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THE JOURNAL OF GENERAL MICROBIOLOGY

THE PREPARATION OF PAPERS

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

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

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(1) Papers should be written with the utmost conciseness consistent with clarity. The best English for the purpose of the *Journal* is that which gives the sense in the fewest short words, spelt according to the *Shorter Oxford English Dictionary*.

(2) A paper should be written only when a piece of work is rounded off. Authors should not be seduced into writing a series of papers on the same subject as results come to hand. It is better to wait until a comprehensive paper can be written.

(3) Authors should state the objective when the work was undertaken, how they did it and the conclusions they draw. A section labelled 'Discussion' should be strictly limited to discussing the results if this is necessary, and not to recapitulation.

(4) Figures and tables should be selected to illustrate the points made if they cannot be described in the text, to summarize, or to record important quantitative results.

(5) Authors should remember that in preparing their typescript they are giving instructions to the printer (about layout, etc.) as well as attempting to convey their meaning to their readers.

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A manuscript should be submitted in double-spaced typing with wide margins, and on paper suitable for ink corrections. The paper must be written in English and should, in general, be divided into (*a*) Summary; (*b*) Introduction; (*c*) Methods; (*d*) Results; (*e*) Discussion (if any) and general conclusions; (*f*) Acknowledgements; (*g*) References.

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Description of Solutions. The concentrations of solutions are preferably defined in terms of normality (N) or molarity (M). The term '%' must be used in its correct sense, i.e. g./100 g. of solution; otherwise '%(v/v)' and '%(w/v)' must be used when the figure is larger than 1 %.

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Chemical Nomenclature. Follow the 1969 revision of *Policy of the Journal and Instructions to Authors*, The Biochemical Society, 7 Warwick Court, London, W.C.1.

Nomenclature of Enzymes. The system published in *Report of the Commission of Enzymes of the International Union of Biochemistry*, Oxford: Pergamon Press, 50*s*., is used.

Nomenclature in Bacterial Genetics. The proposal by M. Demerec, E. A. Adelberg, A. J. Clark and P. E. Hartman published in *Genetics* (1966), **54**, 61 has been reprinted in *J. gen. Microbiol.* (1968), **50**, 1 as a useful guide to authors. The general principles laid down should be followed wherever practicable.

Nomenclature and Description of Micro-organisms. The correct name of the organisms, conforming with international rules of nomenclature, must be used; if desired, synonyms may be added in brackets when the name is first mentioned. Names of bacteria must conform with the Bacteriological Code and the opinions issued by the International Committee on Bacteriological Nomenclature. Names of algae and fungi must conform with the International Rules of Botanical Nomenclature. Names of protozoa must conform with the International Code of Zoological Nomenclature. Bacteriological Code, Iowa State College Press, Ames, Iowa, U.S.A. (1958); Botanical Code, International Bureau of Plant Taxonomy and Nomenclature, 106 Lange Nieuwstraat, Utrecht, Netherlands (1952); Zoological Code, International Trust for Zoological Nomenclature, London (1961). One or two small changes have been made to these rules at later International Congresses.

The following books may be found useful:

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

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

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

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

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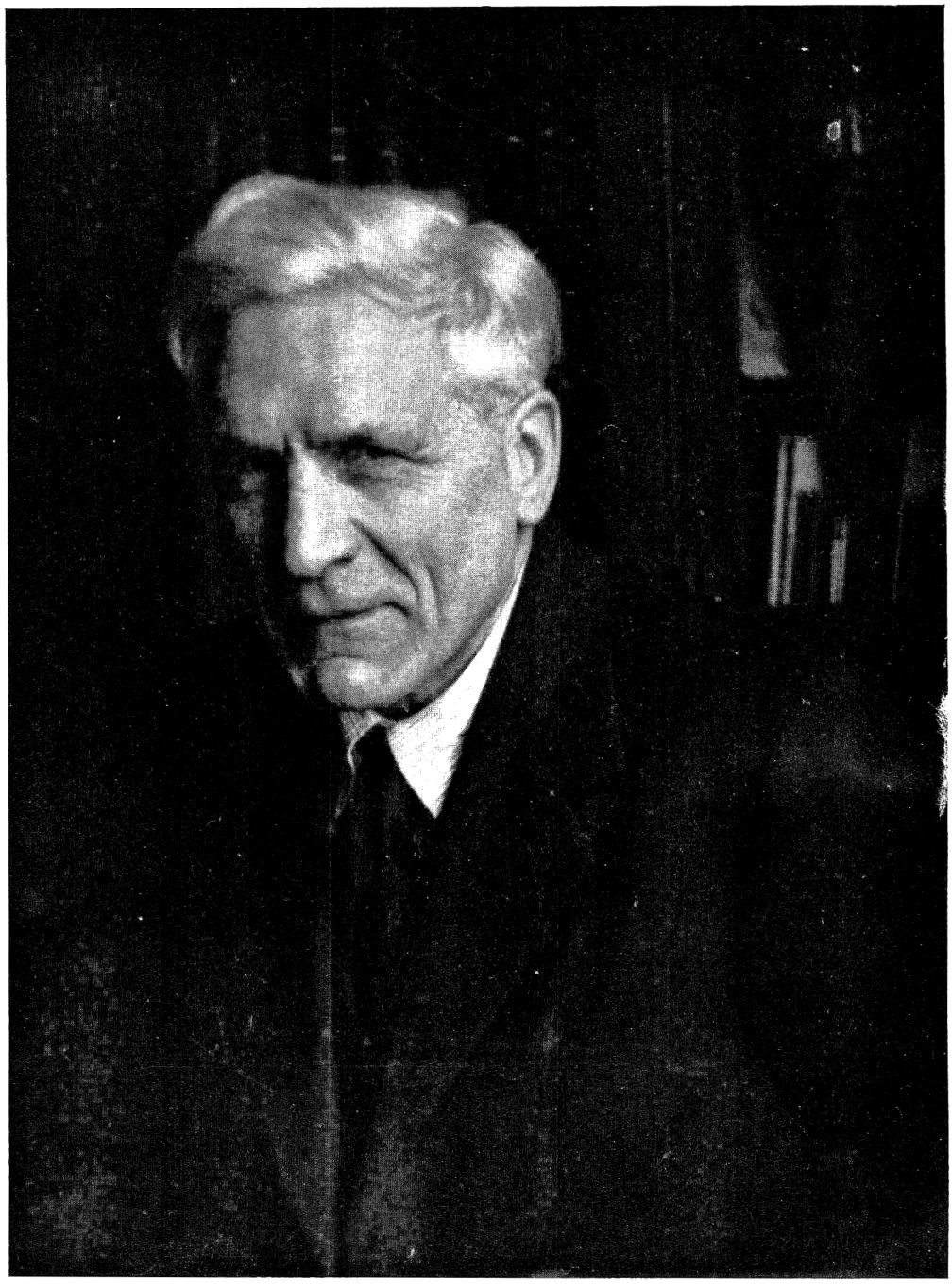

James Watt M-Leod

(*Facing p.* 1)

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With 1 plate
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The 82nd Birthday of James Walter McLeod

JAMES WALTER MCLEOD, president of the Society for General Microbiology from 1949 to 1952, was born on 2 January, 1887, at Dumbarton. He is a striking personality in many ways, and always has inspired deep affection and loyalty of those who came into contact with him.

He graduated M.B., Ch.B. with commendation from the University of Glasgow in 1908 and after holding two house appointments at Glasgow went as a ship's surgeon on a trip to India. After his return in 1909 he was appointed Coats scholar and in 1910 Carnegie scholar in the Pathology Department of Robert Muir, where he worked under Carl Browning on bacterial haemolysins with special reference to streptococcal haemolysin. In 1912 he was appointed assistant lecturer in pathology at Charing Cross Medical School from where in 1914 he joined the R.A.M.C. as temporary lieutenant, and later as captain in charge of the 8th mobile laboratory. In this capacity he successfully mixed valour with research for he was mentioned four times in despatches, was awarded the military O.B.E., and published papers on trench fever, trench nephritis, bacillary dysentery and the bacteriology of epidemic influenza. In 1919 he was appointed Lecturer in Bacteriology in the School of Medicine at Leeds, and from 1920 on was engaged in work on bacterial oxidation-reduction and respiration, particularly of anaerobes. The ten years devoted to these studies culminated in McLeod's contributions to Falk's 'Newer Knowledge of Bacteriology', 1928, and to the Medical Research Council's 'System of Bacteriology', 1930. The practical result dating from this period best known and used all over the world in diagnostic laboratories is the oxidase reaction for the recognition of the gonococcus in mixed cultures. A natural corollary to the improvement of diagnostic procedures were efforts directed towards the improvement of culture media, notably of the gonococcus and the diphtheria bacillus. In turn, the intensive use of his heated blood tellurite medium for the isolation of *Corynebacterium diphtheriae* led him to the realisation that there exist three main types of the diphtheria bacillus which he could relate to clinical severity and named accordingly. The wide interest this aroused with both favourable and contradictory reports from all countries, kept him busily engaged on this subject for nearly 20 years which were interspersed with papers on the differential diagnosis of *Clostridium oedematiens*, tetanus infection in hospital arising from dust in surgical theatres, the value of antiseptics as prophylactic applications to recent experimental wounds, the inhibiting factor of the action of sulphonamides in nutrient media, and the distribution of the whooping cough bacillus in contacts of cases. With all this activity McLeod made a major contribution to the application of scientific methods and knowledge to diagnostic bacteriological procedures from which patients and clinicians have derived great benefit.

This was also the period when J. W. McLeod received the honours of being elected a Corresponding Member of the Soc. de Biologie, a Fellow of the Royal Society, and an honorary Sc.D. of Trinity College, Dublin.

It was inevitable that a man known for his scrupulous fairness had high administrative duties thrust upon him. Thus, from 1948 until his retirement from the Chair of

Bacteriology in 1952, he was Dean of the Medical School at Leeds which left him little time for work at the laboratory bench. After a short period of rest in the Scottish countryside he returned to laboratory work in Edinburgh, where ever since he has been immersed in the bacteriological problems of chronic urinary infection of paraplegics, and of staphylococcal toxins.

J. W. McLeod's spare time has always been taken up by affairs of the Presbyterian Church and the Boy's Brigade, and in his earlier days with athletics, rugby, cricket and hockey. There are many colourful stories about him current, one of the best of which relates that he, a strict teetotaller, narrowly escaped arrest for drunken conduct in public when on a dark crisp winter's night with firm snow underfoot he went down the slope of Mount Preston from his home to the Department on a toboggan singing at the top of his voice and was stopped by a police constable. At the age of 62 when he had missed his connexion at York in the early hours of the morning, he thought nothing of walking to Leeds carrying his suitcase. A chronic hip ailment has, of late, curtailed his vigorous physical activities and has necessitated a major operation which has interrupted his bench work. All his friends, past and present colleagues and associates who have been inspired by J. W. McLeod's enthusiasm, fairness, deep humanity and generosity are glad that a turbulent but complete recovery period has enabled him to continue as an active bacteriologist.

K.E.C. and K.Z.

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Studies on *Stigmatella aurantiaca* (Myxobacterales)

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(*Accepted for publication* 6 *May* 1969)

SUMMARY

We have isolated the fruiting myxobacterium *Stigmatella aurantiaca* from wood and bark, and have examined some aspects of its ecology, morphology, physiology and taxonomy. Vegetative cells were Gram-negative, spindle-shaped rods 5·2 to 8·4 μ long and 0·65 to 0·74 μ wide. The myxospores were optically refractile short rods measuring about $3{\cdot}2 \times 1{\cdot}0$ μ. Fruiting bodies were usually bright orange or red brown and consisted of a stalk 60 to 140 μ high bearing 1 to 20 cysts at its top. Most cysts were spherical or ovoid, measuring 40 to 60 μ by 25 to 45 μ. *S. aurantiaca* was cultivated in liquid culture in a dispersed vegetative state or on solid media, producing either fruiting bodies or vegetative forms. Optimal vegetative growth was obtained with a medium containing Casitone, glucose and salts. The optimal temperature for growth was 30°. Cultures could be preserved by storage of vegetative forms at −60° or of dried myxospores or fruiting bodies at −18°. Dried myxospores were resistant to desiccation and elevated temperatures. We propose that *Stigmatella*, which had been incorporated into the genus *Chondromyces*, be re-established as a separate genus within the Polyangiaceae. We further propose the inclusion of *Chondromyces* among the Sorangiaceae.

INTRODUCTION

When Thaxter in 1892 recognized *Stigmatella aurantiaca* (*Chondromyces aurantiacus*) as a myxobacterium, it had already been described three times as a member of the *fungi imperfecti* (Berkeley, 1857; Berkeley & Broome, 1873; Kalchbrenner & Cooke, 1880). Thaxter said: 'With the exception of *Myxococcus rubescens* this is the commonest member of the group [i.e. myxobacteria] and must have been met with by anyone who has sought for Myxomycetes on decaying wood, where though very minute it is conspicuous from its bright colour'. It would be a mistake, however, to conclude from this that *S. aurantiaca* is now a familiar or well-characterized member of the fruiting myxobacteria. European workers only rarely encountered *S. aurantiaca* (Quehl, 1906; Zukal, 1897) and Krzemieniewska & Krzemieniewski wrote (1946): 'During a period of about 15 years we were not once able to observe it' (i.e. *C. aurantiacus*). They finally obtained it from rotting beech wood and pine twigs.

When, in the 1940s, the myxobacteria once again became the subject of investigations in North America, the organism no longer appeared to be abundant there either. The contradiction was eventually resolved by Nellis (1962), who confirmed Thaxter's observation that *Stigmatella aurantiaca* could easily be obtained when rotting wood was used for its isolation rather than the soil and dung used by most of the later

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investigators. Our results support these findings. When we isolated and cultivated *S. aurantiaca* and became familiar with its properties, it appeared that this organism could not be closely related to *Chondromyces crocatus* and *C. apiculatus* with which it had been united in one genus. The purposes of the present report are to document this statement, and to expand the existing description of *S. aurantiaca* (Solntseva, 1942).

METHODS

Media. Stock cultures were grown on C/10 agar: Casitone (Difco), 0·3 %; $CaCl_2$, 9 mM; agar (Difco), 1·5 % (w/v); or on yeast agar: baker's yeast, 1·0 % (fresh weight of yeast cake); $CaCl_2$, 9 mM; agar, 1·5 % (w/v). Water agar contained agar, 1·5 % (w/v); $CaCl_2$, 9 mM. Liquid cultures were kept in modified CT medium containing Casitone, 1·0 %; $MgSO_4$, 10 mM added aseptically after autoclaving. When sugars were included, these were autoclaved separately. Liquid media for testing different N-sources always included 10 mM-$MgSO_4$. *Sarcina lutea* was cultivated on yeast-extract agar containing Casitone, 0·3 %; yeast extract (Difco), 0·5 %; agar, 1·5 % (w/v). All agar media were adjusted to pH 7·2 with KOH. The liquid media were usually used at their original value of pH 6·8; when N-sources other than Casitone were used, the media were adjusted to pH 7·0. Soft agar for pour plates was C/10 agar but with 0·5 % agar.

Cultivation. All cultures were kept at 30°: the plate cultures in a dark incubator at high humidity, the liquid cultures on a reciprocal water-bath shaker (120 strokes/min.). Liquid cultures were grown either in 250 ml. conical flasks or in nephelometer flasks, both containing 25 ml. medium/flask. Growth was determined by measuring extinction in a Klett-Summerson colorimeter at 540 nm. The results obtained with this method were sometimes misleading, e.g. when the organisms lysed because of unfavourable conditions, producing a dark pigment. In doubtful cases the cultures were examined with the microscope, and, when necessary, growth was determined by counting organisms in a Petroff-Hausser chamber.

Experimental methods. For freeze-drying, organisms were suspended in skim milk, put into ampoules, shell frozen, and dried in high vacuum.

Determination of the pH optimum of growth was made in liquid medium with 0·25 % Casitone and 8 mM-$MgSO_4$, to which was added 20 mM-K + Na phosphate buffer at different pH values. To keep the concentration of monovalent cations constant, compensatory amounts of NaCl were added. NaCl alone at 20 mM had no pronounced influence on growth.

Resistance to ultrasonic treatment was tested by treating 5 ml. batches in a MSE ultrasonic vibrator at maximal energy output (about 20 kcyc.); the samples were kept in an ice bath during treatment. When treatment for several minutes was required, this was done intermittently to avoid heating. Decrease of number of organisms was examined by counting in a Petroff-Hausser chamber.

Heat resistance was determined by heating samples in a water bath. To allow rapid temperature equilibration, only 0·2 to 0·5 ml. amounts of suspensions were used, and they were put into preheated tubes. The heated organisms were streaked on C/10 agar. To test heat resistance of dry myxospores, 0·2 ml. samples of a spore suspension in modified CT medium were put in sterile tubes and dried in high vacuum for 10 hr; the spores formed a thin film on the bottom of the tube so that rapid heat transfer was guaranteed. After heating, 0·5 ml. of sterile water was added, and the resuspended

myxospores streaked on C/10 agar. These experiments were only roughly quantitative, i.e. survival was judged from growth delay and amount of growth, as compared with controls.

RESULTS AND DISCUSSION

Isolation and ecology

Rotting soft wood (Salix, Populus, Acer) and bark from fallen trees were collected during the summer and autumn months in and around Minneapolis, Minnesota. The specimens were put into large Petri dishes lined with filter paper, and were moistened with distilled water so that they were wet but not soaked. The crude cultures were incubated at 30° and examined daily with a dissecting microscope for fruiting bodies. When these appeared at all they always did so during the first few days of incubation. *Stigmatella aurantiaca* appeared quite regularly, usually forming 10 to 100 fruiting bodies on one piece of wood. It was often accompanied by Chondromyces or other less conspicuous myxobacteria.

Occasionally fruiting bodies were observed in nature. In one instance a large log showed bright orange patches consisting of tens of thousands of fruiting bodies of *Stigmatella aurantiaca*. Nellis (1962) pointed out that *S. aurantiaca* could easily be isolated from specimens which were completely dry upon collection. We found that pieces of wood with fruiting bodies could be stored dry in the laboratory for more than 1 year, and the organism readily isolated when the wood was moistened and incubated for 1 to 2 days. *Stigmatella aurantiaca* seems to live primarily in the surface layers of the decaying material; samples taken from the interior of rotting trunks did not yield cultures.

For isolation, fruiting bodies were transferred to streaks of living *Sarcina lutea* on water agar. After incubation for 1 to 2 days at 30°, small swarms had developed, which lysed the sarcinas rapidly. From the edge of these swarms transfers were made to fresh bacterial streaks. A few passages eliminated amoebae, nematodes and fungi. Invariably, however, bacterial contaminants remained within the slime layers of the swarm, in close association with the myxobacteria, as also described for Chondromyces species (Abadie, 1966). The contaminants were usually small non-motile rods carried along with the myxobacterial cells; they were found throughout the swarm, even at the rapidly migrating edge. Pure cultures of *Stigmatella aurantiaca* were obtained by three different methods. (1) Swarm material taken from C/10 agar was suspended in 0·3 % Casitone in a screw-cap tube and shaken vigorously with glass beads (0·5 to 1·0 mm.), the supernatant fluid diluted appropriately, mixed with molten soft agar and poured as a thin layer on top of a C/10 agar base. This technique is a modification of one described by McCurdy (1963). (2) Fruiting bodies, which were readily formed on streaks of *S. lutea* or when swarm material was transferred from C/10 agar to water agar, were exposed to ultrasonic treatment for 2 to 3 min. A relatively uniform suspension of myxospores was then obtained which could be plated as above. The ultrasonic treatment also killed many of the contaminating organisms. (3) Fruiting bodies were suspended in sterile distilled water, heated in a water bath at 57° for 15 to 20 min. and then transferred to C/10 agar. While myxospores survived the heat treatment, the contaminants were usually all killed (Solntseva, 1939). From several pure strains, one clone, croCl 1, obtained by method 1, was chosen for detailed study.

In our experience *Stigmatella aurantiaca* did not appear in cultures prepared from dung or soil, which yielded a variety of other closely related myxobacteria. Other investigators have reported similar results (Krzemieniewska & Krzemieniewski, 1946; Nellis, 1962). Though *S. aurantiaca* has been found in soil and on dung (Agnihothrudu, Barua & Barua, 1959; Krzemieniewska & Krzemieniewski, 1927; Krzemieniewska & Krzemieniewski, 1930; Nellis, 1962; Solntseva, 1942) it seems to prefer rotting wood or bark as a habitat; at least it can be obtained from these sources much more readily. The basis for this preference is not clear, especially since our physiological studies with pure strains have shown that *S. aurantiaca* is very similar in its requirements and properties to *Podangium erectum* and *Polyangium fuscum*, both of which are quite commonly isolated from soil and dung. Like the other fruiting myxobacteria, *S. aurantiaca* can be cultivated easily on C/10 agar or yeast agar; it readily forms fruiting bodies on these agars, is strongly bacteriolytic, and does not produce cellulases.

Cellular morphology

The vegetative forms of *Stigmatella aurantiaca* are spindle-shaped rods, 5·2 to 8·4 μ long and 0·65 to 0·74 μ wide (Pl. 1, fig. 1). Their shape is different from that of the cylindrical blunt-ended vegetative rods of *Chondromyces crocatus* (Pl. 1, fig. 2), and a *Sorangium* sp. (Pl. 1, fig. 3). They resemble the vegetative forms of Podangium and the somewhat longer and more slender forms of Podangium.

Vegetative forms of *Stigmatella aurantiaca* readily formed spheroplasts, e.g. in old liquid cultures, or when suspended in CsCl solution (above 10 mM). We did not purify the CsCl used and its toxicity may have been due to contaminating materials. In contrast to *Myxococcus xanthus* (Mason & Powelson, 1958), *S. aurantiaca* did not form spheroplasts on exposure to 10 mM-Na^+ or K^+.

The myxospores of *Stigmatella aurantiaca* were optically refractile short rods, often slightly bent, with rounded ends (Pl. 1, fig. 4, 5). In this respect as well, it differs from Chondromyces and Sorangium species, where the rods within fruiting body cysts appear very similar to the vegetative forms. Myxospores from fruiting bodies of *S. aurantiaca* measured about 3·2 × 1·0 μ (2·6 to 3·5 × 0·9 to 1·2 μ) but were occasionally even shorter and fatter. They are surrounded by a thin capsule as shown by electron microscopy (Reichenbach, Voelz & Dworkin, 1969), whereas the myxospores of Chondromyces are not encapsulated (McCurdy, 1967). The fine structure of vegetative forms and myxospores of *S. aurantiaca* has been described elsewhere (Reichenbach *et al.* 1969).

Fruiting-body structure

Fruiting bodies of *Stigmatella aurantiaca* consisted of a stalk 60 to 140 μ high and bearing 1 to 20 cysts at its top (Pl. 2, fig. 6). The fruiting body was 130 to 200 μ high. The cluster of cysts had a diameter of 120 to 220 μ.

The stalk, whose width ranged between 35 and 100 μ, usually broadened considerably toward its base. Sometimes it formed a narrow board-like ridge rather than a tapering column (Pl. 2, fig. 7). It was only rarely branched, but often several stalks arose from a common base. The stalk consisted largely of hardened slime, in which some cells were always embedded. It was sometimes unpigmented and opaque, but was often yellowish or brownish. Occasionally, rudimentary fruiting bodies bearing only a single cyst were formed (Pl. 2, fig. 8).

The cysts were spherical, ovoid, pear-shaped or subcylindrical, and usually narrowed at the base into a white slime pedicel about 10 μ wide and 10 to 20 μ long, or sometimes longer. Occasionally, no pedicel was developed. The dimensions of the cysts varied from 25 to 102 × 16 to 70 μ; most cysts measured 40 to 60 × 25 to 45 μ. The fruiting bodies were usually bright orange or red brown, but the pigmentation varied from light yellow-brown to dark chestnut-brown or almost black. This variability in cyst size and colour was observed with the same strain even under apparently identical culture conditions.

The cyst was surrounded by a tough wall which had an irregularly wrinkled surface. All the pigment seemed to be contained in this wall. The myxospores inside the cyst were embedded in a slimy matrix and not arranged in any specific pattern. Electron-microscope observations on the fruiting body structure of *Stigmatella aurantiaca* will be published later (Voelz & Reichenbach, in preparation).

Swarm structure

The swarm of *Stigmatella aurantiaca* is typical for the Podangium–Polyangium group. It developed an irregular edge (Pl. 2, fig. 9) and lacked a pronounced marginal ridge. The swarm showed well-developed radial streams and often a pattern of oscillating waves which were originally thought to be typical for the Myxococcaceae (Reichenbach, 1965). Although the majority of cells were concentrated in the marginal area, the central parts of the swarm, especially along the radial streams, remained densely populated even at late stages of swarm development. The cells deposited a tough slime layer, but the surface of the agar was only slightly changed. The swarm sometimes appeared unpigmented; however, when the cells were concentrated, a pink pigmentation was visible. On C/10 agar older swarms were often light to dark brown, and on yeast agar, yellow or yellow brown.

The swarms of Chondromyces and Sorangium species are quite different (Reichenbach, Kuczka & Heunert, 1964). There the vegetative cells concentrate at the relatively smooth swarm edge often forming a heavy ridge, which may be bright yellow or orange. These swarms, at least in their central parts, are usually sunk into the agar forming a shallow pit, with the cells generally penetrating into the substrate. There is no tough slime layer, but the agar surface is heavily etched, deep holes and trenches often being produced by the activity of the cells.

Cultivation and growth requirements

Stigmatella aurantiaca grew quite well on two of the standard media for myxobacteria (C/10 agar, yeast agar) though fruiting bodies were not formed on either substrate. Fruiting bodies were readily obtained when swarm material was transferred to a streak of living *Sarcina lutea* on water agar. When these cultures were incubated at 30°, the early stages of fruiting-body formation were observed after 10 to 15 hr and mature fruiting bodies were present after 24 hr. The sarcinas as well as the yeast cells in yeast agar were lysed by the myxobacterium, producing large clear zones.

Stigmatella aurantiaca also grew in liquid CT medium shaken at 30°. In the initial cultures most of the population grew in clumps, but after a few passages growth occurred as an even suspension. This was true of all three strains tested, though the number of passages required to yield dispersed growth differed. Once a strain showing dispersed growth was established, it could also grow in static liquid culture and

still form a uniform suspension. Such strains showed a completely different swarm morphology when transferred back to agar plates from a liquid culture; the cells spread only slowly over the substrate, the swarms appeared soft and slimy, without the typical surface pattern, and no fruiting bodies were formed when transfers were made to water agar. Even after many transfers from plate to plate, reversion to the original type was not observed.

