gas.

Physiological characteristics. The Calvin cycle is present in all phototrophic bacteria examined. Under photo-organotrophic condition, the key enzymes of the cycle can be repressed. Most green sulphur bacteria are obligately autotrophic. The presence of ferredoxin-dependent pyruvate and ketoglutarate synthatases suggest the operation of a reversed Krebs cycle (Evans, M. C. W., Buchanan, B. B. & Arnon, D. I. (1966), *Proc. natn. Acad. Sci., U.S.A.* **55**, 928). The inability of a number of green and purple sulphur bacteria to grow in the absence of reduced sulphur compounds with H_2 as the only electron donor is due to the lack of an assimilatory sulphate reduction mechanism. The first step of the thiosulphate metabolism in *Chromatium* was shown to be the reductive cleavage of the two sulphur atoms, excluding a polythionate pathway (Smith, A. J. & Lascelles, J. (1966). *J. gen. Microbiol.* **42**, 357). The anaerobic, energy-yielding dark metabolism of *Chromatium* was shown to include a conversion of polysaccharide and sulphur to poly-β-hydroxybutyrate, CO_2 and H_2S (van Gemerden, H. (1967), thesis, Leiden).

Psychrophilic Microbes. By A. H. Rose (*School of Biological Sciences, Bath University of Technology, Claverton Down, Bath*)

The existence of microbes which are capable of growing well at near-zero temperatures has been recognized for almost a century. For a long time these microbes were regarded as curiosities—some would say exotic—because they grew at temperatures that were below the minimum for growth of mesophiles; for this reason they were termed 'psychrophiles'. It was only after the temperature characteristics of microbes from oceans, lakes, rivers and soils had been studied that it was realized that psychrophiles are not rare or exotic, and are probably more widespread than mesophilic microbes.

There are three aspects to the physiology of psychrophiles which distinguish these organisms from other microbes. Some psychrophiles—the obligate strains—have not only lower minimum temperatures but also lower maximum temperatures for growth compared with mesophiles. The physiological bases of the low maximum temperatures for growth in those obligate psychrophiles so far studied vary in different organisms, and range from temperature-sensitive lesions in the synthesis and/or activity of respiratory enzymes (Evison, L. M. & Rose, A. H. (1965), *J. gen. Microbiol.* **40**, 349) to abnormal temperature sensitivity in cytoplasmic membranes (Hagen, P. O., Kushner, D. & Gibbons, N. E. (1964), *Can. J. Microbiol.* **10**, 813).

Another curious physiological property of psychrophiles is that, among bacteria, the psychrophilic habit is very largely confined to Gram-negative organisms. Gram-positive psychrophilic bacteria have been reported (Larkin, J. M. & Stokes, J. L. (1966), *J. Bact.* **91**, 1667), but they are rare. The physiological basis of the differentiation obtained with the Gram stain is thought to reside in the size of the pores in the cell wall peptidoglycan and, since these wall components are synthesized in the cytoplasmic membrane, it would seem that the psychrophilic habit may be a reflexion of differences in the structure and function of certain membrane components. However, the nature of any such differences remains clouded in mystery.

The third and most important physiological property of psychrophiles as compared with mesophiles is the ability of the former to grow well at $0°$; most mesophiles have a minimum temperature for growth of 5 to $10°$. Two main explanations have been forwarded to explain the physiological basis of the minimum temperature for growth in mesophiles. The first of these proposes that solute uptake in mesophiles is inactivated at temperatures between 5 and $10°$, whereas these processes in psychrophiles can operate down to and perhaps below $-2°$ (Baxter, R. M. & Gibbons, N. E. (1962), *Can. J. Microbiol.* **8**, 511; Farrell, J. & Rose, A. H. (1968), *A. Rev. Microbiol.* **21**, 101). The second hypothesis is that enzymes in psychrophiles are better adapted to functioning at near-zero temperatures than are those in mesophiles, presumably because they are less easily denatured by cold (Stokes, J. L. & Larkin, J. M. (1968), *J. Bact.* **95**, 95). At present, it is impossible to state whether either one of these phenomena explains the minimum temperature for growth of a particular mesophilic microbe.

Thermophilic Bacteria. By L. L. Campbell. (*Department of Microbiology, University of Illinois, Urbana, U.S.A.*)

Thermophilic bacteria are characterized by their ability to grow at (and in many cases require) temperatures which inactivate the cellular components of most forms of life. Two general explanations have been offered. The most obvious is that the essential cell components of thermophilic bacteria are more heat stable than those of mesophilic bacteria. Another explanation is that the cells are capable of rapid resynthesis of the inactivated cellular components. The enzymes and certain cellular proteins of thermophilic bacteria can be placed into three general groups with respect to their thermal stability: (1) Those that are stable at the temperature of production but are inactivated at slightly higher temperatures; (2) those that are inactivated at the temperature of production in the absence of substrate (the substrate-protected class); and (3) those that are intrinsically heat stable.

The ribosomes of *Bacillus stearothermophilus* undergo thermal denaturation at temperatures markedly higher than those obtained from mesophilic bacteria. Data from several laboratories make improbable the possibility that the increased heat stability of thermophile ribosomes arises from ribosomal RNA per se. Differences in the primary structure of the ribosomal proteins might explain the greater thermostability of the ribosomes of *B. stearothermophilus*. The evidence available does not rule out this possibility although it appears more likely that the explanation lies in the fine structure of the RNA-ribosomal protein interactions. These possibilities are currently being studied by several investigators.

The Special Physiology of Hydrocarbon Oxidizing Bacteria. By J. R. QUAYLE (*Department of Microbiology, University of Sheffield*)

The ability to utilize hydrocarbons is a property that is widespread amongst many different kinds of micro-organism; there are, nevertheless, some generalizations which can be made about the genera which seem to predominate when the organisms are isolated by elective culture on hydrocarbon media. When alkanes are used as growth substrate, the most commonly encountered organisms appear to be species of *Brevibacterium*, *Corynebacterium*, *Mycobacterium*, *Nocardia* and *Streptomyces*, and to a less extent *Achromobacter*, *Flavobacterium* and *Pseudomonas*. Based on the extensive survey published by Fuhs (Fuhs, G. W. (1961), *Arch. Mikrobiol.* 39, 374), an examination is made of the range of hydrocarbons reported to be utilized by micro-organisms. Straight-chain alkanes containing 9–16 carbon atoms appear to be the most favoured substrates for growth. Compared with higher alkanes, growth on methane seems to be a more restricted property. The inability, so far, of any fully authenticated methane utilizer to grow on any substrate other than methane or methanol is discussed.

The feature common to all hydrocarbon substrates, by definition, is their reduction level and absence of oxygen in the molecule. Since the central clearing house of intermediary metabolism involves oxygen-containing intermediary metabolites, all metabolism of hydrocarbon substrates will at some stage involve oxygenation of the molecule. The obligatory nature of this process at an early stage (if not the initial stage) of the metabolism of the substrate thus constitutes a feature of special physiology of these organisms. Oxygenation of aromatic and aliphatic hydrocarbons by hydroxylase, mono-oxygenase and di-oxygenase enzymes, as well as the role of these enzymes in cleavage of the aromatic ring, are reviewed.

The unique properties of hydrocarbons as growth substrates may result in special features of growth kinetics and cell structure; these are areas which call for attention at the present time.

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