Suspension cultures of strains which give dispersed growth were used to study the nutritional requirements of *Stigmatella aurantiaca* in more detail. Optimal growth was obtained with a medium containing: 1 % Casitone, 30 mM-glucose or 20 mM-sucrose, 12·5 mM-$MgSO_4$, 10 mM-PO_4 buffer (pH 7·2). Omission of sugar and phosphate resulted in a relatively small decrease in growth. The generation time at 30° was 7 to 8 hr for both variants of the medium. In unbuffered sugar-free Casitone medium the pH was shifted during growth from 6·8 to 7·8. Soon after the stationary phase was reached, the cells lysed and the culture became black within 1 to 2 hr. When sugars were present, the pH decreased to 5·8 to 6·2, and the culture in stationary phase remained brownish or became bright ochre. At this stage the cells usually converted to myxospores, presumably because of the acid produced. Induction of myxospore formation will be described in a subsequent paper (Reichenbach & Dworkin, in preparation).

Growth of *Stigmatella aurantiaca* required a complex N-source which was best provided by enzymic hydrolysates of protein, e.g. Casitone. The optimal concentration was 1 %. At 2 % (w/v), as used in CT medium for *Myxococcus xanthus* (Dworkin, 1962), growth was inhibited and there was a sharp decrease below 0·5 % (Fig. 1). Compared with Casitone under the same conditions, much less growth was obtained with Tryptone (Difco) or vitamin-free Casitone (Difco), and no growth at all with Casamino acids (Difco) or L-asparagine (20 mM with 15 mM-sucrose). Addition of 30 mM-glucose or 20 mM-sucrose to Casitone medium resulted in higher population densities and a decrease from pH 7·0 to pH 5·8 to 6·2 in the culture during growth. This effect was unexpected, as growth of *M. xanthus* was not improved by sugars (Dworkin, 1962), nor could we find any response to glucose with a variety of strains of *Myxococcus fulvus*, *M. virescens*, *M. xanthus*, *Chondrococcus coralloides* and *C. macrosporus*.

When glucose was added to Casitone medium before sterilization growth was markedly inhibited, as compared with cultures containing either separately autoclaved glucose or without glucose. However, there was still considerable development, and the pH value became almost as low as in the control with separately sterilized glucose, indicating that some glucose was metabolized.

As with other myxobacteria, growth of *Stigmatella aurantiaca* depends on the presence of divalent cations in fairly high concentrations. In plate cultures Ca^{2+} fulfilled this requirement. In liquid suspension cultures, however, only Mg^{2+} was used, since Ca^{2+} caused the cells to clump and settle on the glass walls of the flask. The optimal concentration of Mg^{2+} in 1 % Casitone medium was about 12·5 mM (Fig. 1).

It was not necessary to add phosphate to the Casitone medium, since Casitone contains sufficient phosphate; pH 7·0 to 7·2 was optimum for growth; there was no growth at pH 6·4 and a marked decrease at pH 6·6; there was still good growth at pH 7·6 (Fig. 1).

The optimal temperature for growth was about 30°; some growth was possible at a temperature as high as 37°.

The poor growth of *Stigmatella aurantiaca* in vitamin-free Casitone suggested a requirement for growth factors. However, growth was not improved by addition of yeast extract, mixtures of water-soluble vitamins or of trace elements. It is possible that the vitamin-free Casitone used contained a growth inhibitory substance.

Preservation of cultures

Vegetative swarms of *Stigmatella aurantiaca* on C/10 agar or yeast agar usually lost viability after 2 to 3 weeks of incubation at 30°. Liquid cultures in the absence of acid production lysed after 3 to 4 days. A convenient method for preserving myxobacterial cultures was accordingly sought. Storage of cultures at 4° was not effective even though growth and swarming ceased. When fruiting bodies were formed, however, successful transfers could be made from cultures kept for 14 months at 4°.

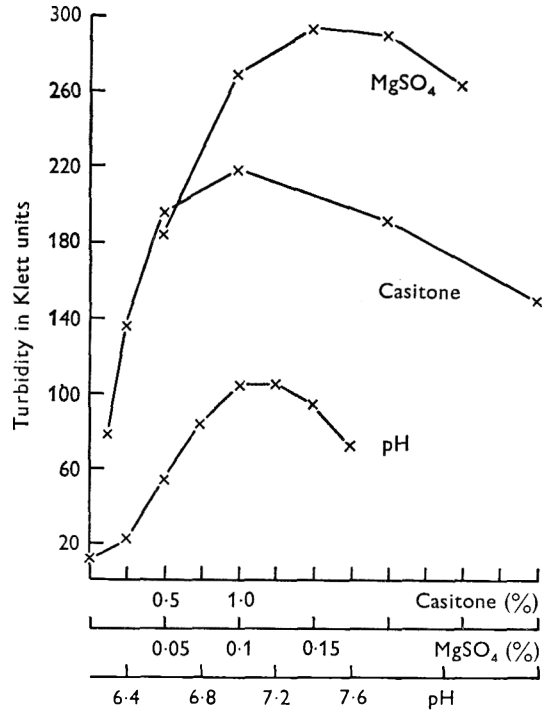

Fig. 1. Growth response of *Stigmatella aurantiaca* suspension cultures to different pH values and concentrations of Casitone and $MgSO_4$. Growth was determined turbidimetrically after incubation for 55 hr. Media: (*a*) different concentrations of Casitone + 0·1 % $MgSO_4$, pH 6·8; (*b*) 1 % Casitone + various concentrations of $MgSO_4$, pH 6·8; (*c*) 0·25 % Casitone + 0·1 % $MgSO_4$ + 0·02 M-phosphate buffer at various pH values.

Excellent results were obtained with freezing. Samples (2 ml.), from liquid cultures in the exponential phase of growth, were placed in screw-cap tubes, frozen immediately in solid CO_2 + acetone and stored at −60°. These cultures were thawed at room temperature and inoculated into liquid medium; within 24 hr dense cultures had usually arisen. The organism remained viable in the frozen state for at least 12 months. It was important, however, that the suspension be frozen rapidly and stored at very

low temperatures; cells thawed after a few days at $-20°$ invariably lysed. Freezing was also used to store slope agar cultures. Swarms on C/10 agar slopes quick-frozen and stored at $-60°$ were still viable after 18 months. The freezing and thawing process caused the agar to become quite soft, but the swarm sheet remained intact and could easily be removed. It should be emphasized that both freezing techniques are effective for the preservation of most myxobacteria, e.g. Myxococcus, Chondrococcus, Polyangium and Podangium species, with the notable exception of *Chondromyces apiculatus* (the only Chondromyces species tested) and Sorangium species. *Chondromyces apiculatus* swarms (four strains) barely survived a few months in the frozen state while Sorangium swarms were always killed by freezing and thawing unless fruiting bodies were present. Freeze-drying was unreliable for the preservation of *Stigmatella aurantiaca* as well as for other fruiting myxobacteria.

The most convenient and effective method for the preservation of *Stigmatella aurantiaca* or fruiting myxobacteria in general was the storage of dry myxospores or fruiting bodies. A dense suspension of myxospores dropped on small pieces of filter paper in a Petri dish was dried in a desiccator. (Myxospores of *S. aurantiaca* could be obtained by the glycerol technique devised for *Myxococcus xanthus*; Dworkin & Gibson, 1964). The dried specimens were placed in screw-cap tubes and stored at room temperature or in a freezer. Myxospores thus kept at $-18°$ were viable after 17 months.

Fruiting bodies could be grown directly on filter paper; pieces of filter paper (about 25×15 mm) were laid on water agar and inoculated heavily with swarm material from a growth culture. Within 24 hr fruiting bodies were formed, which were then allowed to mature for 3 to 5 days. The filter-paper strips were placed in screw-cap tubes and dried *in vacuo*. Such fruiting bodies were still viable after 20 months at room temperature.

Resistance properties of myxospores

Vegetative forms of *Stigmatella aurantiaca* were very sensitive to ultrasonic treatment. Starting with $4{\cdot}5 \times 10^8$ cells/ml., less than 1 % remained viable after treatment for 30 sec. When myxospores were treated under comparable conditions, 70 % remained viable after 1 min., 30 % after 3 min. and 5 % after 6 min.

Vegetative forms of *Stigmatella aurantiaca* were quickly killed upon drying, even under careful conditions of freeze-drying. Myxospores, on the other hand, were completely resistant to drying. A similar situation exists with *Myxococcus xanthus* (Sudo & Dworkin, 1969). Myxospores can be stored in the dry state for months without losing their capacity to germinate when moistened. It seems likely that the resistance to drying is an important biological function of myxospores, for myxobacteria are frequently in environments which periodically become dry. Heating a suspension of *S. aurantiaca* myxospores at 51° for 15 min. killed all the myxospores; however, when they were heated in the dry state they easily withstood temperatures of 61° and 68° for 15 min., and were killed slowly at 74° and 83°; a few myxospores even survived 15 min. at 90°. A similar difference in heat resistance between dry and wet spores was shown for *Myxococcus fulvus* (Baur, 1905). Thus, it is clear that dry myxospores are easily capable of withstanding any temperatures they may be exposed to in their natural environment.

Fruiting-body formation

Fruiting-body formation by *Stigmatella aurantiaca* could be induced by transferring swarm material from growth-supporting agar media, e.g. C/10 agar or yeast agar, to

water agar or to filter paper placed on water agar. When such plates were incubated at 30°, the differentiation of cysts had begun by 15 hr. The cysts were intially translucent, but soon became pigmented and opaque; at about 24 hr the fruiting bodies appeared to be mature.

It was not possible to maintain ability to form fruiting bodies in a culture which had been repeatedly grown and transferred on rich media which prevented fruiting. This loss of fruiting ability by a culture which had been maintained on rich media is an unexplained phenomenon common among the myxobacteria.

Taxonomy

Stigmatella aurantiaca is presently included in the genus *Chondromyces* as *Chondromyces aurantiacus*. *Stigmatella aurantiaca* differs from *Chondromyces crocatus*, the type species of the genus, in the shape of the vegetative cells, in the way myxospores are formed (in Chondromyces there is either no visible difference between vegetative cells and myxospores, or at most a slight shrinkage), in swarm morphology, and even in some characteristic features of the otherwise quite similar fruiting bodies. We therefore propose that these two organisms be classified in separate genera.

The genera *Stigmatella* and *Chondromyces* were proposed by Berkeley (1857), for organisms which he assumed to be *fungi imperfecti*; the original descriptions consisted only of the line drawings of the type species, *Stigmatella aurantiaca* and *Chondromyces crocatus*. These drawings as well as a later description (Berkeley, 1874) were based on specimens collected by P. Ravenel in South Carolina, U.S.A., and kept in the Curtis herbarium. Berkeley also described *S. aurantiaca* a second time as a new species of Hyphomycetes, *Stilbum rhytidosporum* (Berkeley & Broome, 1873). A third description of the same organism came from Kalchbrenner & Cooke (1880), who presented it as a new species and a new genus, *Polycephalum aurantiacum*, believed to be closely related to the fungal genus *Stilbum*.

Stigmatella aurantiaca was recognized as a myxobacterium by Thaxter (1892), who also realized that this organism was identical with the various '*fungi imperfecti*' mentioned above. The identity was later confirmed by Massee (1893), who examined the original material on which the various descriptions were based.

Thaxter united the genera *Chondromyces* and *Stigmatella*, eliminating the name *Stigmatella* which had been published after *Chondromyces*. This merger was based entirely on the similarity of the fruiting bodies of both organisms. Thaxter incorrectly described the vegetative cells of *C. crocatus* as 'cylindrical or tapering slightly' (1892). The fact that there are two rather different cell types among myxobacteria was realized only much later by Krzemieniewska & Krzemieniewski (1928). They also called attention to the similarity between the vegetative cells of *Chondromyces crocatus* and Sorangium and to the fact that *Stigmatella aurantiaca* cells are different from both, though their statement that 'such cells [as in *C. crocatus*] are not to be found in any other *Chondromyces* species' (1946) is not correct.

If the genus *Chondromyces* is split, the original generic name of *Stigmatella* once again becomes valid. It is unlikely that this would cause any nomenclatural confusion as the generic name was used only once again, i.e. by Saccardo (1886), for *Sphaerocreas pubescens* = *Stigmatella pubescens*, an unjustified synonomy which was subsequently rejected by Thaxter (1892). There are some fungal genera with similar names, as

Stigmatea Mont., *Stigmea* Fr., *Stigmella* Lev., and *Stigmatula* Sacc., but these names are sufficiently different from *Stigmatella* to exclude confusion.

The genus *Stigmatella* may then be redefined as: myxobacteria with vegetative cells which have tapering ends, and which change considerably in shape during myxospore formation; myxospores short fat rods, optically refractile, with a thin capsule; fruiting bodies consist of a slime stalk (which is not branched and does not exceed 200 to 300 μ in length), bearing a cluster of cysts at its top; cysts with a tough cyst wall. Type species: *Stigmatella aurantiaca* Berk. & Curt. 1857.

The genus *Chondromyces* comprises: myxobacteria with vegetative cells which have blunt, rounded ends, of the Sorangium type; they change little in size and shape during myxospore formation; myxospores look like vegetative cells (have no capsule); fruiting bodies with slime stalks (500 μ or more long, with longitudinal streaks), branched or unbranched, with clusters of cysts at the top; cysts with tough cyst wall. Type species: *Chondromyces crocatus* Berk. & Curt. 1857. (Characters given in parentheses have still to be examined with more strains and species before their taxonomic weight can be validated). Three other species which undoubtedly belong to the genus *Stigmatella* have been named, namely *C. brunneus* (Krzemieniewska & Krzemieniewski, 1946), *C. cylindricus* (Krzemieniewska & Krzemieniewski, 1930), and *C. medius* (Krzemieniewska & Krzemieniewski, 1930). These three species are supposed to differ from *Stigmatella aurantiaca* in shape, size, and colour of their cysts. However, in the light of the variability we found with our strains, we believe that the above mentioned species can be regarded at best as varieties of *S. aurantiaca*.

Stigmatella aurantiaca is closely related to *Podangium erectum* as realized by both Thaxter (1897) and the Krzemieniewskis (1927). Actually, *S. aurantiaca* sometimes produces atypical fruiting bodies with single cysts on top of the cystophore (Pl. 2, fig. 8). These can hardly be distinguished from fruiting bodies of *P. erectum*. Thus, locating the genus *Stigmatella* within the family Polyangiaceae has a sound basis. Within this family, the genus *Stigmatella* has the most complex fruiting bodies and marks the end of a continuous morphological sequence which begins with the unstalked cysts of *Polyangium fuscum*. The genus *Chondromyces* on the other hand may be justifiably transferred into the family Sorangiaceae, for the genera *Chondromyces* and *Sorangium* have much in common: both share the same type of vegetative cells; in both, myxospores do not differ morphologically from vegetative rods; both have a similar growth pattern, with swarms sinking into the agar to form shallow pits with pronounced marginal ridges. Even the pigmentation is often the same—a bright yellow orange which is unusual among the myxobacteria. The similarity in fruiting-body structure between the genera *Stigmatella* and *Chondromyces* can probably be regarded as a result of evolutionary convergence.

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EXPLANATION OF PLATES

PLATE 1

Fig. 1. Vegetative cells of *Stigmatella aurantiaca*. Living cells, *in situ* on agar surface in moist chamber. Several dividing cells can be seen. The flexibility of the rods is evident. Total magnification × 2800. Phase-contrast. Zeiss microscope.

Fig. 2. Vegetative cells of *Chondromyces crocatus*. Living cells, *in situ* on agar surface in moist chamber. Total magnification × 2190. Phase-contrast. Zeiss microscope.

Fig. 3. Vegetative cells of a cellulose-decomposing *Sorangium* strain. Living cells, *in situ* on agar surface in moist chamber. Total magnification × 1820. Phase-contrast. Zeiss microscope.

Fig. 4. Myxospores of *Stigmatella aurantiaca*. Living cells, formed on agar surface in moist chamber, *in situ*. Total magnification × 2750. Phase-contrast. Zeiss microscope.

Fig. 5. Myxospores of *Stigmatella aurantiaca*. Living cells on agar surface in moist chamber, *in situ*. With the × 40 objective the high optical refractility of the myxospores becomes apparent. Optical refractility is not obvious in fig. 4, which was taken with a × 100 oil-immersion objective. Total magnification × 1125. Phase-contrast. Zeiss microscope.

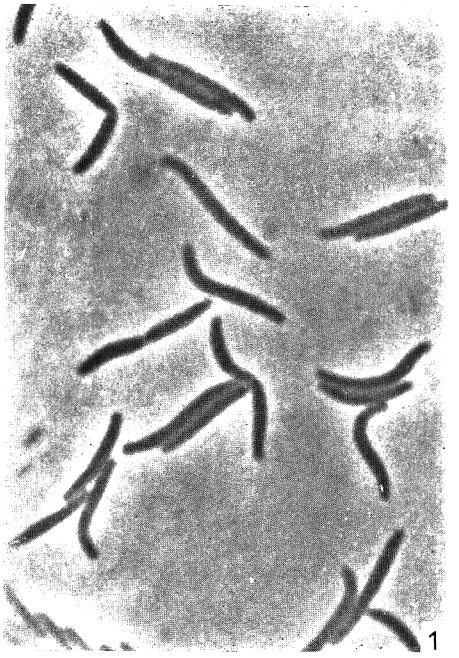
PLATE 2

Fig. 6. Fruiting body *Stigmatella aurantiaca*. On agar surface. Pure culture. Total magnification × 340. Zeiss microscope.

Fig. 7. Atypical fruiting body of *Stigmatella aurantiaca*. With a flat board-like stalk. On agar surface. Pure culture. Total magnification × 200. Zeiss microscope.

Fig. 8. Rudimentary fruiting body of *Stigmatella aurantiaca*. With one single cyst on top of slime stalk. On agar surface. Pure culture. Total magnification × 420. Zeiss microscope.

Fig. 9. *Stigmatella aurantiaca*, swarm edge. Pure culture grown on plain water agar. Total magnification × 95. Zeiss microscope.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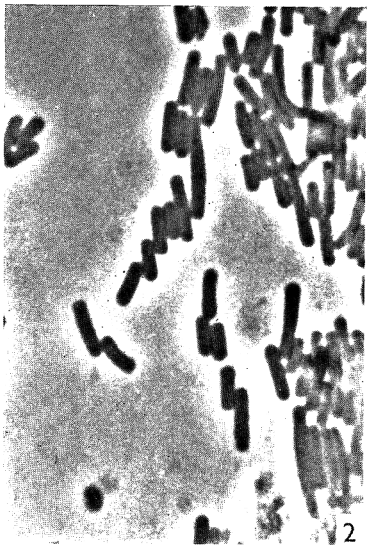
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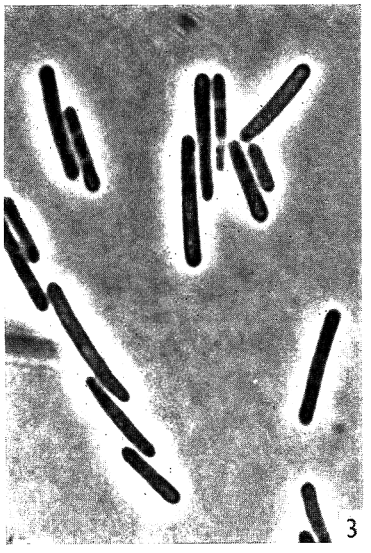
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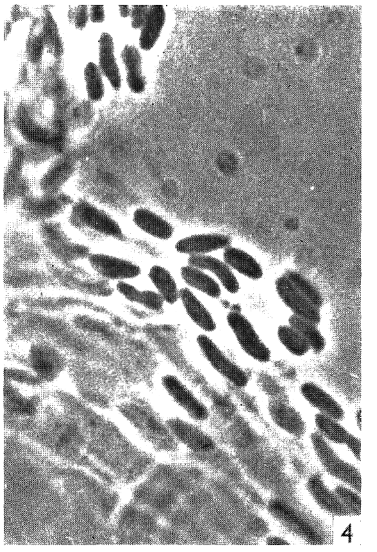
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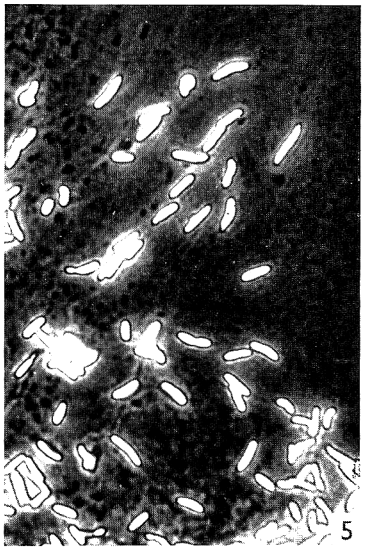
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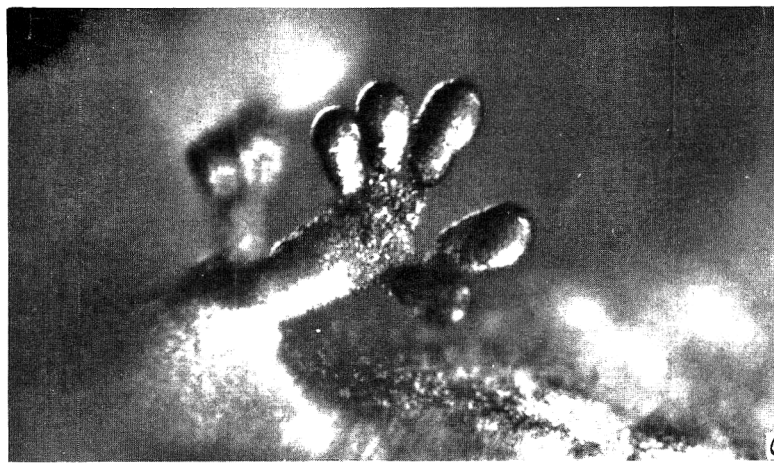
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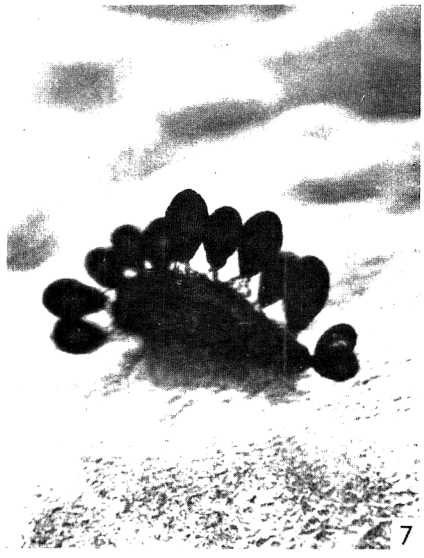
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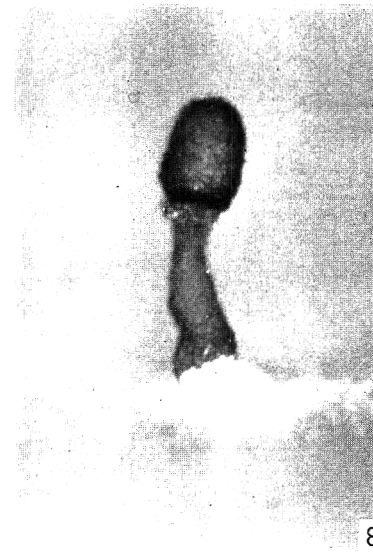
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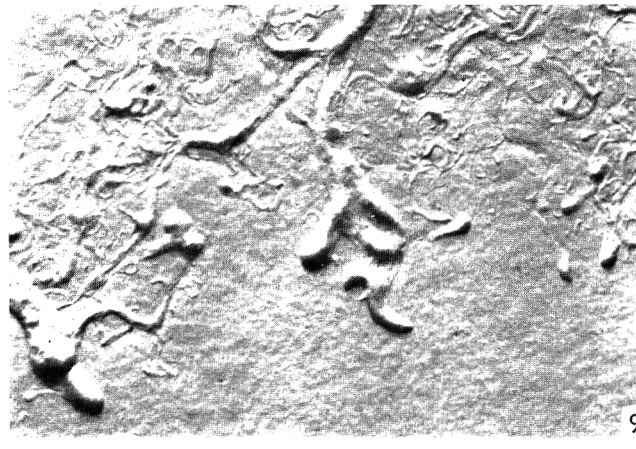
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Thiamine Limited Steady State Growth of the Yeast *Cryptococcus albidus*

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SUMMARY

The concentration/growth rate relationship of thiamine-requiring *Cryptococcus albidus* was examined. Data suggest a diffusion-limited mechanism characterized by an apparent Michaelis constant for growth of $4{\cdot}7 \times 10^{-13}$ M. This relationship was obtained from continuous culture at steady state and tested by a non-steady state procedure and in batch growth. It was concluded that most natural water systems have sufficient thiamine to support some thiamine-requiring micro-organisms at above rate limiting concentrations.

INTRODUCTION

While the concentration controlled rate of thiamine incorporation has been studied in yeast (Ziro, 1955) and bacteria (Neujahr, 1966), the concentrations of vitamins over which vitamin-limited growth rates of heterotrophs are controlled remain in question. Vitamins are present in most natural, terrestrial and marine systems, and their distribution in sea water has been measured (Holm-Hansen, Strickland & Williams, 1966; Neujahr, 1966; Natarajan & Dugdale, 1966). One result of the rather ubiquitous distribution of vitamins in measurable quantities is that most species of micro-organisms absorb part of their vitamin-coenzyme complement from the surroundings and consequently develop vitamin requirements. The facility with which micro-organisms incorporate required vitamins becomes one factor in nutritional chemistry upon which the resulting ecosystem depends. The following paper describes steady state growth rates of a marine yeast, *Cryptococcus albidus*, with respect to thiamine concentration.

METHODS

Organism and cultivation. Cryptococcus albidus, the thiamine bioassay organism of Natarajan & Dugdale (1966), was selected because of its thiamine requirement, marine origin and amenability to continuous culture. Growth was in a 500 ml. reactor vessel supplied with a glucose + mineral salts medium at a controlled rate from a 20-l. carboy at 25° as described elsewhere (Button, 1969*a*). Although oscillations in the organism population density occurred under some nutritional conditions, such as manganese deficiency (D. K. Button, unpublished data), the thiamine-limited steady state was smooth and constant.

Thiamine-free glucose was prepared by irradiating the required amount of recrystallized glucose in a small quartz tube for 1 hr with an ultraviolet lamp. The half-life of thiamine is of the order of minutes under these conditions (Button, 1968). The feed

carboy and mineral salts were subjected to several hours of irradiation. Purified glucose was added to 100 mg./l., and the medium immediately autoclaved.

Limiting substrate. A stock solution of thiamine, which is stable in acid solution (Koser, 1968), was autoclaved for 5 min. at pH 2, 121°, and kept in 10^{-7} M solution. Sterility tests were occasionally made by spreading a sample on nutrient agar plates. Bioassay showed that freshly prepared thiamine solution had the same activity as 2-year-old stock. The stock solution was diluted as required and added directly to the feed carboy.

Population measurements. Populations were determined by standard plate count techniques and by electronic counting and sizing (Coulter Counter Model B, Hialeah, Florida). Normally 10 to 15 volumes were allowed to pass through the reactor in the process of establishing the steady state values reported.

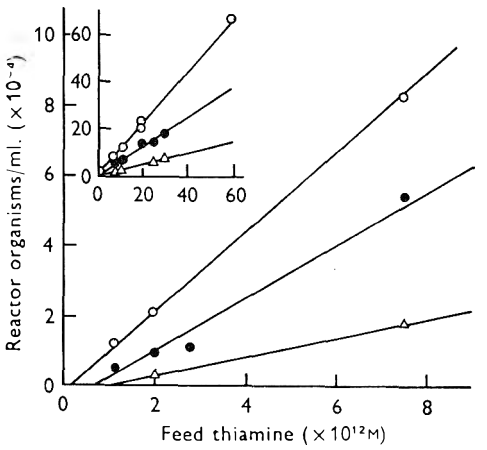

Fig. 1

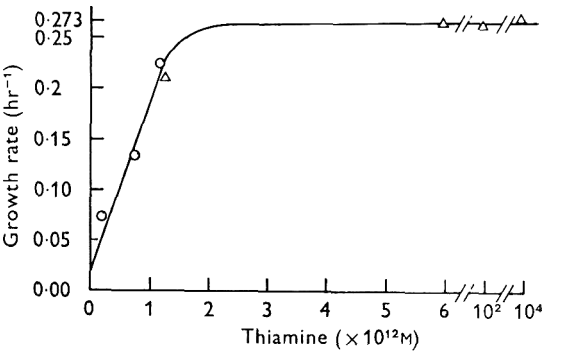

Fig. 2

Fig. 1. *Cryptococcus albidus*. Steady state thiamine limited reactor populations at dilution rates equivalent to 30%, ○; 50%, ●; and 80%, △, of a dilution rate equivalent to the maximum growth rate of 0·273 hr^{-1}. The inset extends the concentration range of the data.

Fig. 2. Relationship between thiamine concentration and growth velocity of *Cryptococcus albidus*. Circles represent intercepts from Fig. 1. Triangles are taken from washout data according to equation (3).

RESULTS

Thiamine concentrations controlling growth velocities in their mid-velocity range were determined by the zero population extrapolation point by using a plot of input thiamine versus steady state population (Button & Garver, 1966). Figure 1 shows the data used for extrapolation at growth rates corresponding to 30, 50, and 80% of μ_{max}, the maximum growth rate of *Cryptococcus albidus* under conditions specified. The extrapolated values, equivalent to extracellular thiamine concentrations, were 1×10^{-13}, 6×10^{-13} and 8×10^{-13} M, respectively. A complete set of data over a large concentration range is shown in the inset. Intercepts of the population data on the substrate axis are replotted in Fig. 2.

This determination of extracellular thiamine concentrations at various growth rates was tested in two ways. The first method was designed to yield concentration informa-

tion at velocities approaching the maximum growth rate. After an initial steady state had been reached, the reactor population was adjusted to some low value with respect to thiamine concentration by an increase in feed rate for a suitable period. After obtaining the desired population density, the feed rate was readjusted to a value near the expected growth rate, and a period of several hours was allowed for growth rate stabilization. Then the rate of reactor population change was measured and the growth rate calculated from the usual equation describing population growth in a perfectly mixed continuous flow reactor diluted by sterile feed:

$$dx/dt = \mu X - rX, \tag{1}$$

where μ is the specific growth rate and r the feed rate into the reactor divided by its volume, each having units of time^{-1}. Integrating between initial and final organism concentrations, X_0 and X, over a time interval, t, yields the exponential relationship

$$X = X_0 e^{(\mu - r)t}, \tag{2}$$

where $\mu - r$ is the slope of a line describing ln X with respect to time. If the dilution rate is set approximately equal to the expected growth rate then μ can be determined to the same order of accuracy as the feed rate, since the reactor is filled and its volume fixed. The pump-controlled feed rate variation was less than 1 %. Extracellular thiamine concentration is simply the difference between the input thiamine concentration, S_0, and the organism concentration, X, times the appropriate organism yield, γ, as shown in equation (3).

$$S = S_0 - X/\gamma. \tag{3}$$

Table 1 relates organism yield to thiamine used. These data were obtained from electronic counter size distributions and yields calculated from the slopes of the lines from Fig. 1.

Table 1. *Cryptococcus albidus: influence of specific growth rate (dilution rate) on organism size and growth yield from thiamine*

Dilution rate (% μ_{max})	Organism volume (average)	Thiamine molecules/organism	g. organism/ g. thiamine
15	—	$1{\cdot}7 \times 10^4$	—
30	38 μm^3	$5{\cdot}3 \times 10^4$	$29{\cdot}0 \times 10^5$
50	40 μm^3	$9{\cdot}0 \times 10^4$	$18{\cdot}0 \times 10^5$
80	70 μm^3	$24{\cdot}0 \times 10^4$	$1{\cdot}2 \times 10^5$

Growth rate data obtained by this method and corrected for thiamine used according to Table 1 are shown as triangles in Fig. 2. The growth rate data at high thiamine concentrations determined from batch growth curves were identical with those shown in Fig. 2 above 6×10^{-12} M. Concentrations of thiamine as high as 1×10^{-6} M showed no inhibition. An example of a non-steady state experiment at lower thiamine concentrations is shown in Fig. 3. This experiment yielded data in the region of the Michaelis constant and is plotted at 0·21 hr^{-1} in Fig. 2. Although this type of experiment was designed for precision at higher growth velocities, the agreement between these data and the extrapolation data shown as circles in Fig. 2 is reasonably good. The data reported in Fig. 2, when computed fit to a hyperbola, yielded an apparent Michaelis constant for growth, K_s, of $4{\cdot}7 \times 10^{-13}$ M with a variance of $0{\cdot}4 \times 10^{-13}$ M and a maximum growth velocity of 0·273 hr^{-1} with a variance of 0·012 hr^{-1}.

Steady state nutrient limited cultures normally respond to a change in limiting

nutrient concentration by a major change in division rate. This phenomenon was used to test the reported Michaelis constant by a second method. A small number of organisms from a steady state thiamine limited culture growing at half-maximum rate was introduced into 100 ml. sterile quartz tubes containing various concentrations of thiamine in a medium which had been purified by irradiation. Care was taken to effect a rapid transfer of organisms from steady state and to avoid temperature fluctuation. The cultures were shaken in an incubator at 25° and the rate of population change measured as shown in Fig. 4. The growth rate of the inoculum in continuous culture was 0·132 hr^{-1}. Of the thiamine concentrations used, that which effected the least change in growth rate after rapid transfer to the batch system was 1×10^{-13} M.

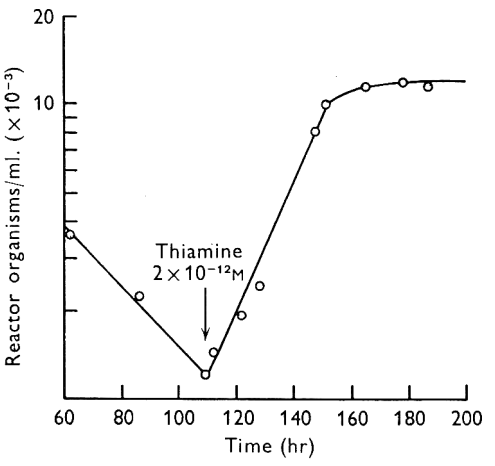

Fig. 3

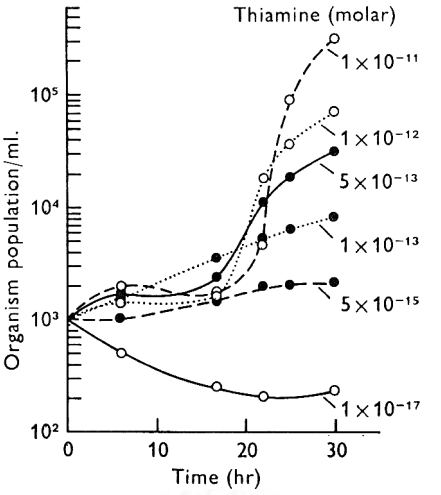

Fig. 4

Fig. 3. *Cryptococcus albidus*. Reactor population, dilution rate 0·132 hr^{-1}. The arrow indicates a change of feed thiamine level from 5×10^{-13} M to $2{\cdot}5 \times 10^{-12}$ M.

Fig. 4. Batch growth curves of *Cryptococcus albidus* at initial thiamine concentrations indicated. An inoculum of 1000 organisms/ml. was obtained from a steady state continuous culture diluted at a rate of 0·132 hr^{-1}.

The resulting growth rate with this concentration was 0·099 hr^{-1}, midway between the inoculum growth rate from continuous culture and the value 0·041 hr^{-1} predicted by the Michaelis–Menten equation using K_s reported above. These batch growth rates are thus compatible with the Michaelis constant determined at steady state from continuous culture data. Higher concentrations consistently produced non-steady state behaviour as shown by the non-linearity of the exponential curve in Fig. 2. After the lag induced by the increase in limiting substrate the growth sustained a rate greater than μ_{max} for a few divisions. This new division rate also follows a saturation curve with respect to initial thiamine concentration. The concentration producing the half maximal rate for this phenomenon is 1×10^{-12} M.

Effects of temperature, pH value and mixing energy on the growth velocity/concentration relationship were examined by arranging a thiamine limited steady state population in continuous culture at sufficiently low population density so that a significant portion of the thiamine supplied by the feed remained in the extracellular

phase. Changes in the concentration/velocity relationship are then reflected by changes in the steady state organism population. When the feed of a continuous culture system at pH 4 was titrated to pH 6 while in operation at half maximum growth rate, no change in the extinction value 0·004 the population density was observed.

The effect of temperature on the population is shown in Table 2. The growth rate was set at 80 % of μ_{max} at the lower temperature so that the population over the whole range of temperatures could be observed at the same specific growth rate, 0·218 hr^{-1}. The decrease in population density at the highest temperature is accounted for by a shift of the size distribution toward larger organisms at the higher temperature. These data showed no increase in mass of organism derived from a given thiamine concentration with increasing temperature.

Table 2. *Cryptococcus albidus: influence of temperature on number of organisms/ml. (growth rate = 0·80* μ_{max})

Temperature	Organisms/ml.
17·0°	24,400
25·0°	18,600
29·5°	8,488

Table 3. *Cryptococcus albidus: effect of power input (mixing efficiency) on organism concentration with growth rate = 0·80* μ_{max}

Watts per l.	Organisms/ml.
0·1	9,520
1·4	15,120

A change in reactor stirring rate did, however, affect the total population. Table 3 shows the population under identical concentrations of thiamine limited steady state growth, but at different stirring rates of 856 and 85 rev./min. Stirring was provided by a 1-in. (2·54 cm.) teflon-coated magnet. Growth rate was half maximum and agitation was changed by varying the speed of the magnetic stirrer as described by Borkowski & Johnson (1967). A significant difference in population density was consistently observed with changes in agitation power input.

DISCUSSION

The value of $4{\cdot}7 \times 10^{-13}$ M was established as the mid-range for thiamine concentration controlled growth rate of *Cryptococcus albidus*. This value was confirmed by two independent methods, since the concentrations involved were much lower than those usually measured when examining growth kinetics; the results were compatible with the value reported. Concentration data in the region of μ_{max} were fairly precise and suggested deviation from this hyperbolic relationship. Although the data in the region of the Michaelis constant were less precise, the data in this region are best fit with a straight line as shown in Fig. 2 and are suggestive of a diffusion limited mechanism. Deviation from the origin is within the experimental error reported; however, this deviation can also reflect the degree of contamination by background thiamine. The concept of a diffusion limited mechanism was supported by the fact that changes in

temperature or pH value did not affect the velocity of the rate limiting step. More energetic mixing did however increase the efficiency of thiamine absorption. As reported elsewhere (Button, 1969*b*), the effective concentration of thiamine in the bulk of the medium is approximately doubled by mixing in this diffusion limited system over the normal growth rate range of the yeast. Diffusion to and across the cell wall appears to be sufficient at the concentrations reported to provide the thiamine flux observed. Borkowski & Johnson (1967) provided a useful derivation and discussion concerning oxygen supply which can be applied to this calculation.

Short-term division rates were significantly faster than the long-term value of μ_{max} when a thiamine limited culture was supplied with excess thiamine. This suggests the accumulation during thiamine limited growth of some component whose supply is normally the rate limiting step. The Michaelis constant for growth is directly related to μ_{max}, hence the short-term high growth rate is associated with a slightly larger Michaelis constant.

Most organic nutrients are actively transported into the organism (Müller, 1967). If there is an initial common carrier (Hengstenberg, Egan & Morse, 1967) one can calculate a lower limit for the amount of carrier required to give a given flux at the reported concentrations of substrate (Amdur & Hammes, 1966). By using the upper limit for bimolecular reactions in liquids of 10^9 M^{-1} sec.$^{-1}$ and a carrier molecular weight of 30,000 the observed flux would require that the organism be composed of only 0·01 % of this carrier. A carrier concentration of around 1 % would bring the reaction rate down to within the range of many enzymes. Thus the Michaelis constant of $4{\cdot}7 \times 10^{-13}$ M is reasonable for either a diffusion or an active transport mechanism, even though it is a lower concentration than usually considered in nutritional chemistry.

Although other workers (Jannasch, 1967; R. D. Hamilton, personal communication) have reported that the yield constants in their systems were not constant, we did not find that to be the case, as shown by the linearity of the data shown in Fig. 1. The yield constant does change with growth rate because larger amounts of the coenzyme are required to catalyse the higher metabolic rates at the faster growth rates, but it is constant at a given growth rate.

Concentrations of thiamine in the oceans are in the range of 10^{-10} to 10^{-9} M (Natarajan & Dugdale, 1966; Carlucci & Silbernagel, 1967). In some regions the concentration ranged below their limits of detection of about 10^{-13} M. This is still 100 times the thiamine concentration of kinetic control for *Cryptococcus albidus*. Thus where thiamine is an important factor in the ecology of heterotrophic systems it is probably in a range below current means of analytical detection.

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An Ultrastructural Study of the Hyphae, Endoconidia and Chlamydospores of *Thielaviopsis basicola*

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(*Accepted for publication* 12 *May* 1969)

SUMMARY

The cell wall of hyphae of *Thielaviopsis basicola* consisted of an inner transparent layer and an outer electron-dense layer. The cell wall of the endoconidia was usually composed of a single transparent layer; at the tip of the endoconidia distal from the phialide the cell wall protruded into the cytoplasm. Hyphal tubes that contained endoconidia were devoid of cytoplasmic components. The only cytoplasmic component recognizable in the phialide was the endoplasmic reticulum. Two complex cell-wall systems were observed in chlamydospores. An outer cell wall enclosed the whole chlamydospore and each individual compartment possessed its own separate cell wall. Both cell walls were composed of an outer very electron-dense layer and an inner layer which appeared to be electron-transparent.

INTRODUCTION

This study of *Thielaviopsis basicola* (Berk. and Br.) Ferraris was undertaken in an effort to determine the ultrastructure of the hyphae and two types of propagules (endoconidia and chlamydospores) produced by this fungus. *T. basicola*, which was first described by Berkeley & Broome in 1850, is a soil-inhabiting parasite that causes serious black root rot in the seed beds of European tobacco growers and also damages plants in the field. The conidia of *T. basicola* develop in the interior of the phialide, are successively pushed into a tube-like hypha and are eventually liberated through the apex of this hypha (Brierley, 1915).

Chlamydospores are characterized by a thick cell wall that encloses chains of cells or compartments that contain cytoplasmic components in high densities. In *Thielaviopsis basicola* chlamydospores may be formed either terminally or laterally on hyphae. Patrick, Toussoun & Thorpe (1965) demonstrated that disruption of a chlamydospore upon maturation is a prerequisite for germination of the individual compartments. Christias & Baker (1967) reported that this disruption can be induced by the action of the enzyme chitinase.

METHODS

Organism. Thielaviopsis basicola strain 653, classified by Stover (1956) as a brown form of *T. basicola*, was chosen for study because it forms numerous chlamydospores and endoconidia in short periods of time. It is stable in cultures and is pathogenic to tobacco. Our stock was obtained from the fungus collection of the Tobacco Research

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Centre SOTA, Nyon, Switzerland. It was originally isolated in June 1967 from rootlets of Burley var. SOTA 27 grown in Corcelles, Switzerland.

Media. The organism was isolated from tobacco seedlings on the VDYA-PCNB medium of Papavizas (1964). Malt agar medium (2 % malt extract and 2 % Difco agar) was used for the maintenance of stocks and also for culture of the cellular material used for electron microscopy.

Cultural conditions. The fungus was grown in Petri dishes at 25°. After incubation for 4 days a dark halo formed approximately 1 cm. from the point of inoculation at the centre of the Petri dish. The mycelia in this area were of the type that contains endoconidia. A portion of this halo was placed in warm (40°) 2 % agar solution which solidified immediately and thus prevented dispersion of the endoconidia.

Microscopy. Small blocks cut from the agar were kept in phosphate-buffered 2 % $KMnO_4$ (0·1 M, pH 7·4) for 2·5 hr at 4° then washed in the buffer and allowed to stand 4 hr in the last wash.

For electron microscopy the blocks were dehydrated in a series of acetone + water solutions, stained with uranyl acetate, and embedded in Vestopal W by the method of Kellenberger & Ryter (1958). Ultrathin sections were cut with glass knives on a Porter-Blum ultramicrotome and mounted on Parlodion-covered grids. All observations were made with an Hitachi HS-7S electron microscope.

RESULTS

Light microscopy

Under the light microscope, unstained endoconidia could be seen within the hyphal tubes (Pl. 1, fig. 1). The walls of these cells appeared to be structurally distinct from the wall of the hyphal tube. Hyphal tubes from which the endoconidia had been liberated appeared as empty cylinders.

Chlamydospores, composed of several thick-walled compartments, were attached either at intercalary positions or at the tip of the hyphae that gave rise to them (Pl. 1, fig. 2). Some were also observed to be lateral to hyphae. A few hyaline basal cells were visible between the hyphae and the dark spores of the chlamydospores.

Electron microscopy

Hyphae. The hyphal cytoplasm contained mitochondria, nuclei and a well developed endoplasmic reticulum (Pl. 1, fig. 3). These structures were similar to those described for other fungi (Moore, 1965). One differentiating feature was the ultrastructure of the hyphal cell wall which was composed of two portions: an outer electron-dense layer and an inner transparent layer.

Endoconidia and free conidia. The fine structure of endoconidia and liberated conidia was identical (Pl. 1, fig. 4; Pl. 2, fig. 5, 6, 7). The cell wall of conidia lacked the electron-dense portion seen in the cell walls of hyphae. The cell walls of some conidia had a slightly electron-dense outer portion (Pl. 2, fig. 6) but the electron-density of this portion was always considerably less than that of hyphal cell walls. In sections cut parallel to the longest axis of a conidium one centrally located nucleus could be seen flanked usually by two vacuoles and clusters of vesicles at the tips of the cell (Pl. 1, fig. 4; Pl. 2, figs. 6, 7). These vesicles, which contain an unknown storage

product, appear as blank white spaces in permanganate-fixed material. Near the vacuoles and groups of vesicles, at the tip of the endoconidium that is distal to the phialide, the cell wall protruded into the cytoplasm (Pl. 1, fig. 4; Pl. 2, fig. 5, 6).

Hyphal tubes. The hyphal tubes that contained endoconidia were composed of cells which were always devoid of cytoplasmic components (Pl. 2, fig. 5, 6, 7). The walls of these cells were never attached to the walls of the endoconidia and were identical to the cell walls of vegetative hyphae.

Phialides. The phialides are large structures enclosed in the hyphal tubes (Pl. 2, fig. 6). Their cell walls were essentially the same as those of the endoconidia. Their endoplasmic reticulum was clearly recognizable, but no other cytoplasmic components were visible. The cell walls of the phialides appeared to be essentially the same as those of the endoconidia. No evidence was ever found of an attachment between the wall of the phialide and the wall of a hyphal tube.

Chlamydospores. Two complex cell-wall systems were observed in chlamydospores. An outer cell wall enclosed the whole chlamydospore and thus surrounded all the compartments contained within. Each individual compartment possessed its own separate cell wall (Pl. 3, fig. 8, 9, 10). The cell walls which surrounded the whole chlamydospore and the individual compartments were composed of at least two layers, an outer very electron-dense layer and an inner layer which appeared to be electron-transparent.

Plate 3, fig. 8 shows the characteristic density of the cytoplasmic components. This particular section transversed four compartments of the chlamydospore. A nucleus is visible in one of the cells, as well as numerous mitochondria. The endoplasmic reticulum is very extensive and is composed of parallel strands that are separated by an electron-transparent lumen (Pl. 3, fig. 9).

Plate 3, fig. 10 shows an electron micrograph of a 4-day-old chlamydospore with an intercompartmental pore that permits the exchange of cytoplasm between the two adjacent cells.

DISCUSSION

The exact significance of the protrusion of the cell wall into the cytoplasm at the tip of the endoconidia distal from the phialide is not definitely known. It may be a remnant of previous connection with a neighbouring conidium during formation.

The outer wall of each chlamydospore surrounds several individual compartments, each of which has its own cell wall. The function of the surrounding outer wall may be to retain the integrity of the chlamydospore until maturation and subsequent liberation of the individual compartments. Separation of compartments is necessary for germination (Patrick *et al.* 1965). It seems probable that the outer wall breaks down when the compartments mature. Since chitinase is able to bring about the disruption of chlamydospores (Christias & Baker, 1967), one can theorize that the surrounding outer wall may be more susceptible to the action of this enzyme than are the walls of the individual compartments.

The pore present between the compartments of young chlamydospores allowed cytoplasmic exchange between compartments. It is quite possible that these pores do not exist in older chlamydospores. The fact that the fine structure of these pores is identical with that described by Moore & McAlear (1962) in Ascomycetes lends support to the hypothesis that Thielaviopsis is the imperfect form of an Ascomycete.

The content of the vacuoles and vesicles usually present at each end of the endoconidia is still unknown.

Hyphal tubes in which endoconidia were seen always lacked cytoplasmic components. This lack is similar to that seen in the host mycelia of the intramycelial mycelia of the 'clock' mutant (Lowry & Sussman, 1966) and the intraconidial conidia of 'spray' mutant (DelVecchio & Turian, 1968) of *Neurospora crassa*.

The phialide, observed as a rather large structure enclosed in the hyphal tube surprisingly seemed to be composed of moribund cytoplasm. However, traces of the endoplasmic reticulum were found.

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EXPLANATION OF PLATES

Light micrographs of *Thielaviopsis basicola*. Abbreviations used: *B* = basal cells of chlamydospores; *C* = conidiophore; *EC* = endoconidia; *HT* = hyphal tube which encloses endoconidia; *P* = phialide; *Cc* = compartments of chlamydospores.

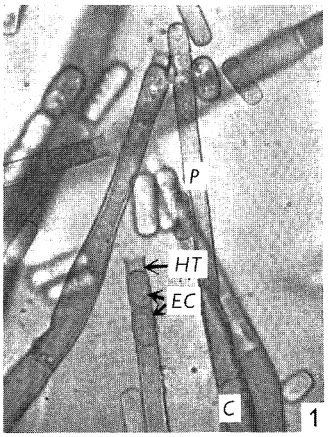
Electronmicrographs. Abbreviations used: *CIC* = chlamydospore inner cell wall; *COC* = chlamydospore outer cell wall; *ER* = endoplasmic reticulum; *HIC* = hyphal inner cell wall portion; *HOC* = hyphal outer cell wall portion; *M* = mitochondria; *N* = nucleus; *P* = phialide; *Pr* = protuberance of cell wall into the conidial cytoplasm; *SV* = storage vesicles; *V* = vacuole.

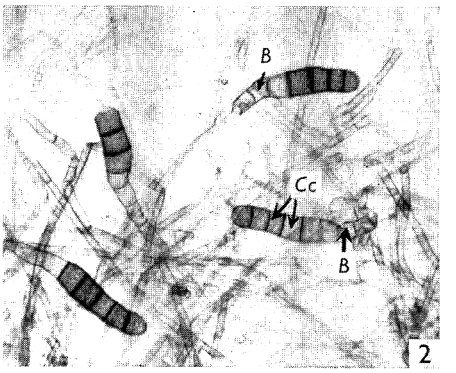
PLATE 1

Fig. 1. Endoconidia (*EC*) enclosed in hyphal tube (*HT*). A phialide (*P*) can be noted at the base of the hyphal tube, and immediately beneath the phialide a conidiophore (*C*). × 635.

Fig. 2. Chlamydospores which contain individual compartments (*Cc*) and also basal cells (*B*). × 250.

Fig. 3. Cell wall of a hypha composed of an electron-dense layer (*HOC*) and an inner electron-transparent layer (*HIC*). × 12,250.


P
HT
EC
C
1


B
Cc
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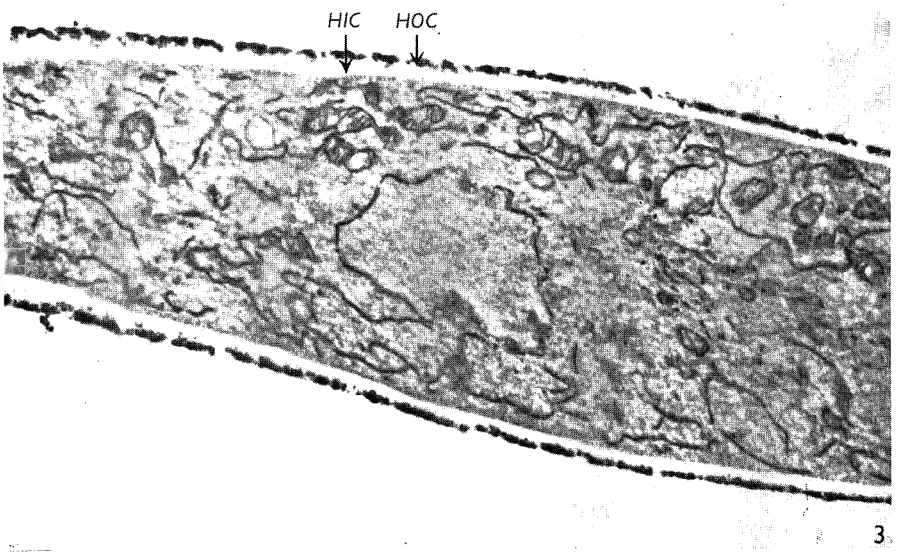
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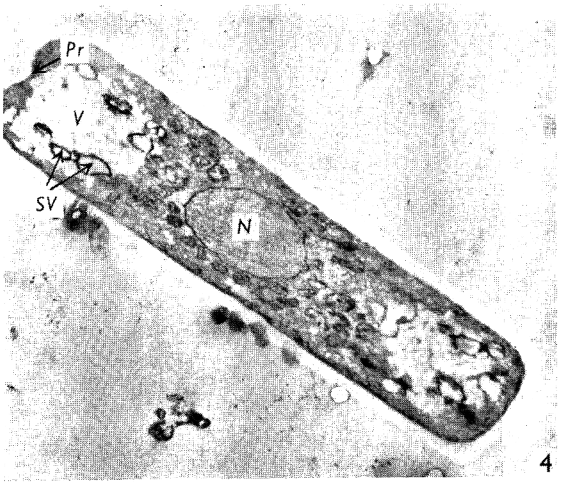
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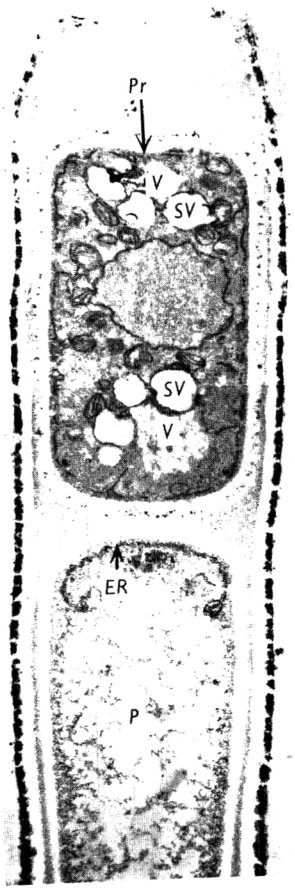

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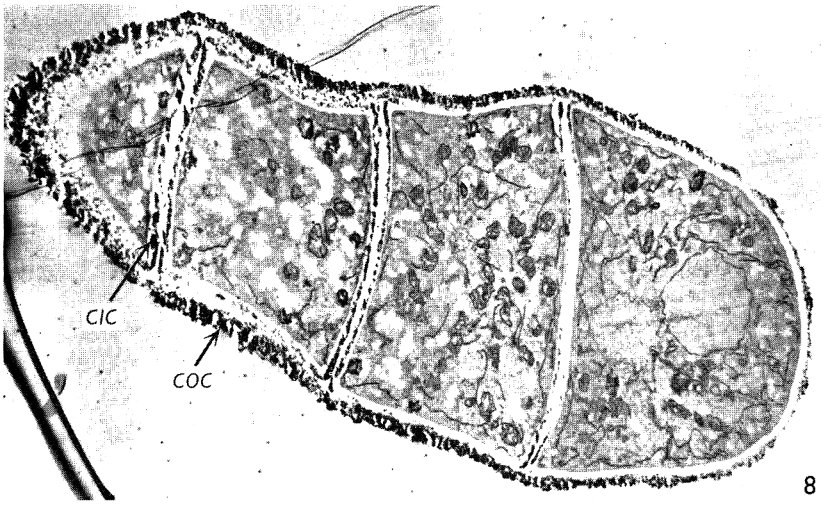

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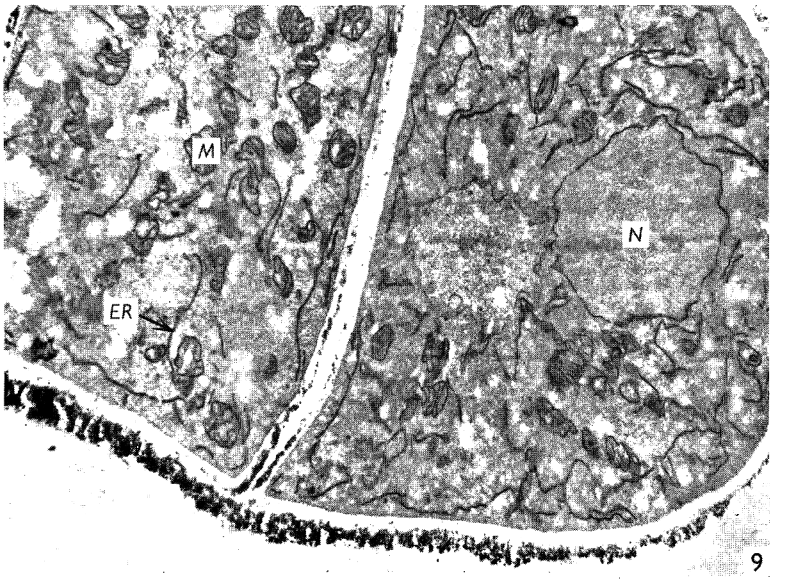

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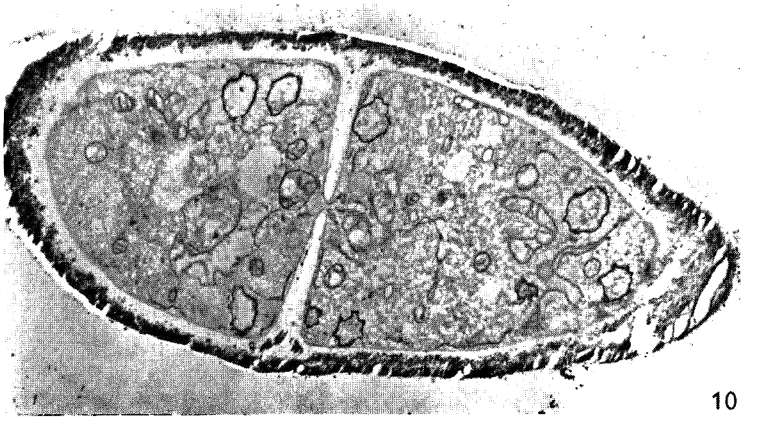

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Fig. 4. Liberated conidium with a centrally located nucleus (*N*) flanked by vacuoles (*V*) and storage vesicles (*SV*). Note the protuberance (*Pr*) of the cell wall into the cytoplasm. The wall appears as a single transparent layer. × 8750.

PLATE 2

Fig. 5. Section of an endoconidium contained in a hyphal tube. The protuberance (*Pr*) found in many conidia can be seen. The cell wall of the endoconidium is distinct from that of the hyphal tube which resembles the cell wall of normal vegetative hyphae. The hyphal tube surrounding the endoconidium lacks cytoplasmic components. × 14,350.

Fig. 6. An endoconidium at the apex of its hyphal tube. A slight protuberance (*Pr*) can be seen in the conidium, as well as vacuoles (*V*) and storage vesicles (*SV*). Traces of endoplasmic reticulum are visible in the phialide (*P*) that is situated just under the endoconidium. The outer portion of the cell wall of the endoconidium is slightly darkened. × 11,550.

Fig. 7. An endoconidium in process of leaving its hyphal tube. The space characteristically seen between endoconidia is clearly shown. × 9800.

PLATE 3

Fig. 8. Section through four compartments of a chlamydospore. Each compartment has its own cell wall (*CIC*); the entire chlamydospore is enclosed in an outer cell wall (*COC*). × 6800.

Fig. 9. The cytoplasm of a chlamydospore containing nucleus (*N*), mitochondria (*M*) and an extensive endoplasmic reticulum (*ER*). × 11,550.

Fig. 10. Chlamydospore with a pore between two compartments. × 9540.

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The Supply of Cholesterol and Fatty Acids for the Growth of Mycoplasmas

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SUMMARY

Mycoplasma strain Y, serologically and biochemically related to *Mycoplasma mycoides*, grew well in a medium containing unesterified fatty acids, cholesterol and some samples of fatty acid-poor bovine serum albumin. With other samples of serum albumin, growth was accompanied by cellular lysis. Heating albumin solutions, treating them with charcoal, or varying the ratio of fatty acids to albumin, the concentration of cholesterol, or the order in which the lipid components were added to the medium, all influenced the amount of lysis. These effects may be due to varying interaction between fatty acids and cholesterol and between fatty acids and albumin. When fatty acids are firmly bound to albumin, their interaction with cholesterol is prevented, resulting in a cholesterol deficiency. Lysis was prevented by the addition of a heat-stable defatted serum protein fraction (fraction C), or a pronase digest of fraction C, both of which dispersed cholesterol.

INTRODUCTION

Media of partially defined composition for growth of *Mycoplasma mycoides* strain V5, and Mycoplasma strain Y were described as media A and B (Rodwell, 1956, 1960; Rodwell & Abbot, 1961). These media contained a defatted serum protein fraction (fraction C). Deficiencies of glycerol, cholesterol or fatty acids resulted in marked morphological changes, cell death and lysis (Rodwell & Abbot, 1961). Medium C (Rodwell, 1967) containing defatted bovine serum albumin (BSA) and a pronase digest of BSA as undefined components, gave good growth of strain Y, and was used to study the fatty acid growth requirements. The problem of supplying fatty acids in a non-toxic form, and of cholesterol in an available form, is considered further in this paper.

METHODS

Organism. Strain Y, isolated from a goat (Laws, 1956), is serologically and biochemically related to *Mycoplasma mycoides*, but differs in that its growth is not improved by aeration.

Growth assays. Mycoplasmas for inoculating cultures were grown in BVFOS medium (Turner, Campbell & Dick, 1935). The cultures were centrifuged, and the mycoplasmas suspended in an equal volume of 0·02 M-tris-HCl buffer containing 0·01 M-$MgSO_4$ (TM). The suspensions were diluted 1/10 or 1/100 in TM and one drop of diluted suspension containing approximately 10^7 or 10^6 viable particles was

inoculated into 5 ml. volumes of test media in aluminium capped, 18 mm. diameter, optically matched test tubes.

Measurement of growth. Growth was measured at intervals during incubation at 37° by turbidity at 660 mμ. In some experiments, where it was suspected that turbidity might be an unreliable measure of growth, the synthesis of DNA was also measured by the incorporation of ^3H thymidine as described by Byfield & Scherbaum (1966). Samples of the cultures (90 μl.) were absorbed on discs of Whatman 3 MM filter paper, which were collected in cold 5 % (w/v) trichloracetic acid solution, and processed together at the end of the experiment.

Cultures were examined microscopically by dark-field illumination to assess the effects of nutrition on cellular morphology. Experience has shown (Rodwell & Abbot, 1961) that extensive lysis (as revealed by electron microscopy) during growth of Mycoplasma cultures was always accompanied by a decreased turbidity, an increased viscosity—probably due to liberated DNA—and a tendency of the cells to aggregate into a sticky deposit.

Composition of media. Medium C was described previously (Rodwell, 1967).

Medium C1 had the same composition except that L-alanyl-L-alanyl-L-alanine (L-ala$_3$) replaced the pronase digest of bovine serum albumin (BSA), and glutamic acid, aspartic acid, cystine, biotin, leucovorin and vitamin B_{12} were omitted. It had the following composition: $Na_2HPO_4 + NaH_2PO_4$, pH 7·8, 0·14 M; KCl, 0·01 M; $MgSO_4$, 0·001 M; glucose, 0·04 M; glycerol, 5 mM; spermine, 0·1 mM; cholesterol, 0·08 mM; sodium oleate, 0·05 mM; sodium palmitate, 0·05 mM; 'fatty acid poor' bovine serum albumin (BSA), 0·8 g./l.; adenine, guanine and uracil, 10 mg./l. each; thymine 5·0 mg./l.; coenzyme A, riboflavin, nicotinamide and thiamine, 1·0 mg./l. each; DL α-lipoic acid, 0·1 mg./l.; L-asparagine, L-glutamine, L-arginine, L-lysine, L-histidine, L-leucine, L-isoleucine and L-proline, 1·0 mM each; DL-phenylalanine, DL-valine, DL-methionine, DL-serine, DL-threonine, DL-tryptophane and glycine, 2·0 mM each; L-cysteine, 1·3 mM; L-ala$_3$ 0·3 mM. Glucose, riboflavin, BSA, glutamine and cysteine were added from sterile solutions to previously autoclaved medium. Glutamine and cysteine solutions were freshly prepared. Cholesterol was added as an aqueous dispersion prepared by the addition of 1 vol. of a solution in ethanol to 19 vol. stirred water at 65°. Coenzyme A was added before autoclaving from a 0·1 mg./ml. solution containing dithiothreitol 1·0 mg./ml. in phosphate buffer, pH 7·0.

In medium C2, L-alanine (1 mM) was substituted for L-ala$_3$, the concentration of cholesterol was increased to 0·1 mM, 'fatty acid poor' BSA was replaced by charcoal treated BSA (BSA-Ch) and pronase digest of fraction C (PC) 0·4 g./l. was added.

Materials. Fraction C was prepared as described by Rodwell & Abbot (1961). Carbohydrate reacting in the anthrone reaction was not detected in the product. A washed lipid extract (Folch, Lees & Sloane Stanley, 1957) of one batch contained 0·38 μg. atom of phosphorus and a total of 0·9 μmole fatty acids (mainly palmitic, stearic and oleic acids) per g. fraction C. After extraction with chloroform + methanol + N-hydrochloric acid (13·3 + 6·7 + 1·0), the washed lipid extract contained a total of 2·6 μmoles fatty acids per g. fraction C, while after hydrolysis of fraction C in 6 N-HCl for 16 hr at 100°, 8·2 μmoles fatty acids per g. were extracted. If these were present in phospholipids, this would correspond to a total of approximately 0·3 % phospholipid,

most of which is firmly bound. The total phosphorus content was 12·0 μg. atom per g. fraction C.

Pronase digest of fraction C (PC) was made by incubating a solution of fraction C (100 mg.) at pH 7·5 with 1·0 mg. pronase (Calbiochem, Los Angeles, California) at 37° for 24 hr. The pH was readjusted to 7·5, another 1·0 mg. pronase added and incubation continued for a second 24 hr period.

Samples of human and bovine serum albumin were obtained from Calbiochem; Pentex Incorporated, Kankakee, Illinois; Armour Ltd., Eastbourne, England; and Sigma Co., St Louis, Missouri. 'Fatty acid poor BSA' was manufactured by Pentex Incorporated. All these were depleted of bound fatty acids either by Goodman's (1957) method or by charcoal treatment (Chen, 1967). The charcoal (Nuchar C190) was treated *in vacuo* in 10 N-HCl for 1 to 2 hr and washed with water. Charcoal could then be removed from albumin solutions by filtration. The treated solutions (BSA-Ch) were sterilized by filtration through a Seitz filter pad which had been extracted successively with chloroform + methanol, ethanol + acetic acid, ethanol and water. Protein was determined in the filtrate either by the method of Lowry, Rosebrough, Farr & Randall (1951) or by extinction at 280 mμ.

Amino acids (A Grade) were obtained from Calbiochem; fatty acids and cholesterol from Applied Science Inc., Pennsylvania, and alanyl peptides from Yeda Research and Development Co., Rehovoth, Israel. Cholesterol-4 ^{14}C was obtained from the Radiochemical Centre, Amersham, Bucks. and purified by thin layer chromatography (ITLC-SA, Gelman Instrument Co., Michigan; solvent isooctane + diethyl ether, 9:1). The main radioactive band was located by radioautography and eluted with ethanol.

Analytical methods. Cholesterol was determined by the method of Wycoff & Parsons (1957), phosphorus by that of Bartlett (1959) as modified by Marinetti (1962), and radioactivity with a Packard Tri-Carb liquid scintillation spectrometer in a dioxane system (Bray, 1960). Fatty acids were estimated by gas liquid chromatography as described previously (Rodwell, 1968) except that for the analysis of fatty acids in lipid extracts of fraction C, the methyl esters were prepared by trans-esterification with boron trifluoride in methanol at 64° for 2 hr.

Paper electrophoresis was performed in barbiturate buffer, pH 8·6, in an apparatus manufactured by LKB-Produkter, Sweden. The paper strips were stained for protein with brom phenol blue; ^{14}C-cholesterol was located by radioautography.

RESULTS

Growth of Mycoplasma strain Y in medium C. Medium C gave excellent growth of this strain from an inoculum of 1 to 10 viable particles. The maximum yield, which was undiminished in serial subculture, was 0·35 mg. dry weight, and the maximum viable count was $6{\cdot}0 \times 10^9$ colony forming units (c.f.u.) per ml. culture. Growth curves determined by viable counts, turbidity and by ^{3}H thymidine incorporation are shown (Fig. 1). The mean doubling time as determined by all three methods was 1·6 to 1·7 hr. Thymidine was incorporated at an exponential rate and then ceased abruptly. The turbidity continued to increase (at a decreasing rate) for about 4 hr longer, perhaps because the mycoplasmas increased in mass after DNA replication ceased. In this experiment viable counts were discontinued after 24 hr incubation, but in other

experiments the count reached a maximum at about the same time as the turbidity. Increase in the number of viable particles after the cessation of DNA replication might be caused by the breaking up of filaments containing several 'DNA complements'. Medium C1 gave the same growth rate and yield, and was used in subsequent experiments.

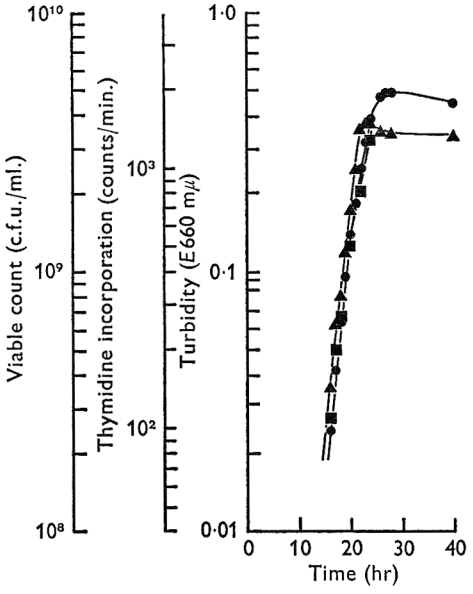

Fig. 1

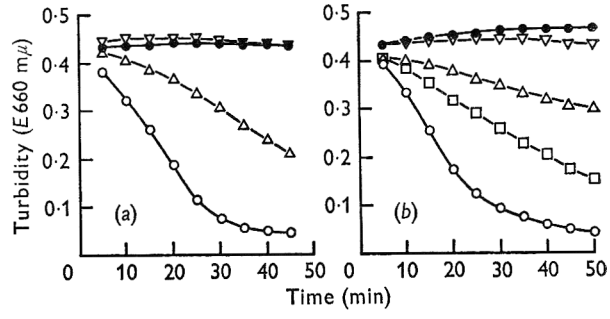

Fig. 2

Fig. 1. Growth of Mycoplasma strain Y in medium C. Medium C (100 ml.), modified by the substitution of ^{3}H thymidine (2 mg. l., specific activity 30 μc. μmole) for thymine, was seeded with 2×10^6 colony forming units and dispensed in 5 ml. volumes in optically matched test tubes. Viable cell counts, ■; turbidity, ●; and ^{3}H thymidine incorporation, ▲, were determined on replicate tubes during incubation at 37°.

Fig. 2. Inhibition of oleate-induced lysis of Mycoplasma strain Y by bovine serum albumin (BSA). (*a*) Fraction C and pronase digest of fraction C (PC). (*b*). Phosphate buffer, 0·05 M, pH 7·4; sodium chloride, 0·25 M; sodium oleate, $2{\cdot}5 \times 10^{-5}$ M, and BSA, fraction C or PC were incubated at 37° for 10 min. in optically matched test tubes; cell suspension added and turbidity measured during incubation at 37°. (*a*) ●, No oleate or BSA; ○, oleate; △, oleate +BSA 0·08 mg. ml. (molar ratio 15:1); ▽, oleate+BSA, 0·2 mg. ml. (molar ratio 6:1). (*b*) ●, No oleate or fraction C; ○, oleate; △, oleate+0·05 mg. ml. fraction C; ▽, oleate+ 0·2 mg./ml. fraction C; □, oleate+0·2 mg. ml. PC.

Growth-promoting properties of serum albumin

It was concluded (Rodwell, 1967) that in medium C1, in which unesterified fatty acids are added in a total concentration of 0·1 mM, albumin is required to bind fatty acids. The concentration of BSA required for growth increased with increasing fatty acid concentrations, and albumin was not required when the concentration of fatty acids was decreased below a critical value as in medium D. Mycoplasma cells are rapidly lysed by long chain monoenoic fatty acids in concentrations lower than those required for optimum growth, and lysis is prevented by BSA (Fig. 2*a*). It was found subsequently that not all samples of serum albumin were equally effective in promoting growth of strain Y in medium C1. Two crystalline samples and six preparations of

fraction V were tested, some of them before and after fatty acid depletion by Goodman's procedure or by charcoal treatment. Of these, only two gave good growth (equivalent to growth of strain Y in best media), whereas all of the others gave growth accompanied by various degrees of cellular lysis. The amount of lysis was greater with increasing albumin concentration (i.e. with decreasing fatty acid-albumin ratios, Fig. 3). but not when the concentrations of fatty acids were increased in proportion. Increasing the cholesterol concentration to 0·1 mM or heating the albumin solutions at 56° for 30 min. decreased the amount of lysis and growth was now better at a lower fatty acid:albumin ratio (Fig. 3). Two samples of 'fatty acid poor BSA' which originally gave growth without appreciable lysis, gave growth with lysis after charcoal treatment.

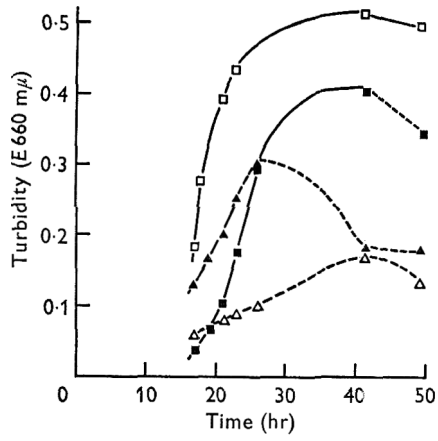

Fig. 3

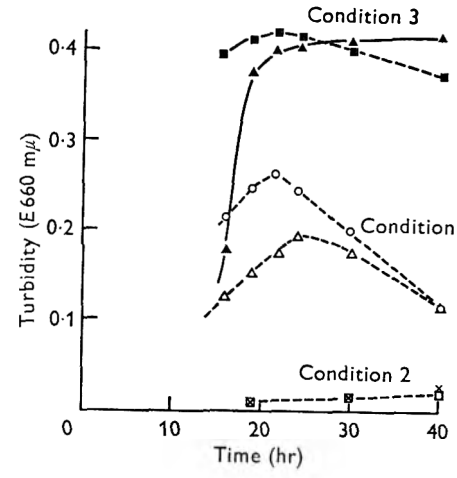

Fig. 4

Fig. 3. Effect of heating at 56° for 30 min. on the growth-promoting activity of bovine serum albumin (BSA) for Mycoplasma strain Y in medium C 1. ▲, BSA solution unheated, 1·0 mg./ml.; △, BSA solution unheated, 2·0 mg./ml.; ■, BSA solution heated, 1·0 mg./ml.; □, BSA solution heated, 2·0 mg./ml. ---, indicates lysis. The BSA was a crystalline sample; the molar ratio of fatty acids to BSA was 7:1 for a BSA concentration of 1·0 mg./ml.

Fig. 4. Effect of the order in which fatty acids and cholesterol dispersion were added to medium C 1 on the growth of Mycoplasma strain Y. In condition 1, cholesterol dispersion and sodium salts of fatty acids were added before autoclaving the medium; BSA-Ch solution was added after autoclaving. In condition 2, cholesterol dispersion was added before autoclaving the medium; the fatty acid salts were incubated with the BSA-Ch solution at 37° for 30 min., and the mixture added to the autoclaved medium. In condition 3, fatty acids were added as BSA-Ch fatty acid mixtures as in condition 2, but the cholesterol dispersion was autoclaved separately and added last. △, Condition 1, BSA-Ch 0·9 mg./ml.; ○, condition 1, BSA-Ch 1·8 mg./ml.; □, condition 2, BSA-Ch 0·9 mg./ml.; ×, condition 2, BSA-Ch 1·8 mg./ml.; ▲, condition 3, BSA-Ch 0·9 mg./ml.; ■, condition 3, BSA-Ch 1·8 mg./ml. ---, indicates lysis.

Experiments in which the order in which cholesterol and fatty acids were added to the medium was varied suggested that these results might be explained by varying degrees of interaction between fatty acids and cholesterol on the one hand, and between fatty acids and albumin on the other. It may be seen (Fig. 4) that when cholesterol and fatty acids were autoclaved in the medium (condition 1) there was growth with lysis; when cholesterol was autoclaved in the medium in the absence of

fatty acids (condition 2), there was almost no growth; but when the cholesterol dispersion was autoclaved in the absence of salts and added to the medium last (condition 3) there was good growth with little apparent lysis. Turbidity, proportional to the amount of cholesterol, developed during autoclaving in condition 2, and was presumably due to cholesterol precipitation.

Fraction C and pronase digest of fraction C (PC)

BSA could be replaced for growth of strain Y in medium C 1 by the heat-stable, defatted serum fraction C, but growth was slower. Fraction C had about the same activity as BSA in preventing lysis of cells by oleate (Fig. 2*b*). When added before

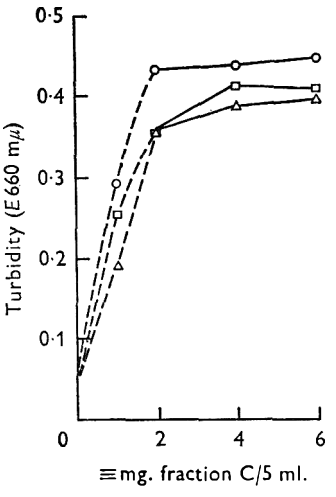

Fig. 5

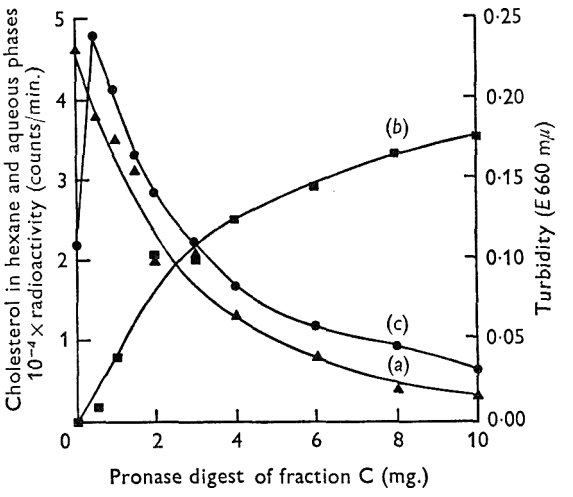

Fig. 6

Fig. 5. Effect of fraction C, pronase digest of fraction C (PC) and dialysed PC on the growth of Mycoplasma strain Y in medium C 1 containing charcoal-treated bovine serum albumin (BSA-Ch). Fraction C (or PC), fatty acids and cholesterol were added before, and BSA-Ch after the medium was autoclaved. The cultures were incubated for 22 hr. ○, Fraction C; □, PC; △, PC after dialysis. ---, indicates lysis.

Fig. 6. Effect of pronase digest of fraction C (PC) on the distribution of cholesterol between hexane and aqueous phases after shaking with hexane, and on the turbidity of cholesterol dispersions after autoclaving in buffer solution: curve (*a*) cholesterol in hexane phase; curve (*b*) cholesterol in aqueous phase; curve (*c*) turbidity. For curves (*a*) and (*b*), 0·7 mmole $Na_2HPO_4 + NaH_2PO_4$ buffer, pH 7·8; aqueous dispersion of ^{14}C-labelled cholesterol, 1·0 μmole and PC (total volume 6·0 ml.) were incubated at 37° for 30 min. Hexane (2·5 ml.) was added, the tubes shaken vigorously and centrifuged at 35,000 rev./min. for 1 hr (Spinco Model L, rotor 40). Radioactivity was determined in the hexane and aqueous phases. For curve (*c*), 0·7 μmole $Na_2HPO_4 + NaH_2PO_4$ buffer, pH 7·8; aqueous cholesterol dispersion, 1·0 μmole and PC (total volume 6·0 ml.) were autoclaved at 10 lb. for 10 min. and the turbidity measured at 420 mμ in 1 cm. cuvettes.

autoclaving, it also prevented lysis in media containing BSA-Ch. Its ability to replace BSA for growth, and its fatty acid-binding activity, were largely destroyed by pronase digestion (Fig. 2*b*), but its ability to prevent lysis during growth in medium containing BSA-Ch was almost unaffected (Fig. 5). With BSA-Ch and either fraction C or PC, the order in which cholesterol and fatty acids were added made no apparent difference to growth.

Fraction C disperses cholesterol. It prevented the precipitation of cholesterol when aqueous dispersions were heated in the presence of salts. When cholesterol dispersions were mixed with fraction C and buffer solution, and the mixture shaken with hexane, the cholesterol remained in the aqueous phase. Cholesterol-dispersing activity, in contrast to fatty acid-binding activity, was little changed by pronase digestion. The distribution of cholesterol (determined by radioactivity measurements) in the hexane and aqueous phases when a ^{14}C-labelled cholesterol dispersion was mixed with buffer

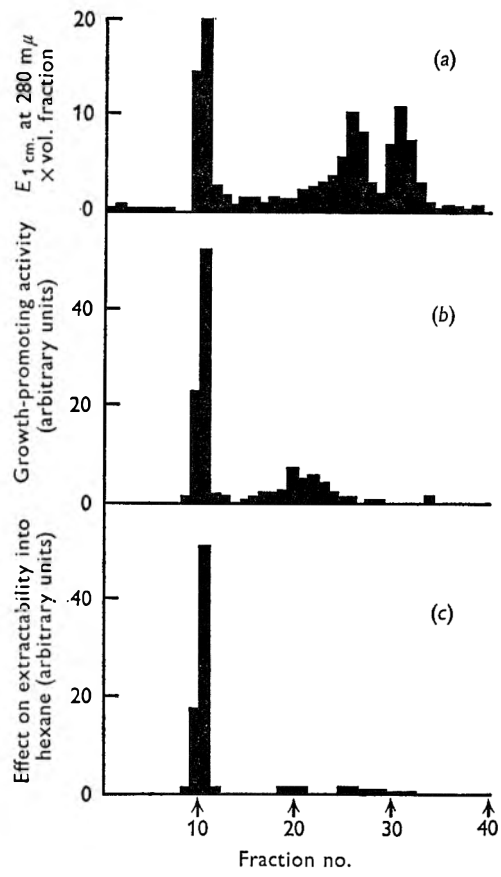

Fig. 7. Chromatography of pronase digest of fraction C (PC) on a Sephadex G50 column. PC (80 mg.) in 1·0 ml. 0·02 M-ammonium bicarbonate was applied to a column of 80 ml. bed volume. The column was eluted with 0·02 M-ammonium bicarbonate, 2·7 ml. fractions collected, and assayed for extinction at 280 mμ (*a*); growth-promoting activity in medium C2 (*b*); and cholesterol-dispersing activity (*c*). Cholesterol-dispersing activity was measured by the effect on the distribution of cholesterol between hexane and aqueous phases under the conditions described in Fig. 6, cholesterol being determined colorimetrically in the hexane phases. The units of growth-promoting activity and cholesterol-dispersing activity were the amounts having activity equal to 1 mg. of PC.

and various amounts of PC and the mixtures then shaken with hexane is shown in Fig. 6 (curves *a* and *b*), and the effect of PC on turbidity when an aqueous cholesterol dispersion was autoclaved in buffer solution in Fig. 6 (curve *c*). In the presence of PC, only about 80 % of the radioactivity was recovered in the hexane and aqueous phases;

the remainder was probably present in an interfacial gel layer which was not analysed. The discrepancy and the amount of interfacial layer was even larger with fraction C. The turbidity after autoclaving without PC was less than with a low concentration of PC. Cholesterol precipitated in large aggregates after autoclaving without PC; with low concentrations of PC the mixtures were opalescent and with higher concentrations they were clear.

The growth-promoting activity of PC was almost unchanged after dialysis (Fig. 5). PC was fractionated on a column of Sephadex G50 and the fractions assayed for material absorbing at 280 mμ (Fig. 7*a*), for growth-promoting activity (Fig. 7*b*), and for their effect on the distribution of cholesterol between hexane and aqueous phases (Fig. 7*c*). Most of the growth-promoting activity, and the cholesterol-dispersing activity, were eluted in the void volume with about a twofold increase in specific activity.

Further purification of fraction C was obtained by iso-electric precipitation. After two precipitations at pH 4·8, all of the growth-promoting activity was recovered in the fraction insoluble at pH 4·8 (fraction D), with about a twofold increase in specific activity. Much of the inactive material absorbing at 280 mμ in the fractionation of PC (Fig. 7) was thus probably derived from inactive material present in fraction C. Fraction D contained about 75 % of the fatty acids present in fraction C.

Fraction D gave a broad diffuse band extending about 2 cm. from the origin after paper electrophoresis. A mixture of fraction D in 0·1 M-phosphate buffer, pH 7·4, and ^{14}C-cholesterol dispersion was incubated at 37° for 30 min. After paper electrophoresis of the mixture, the cholesterol remained in a narrow band at the origin, and was almost entirely separated from the protein.

DISCUSSION

The provision of cholesterol in an assimilable form and of fatty acids in a non-toxic form for the growth of mycoplasmas presents a problem. On the one hand, unesterified fatty acids cause rapid lysis by interacting with the cell membranes, and on the other, a fatty acid deficiency results in lysis during growth in an otherwise complete medium, attributable to impaired membrane synthesis. The problem is more difficult in the case of strains producing high yields of organisms for which fatty acids and sterol must be provided in relatively high concentrations. Strain Y, for example, incorporates about 60 μmoles fatty acids and 30 μmoles cholesterol per l. of culture during growth in medium C2.

Medium C contained BSA to bind fatty acids (Rodwell, 1967). It is likely that, in this medium, cholesterol is dispersed by an interaction with fatty acids. Some samples of BSA gave growth accompanied by cellular lysis, and other samples which originally gave growth without lysis, gave growth with lysis after charcoal treatment. These samples may bind fatty acids more firmly, thus preventing their interaction with cholesterol. The poor growth and lysis may therefore be due to a cholesterol deficiency. The availability of fatty acids for growth may also differ with different albumin preparations. Kessler, Demeny & Sobotka (1967) showed a twofold difference in the rate at which palmitate was taken up by tissues from fatty acid + albumin complexes formed by two different methods.

Smith, Lecce & Lynn (1954) described a defatted serum lipoprotein for growth of some strains of sterol-requiring mycoplasmas, and Smith & Boughton (1960) postu-

lated that it was required for cholesterol incorporation. Rodwell (1956) described a heat-stable defatted serum protein fraction (fraction C) and suggested that fraction C, cholesterol and fatty acids might interact to provide a non-toxic source of fatty acids for growth of *Mycoplasma mycoides*. Rodwell & Abbot (1961) later suggested that fraction C might also be required for cholesterol incorporation. Evidence that it binds fatty acids and disperses cholesterol is presented in this paper.

Fraction C is thought to consist largely of a defatted serum lipoprotein. Cholesterol was bound by the apoprotein of human high-density serum lipoprotein only when phospholipids were also bound (Sodhi & Gould, 1967). Fraction C contains small amounts of firmly bound lipid, but whether this is essential for the cholesterol interaction, the nature of the interaction, and whether fraction C is related to the apoprotein of a serum lipoprotein, were not determined.

I wish to thank Mr B. Zwolak for his capable technical help.

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A Defined Medium for Mycoplasma Strain Y

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SUMMARY

In a defined medium for Mycoplasma strain Y, a mixture of the diacetoxysuccinoyl esters of monoolein and monopalmitin replaced unesterified fatty acids and serum protein fractions, enabling the minimal growth requirements to be determined. Other Mycoplasma strains did not grow in media in which these esters replaced fatty acids and protein fractions.

INTRODUCTION

Good growth of Mycoplasma strain Y was obtained in a medium containing defatted bovine serum albumin to bind fatty acids and a heat-stable serum protein fraction to disperse cholesterol (medium C2, Rodwell, 1969). The amino acid, peptide and growth factor requirements of strain Y were determined in a protein free medium (medium D, Rodwell, 1967) in which fatty acids were added in growth limiting concentrations. Growth in medium D was poor and might have been limited by nutrients other than fatty acids, while the protein supplements might have contributed unrecognised nutrients in medium C2. A completely defined medium giving good growth of strain Y is described in this paper. Some observations on the nutrition of other strains are also reported.

METHODS

Organisms. Strain Y, isolated from a goat (Laws, 1956), resembles *Mycoplasma mycoides* in its nutrition and metabolism (Rodwell, 1960, 1967), but differs in that its growth is not improved by aeration. The following strains were also used: V5, GLADYSDALE and KH_3J of *M. mycoides*; the CHU strain of *M. mycoides* var. *capri;* strains N29 and L2917, isolated from bovine arthritis, and S6 (*M. gallisepticum*).

Growth assays. These were performed as described previously (Rodwell, 1969). Cultures of strain Y were incubated vertically without agitation at 37°; cultures of the other strains were incubated in the same way and also in an inclined rack which was rotated at 10 rev./min. (rotated tube cultures).

Growth was measured by turbidity at 660 mμ; by the incorporation of ^{3}H thymidine (Rodwell, 1969), or by protein determination, by the method of Lowry, Rosebrough, Farr & Randall (1951) with crystalline bovine serum albumin dried to constant weight as standard, in cells washed in 0·25 M-sodium chloride + 0·02 M-sodium phosphate (pH 7·4) + 0·01 M-magnesium sulphate.

In some cases, an accumulation of pyruvate during growth suggested a defect in the pyruvate oxidase system, and cells were then examined manometrically for pyru-

vate oxidase activity. The preparation of cell suspensions, and amino acid supplement for manometric experiments, were described previously (Rodwell, 1967).

Media. Media C, C1 and C2 were as described (Rodwell, 1967, 1969). Medium C3 had the same composition as medium C except that the concentrations of glycerol and of 'fatty acid poor' BSA were increased to 0·02 M and 2·7 g./l. respectively, that of sodium phosphate was decreased to 0·11 M and sodium DL-lactate (0·03 M) and fraction C (0·8 g./l.) were added.

Alanyl peptides were obtained from Yeda Research and Development Co., Rehovoth, Israel. TEM-4T was a product of Hachmeister Co., Pittsburg, Pennsylvania. It had a fatty acid composition of 28 mole palmitic acid, 19 mole stearic acid, 42 mole oleic acid and smaller amounts of palmitoleic and linoleic acids per 100 mole fatty acid. Other medium components were as described previously (Rodwell, 1969).

Synthesis of diacetoxysuccinoyl esters of monoglycerides. Monopalmitin was synthesized by reaction of DL-isopropylidene glycerol with palmitoyl chloride (Baer & Fischer, 1945). The product (m.p. 76–76·5°) was chromatographically pure by thin layer chromatography (t.l.c.). Monoolein was synthesized by direct esterification of glycerol (Hartman, 1957). The product (m.p. 33·5–34·5°) contained no diglyceride or other impurities detectable by t.l.c.

Reaction of diacetyl D-tartaric acid anhydride (m.p. 133·4°) with equimolar amounts of the monoglycerides at 110° in the absence of a cosolvent gave a complex mixture of products. Reaction in boiling chloroform gave a less complex mixture, while in the presence of pyridine it proceeded rapidly and gave only two major products. A solution of monoolein (2 mmoles) in 10 ml. dry chloroform was concentrated by distillation to 5 ml. Diacetyl tartaric acid anhydride (2·1 mmoles) and dry pyridine (4 mmoles) were then added. After 30 min. at 65°, a thin layer chromatogram—solvent chloroform + methanol + acetic acid + water (84 + 15 + 5 + 2 by vol.)—of the reaction mixture showed, in decreasing order of mobility, a small amount of unreacted monoglyceride, traces of unknown products, a very heavy spot (fraction OT/1) and a smaller amount of another product (fraction OT/2). Fractions OT/1 and OT/2 were purified by chromatography on a column of silicic acid. The column (40 g. silicic acid) was prepared and washed in chloroform + methanol (100 + 2 v/v), and the sample (1 g. reaction products) applied in the same solvent. The components were eluted with 600 ml. chloroform + methanol (100 + 2) which eluted unreacted monoolein, traces of unknown products and fraction OT/1; and 300 ml. chloroform + methanol (100 + 5) which eluted fraction OT/2. Fractions (25 ml.) were collected and examined by t.l.c., and those containing the pure components OT/1 and OT/2 were combined. The residues after evaporation of solvents were dissolved in methanol, and pyridine was removed by stirring with an excess of Dowex 50 (H^+) ion exchange resin washed and suspended in methanol. The yields of fractions OT/1 and OT/2 were about 50 and 25 % respectively of the starting materials. The corresponding monopalmitin derivatives, which migrated like OT/1 and OT/2 on t.l.c. plates, were prepared in the same way (fractions PT/1 and PT/2). The m.p. of fractions OT/1 (−8° to −6°) and PT/1 (34° to 36°) were similar to published values for 1-*O*-(2′(R),3′(R)-diacetoxysuccinoyl)-3-*O*-oleoylglycerol (IIA), and the palmitoyl homologue (IB) respectively (Birnbaum, 1955). It was suspected that fractions OT/2 and PT/2 might be 1,2-di-*O*-(2′(R), 3′(R)-diacetoxysuccinoyl)-3-*O*-oleoylglycerol(IIA), as and the palmitoyl homologue (IIB). IA and IB would have one titratable carboxyl group and would consume five equivalents of alkali on saponification, while IIA and

IIB would have two titratable carboxyl groups, and consume nine equivalents of alkali on saponification (Fig. 1).

Neutralization equivalents were determined by potentiometric titration of solutions in 50 % (v/v) aqueous ethanol with aqueous sodium hydroxide, and saponification equivalents after saponification in aqueous ethanolic sodium hydroxide at 80° for 1 hr. The values found (Table 1) agree closely with those calculated for IA and IB, and IIA and IIB.

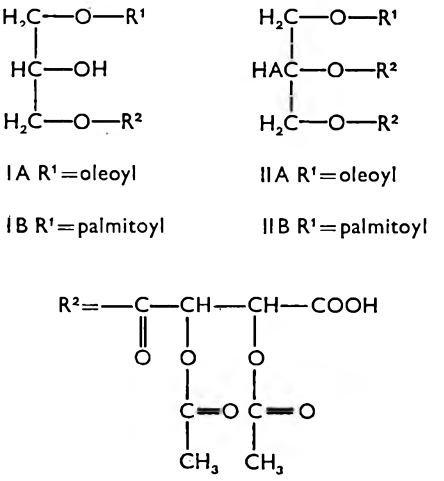

Fig. 1. Structures of diacetoxysuccinoyl esters of monoglycerides.

Table 1. *Neutralization and saponification equivalents of diacetoxysuccinoyl esters of monoolein and monopalmitin*

Fraction	Compound	Neutn. equiv. Calc.	Neutn. equiv. Found	Sapon. equiv. Calc.	Sapon. equiv. Found
OT/1	1-*O*-(2′(R),3′(R)-diacetoxysuccinoyl)-3-*O*-oleoylglycerol	573	567	114	114.5
OT/2	1,2-di-*O*-(2′(R),3′(R)-diacetoxysuccinoyl)-3-*O*-oleoylglycerol	395	400	87·7	88·7
PT/1	1-*O*-(2′(R),3′(R)-diacetoxysuccinoyl)-3-*O*-palmitoyl-glycerol	547	552	109	111
PT/2	1,2-di-*O*-(2′(R),3′(R)-diacetoxysuccinoyl)-3-*O*-palmitoylglycerol	381	375	84·8	86·0

RESULTS

Replacement of proteins and fatty acids by diacetoxysuccinoyl esters of monoglycerides

BSA and unesterified fatty acids were replaced for growth of strain Y in medium C1 by TEM-4T, a complex mixture of diacetoxysuccinoyl esters of mono- and diglycerides (Fig. 2). The minimum concentration of TEM-4T required for maximum growth was about 50 mg./l. This amount would provide, after complete hydrolysis, a total of 85 μmoles fatty acids. With this concentration of TEM-4T the concentration of cholesterol had to be increased before any growth occurred. The medium was

then slightly turbid, and some highly refractile microcrystals formed during subsequent incubation.

TEM-4T was replaced (Fig. 2) by an equimolar mixture of IA and IB and also by a mixture of IIA and IIB. The concentrations of the mixtures, and the proportions of these to cholesterol, were varied: the results shown in Fig. 2 are for the optimum concentrations. Growth with the synthetic homologues was not quite as good as with TEM-4T. They appeared to be more toxic in that the growth-inhibitory concentration of the mixtures was a little lower than with TEM-4T, but the growth was almost as good as with TEM-4T, and the results obtained by turbidity measurement were confirmed by thymidine incorporation. The yields of cell protein at maximum turbidity were 0·20, 0·18 and 0·16 mg./ml. culture for media containing TEM-4T, IA+IB, and IIA+IIB respectively.

Growth of strain Y was obtained also when aqueous dispersions of monoolein and monopalmitin were substituted for TEM-4T. Media with these were very turbid and growth had to be measured by thymidine incorporation. Growth was slower and the amount of thymidine incorporated was about 60% of that in a parallel culture in TEM-4T medium.

Nutritional requirements of strain Y

Since it is unlikely that TEM-4T would be contaminated with amino acids and water soluble growth factors, requirements for these were in most cases determined in a medium E in which it replaced fatty acids and protein fractions.

Vitamins and coenzymes. Requirements for thiamine, riboflavin and nicotinamide (or nicotinic acid) in a partially defined medium were described previously. Coenzyme A was essential in the TEM-4T medium and could not be replaced by pantothenate, pantethine or pantetheine. Pyruvate accumulated during growth in a partially defined medium (medium B2, Rodwell, 1963). This medium probably contained growth-limiting amounts of coenzyme A or other utilizable precursor contaminating one or other of the undefined components. Mycoplasmas grown in it lacked pyruvate oxidase activity, but oxidised pyruvate normally in the presence of coenzyme A (Fig. 3). The abrupt increase in the rate of oxygen uptake with pyruvate as substrate (Fig. 3) did not indicate a change in the rate of pyruvate oxidation, but was due to the accumulation, and subsequent oxidation, of lactate (Rodwell, 1967). With growth-limiting amounts of coenzyme A, growth was accompanied by cellular lysis, presumably because of a failure to incorporate fatty acids.

Growth stimulation by α-lipoic acid in a partially defined medium was found previously (Rodwell, 1960). The effect of α-lipoic acid on growth in medium C2 is shown in Fig. 4. While it stimulated growth in primary culture, it had little or no effect on growth in subculture in the same medium. The pyruvate oxidase activity of mycoplasmas harvested after 20 hr incubation from replicate subcultures in media with and without α-lipoic acid was examined (Fig. 5). Mycoplasmas from the deficient culture had no pyruvate oxidase activity, but oxidized it normally in the presence of α-lipoic acid. There was no growth response to α-lipoic acid in medium E either in primary culture, or in subculture. Thus it appears that the functioning of the pyruvate oxidase system was not essential for growth of this strain—at least in non-aerated cultures.

Pyridoxin or other vitamin B6 derivatives were not required in the TEM-4T medium

in which alanine was provided by L-alanyl-L-alanyl-L-alanine (L-ala$_3$). Strain Y grew slowly without alanine or alanyl peptides (see below) and, under conditions where growth was made dependent on alanine synthesis, i.e. in the absence of alanine or of alanyl peptides, growth was stimulated by pyridoxamine (Fig. 6). Growth under these conditions was no better when other derivatives of pyridoxin, including pyridoxal phosphate, were substituted for pyridoxamine. Requirements for biotin, folinic acid or vitamin B_{12} were not found in any medium.

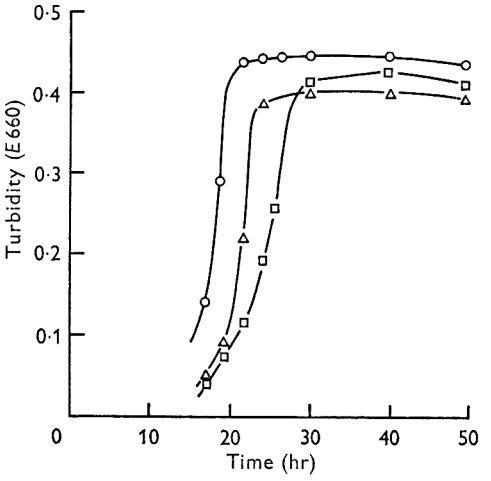

Fig. 2

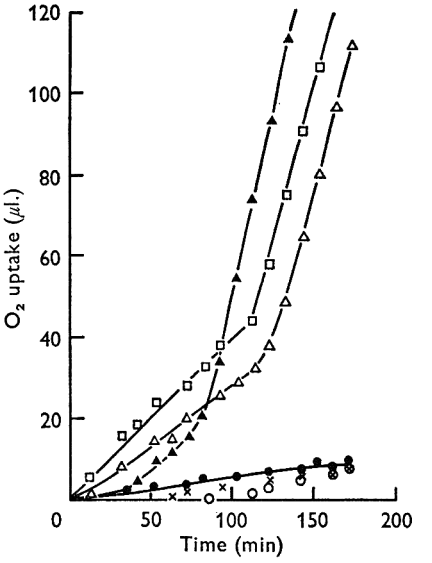

Fig. 3

Fig. 2. Replacement of BSA, fraction C and unesterified fatty acids by TEM-4T or synthetic homologues for growth of Mycoplasma strain Y. The concentration of cholesterol in medium C2 was increased to 200 µM. BSA, pronase digest of fraction C (PC) and unesterified fatty acids were replaced by: ○, TEM-4T, 50 mg./l.; □, 1A and 1B, 40 µM each; △, IIA and IIB, 30 µM each.

Fig. 3. Effect of coenzyme A deficiency on the pyruvate oxidase activity of Mycoplasma strain Y. Mycoplasmas were grown in medium B2 (Rodwell, 1963) with coenzyme A (coA cells) and without coenzyme A (coA-deficient cells). Manometer flasks contained: $Na_2HPO + KH_2PO_4$, pH 7·4, 200 µmoles; sodium pyruvate, 20 µmoles; coenzyme A (coA), 20 µg; amino acids, 3·5 mg.; coA cells, 1·4 mg.; coA deficient cells, 1·25 mg.; KOH in centre well. Fluid volume 2·5 ml. ▲, coA-deficient cells with coA; ○, coA-deficient cells without coA; □, coA cells with coA; △, coA cells without coA; ●, coA-deficient cells with coA, but no pyruvate; ×, coA cells with coA, but no pyruvate.

Amino acid requirements. Absolute requirements for all amino acids except glutamic acid, aspartic acid and cystine were reported previously in medium D (Rodwell, 1967). This was confirmed in the TEM-4T medium in which alanine was provided by the tripeptide (L-ala$_3$). In contrast to the results obtained earlier, there was good growth when L-alanine at a concentration of 1 mM was substituted for alanyl peptides, and slow growth in the absence of alanine or alanyl peptides (Fig. 6). Higher concentrations of L-alanine (8 mM) inhibited growth, and this inhibition was partly reversed by increasing the glycine concentration. Growth with the tripeptide (L-ala$_3$) and tetrapeptide (L-ala$_4$) was a little faster than with the free amino acid, but the dipeptide L-alanyl alanine inhibited growth.

Minimal medium. The inorganic requirements, and those for fatty acids, sterol, spermine, nucleic acid precursors and glycerol have been discussed previously and were not considered further. The composition of the minimal medium giving the best growth of strain Y is listed in Table 2.

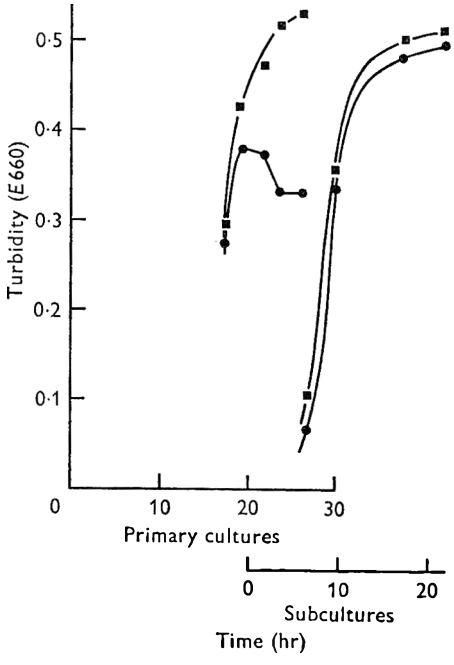

Fig. 4

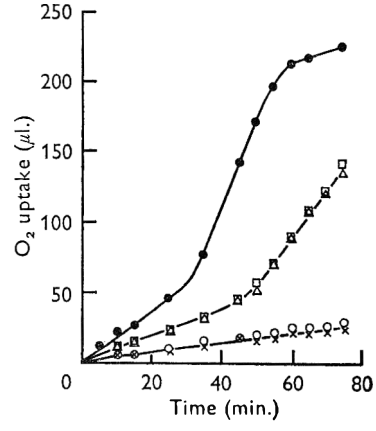

Fig. 5

Fig. 4. Effect of α-lipoic acid on growth of Mycoplasma strain Y. Cultures in medium C2. ■, With DL-α-lipoic acid (0·2 µg ml.); ●, no α-lipoic acid.

Fig. 5. Effect of α-lipoic acid deficiency on pyruvate oxidase activity of Mycoplasma strain Y. Mycoplasmas were from subcultures for 20 hr in medium C2 with α-lipoic acid (α-lipoic acid cells), and without α-lipoic acid (deficient cells)—see Fig. 4. Manometer flasks contained: $Na_2HPO_4 + KH_2PO_4$, pH 7·4, 200 µmoles; sodium pyruvate, 20 µmoles; DL-α-lipoic acid, 10 µg., amino acids 3·5 mg.; α-lipoic acid cells, 2·2 mg.; deficient cells, 3·8 mg.; KOH in centre well; fluid volume 2·5 ml. ●, Deficient cells with α-lipoic acid; ×, deficient cells without α-lipoic acid; ○, deficient cells with α-lipoic acid but no pyruvate; □, α-lipoic acid cells with α-lipoic acid; △, α-lipoic acid cells without α-lipoic acid.

Nutrition of other strains of Mycoplasma

Mycoplasma mycoides strain V5 did not grow in media in which TEM-4T replaced fatty acids and the protein fractions, even with the addition of a pronase digest of BSA to provide peptides and other growth factors. Several strains, including V5, were tested for growth in medium C; none grew well, or in rotated tube cultures in medium C modified for aerobic growth conditions by increasing the glycerol concentration and adding lactate. Two strains of *M. mycoides* (V5 and GLADYSDALE), the bovine arthritis strains (N29 and L2917), and a strain of *M. mycoides* var. *capri* (CHU) grew well in rotated tube cultures in medium C3, the growth rate and yield of organisms being about the same as that obtained with strain Y in medium C2. *Mycoplasma*

Table 2. *Composition of defined medium E for growth of Mycoplasma strain* Y

Additions	Concentration
Na_2HPO_4	0·14 M
KCl	0·01 M
$MgSO_4$	0·001 M
Glucose*	0·04 M
Spermine	0·1 mM
Cholesterol†	0·2 mM
Glycerol	5·0 mM
Diacetoxysuccinoyl esters of monoolein and monopalmitin (or TEM-4T)†	0·04 mM (or 50 mg./l. of TEM-4T)
Adenine, guanine, uracil	10·0 mg/l. each
Thymine	5·0 mg./l.
Coenzyme A‡, riboflavin*, nicotinamide, thiamine	1·0 mg./l. each
DL-α-lipoic acid	0·1 mg./l.
L-asparagine, L-glutamine*, L-arginine, L-lysine, L-histidine, L-leucine, L-isoleucine, L-proline	1·0 mM each
DL-phenylalanine, DL-valine, DL-methionine, DL-serine, DL-threonine, DL-tryptophan, glycine	2·0 mM each
L-cysteine*	1·3 mM
L-alanylal-L-anyl-L-alanine	0·3 mM

* Added from sterile solutions to previously autoclaved medium. Glutamine and cysteine solutions were freshly prepared.

† Aqueous dispersions prepared by the addition of solutions in ethanol to stirred water at 65° for cholesterol, or at 20° for TEM-4T and homologues.

‡ Added before autoclaving from a 0·1 mg./ml. solution containing dithiothreitol, 1·0 mg./ml. in phosphate buffer, pH 7·0.

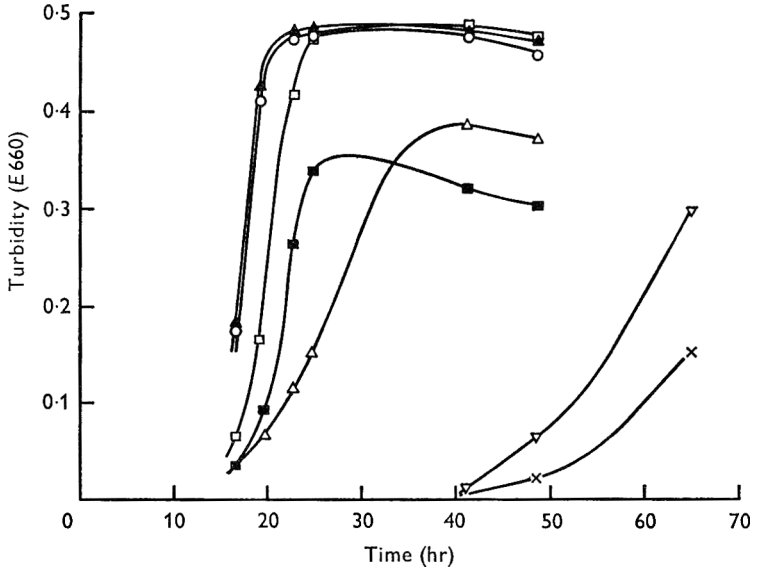

Fig. 6. Effect of L-alanine and L-alanyl peptides on growth of Mycoplasma strain Y. Medium E contained TEM-4T and L-alanine, peptides and pyridoxamine as indicated. ×, without L-ala, peptides or pyridoxamine; ▽, without L-ala or peptides; □, L-ala, 1 mM; ■, L-ala, 8 mM; △, L-ala_2, 1 mM in ala; ○, L-ala_3, 1 mM in ala; ▲, L-ala_4, 1 mM in ala.

mycoides strain KH$_3$J grew poorly, while the S6 strain of *M. gallisepticum* gave no growth. The strains of *M. mycoides* and the bovine arthritis strains grew in medium C3 as extremely long, branched filaments.

Glycerol was essential for the *Mycoplasma mycoides* strains and for the strain of *M. mycoides* var. *capri*. Exogenous glycerol was not essential for the bovine arthritis strains, but they grew poorly without it, and the cells underwent lysis. Requirements for other nutrients were not determined, apart from the observations already reported for strain V5 (Rodwell, 1960).

DISCUSSION

The protein supplements might have contributed unrecognized nutrients for growth of strain Y in medium C2. To eliminate these it was necessary to substitute fatty acids in a non-toxic, chemically defined form, which would at the same time disperse cholesterol. Mixtures of 'Tweens' (polyoxyethylene sorbitan mono esters of fatty acids) were tried without success. Lund & Shorb (1966) described a partially defined medium in which TEM-4T provided fatty acids for growth of Mycoplasma strain J. TEM-4T contains a balanced mixture of saturated and unsaturated fatty acids, and gave good growth of strain Y. The concentration required for optimum growth was much greater than in the medium of Lund & Shorb, and the cholesterol concentration had to be increased in proportion. It is possible that an interaction between TEM-4T and cholesterol modifies the surface active properties of the glycerides and disperses cholesterol. TEM-4T was in turn replaced by synthetic homologues, enabling the minimal nutritional requirements to be determined.

TEM-4T and the synthetic homologues do not seem to be completely non-toxic for mycoplasmas. Strain V5 required both BSA (at an increased concentration) and fraction C (or PC) for good growth, and these could not be replaced by TEM-4T. While strain V5, and the other strains which grew well in medium C3, may require unrecognized nutrients (supplied by the protein supplements), it is also possible that TEM-4T is not a suitable source of fatty acids for them.

Other strains might be grown in partially defined media by including more complex mixtures of nucleic acid precursors (such as are required by *Mycoplasma laidlawii* strain B, Tourtellotte, Morowitz & Kasimer, 1964), by altering the proportions of amino acids, or by adding arginine in high concentration as an energy source for growth of non-fermentative strains having the arginine dihydrolase pathway (Barile, Schimke & Riggs, 1966).

I wish to thank Professor J. M. Swan for advice on the synthesis and nomenclature of the diacetoxysuccinoyl esters of monoglycerides, Dr H. Birnbaum for supplying me with a sample of TEM-4T and Mr B. Zwolak for his capable technical help.

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Sources of Carbon and Energy used by *Coprinus lagopus sensu* Buller

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SUMMARY

The sources of carbon + energy utilized by a strain of *Coprinus lagopus* which is in wide use in biochemical and genetic experiments have been determined. Over 120 compounds were tested for growth in liquid medium. Only acetate, fructose, glucose, maltose, mannitol, mannose, xylose and the polymers cellulose and starch supported growth as sole carbon + energy sources at 10 mM concentration. Amino acids were used as sources of carbon, but only in the presence of equimolar amounts of glucose. Suggestive evidence was obtained for inducible enzyme systems concerned with the utilization of ethanol, lactose and alginate.

INTRODUCTION

The carbon nutrition of the Basidiomycete genus *Coprinus* has received attention from a number of workers in recent years (Leonian & Lilly, 1938; Voderberg, 1948; Fries, 1955; Madelin, 1956*a*). For a variety of reasons, however, it was decided to repeat and extend some of what has already been done. There is reason to believe (Fries, 1955; and see Discussion) that, with respect to nutrition in general, a good deal of polymorphism may occur among isolates of the same species. The nomenclature of *Coprinus lagopus* and its allies is considerably confused so that the identity of the material used in previous studies is strictly speaking uncertain. Also, most previous investigations have been particularly related to studies of fruiting behaviour, and have thus been mainly concerned with the physiology of the dikaryon. The present report lays emphasis on monokaryons of an isolate which is well characterized genetically (Day & Anderson, 1961; Moore, 1967), with the intention of providing a reliable basis for more detailed biochemical and genetical analyses of carbon metabolism.

METHODS

Organism. The strains used belonged to a backcrossed wild-type line prepared by D. H. Morgan from the strains originally isolated from the wild by P. R. Day (Day & Anderson, 1961). They had the stock numbers BC9/66 (mating type A_6B_6) and BC9/55 (A_5B_5) and were isolated after nine generations of backcrossing to Day's H9 wild type. These isolates agree extremely well with the description of *Coprinus lagopus* given by Buller (1924) and his example of the use of this name has been followed in many publications dealing with genetic phenomena in these strains (e.g. Day & Anderson, 1961; Morgan, 1966; Cowan & Lewis, 1966; Casselton & Lewis, 1967; Moore, 1967).

Media. The basal liquid medium (referred to as NCM) contained: 30 mM-NH_4Cl;

10 mM-Na_2HPO_4; 10 mM-KH_2PO_4; 2 mM-Na_2SO_4; $1{\cdot}5 \times 10^{-6}$ M-thiamine hydrochloride; no trace elements were added to this medium. The medium was at pH 6·8 after autoclaving for 10 min. at 121°. Substances tested for availability as sole sources of carbon + energy originated from commercial firms; most were added in the solid form to the basal medium immediately before sterilization. All carbohydrates, labile and volatile substances were added as filter-sterilized solutions to appropriately concentrated sterile NCM. Unless otherwise stated all potential carbon + energy sources were added to a final concentration of 10 mM. Tests involving sparingly soluble substances essentially involved saturated solutions. Polymers were used at the rate of 1·8 mg./ml.

Inocula were 2 mm. diameter discs cut from the margins of 6-day colonies growing on NCM + 5 mM-glucose. The mycelial discs were cut 24 hr before use and incubated at 37° until used on Coprinus complete medium (Moore, 1968). This period of incubation resulted in the production of a small amount of fresh aerial mycelium which considerably eased the task of floating inocula onto the liquid medium surface (Norkrans, 1953).

Cultivation in liquid medium. The liquid media were used in 20 ml. quantities in 9 cm. glass Petri plates. A single inoculum disc was floated on each and the plates incubated statically at 37° for 14 days. Five such plates (i.e. a total of 100 ml. medium + 5 inoculum discs) constituted a single test; five replicates of each test were generally made.

Harvesting was done by filtration through weighed Oxoid membrane filters, the harvest being washed with 200 ml. of cold water during filtration. Sparingly soluble compounds were removed by repeated washings with large volumes of warm water. The membranes and harvest were dried to constant weight at 40°.

RESULTS AND DISCUSSION

The response of the haploid wild-type monokaryon, BC9/66, to the compounds tested individually as carbon + energy sources is summarized in Tables 1 and 2. Although at least some variation in mycelial yield was evident in tests involving utilizable compounds there was an unmistakable and consistent difference between substances which were not utilized and those that were used either well or weakly. Of the 124 compounds tested as sole sources of carbon + energy only eight were used by this organism at 10 mM concentrations.

A comparison of the yields of mycelium obtained from the monokaryons BC9/66 and BC9/55 and their joint dikaryon on a selection of potential carbon + energy sources is shown in Table 3. These figures, and the data in subsequent Tables, come from experiments in which attention was concentrated on a much more limited selection of compounds than was used in the initial screening tests.

The BC9/66 monokaryon was tested for growth on different concentrations of some potential carbon + energy sources (Table 4*a*) and the growth of all three mycelia compared on different glucose concentrations (Table 4*b*). It was evident from these experiments that lactose, and possible sucrose, though classified as not utilized in the initial screening tests, could nevertheless support at least some growth at high concentrations. Attempts were therefore made to see whether growth could be obtained on these and related compounds in supplemented media.

Table 1. *Compounds which did not support the growth of Coprinus lagopus BC9/66 when present as sole source of carbon + energy*

(i) Sugars and related compounds

Alginate	Glucose-6-phosphate	Mucate
D-arabinose	2-deoxy-D-glucose	Raffinose
L-arabinose	Glucuronate	Rhamnose
Ascorbate	Glycerol	Ribose
Dihydroxyacetone	Inositol	Sorbose
Galactose	Lactose	Stachyose
Glucosamine	Melibiose	Sucrose

(ii) Amino acids and related compounds

L-alanine	L-glutamine	L-ornithine
L-arginine	Glycine	L-phenylalanine
L-asparagine	Glycylglycine	L-proline
L-aspartate	L-histidine	L-serine
Creatine	L-hydroxyproline	L-threonine
L-cysteine	L-leucine	L-tryptophan
L-cystine	L-isoleucine	L-tyrosine
L-glutamate	L-lysine	L-valine
	L-methionine	

(iii) Normal alcohols, mono- and dicarboxylic acids

Methanol	Formate	
Ethanol		Oxalate
Propanol	Propionate	Malonate
Butanol	Butyrate	Succinate
Pentanol	Valerate	Glutarate
Hexanol	Caproate	Adipate

(iv) Aliphatic and related compounds

Acetaldehyde	Ethyl acetate	Linolenate
Acetone	Fumarate	Malate
Aconitate	D,L-glycerate	Maleate
Acrylamide	Glycollate	Oxalacetate
Isobutanol	Glyoxylate	2-oxoglutarate
Citrate	Lactate	Isopropanol
Isocitrate	Linoleate	Pyruvate
Ethanolamine		Tartrate

(v) Aromatic compounds

Benzaldehyde	Benzyl alcohol	Phenol
Benzoate	D-mandelate	Phenylpyruvate

(vi) Purines, Pyrimidines and derivatives

Adenine	DNA (thymus)	Thymine
Allantoin	Guanine	Uracil
Cytosine	Hypoxanthine	Urea
5-methylcytosine	Inosine	Xanthine
	RNA (yeast)	

(vii) Heterocyclic, alicyclic and related compounds

Biotin	Nicotinamide	Pyridoxine
Folate	Nicotinate	Quinate
Indole		Thiamine

Experiments with the BC9/66 monokaryon and testing for growth on NCM + 10 mM glucose + 10 mM test carbon source (Table 5) showed that many of the compounds which did not support growth when present as sole sources of carbon + energy also

failed to support growth when mixed with equimolar glucose. On the other hand some compounds did show a significant synergistic response. This pattern was also evident with a medium consisting of NCM + yeast extract + vitamin mixture + trace elements which could reasonably be supposed to contain any additive whose absence from the basal medium might restrict utilization (Table 6).

Table 2. *Compounds utilized as sole source of carbon + energy by Coprinus lagopus BC9/66*

Incubation: 14 days at 37°

Carbon source	Mycelial dry weight (mg./100 ml.)
NCM (no carbon source)	3·4 ± 0·6
Acetate	7·4 ± 2·8
Cellulose	22·3 ± 2·6
Fructose	22·3 ± 1·8
Glucose	38·1 ± 2·4
Maltose	22·8 ± 2·0
Mannitol	19·7 ± 2·2
Mannose	15·2 ± 1·7
Starch	17·3 ± 1·9
Xylose	12·8 ± 1·2

Table 3. *Comparison of the growth of Coprinus lagopus BC9/66 and BC9/55 monokaryons and their joint dikaryon on selected carbon sources*

Incubation: 14 days at 37°

Carbon source	Mycelial dry weight (mg./100 ml.)		
	BC9/66	BC9/55	Dikaryon
NCM (no carbon source)	3·4 ± 0·6	2·0 ± 0·9	2·7 ± 0·8
Acetate	7·4 ± 2·8	7·9 ± 2·5	7·2 ± 1·9
L-alanine	3·0 ± 0·5	0·4	3·8 ± 0·5
D-arabinose	0·4	2·9 ± 0·6	3·6 ± 0·8
Asparagine	5·9 ± 2·7	2·9 ± 0·5	3·9 ± 2·5
Benzoate	1·3 ± 0·6	3·0 ± 0·7	2·4 ± 0·5
Fructose	22·3 ± 1·8	23·0 ± 1·2	28·0 ± 2·1
Glucose	38·1 ± 2·4	37·9 ± 3·1	39·2 ± 3·2
Glycine	0·8	1·6 ± 0·3	3·9 ± 0·6
Lactose	2·4 ± 0·3	2·8 ± 0·8	3·6 ± 0·9
Lysine	0·4	1·6 ± 0·1	2·1 ± 0·2
Maltose	22·8 ± 2·0	25·3 ± 3·0	24·0 ± 2·4
Nicotinate	2·9 ± 0·2	3·8 ± 0·8	5·8 ± 1·2
RNA (yeast)	3·9 ± 0·9	3·1 ± 0·5	2·2 ± 0·4
Succinate	1·4 ± 0·7	2·8 ± 0·3	2·2 ± 0·4
Sucrose	1·9 ± 0·3	1·7 ± 0·3	2·0 ± 0·8
Tyrosine	0.2	2·0 ± 0·1	2·1 ± 0·7

Although only eight of the 124 compounds initially tested served as sole carbon + energy sources (Table 2) it remains likely that wider screening or the use of different basal media and growth conditions would reveal additional compounds able to support growth. A particular difficulty with experiments such as these is, of course, that it is not known whether some of the compounds may have failed to support growth

because of inability to act as inducers for the appropriate enzymes, or because of their not being transported into the cell.

Such difficulties apart, there is a clear indication that the *Coprinus lagopus* monokaryon BC9/66 was extremely restricted in the number of compounds it would accept

Table 4. *Effect of different initial concentrations of some potential carbon sources on the growth of Coprinus lagopus*

Incubation: 14 days at 37°

(*a*) Growth of the BC9/66 monokaryon

	Substrate concentration (mM)					
	1	3	10	30	100	300
	Mycelial dry weight (mg./100 ml.)					
Acetate	3·2	4·0	8·2	15·8	29·4	0·2
Benzoate	0·9	3·1	2·3	2·1	3·1	1·1
Fructose	14·4	18·4	26·5	52·0	66·7	79·5
Galactose	0·8	1·2	1·1	1·6	1·0	1·5
Glucose	10·7	12·2	40·5	106·0	120·5	124·6
Lactose	2·6	3·9	3·5	6·0	9·5	13·2
Raffinose	1·9	0·4	3·2	0·8	2·3	3·0
Sorbose	2·4	0·5	1·9	0·9	1·3	2·3
Succinate	2·1	1·1	1·4	0·6	1·9	1·3
Sucrose	2·4	2·5	2·6	3·3	2·2	40·7

(*b*) Growth of the BC9/66 and BC9/55 monokaryons and their joint dikaryon on different initial glucose concentrations

BC9/66	10·3	19·0	36·4	99·6	121·0	120·4
BC9/55	2·0	9·6	35·6	84·2	98·4	109·8
Dikaryon	2·2	13·0	35·1	88·9	104·6	114·3

Table 5. *Growth of Coprinus lagopus BC9/66 on some potential carbon sources in medium also containing 10 mM glucose*

Incubation: 14 days at 37°

Carbon source added	Mycelial dry weight (mg. 100 ml.)	Carbon source added	Mycelial dry weight (mg. 100 ml.)
No addition*	38·1 ± 2·4	Galactose	41·4 ± 2·1
Adenine	5·4 ± 0·8	Lactose	65·3 ± 2·2†
Acetate	53·0 ± 1·9†	Maltose	77·5 ± 4·3
Alanine	65·4 ± 1·5†	Phenylalanine	57·0 ± 4·7†
Asparagine	78·0 ± 1·1†	Raffinose	44·9 ± 7·1
Benzoate	4·0 ± 1·0	Serine	54·7 ± 1·1†
Citrate	16·6 ± 1·4	Sorbose	42·4 ± 5·2
Ethanol	70·5 ± 9·3†	Succinate	37·5 ± 3·1
Fructose	76·0 ± 3·4	Sucrose	25·5 ± 2·2

* 'No addition' in this Table = NCM + 10 mM glucose; all other carbon sources also added to 10 mM concentration.

† Signifies a significant synergistic response.

as sole carbon + energy source. It was also quite clear that there was no very significant difference between the response patterns of the two monokaryons and their joint dikaryon. Madelin (1956*a*) reported *C. lagopus* to be cellulolytic and Fries (1955) suggested that Coprinus species which are strongly coprophilic are capable of decom-

posing both cellulose and lignin. Since the strain under test here is indeed strongly coprophilic and was able to use cellulose, it is not surprising that the compounds which were most effectively used as sole carbon sources were closely related to these polymers.

The results presented here are in fair agreement with those reported by Madelin (1956*a*) for *Coprinus lagopus* and Fries (1955) for various Coprinus species. Chang (1967) reported an isolate of *C. cinereus* which was able to utilize cellobiose, cellulose, glucose, mannose, xylan and xylose. Some differences between the results of the present and previous studies are evident. Madelin (1956*a*) found a small but significant

Table 6. *Effect of some potential carbon sources on the growth of Coprinus lagopus BC9/66 in fully supplemented medium*

Incubation: 14 days at 37°

Carbon source added	Mycelial dry weight (mg./100 ml.)	Carbon source added	Mycelial dry weight (mg./100 ml.)
SNCM*	43·3 ± 4·9	Glucose	185·0 ± 5·1†
Acetate	67·9 ± 5·9†	Lactose	140·9 ± 4·3†
Alginate	101·5 ± 1·2†	Maltose	123·8 ± 6·4†
Arabinose	67·1 ± 7·5†	Nicotinamide	42·9 ± 5·7
Asparagine	140·2 ± 4·9†	Raffinose	54·3 ± 3·2
Benzoate	29·4 ± 5·1	Sorbose	43·1 ± 1·7
Fructose	171·2 ± 1·0†	Succinate	41·0 ± 5·7
Galactose	51·4 ± 4·7	Sucrose	51·8 ± 4·7

* SNCM = NCM + yeast extract (0·07%, w v) + vitamin mixture + trace elements.
† Indicates a significant increase in growth.

amount of growth with glycerol and with alanine: this was not confirmed in the present work. However, it should be noted that Madelin used higher concentrations of individual carbon sources and a different basal medium. Certainly, alanine supported very vigorous growth of the BC9/66 monokaryon when mixed with glucose (Table 5). Other differences also became obvious during the present experiments. In particular, strains BC9/66 and its relatives grew well with ammonium-nitrogen or asparagine-nitrogen, and fairly well, although the growth was very variable, on nitrate-nitrogen. Madelin (1956*a*) reported his strain of *C. lagopus* to show little or no growth with nitrate or asparagine as sole nitrogen source, though it would utilize ammonium-nitrogen or alanine-nitrogen. It should, perhaps, be emphasized here that Madelin states that his isolate agreed well with Buller's (1924) description of *C. lagopus*, and the strains used in the present work also agree well with that description. However, Madelin used solid media as against the liquid medium used here, and more significantly the strain used by him had a temperature optimum of 20 to 25° (Madelin, 1956*b*) as compared with a temperature optimum of 37° for the BC9/66 monokaryon and its relatives. The list of discrepancies appearing in the literature on the nutrition of *C. lagopus* can be further extended. Voderberg (1948) reported her strain of *C. lagopus* as being able to utilize nitrate-, ammonium- or organic-nitrogen, but as requiring the presence of powdered straw for the utilization of simple carbohydrates. On the other hand, the *C. lagopus* studied by Leonian & Lilly (1938) readily utilized glucose as sole carbon + energy source and amino acids as nitrogen source, though it was incapable of making use of either nitrate- or ammonium-nitrogen. It is thus quite evident that if these various isolates are indeed identical then either very considerable

differences exist between the strains or the organism is extremely sensitive to alterations in cultural conditions. Conflicting findings such as these, taken together with the taxonomic difficulties previously noted, emphasize the necessity for adequate characterization of the nutritional versatility of each isolate.

Defined media containing a single carbon source are an inadequate representation of the conditions likely to be met by the fungus in nature. Growth in mixtures of substrates, though still a compromise, is more likely to give a true indication of the metabolic versatility of the organism. In the presence of glucose this organism showed considerable growth with a number of compounds not otherwise utilized as sole carbon sources (Table 5). The majority of such responses are most easily explained in terms of sparing reactions. Evidently the provision of an alternative initial source of energy, in the form of glucose, enabled amino acids to be accepted into the metabolic sequence and their carbon skeletons utilized. Stimulation of growth by the extra nitrogen offered by the amino acids is insufficient to account for the extent of the response seen. An active and probably inducible enzyme system for the utilization of ethanol is indicated by the response to that compound. An inducible enzyme system is also indicated (Tables 5, 6) for the utilization of alginate (polymannuronate) and of lactose. Galactose was definitely not used either as sole carbon source (Tables 1, 4) or in supplemented media (Tables 5, 6). Madelin (1956*a*) reported lactose and galactose to be inhibitory to the growth of his *Coprinus lagopus*. This was certainly not the case with the BC9/66 monokaryon used here. Another distinction is that Madelin obtained an increase in growth by the addition of galactose to glucose-containing medium, though lactose retained some inhibitory properties in glucose medium. These results should be compared with those summarized in Tables 4, 5, 6 above. The position of sucrose is uncertain. None of the Coprinus species tested by Fries (1955) responded to 'saccharose' and Madelin (1956*a*) reported no growth of his *C. lagopus* with this disaccharide. Significant growth was obtained repeatedly in my experiments on the highest sucrose concentration tested (300 mM; Table 4*a*) though not in supplemented media with sucrose at 10 mM (Tables 5, 6). The reason for this pattern of response is unclear. It is most likely that the sucrose was contaminated by free hexose, this only becoming significant at the high concentrations used in the experiments summarized in Table 4. It would only require about 1–2 % contamination by hexose to give the mycelial yields obtained.

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Host-controlled Restriction Mutants of *Salmonella typhimurium*

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SUMMARY

Forty-eight independent restriction-deficient mutations of *Salmonella typhimurium* LT2 were isolated by using selective and non-selective methods. With phage P 22 it was shown that some mutations affected the restriction capacity only, while others affected both restriction and modification. The host-restriction of *S. typhimurium* decreased the recovery of F-*lac*$^+$ infected cells and decreased the yield of recombinants in bacterial mating and in phage P 22-mediated transduction.

INTRODUCTION

Host specificity is a process by which bacterial cells control the acceptance of foreign DNA. Two strain-specific mechanisms are involved in the achievement of this control. (1) The DNA synthesized within the cell can be altered in a non-inheritable fashion (*modification*). In at least one case this modification has been shown to be the methylation of a DNA base (Arber & Smith, 1966). (2) Upon entry into the cell, foreign DNA which does not carry the appropriate modification can be rapidly degraded (*restriction*). The primary step in this degradation is due to a specific endonuclease which causes a discrete number of double-chain scissions in the DNA (Meselson & Yuan, 1968). Strain-specific sites, probably base sequences, along the DNA, must be present and recognized before either restriction or modification can be exercised (Arber & Kühnlein, 1967).

Most of our present understanding of host specificity comes from genetical and biochemical studies in *Escherichia coli* in which differences in host specificity between strains were described several years ago and in which it was possible to isolate and study host specificity deficient mutants (review: Arber & Linn, 1969). *Salmonella typhimurium* LT2 and LT7 strains are extensively used in genetical studies, yet no difference in host specificity has been described between them. The existence in *S. typhimurium* of a barrier to the acceptance of DNA carrying *E. coli* host specificity was nevertheless suggested by some incidental observations (Zinder, 1960; Miyake, 1962). Furthermore, it has been shown that the fertility (*fer*) mutation in *S. typhimurium* results in the loss of previously undetected host restriction towards the DNA of *E. coli* (Colson & Colson, 1967; Okada, Watanabe & Miyake, 1968). The key observation in these studies was that the restriction which affects *E. coli* DNA also affects phage P 22 DNA. Most *fer* mutants lose both the restriction and the modification functions, and lysates of phage P 22 produced by them form plaques less efficiently (10^{-4}) when plated on *S. typhimurium* LT7 or LT2 than on the *fer* mutants themselves.

The experiments presented in this paper constitute the first step in an investigation of the genetic control of the host specificity that was revealed in *Salmonella typhimurium* by the isolation of *fer* mutants. We describe the isolation and the phenotypic characterization of a collection of restriction deficient mutants from *S. typhimurium* strain LT2.

METHODS

Bacteria. Salmonella typhimurium strains LT7 and LT7 *mut* (carrying a mutator gene) were obtained from G. G. Meynell (Lister Institute, London). Restriction deficient mutants of these strains were described previously (Colson & Colson, 1967). *S. typhimurium* LT2:*proC 90* and *proC 110* were obtained from H. O. Smith; SR305 HfrA *hisD* 23 *gal-50* and SB106 *ile-405 rha-461 leu-1003 proA 46 purC 7 str-r* from P. Hartman; SA534 HfrK 4 *serA 13*, SA195 *proAB 47 purE 66*, and *argD 6* from K. Sanderson; SL1027 *metA metE trpB xyl str-r* from B. Stocker.

Escherichia coli K12; W1655 *met lac* F-*lac*$^+$; a derivative of C600 lacking the K host specificity (Colson, Glover, Symonds & Stacey, 1965). *E. coli* B: a derivative of BC251 lacking the B host specificity (Glover & Colson, 1969).

Bacteriophages. Phage P 22 wild type was obtained from P. Fredericq. A *c 2* mutant was provided by G. G. Meynell. P22 *int-11*, lacking a function necessary for the integration of the prophage into the bacterial chromosome (Smith & Levine, 1967) was donated by H. O. Smith.

Media. Nutrient broth contained Difco Bacto Tryptone, 10 g.; NaCl, 8 g.; distilled water, 1 l. Nutrient agar was nutrient broth solidified with 1·5% (w/v) Difco Bacto agar. Phage agar was broth containing 1% (w/v) Difco Bacto agar and was used as bottom layer for phage assay in soft agar overlays. Soft agar was 0·6% (w/v) Difco Bacto agar in distilled water. EMB lactose agar, was as Arber & Morse (1965). Minimal medium (g./l.): NH_4Cl, 5; NH_4NO_3, 1; Na_2SO_4, 2; K_2HPO_4, 3; KH_2PO_4, 1; $MgSO_4.7H_2O$, 0·1; this medium was solidified with 1·5% (w/v) Difco Bacto agar and supplemented with 0·02 g./l. of appropriate L-amino acids or adenine when necessary. Dilution buffer contained (g./l.): KH_2PO_4, 3; Na_2HPO_4, 7; NaCl, 4; $MgSO_4.7H_2O$, 0·2.

Mutagenic treatment. Exponentially growing broth cultures were incubated at 37° for 25 min. in the presence of *N'-N*-methyl-*N*-nitrosoguanidine (NTG) 30 μg./ml. NTG was removed by twice washing in buffer. The cells were then diluted 1/100 in fresh broth and incubated to allow expression of the mutations.

Terminology. According to Arber & Linn (1969) the following terminology for host specificity will be used. The host specificity system of *Salmonella typhimurium* LT7 and LT2 will be noted as 'LT'. The phenotypes will be characterized as r_{LT} for restriction and m_{LT} for modification, with the superscript plus or minus. Thus $r^+_{LT}m^+_{LT}$ characterizes wild type, while $r^-_{LT}m^+_{LT}$ and $r^-_{LT}m^-_{LT}$ will indicate respectively strains lacking the LT restriction, and both the LT restriction and the LT modification. The presence or the absence of the LT modification on phage P 22 will be specified by the notation for the LT specificity preceded by a point. Thus P 22.LT characterizes phage P 22 carrying the LT modification, while P 22.0 indicates phage P 22 lacking any detectable modification.

RESULTS

Isolation of restriction—deficient mutants

Stocks of phage P 22 *c 2* lacking the LT modification (P 22 *c 2*.0) were obtained on a previously isolated *fer* mutant of *Salmonella typhimurium* LT7 (Colson & Colson, 1967) and on an *argD* mutant of strain LT2 that was found to lack both restriction and modification. A wide variation in efficiency of plating (e.o.p.) was observed when these phage stocks were plated on NTG-treated cultures of LT2. On most cultures the phage formed plaques at a higher frequency than on control cultures. It was logical to assume that the increase in successful infections originated from infection of restriction-deficient mutants, thus raising the e.o.p. of P 22.0 significatively higher than that obtained under normal condition of restriction. This test was therefore repeated on a large number of independent NTG-treated cultures from several strains of LT2 to assess the efficiency of the mutagenic treatment and to discriminate those cultures which after further selection could be expected to yield mutants. An increase in e.o.p. from 10^{-4} to more than 10^{-3} was commonly observed, indicating the presence of more than 0·1 % of accepting cells. Such powerful mutagenesis allowed simple methods to be used with success for the isolation of the mutants. About 1×10^3 cells from the NTG-treated cultures were spread on nutrient agar plates together with 1×10^5 particles of P 22 *c2*.0. After overnight incubation, the plates were screened for the presence of colonies showing phage infection at their edges. Such colonies were picked, restreaked and tested for their restriction phenotype. About one-third of them were found to be r^-_{LT} (40 out of 126 tested). Most mutants were isolated by this method, but two other selective methods were used.

It was shown that *F-lac* is subject to host-restriction when transferred from *Escherichia coli* to *Salmonella typhimurium* (Colson & Colson, 1967). Transfer could therefore select accepting cells, as does the transfer of an R factor (Okada & Watanabe, 1968). Samples of mutagen-treated cultures of *S. typhimurium* were spread on minimal lactose plates together with samples of exponentially growing *E. coli* W 1655 F-*lac*$^+$*met*. Contra-selection of the donor was achieved by omitting methionine from the medium and by the presence of streptomycin when a *met* recipient strain was used (SL 1027). Some r^-_{LT} were found among the *lac*$^+$ colonies, but only at a frequency of 10 % (6 of 68 tested).

Transduction of bacterial markers from a strain lacking modification was also used as a method to select r^-_{LT} mutants in the recipient. Preliminary experiments had revealed that the frequency of transduction by P 22 was very poor in LT7 when the transducing lysate was grown on LT7 $r^-_{LT}m^-_{LT}$ (Colson & D'Aout, unpublished). However, the fact that most P 22 transduced clones are immune to P 22 under the conditions used meant that scoring for restriction with P 22.0 was impossible. This difficulty was overcome with a P 22 *int* mutant (Smith & Levine, 1967). Such phage mutants are deficient in the integration function and cannot lysogenize by themselves. Lysogenic strains for P 22 *int* can nevertheless be obtained by complementation between *int* and *c* mutants. After induction, such lysogenic cultures produce highly defective lysates which transduce some bacterial markers (*proC*, *purE*) at an abnormally high frequency (Smith, 1968). LT2 *argD* 6 $r^-_{LT}m^-_{LT}$ was lysogenized with P 22 *int-11* and induced according to the methods of Smith (1968). The lysate was utilized to transduce *proC*$^+$ and *purE*$^+$ to NTG-treated strains. Most transduced clones were

found to be P 22-sensitive after one restreaking. Only a few of them (5 of 195 tested) were found to be restriction-deficient.

To ensure that all the mutants for further studies originated from independent mutational events only one r^-_{LT} clone was kept from each NTG treated culture. A total of 48 independent restriction-deficient mutants were isolated in LT2 strains, 37 were obtained from direct screening, 6 after F-*lac*$^+$ infection and 5 by P 22 *int*.0 transduction.

Table 1. *Host specificity phenotypes among 48 restriction-deficient mutants of Salmonella typhimurium*

Strain	No. of mutants with phenotype			
	$r^-_{LT}m^-_{LT}$	$r^-_{LT}m^+_{LT}$	$r^\pm_{LT}m^+_{LT}$	$r^-_{LT}m^\pm_{LT}$
SR 305	0	1	0	1
SA 534	3	1	0	5
SB 106	0	2	0	0
SA 195	2	2	1	4
SL 1027	1	1	0	3
proC 90	9	3	0	4
proC 110	3	0	0	2
Total	18	10	1	19

Restriction ability was determined by plating samples of serial dilutions of P 22.0 with samples of exponentially growing cultures of the mutants. r^-_{LT} corresponds to an e.o.p. of about 1·0; $r^\pm_{LT}$ to an e.o.p. of 10^{-1}. Modification ability was determined by picking and resuspending phage from a single plaque on each mutant and plating serial dilutions of it on the mutant itself and on wild type LT 2. Mutants producing phage with the same e.o.p. on both strains were classified as m^+_{LT}, those from which the phage was restricted about 10^{-4} were scored as m^-_{LT}, those producing phage that was restricted 10^{-1} to 10^{-3} were scored as $m^\pm_{LT}$.

Phenotypic classes of restriction-deficient mutants

Several host specificity phenotypes were observed among the restriction-deficient mutants. These phenotypic classes were distinguished by the degrees of acceptance of P 22.0 and from the subsequent e.o.p. of the phage produced by the mutants when plated on wild-type hosts. Table 1 summarizes the frequency of these classes among the various strains. A weak restriction was found in only one mutant ($r^\pm_{LT}m^+_{LT}$) in which P 22.0 was restricted about 10-fold. Among the others some had completely lost the ability to confer the modification ($r^-_{LT}m^-_{LT}$) while others produced fully modified P 22 ($r^-_{LT}m^+_{LT}$). Quite frequently intermediate abilities to modify were observed. Phage produced by these mutants plated on wild-type hosts with an e.o.p. between 10^{-1} and 10^{-3}. These phenotypes intermediate for modification were classified as $r^-_{LT}m^\pm_{LT}$. Table 2 shows the e.o.p. of P 22 on LT2 and on some of its r^-_{LT} mutants and also on LT7 and on some LT7 mutants isolated previously (Colson & Colson, 1967). These results confirm that there is no difference in host specificity between LT2 and LT7. When phage was grown on wild type LT2 its e.o.p. on LT7 was as good as on LT2, and vice versa. Similarly, when the phage was grown on a m^-_{LT} mutant of either LT2 or LT7 its e.o.p. was decreased by a similar factor on both wild-type strains. The decreased restriction which is commonly observed in LT7 *mut* is caused by a high frequency of mutator-induced r^-_{LT} mutants in the population.

Host restriction of bacterial DNA

Host specificity mutants were isolated in several genetically marked strains and in Hfr donor strains. It was therefore possible to investigate the incidence of restriction in bacterial matings and in transduction. Pairs of otherwise isogenic r^+_{LT} and r^-_{LT} strains were used as recipients in conjugation experiments with two Hfr strains, one of which carried a mutation leading to the $r^-_{LT}m^-_{LT}$ phenotype. Within each set of four crosses, restriction of donor DNA could occur in only one mating pair (Hfr

Table 2. *Efficiencies of plating of P 22 on LT2, LT7 and r^-_{LT} mutants of Salmonella typhimurium*

	Phage lysates prepared from				
	LT 7	LT 7 $r^-_{LT}m^-_{LT}$	LT 2	LT 2 $r^-_{LT}m^+_{LT}$	LT 2 $r^-_{LT}m^-_{LT}$
Host strain	Relative e.o.p.				
LT 7	1	$4{\cdot}0\times10^{-4}$	1	1	$1{\cdot}6\times10^{-4}$
LT 7 $r^-_{LT}m^-_{LT}$	1	1	1	1	1
LT 7 *mut*	1	$6{\cdot}4\times10^{-4}$	1	1	$1{\cdot}0\times10^{-3}$
LT 2	1	$3{\cdot}0\times10^{-4}$	1	1	$1{\cdot}5\times10^{-4}$
LT 2 $r^-_{LT}m^+_{LT}$	1	1	1	1	1
LT 2 $r^-_{LT}m^-_{LT}$	1	1	1	1	1

Phage from a single plaque of P 22 *c 2* grown on each host strain was used to inoculate the same host in soft agar overlays. After incubation, the overlays were harvested, shaken with chloroform and buffer and cleared by centrifugation. The lysates were suitably diluted and plated in overlays with exponentially growing cultures of each strain. The e.o.p. values given are based on counts of several hundred plaques. Differences in titre of less than 20% were not regarded as significant.

Table 3. *Host restriction in bacterial matings in Salmonella typhimurium*

		No. of recombinants per donor cell		
Recipient strain	Selected marker	$r^+_{LT}m^+_{LT}$	$r^-_{LT}m^-_{LT}$	Corrected restriction value
SB 106	*proA*$^+$	$2{\cdot}4\times10^{-3}$	$2{\cdot}4\times10^{-4}$	$5{\cdot}1\times10^{-2}$
SB 106 $r^-_{LT}m^+_{LT}$	*proA*$^+$	$4{\cdot}5\times10^{-3}$	$2{\cdot}3\times10^{-3}$	
SB 106	*leu*$^+$	$4{\cdot}0\times10^{-3}$	$3{\cdot}0\times10^{-4}$	$4{\cdot}1\times10^{-2}$
SB 106 $r^-_{LT}m^+_{LT}$	leu$^+$	$7{\cdot}4\times10^{-3}$	$4{\cdot}0\times10^{-3}$	
proC 90	*proC*$^+$	$5{\cdot}0\times10^{-4}$	$2{\cdot}9\times10^{-5}$	$1{\cdot}5\times10^{-1}$
proC 90 $r^-_{LT}m^-_{LT}$	*proC*$^+$	$8{\cdot}0\times10^{-5}$	$1{\cdot}8\times10^{-4}$	

The donor strains were SA 534 HfrK 4 (order of transfer: *leu-pro-purE*) and a $r^-_{LT}m^-_{LT}$ derivative of it. Exponentially growing cultures of the donor strains were diluted 1/10 into prewarmed broth and mixed with equal volumes of exponentially growing cultures of the recipient strains. The mixtures were centrifuged and placed at 37° in a water bath, without resuspending the pellets. After 60 min. the mixtures were shaken vigorously, diluted and plated on selective media. All selections were made in the presence of streptomycin 100 μg./ml., to which only the donors were sensitive. The donor input was estimated from the colony count on nutrient agar plates seeded with diluted samples of the donor cultures at the start of the matings.

$r^-_{LT}m^-_{LT}\times F^-r^+_{LT}m^+_{LT}$). The degree of restriction was estimated from the difference in the frequency of recombinants obtained between the two crosses in which $r^+_{LT}m^+_{LT}$ and $r^-_{LT}m^-_{LT}$ donors were mated with the same $r^+_{LT}m^+_{LT}$ recipient strain. Errors resulting from possible variations in fertility between individual donor cultures were corrected

for from the difference in the number of recombinants for a given marker obtained in two control crosses in which the same two donor cultures were mated with a single non-restricting recipient strain. The number of recombinants in Hfr $r^-_{LT}m^-_{LT}$ × $F^-r^+_{LT}m^+_{LT}$ crosses was clearly decreased as compared to the yield of the control crosses (Table 3).

The same kind of experiments were performed to assess the restriction of F-*lac*. F-*lac*$^+$ donors were obtained in LT2 and LT2 r^-_{LT} m^-_{LT} after mating with *Escherichia coli* K12 W1655 F-*lac*$^+$. Strain W1655 F-*lac*$^+$ was also used as donor and restriction-deficient mutants of *E. coli* K12 and B were included among the recipients. The yield of *lac*$^+$ clones was decreased about 100-fold in those crosses in which restriction could be exercised (Table 4). In the absence of restriction, transfer of F-*lac*$^+$ is not more effective between LT2 strains than between *E. coli* and LT2. It appears also that restriction-deficient mutants of *E. coli* are even better recipients for F-*lac*$^+$ transferred from LT2 than LT2 itself.

Table 4. *Host restriction of an F-lac$^+$ factor in Salmonella typhimurium and Escherichia coli*

Recipient strain *S. typhimurium*	No. of *lac*$^+$ clones per F-*lac*$^+$ donor			Corrected restriction value
	W1655 F-*lac*$^+$	LT2 F-*lac*$^+$	LT2 $r^-_{LT}m^-_{LT}$ F-*lac*$^+$	
proC 90	$6{\cdot}4\times10^{-7}$	$8{\cdot}5\times10^{-5}$	$4{\cdot}1\times10^{-6}$	$6{\cdot}2\times10^{-2}$
proC 90 $r^-_{LT}m^-_{LT}$	$2{\cdot}1\times10^{-4}$	$1{\cdot}6\times10^{-4}$	$2{\cdot}1\times10^{-4}$	
SL1027	$3{\cdot}2\times10^{-7}$	$2{\cdot}7\times10^{-4}$	$2{\cdot}3\times10^{-6}$	$1{\cdot}2\times10^{-2}$
SL1027 $r^-_{LT}m^-_{LT}$	$1{\cdot}3\times10^{-4}$	$2{\cdot}7\times10^{-4}$	$3{\cdot}8\times10^{-4}$	
E. coli				
B251 r^-_B m^-_B	—	$4{\cdot}4\times10^{-4}$	$1{\cdot}3\times10^{-3}$	—
C600 r^-_K m^-_K	—	$8{\cdot}5\times10^{-4}$	$2{\cdot}0\times10^{-3}$	—

Procedure as in Table 3, except that the male input was measured by plating samples of the donor on EMB lactose medium to exclude the *lac* segregants.

Table 5. *Host restriction in P 22-mediated transduction of Salmonella typhimurium*

Recipient strain	Selected marker	No. of transductants per 10^{10} plaque-forming units		Corrected restriction value
		P22.LT	P22.0	
proC 90	*proC*$^+$	$3{\cdot}9\times10^4$	$3{\cdot}0\times10^2$	$3{\cdot}0\times10^{-3}$
proC 90 $r^-_{LT}m^-_{LT}$	*proC*$^+$	$4{\cdot}5\times10^4$	$2{\cdot}0\times10^4$	
proC 110	*proC*$^+$	$9{\cdot}8\times10^4$	$5{\cdot}4\times10^2$	$4{\cdot}6\times10^{-3}$
proC 110 $r^-_{LT}m^-_{LT}$	*proC*$^+$	$4{\cdot}9\times10^4$	$4{\cdot}2\times10^4$	
SB106	*proA*$^+$	$4{\cdot}8\times10^3$	$2{\cdot}2\times10^2$	$5{\cdot}1\times10^{-2}$
SB106 $r^-_{LT}m^+_{LT}$	*proA*$^+$	$5{\cdot}0\times10^3$	$5{\cdot}5\times10^3$	
SA195	*purE*$^+$	$1{\cdot}2\times10^5$	$1{\cdot}4\times10^2$	$4{\cdot}1\times10^{-3}$
SA195 $r^-_{LT}m^-_{LT}$	*purE*$^+$	$1{\cdot}0\times10^5$	$3{\cdot}4\times10^4$	
SL1027	*trpB*$^+$	$7{\cdot}3\times10^3$	$1{\cdot}3\times10^1$	$1{\cdot}1\times10^{-3}$
SL1027 $r^-_{LT}m^-_{LT}$	*trpB*$^+$	$6{\cdot}2\times10^3$	$3{\cdot}8\times10^3$	

P22 lysates containing about 1×10^{11} plaque-forming u./ml. were obtained by the agar layer method on prototrophic LT7 and LT7 $r^-_{LT}m^-_{LT}$ strains. Exponentially growing cultures of the recipient strains were centrifuged and the deposit suspended in buffer at about 1×10^9 cells/ml. 1×10^{10} P22 particles were added to the suspensions and kept for 15 min. at room temperature before plating on selective media.

A similar set of experiments, in which the frequency of transduction was measured for several markers with P 22.LT and P 22.0 lysates in r^{+}_{LT} and r^{-}_{LT} strains showed that the degree of restriction for bacterial markers in transduction was about 100-fold (Table 5).

DISCUSSION

The isolation and phenotypic characterization of a set of 48 restriction-deficient mutants in *Salmonella typhimurium* LT2 clearly shows that the genetic control of host specificity in *S. typhimurium* is similar to other host specificity systems that have been studied. Similar types of mutants have been obtained in phage P1 (Glover, Schell, Symonds & Stacey, 1963), in an extensive analysis in *Escherichia coli* K 12 and B (Wood, 1966) and in *Pseudomonas aeruginosa* (Rolfe & Holloway, 1968). Host restriction in *Salmonella typhimurium* was found to affect the frequency of recombinants formed in bacterial matings, the frequency of transduction, and also the acceptance of plasmids, in accord with previous observations in *E. coli* (Boyer, 1964; Arber, 1964; Arber & Morse, 1965; Copeland & Bryson, 1966).

It seems therefore that similar mechanisms of control over the acceptance of foreign DNA might be widespread among bacteria and since many experiments involve the transfer of DNA between unrelated bacterial strains, such as those which investigate genetic homology between bacterial species and the possibility of plasmid transfer, in interpreting such experiments the role of host restriction should be borne in mind. A striking example of this was the *fer* mutation of *Salmonella typhimurium* which remained puzzling for some years until it was shown that *fer* mutants were restriction-deficient (Colson & Colson, 1967; Okada, Watanabe & Miyake, 1968).

The genetic basis of the mutations which affect host specificity cannot be inferred directly from the observed phenotypes. In particular, the fact that a single mutation quite often affects both restriction and modification remained unexplained until recently. Progress in understanding the mechanism of host specificity was achieved when it was shown that restriction and modification activities are exercised at particular sites on the DNA, which can be rendered an inadequate substrate for *both* activities by mutation (Arber & Kühnlein, 1967). Recombination and complementation studies lead to the demonstration of a third cistron in addition to restriction and modification (Glover, 1968; Glover & Colson, 1969; Arber & Linn, 1969). Mutations which affect host specificity can therefore occur in any one of at least three genes for which the symbols *hss*, *hsr* and *hsm* have been recently proposed (Arber & Linn, 1969). It is likely that among the mutants described in the present paper, those with an $r^{-}_{LT}m^{+}_{LT}$ phenotype lack the restriction function (*hsr*) while those with an $r^{-}_{LT}m^{-}_{LT}$ phenotype are deficient in the recognition of the sites (*hss*). A possible explanation for the $r^{-}_{LT}m^{\pm}_{LT}$ phenotype is a mutation in the *hss* gene resulting in a lowered affinity for the host specific sites on the DNA. At first sight it would appear that such mutations should affect the degrees of restriction and modification equally, but it must be pointed out that the assay systems for restriction and modification are not equally sensitive. In assaying restriction of P 22.0 as much as 20% of the phage could be restricted and remain undetected as a significant change in efficiency of plating, while in assaying modification as little as 0·1% modified phage in a lysate can easily be detected.

In view of the extensive homology between the genetic maps of *Escherichia coli* and *Salmonella typhimurium* (Sanderson & Demerec, 1965) and the similarity between the

host specificity system investigated here and those of *E. coli*, it is attractive to think that host specificity in *S. typhimurium* may belong to the allelic series of host specificities that are found in *E. coli* and which map close to *thr*. (Boyer, 1964; Colson *et al.* 1965; Glover & Colson, 1969). Experiments to be published will show that this *S. typhimurium* host-specificity system has quite a different chromosomal location close to the *pro* region.

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Transduction of *pro*$^+$ to *Salmonella typhimurium proAB47* by Phage P22

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SUMMARY

The multisite mutant *Salmonella typhimurium proAB 47* was infected with phage P 22 from a suspension mediating restricted transduction of proline markers. Evidence was obtained which supports the conclusion that *proAB 47* forms only a few colonies by the mechanism of restricted transduction, and that the near absence of this class of transductants is responsible for the low frequency of incorporation of the *pro*$^+$ marker by this strain.

INTRODUCTION

Miyake & Demerec (1960) reported, in transductions from a wild-type donor to *Salmonella typhimurium proB* mutants using phage P 22 as vector, that the wild-type marker was incorporated in the multisite mutant *proAB 47* (and also *proAB 126*) significantly less frequently than in other strains. They proposed that in *proAB 47* one or both of the regions between the ends of the deletion and the ends of the relevant transduction fragment was shortened, lowering the chance of incorporation by recombination. An alternative explanation, for which we present evidence in this paper, is that this effect results from deletion of the P 22 prophage attachment site in *proAB 47* (Smith & Levine, 1965).

Smith-Keary (1966) showed that a proline-requiring mutant could be converted to proline-independence either by the attachment of a phage fragment at the P 22 prophage site (Young & Hartman, 1966), analogous to the transduction of galactose markers by phage lambda in *Escherichia coli* (Morse, Lederberg & Lederberg, 1956), or by recombination with a donor fragment as in general transduction. If a transductant of the former type also harboured a normal P 22 prophage, the phage fragment could replicate and, after lysis of the cell, convert numerous auxotrophic cells in the background to *pro*$^+$ satellite colonies; a cell-free suspension of such a colony, termed a 'satellited transductant', could transduce *pro*$^+$, but no other markers, at high frequency.

We propose that the diminished numbers of colonies found in *proAB 47* crosses results from the absence of the class of *pro*$^+$ transductants in which a phage fragment is attached at the P 22 prophage site, which is adjacent to the proline genes. If this is correct, it is predicted that *proAB 47* should be unable to incorporate a wild-type marker transduced by particles from a restricted transducing phage suspension.

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METHODS

The nomenclature, materials and methods were essentially those described by Smith-Keary (1960, 1966). The symbol used to represent a transduction is *a* (×) *b*, where *a* is the recipient and *b* the donor.

RESULTS AND DISCUSSION

proAB 47 was infected with sterile satellited transductant suspensions and also with phage P 22 reared on a wild-type donor; the multisite mutant *proB 25*, in which the deleted region is known not to extend as far as the P 22 prophage attachment site (Miyake & Demerec, 1960) was infected as a control. Table 1 shows that: (*a*) phage reared on a wild-type donor produced less colonies on *proAB 47* than on *proB 25*, confirming the observation of Miyake & Demerec (1960). (*b*) The satellited transductant suspensions (e.g. *ES 5*) produced significant numbers of colonies on *proB 25* but few on *proAB 47*. Also, in contrast to *proB 25*, very few of the *proAB 47* transductants (not more than one per plate) were surrounded by satellites; the latter were wild type. This confirms the prediction that *proAB 47* is rarely converted to wild type by restricted phage suspensions.

Table 1. *Infection of multisite mutants with satellited transductant suspensions*

	Donor*		
Recipient bacteria	*ES* 5	*WT*	Revertants*
proAB 47	91	123	68
proB 25	338	4800	101
	SC 47–5	*WT*	
proAB 47	17730†	209	74
proB 25	13950	14940	171

* Numbers of colonies (larger figures approximate) on 4 enriched minimal medium plates (Howe & Dawson, 1968) after 10 days incubation; *c.* 5×10^7 surviving bacteria per plate. *ES* 5 and *SC* 47–5 are satellited transductant suspensions from an independent proline-requiring mutant (*proB* 401) and from *proAB* 47 respectively; *WT*, phage P 22 reared on a wild-type donor. Multiplicity of infection, 0·05 for *ES* 5 and *SC* 47–5 and 0·5 for *WT*. Each experiment was repeated with independent satellited donors and gave similar results.

† 119 of these 17730 colonies were conspicuously larger than the majority.

proAB 47 and *proB 25* were infected with cell-free suspensions of the rare satellited transductants formed by *proAB 47*. Fragments of donor genomes were now transduced to both auxotrophs at high frequency (e.g. *SC 47–5*, Table 1). A few of the colonies on the *proAB 47* (×) *SC 47–5* plates were conspicuously larger than the majority; these large colonies, and also the *proB 25*(×)*SC 47–5*, *proAB 47*(×)wild-type and *proB 25*(×)wild-type colonies, all grew as stable wild-type clones when subcultured on minimal medium. The smaller colonies from the *proAB 47*(×)*SC 47–5* plates, however, segregated mainly auxotrophs and a few slow-growing cells on subculture; the latter, on further subculture, segregated auxotrophs at high frequency. This indicates that the phage from these rare satellited colonies was unable to convert *proAB 47* cells to stable *pro*$^+$ transductants, supporting the proposal that the P 22 prophage attachment site is at least partially deleted in this mutant, and consistent with the finding of Smith & Levine (1965) and recent data of Rao & Smith (1968) showing that P 22 does not

lysogenize at its normal attachment site in *proAB 47*. A possible explanation of the large number of unstable transductants is that the satellited donors (e.g. *SC 47–5*) liberated populations of transduction fragments which, while unable to be integrated into the *proAB 47* genome, could compensate for the deletion in the latter.

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Transformation of Phage-sensitivity in *Bacillus subtilis*

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SUMMARY

Two methods were developed to demonstrate a small fraction (10^{-4}) of phage-sensitive cells in a phage-resistant population with a high degree of accuracy, using the plexiglass phage titration method (Horváth & Alföldi, 1954).

Phage-sensitivity was transferable by DNA isolated from *Bacillus subtilis* 168 M *trp*$^+$ *phs* (SPO-1 phage-sensitive) to the recipient strain of 168 M *trp phr* (SPO-1 phage-resistant) in transformation experiments. The number of 168 M *trp*$^+$ *phs* transformants was a function of the concentration of the transforming DNA. The *trp*$^+$ and *phs* characters are not linked. The competence curves for the number of 168 M *trp*$^+$ *phr* and 168 M *trp*$^+$ *phs* transformants were similar. The phenotypic lag was found to be 5 hr 30 min. The maximum frequency of 168 M *trp*$^+$ *phs* cells among the transformants was 2 to 5%.

INTRODUCTION

A wide range of bacterial characters are transferable (Ravin, 1961) and Horváth, (1969) succeeded in transforming phage-resistance in *Bacillus subtilis*.

The present investigation demonstrates the transformation of phage-sensitivity.

METHODS

Bacterial and phage strains. For transformation the recipient strain was a spontaneous SPO-1 phage resistant mutant of *Bacillus subtilis* 168 M *trp phs* designated 168 M *trp phr*. Phage-sensitive spontaneous mutants were found among the 168 M *trp phr* bacteria and the rate of mutation was $5{\cdot}82 \times 10^{-5}$ mutations per bacterium per generation calculated according to Luria & Delbrück (1943). DNA was prepared from *B. subtilis* 168 M *trp phs* and 168 M *trp phr* strains after transformation to prototrophy.

Media. Bacterial strains were maintained on potato agar (Spizizen, 1958). The recipient strain was precultivated on minimal glucose yeast agar slope (MGY agar); competent cells were prepared in MGY liquid medium; T medium was used for transformation and MG agar for the selection of 168 M *trp*$^+$ *phr* transformants (Horváth, 1967).

Transforming DNA from the donor strains was prepared by the phenol extraction method of Saito & Miura (1963).

The titration of phage was carried out by the plastic tray method (Horváth & Alföldi, 1954).

Transformation procedure. An overnight culture of 168 M *trp phr* on MGY agar slope was suspended in 10 ml. liquid medium in a 100 ml. Erlenmeyer flask, fitted with a side arm for densitometry measurement. The optical density (OD) of the

bacterial suspension was 0·025, which contained $9·6 \times 10^5$ colony forming u./ml. The culture was incubated in a water bath at 37° on a reciprocal shaker at 100 rev./min. (Horváth, 1967). When the bacterial cells reached the peak of competence (OD 1·5) the cell suspension was diluted in T medium to 0·4 OD. To 0·25 ml. of this suspension 0·75 ml. DNA solution in T medium was added to give a final DNA concentration of 5 μg./ml. and shaken for 30 min. in a water bath at 37°. Finally, 0·25 ml. of this mixture was measured into a 50 ml. Erlenmeyer flask containing 4·75 ml. MG liquid medium with 0·1 % casein hydrolysate and after 14 to 15 hr incubation at 37°, 0·05 ml. of the bacterial suspension was transferred to 2 ml. MG liquid medium containing 0·1 % casein hydrolysate. The suspension was shaken in a water bath at 37°, and after 3 to 4 hr the number of phage-sensitive cells as 168 M *trp*$^+$ *phs* transformants was measured according to the following two methods. This transformation procedure yields qualitative rather than quantitative results.

Measurement of the number of SPO-1 phage-sensitive cells in phage-resistant populations

1. *One-step growth curve method.* 168 M *trp phr* and 168 M *trp phs* cell suspensions in early exponential growth were used (OD 0·3). Different numbers of 168 M *trp phs* cells in 0·5 ml. volumes were added to 1 ml. phage resistant 168 M *trp* cells. 2×10^6 SPO-1 phage particles in 0·5 ml. volumes were then added to each tube. These cultures were shaken in a water bath at 37° for 5 min. 0·1 ml. of these suspension were diluted in 10 ml. MGY liquid media and incubation was continued for 1 hr. Samples were taken and one-step growth curves were determined from the plaque forming units (p.f.u.). The higher the number of phage-sensitive cells in the phage-resistant population, the higher the titre of the plateau. The plaque titre at the plateau in log units was designated T_c for the control phage-resistant bacterial suspension and T_e for the bacterial suspensions which contained phage-sensitive cells. The difference $T_e - T_c$, designated D, was used in the transformation experiments to assay the number of phage-sensitive cells present among the 168 M *trp*$^+$ *phr* transformants.

2. *The phage, anti-phage serum method.* To 0·5 ml. phage-resistant cell suspension after transformation 0·25 ml. SPO-1 phage (4×10^7 p.f.u./ml.) was added and shaken at 37° for 10 min. Then 0·25 ml. anti-SPO-1 phage serum (K value 25) was added and incubation continued for a further 10 min. at 37°. The number of infective centres was then assayed using 168 M *trp phs* as indicator bacteria.

RESULTS AND DISCUSSION

Transformation of phage-sensitivity

To investigate the transformation of phage-sensitivity, undiluted 168 M *trp phr* competent cells were used in the experiment. The D value was 1·357 when transformation was carried out with DNA isolated from 168 M *trp*$^+$ *phs* and 5 % of phage-sensitive cells were found among the *trp*$^+$ transformants. When DNA isolated from 168 M *trp*$^+$ *phr* was used in transformation the number of phage-sensitive cells was very low ($D = -0·153$) and not higher than with the control strain, 168 M *trp phr*.

When transforming DNA was treated with 50 μg./ml. DNase before transformation, no transformants could be detected.

Effect of DNA concentration

Different quantities of transforming DNA isolated from 168 M *trp*$^+$ *phs* cells were added to the undiluted competent 168 M *trp phr* cells, and the *D* values were measured. The number of 168 M *trp*$^+$ *phs* transformants was a function of the DNA concentration. The dose-effect curve calculated from the number of 168 M *trp*$^+$ *phs* transformants was steeper than 45°, which shows that the *trp*$^+$ and *phs* markers are not linked. When markers are far apart, double transformants are found only at saturating levels of DNA (Goodgal, 1961; Michel, Sicard & Ephrussi-Taylor, 1964; Kelly, 1967).

The competence curve

The competence curve based upon the number of 168 M *trp*$^+$ *phr* transformants was the same as that found earlier. The characteristics of the competence curve for 168 M *trp*$^+$ *phs* transformants were also similar to those obtained previously (Horváth, 1967, 1968).

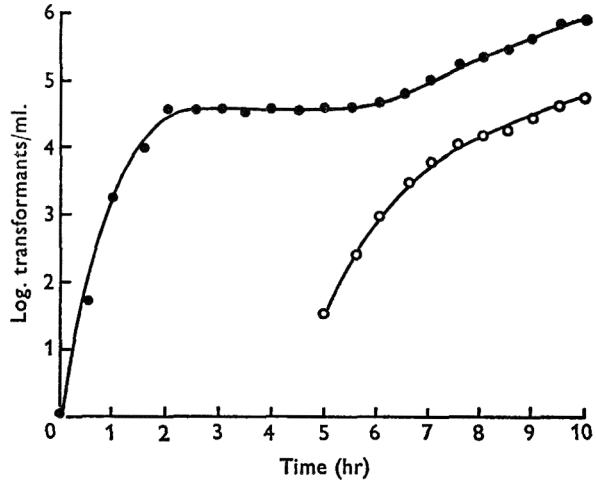

Fig. 1. The number of *trp*$^+$ *phr* and *trp*$^+$ *phs* cells during transformation. ●—— ●, 168 M *trp*$^+$ *phr* transformants; ○——○, 168 M *trp*$^+$ *phs* transformants; 168 M *trp phr* bacteria were transformed with DNA from 168 M *trp*$^+$ *phs*. The number of *trp*$^+$ *phs* transformants was measured using the phage, antiphage serum method. o is the time when DNA was added.

Assay of trp$^+$ *phr and trp*$^+$ *phs bacteria during transformation*

168 M *trp phr* competent cells were used to measure the number of *trp*$^+$ *phr* and *trp*$^+$ *phs* bacteria during transformation. DNA isolated from 168 M *trp*$^+$ *phs* was added to the cell suspension and incubated at 37° for 30 min. 0·5 ml. was then measured into three flasks each containing 9·5 ml. MG liquid medium with 0·1% casein hydrolysate. A control without DNA was included. 0·9 ml. samples were taken during incubation and 0·1 ml. DNase (30 μg.) was then added and incubation continued for 5 min. The *trp*$^+$ *phr* transformants were selected on MG agar. The number of *trp*$^+$ *phs* transformants was measured by the phage, antiphage serum method (Fig. 1).

After subtraction of the control values, the curve for *trp*$^+$ *phs* transformants was obtained. The phenotypic lag was about 5 hr 30 min. A concomitant rise in the number

of *trp*$^+$ *phr* as well as the *trp*$^+$ *phs* transformants was observed during incubation. The frequency of the *trp*$^+$ *phs* among the *trp*$^+$ *phr* transformants was about 3%, calculated from the corrected values.

Sensitivity of trp$^+$ *phr and trp*$^+$ *phs transformants to phage SPO-1*

Suitable bacterial dilutions were spread on MGY agar to obtain 60 to 80 colonies per plate. 4 to 5% of these colonies were SPO-1 phage sensitive in tests employing the replica plating technique (Lederberg & Lederberg, 1952). Six phage-sensitive and ten phage-resistant colonies were tested. The phage-sensitive colonies gave high *D* values and phage-resistant colonies gave very low *D* values. Intermediate *D* values were not observed.

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The Partial Purification and Properties of Endopolygalacturonase and α-L-Arabinofuranosidase secreted by *Sclerotinia fructigena*

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SUMMARY

An endopolygalacturonase (PG) and an α-L-arabinofuranosidase (AF) in culture filtrates of the fungus *Sclerotinia fructigena* were partially purified by ion-exchange chromatography on CM-Sephadex and Ecteola-cellulose columns, and characterized. Molecular weight estimations by gel-filtration and electrophoresis on cellulose acetate strips indicated that both enzymes existed in multiple forms. The PG showed unusual stability to extreme pH values, to classical inhibitors, and to proteolytic enzymes; in the crude filtrate a bimodal temperature/stability curve was obtained.

INTRODUCTION

The role of pectolytic enzymes in the pathogenicity of fungi and bacteria towards higher plants has been reviewed by Wood (1960) and by Bateman & Millar (1966). Pectolytic enzymes are believed to be involved in the brown rots of plant tissues caused by three species of the genus *Sclerotinia*. The importance of 'cytolytic' enzymes in the pathogenicity of fungi was first put forward in the classical paper of de Bary (1886) about *S. libertiana*; Bruschi (cited by Valleau, 1915) was the first to suggest the action of pectolytic enzymes in a fruit-rotting species. Subsequent work with the brown rot fungi was reviewed by Wormald (1954). Cole (1956), who studied the secretion by *S. fructigena* of extracellular pectolytic enzymes, showed a strong parallel between the plant-macerating properties of his preparations and their ability to decrease the viscosity of pectate solutions. In further studies, however, Cole & Wood (1961) concluded that the degree of macerating activity was not always directly related to the degree of polygalacturonase activity alone or together with pectin methyl esterase. They also showed the presence of traces of polygalacturonase in infected apples: an apreciable amount was also detected by Calonge, Fielding, Byrde & Akinrefon (1969).

Much of the earlier work on the pectolytic enzymes of Sclerotinia species and other fungal pathogens was done with crude culture filtrates or with ethanol or acetone precipitates from them; the present work aimed at a fuller purification of the polygalacturonase of *S. fructigena*. During this purification, maceration was found to be associated with a component distinct from polygalacturonase (Byrde & Fielding, 1962); this was later identified as pectin methyl-*trans*-eliminase (Byrde & Fielding, 1968). The presence of an enzyme capable of hydrolysing phenyl-α-L-arabinofuranoside, and initially thought to be involved in maceration, was also demonstrated in these preparations (Byrde & Fielding, 1965) and in infected apple (Calonge *et al.*

1969). The secretion of arabanases by plant pathogenic fungi was reported by Japanese workers (e.g. Kaji, Tagawa & Motoyama, 1965); Fuchs, Jobson & Wonts (1965) surveyed arabanase production by a range of plant pathogens and drew attention to the relatively high secretion of these enzymes by pathogens of leguminous plants rich in arabans. There have been subsequent reports of the secretion of arabanase by Penicillium species (Bush & Codner, 1968) and of an α-L-arabinofuranosidase by *Phytophthora palmivora* (Akinrefon, 1968).

The present paper describes the partial purification and some properties of the polygalacturonase (poly-α-1,4-galacturonide glycanohydrolase; EC. 3.2.1.15) and of the α-L-arabinofuranosidase (α-L-arabinofuranoside arabinohydrolase) secreted by *Sclerotinia fructigena* Aderh. & Ruhl.

METHODS

Materials. Pectinol 10 M (Rohm and Haas, U.S.A.) was used as a reference source of polygalacturonase. Sodium polypectate (Exchange Lemon Products Co., California), 'Brown Ribbon' pectin (12·5% methoxyl) (Union Crystalex Gelatine Ltd., London) and *p*-nitrophenyl-α-L-arabinofuranoside (Fielding & Hough, 1965) were used as substrates. For analytical studies, the pectic materials were purified by washing with acidified 80% (v/v) ethanol in water, followed by ethanol to remove the acid, and dried with ethanol+ether (1:1, v/v). For chromatographic studies, Araban (Koch–Light, Colnbrook, Bucks) and apple fibre (Cole & Wood, 1961) were also used as substrates. Sephadex cross-linked dextrans for gel-filtration and ion-exchange chromatography were obtained from Pharmacia AB, Uppsala. Whatman Ecteola-cellulose powder ET 30 and other modified celluloses were also used. The enzyme inhibitors tested were from commercial sources.

Culture of the fungus. The methods used were as described by Byrde & Fielding (1968) except that, in some of the earlier work, sporulating cultures were grown on potato plugs soaked for 72 hr in aqueous malic acid solution (7·5%, w/v) before autoclaving (Wiltshire, 1920).

Concentration of the enzyme. When desirable, the enzymes in the crude culture filtrate were concentrated by partial 'freezing-out' (Dixon & Webb, 1958) or by tannic acid precipitation followed by washing with cold acetone (Shibata & Nisizawa, 1965). The latter method gave a pale buff-coloured powder which retained high enzyme activities for many months when stored at 4°.

Estimation of enzyme activities. Polygalacturonase (poly-α-1,4-galacturonide glycanohydrolase, PG) (EC. 3.2.1.15), was estimated by one of three methods: (*a*) reducing group assay, based on the estimation of residual iodine with sodium thiosulphate (Jansen & MacDonnell, 1945). Activity was expressed as micro-equivalents of reducing groups liberated per min. at 25°; (*b*) viscosity assay, based on the loss of viscosity of aqueous solutions of pectin or sodium polypectate following decrease in chain length (Wood, 1955). The test solution comprised aqueous pectin or sodium polypectate solution (1% w/v, 5 ml.), appropriate buffer (2 ml.), water (2 ml.) and enzyme preparation (1 ml.). Activity was expressed as $1/t$ where t was the time (min.) for 50% decrease in viscosity; (*c*) an agar plate assay, as described by Dingle, Reid & Solomons (1953) was extensively used for semi-quantitative estimations during purification. The external diameter of the white ring obtained on the pectate agar plate was proportional to the

logarithm of the enzyme concentration over a range of dilutions. Activities were expressed relative to an aqueous solution (1 mg./ml.) of Pectinol 10 M, defined arbitrarily as having 100 units (u.) of activity/ml. Results obtained by this method were shown to be well correlated with those obtained by the viscosity method (see Results).

α-L-Arabinofuranosidase (α-L-arabinofuranoside arabinohydrolase) (AF), *pectin methyl*-trans-*eliminase* (PTE) *and macerating activity* were estimated as described by Byrde & Fielding (1968), who detected no polygalacturonate *trans*-eliminase in the culture filtrate.

Gel filtration. For molecular weight determinations 0·1 M-KCl in 0·05 M-tris buffer (pH 7·4) was used for elution of a Sephadex G 75 column (bead form) following the method of Andrews (1964) and basing the standard curve on nine proteins; 3 ml. fractions were collected. In other experiments, water was used for elution and 10 ml. fractions were collected.

Ion-exchange chromatography. This was done with CM-Sephadex gel (C.50) (Byrde & Fielding, 1968) or Ecteola-cellulose. Before use, Ecteola-cellulose was washed successively with N-NaOH, M-KH_2PO_4 and 0·01 M-phosphate buffer (pH 7·0) to remove impurities showing high absorption at 280 mμ (Bradley & Rich, 1956). Elution was with water and NaCl solutions.

Paper chromatography. Breakdown products were examined on Whatman no. 54 paper with solvents ethyl acetate + acetic acid + water (2 + 1 + 2, by vol.; Jermyn & Isherwood, 1949) or 80% (w/v) phenol in water. Spots were developed by spraying the papers with *p*-anisidine hydrochloride (3%, w/v, in butan-1-ol; Hough, Jones & Wadman, 1950) or with silver nitrate (Trevelyan, Procter & Harrison, 1950).

Electrophoresis on cellulose acetate strips followed the general method described by Kohn (1957). The enzyme solution (50 μl.) was streaked by successive applications with a micro-constriction pipette on a cellulose acetate strip 36 × 5 cm. (Oxoid Ltd., London) in a narrow band approximately 7 cm. from the anode end. The voltage used was 300–400 V, d.c. giving a current of about 2 mA per strip, in 0·05 M-tris buffer (pH 7·6 or 8·5); electrophoresis was at room temperature. After running for approximately 8 hr the sites of enzyme activity were detected in one of three ways: (*a*) for PG, by inverting the moist strip on a plate of pectate agar gel (see estimation methods). After overnight incubation at 25°, the strip was removed and the plate developed with 6 N-HCl to give discrete white zones; (*b*) for AF, the moist strip was sprayed with an aqueous solution of *p*-nitrophenyl-α-L-arabinofuranoside (1 mg./ml.), incubated for 1 hr at 25° and laid on a filter paper moistened with saturated Na_2CO_3 solution for development of the yellow colour; (*c*) alternatively, for semi-quantitative estimation of AF and PG, the strip was removed and divided transversely into segments 1 cm. wide, each of which was further cut into pieces about 2 mm. across and soaked in 1 ml. 0·1 M-acetate buffer (pH 4·7) for 1 hr. Samples were then removed for enzyme assay, using the agar plate method for PG.

RESULTS

Gel filtration and column chromatography. The initial culture filtrate contained PG, AF and PTE activities. The patterns of PG, AF and macerating activity when a crude culture filtrate was applied to Sephadex G 75 and eluted with water are shown in Fig. 1.

Appropriate fractions were dialysed overnight at 4° against distilled water and applied to a column of Ecteola-cellulose. Elution with water followed by 0·1 M-NaCl resulted in the enzyme pattern shown in Fig. 2. When a crude culture filtrate was applied to a column of CM-Sephadex, followed by elution with a sodium chloride gradient, PG and AF were maximal in the early fractions. Macerating activity (due to PTE) followed in two peaks (Fig. 3 of Byrde & Fielding, 1968).

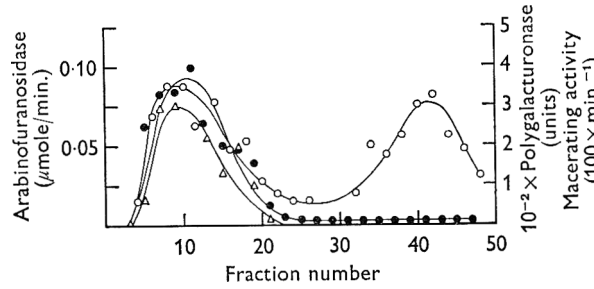

Fig. 1. *Sclerotinia fructigena*. Polygalacturonase (●), arabinofuranosidase (△) and macerating (○) activity of fractions from aqueous elution of Sephadex G 75 dextran gel.

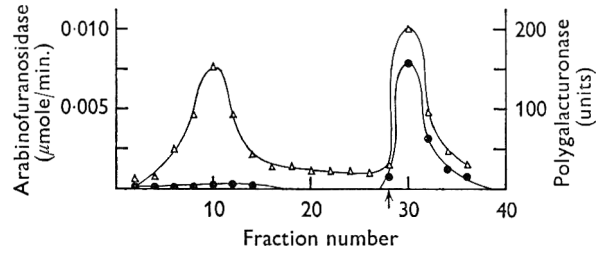

Fig. 2. *S. fructigena*. Polygalacturonase (●) and arabinofuranosidase (△) activities of fractions from Ecteola-cellulose eluted first with water and then (from arrow) with 0·1 M-NaCl.

CM-Sephadex and Ecteola-cellulose column chromatography were used successively for partial purification of PG and AF, to give purification factors of 61 and >24 respectively, and elimination of all but traces of the other enzymes.

For PG, CM-Sephadex was used initially, enabling the PTE component to be eliminated. When dialysed fractions high in PG and AF activity were then passed through Ecteola-cellulose, PG activity was retained by the column and could be eluted in a sharp peak with 0·1 M-NaCl. By contrast with the behaviour when fractions from Sephadex G 75 were applied to Ecteola-cellulose, AF activity was not present in the initial eluate, nor was it present in the 0·1 M-NaCl eluates of high PG activity (Table 1). The uronide concentration (McComb & McCready, 1952) was decreased from 1700 μg./ml. in the culture filtrate to 2 μg./ml. in fraction 26.

For AF purification, the chromatography was done in reverse order. With a tannic acid preparation the PG was retained on the Ecteola-cellulose. The water eluate containing 58% of the AF activity was then applied to a CM-Sephadex column which enabled the AF to be separated from the PTE (Table 2).

Unsuccessful purification methods. Several techniques conventionally used for enzyme purification proved to be unsuitable for the purification of this PG. Poor recoveries

Table 1. *Partial purification of polygalacturonase from Sclerotinia fructigena*

Fraction	Vol. (ml.)	Activity AF (μmole/hr)	Activity PG (u.)	Activity PTE (ΔE_{240}/100 min. at pH 7)	Total activity AF	Total activity PG	Total activity PTE	E_{280}	Sp.act. PG (units/E)	Yield (%)	Purification
Culture filtrate	10	28·4	631	2·34	284	6310	23·4	3·14	200	100	1
Elution from CM-Sephadex (fractions 3–8)	40	2·75	100	0·01	109	4000	0·40	0·368	270	63	1·35
Elution from Ecteola-cellulose (fractions 26–30)	50	0·068	40·7	—	3·4	2035	—	0·003	12,210	31	61

—, Not detected.

Table 2. *Partial purification of arabinofuranosidase from Sclerotinia fructigena*

Fraction	Vol. (ml.)	Activity AF (μmole/hr)	Activity PG (u.)	Activity PTE (ΔE_{240}/100 min. at pH 7)	Total activity AF	Total activity PG	Total activity PTE	E_{280}	Sp.act. AF (units/E)	Yield (%)	Purification
Tannic acid ppt. at 5 mg./ml. (filtered)	19	24·5	1260	0·60	465	23,940	11·4	3·5	7	100	1
Water elution from Ecteola-cellulose (fractions 14–19)	30	8·5	13	0·12	255	390	3·6	0·074	115	55	16·5
0·05 M-NaCl elution from CM-Sephadex (removal of PTE)	80	0·84	—	—	68	—	—	<0·005	>168	15	>24

—, Not detected.

(about 33 %) from the culture filtrate were obtained with acetone, ethanol or ammonium sulphate precipitation, the latter being tried at a range of pH values. Adsorption on, and elution from, pectic acid gel (Demain & Phaff, 1954) gave heavy polysaccharide contamination, whilst calcium phosphate gel, aluminium oxide and glass powder did not adsorb the enzyme. Bentonite adsorbed the activity but this could not be recovered. Of the modified celluloses, carboxymethyl cellulose gave poor adsorption of PG, by contrast with the experience of Mill & Tuttobello (1961) with the PG of *Aspergillus niger*.

DEAE-cellulose appeared less useful than Ecteola-cellulose, whilst elution from TEAE-cellulose resulted in a plateau, rather than a peak, of activity. Zone electrophoresis on starch gel (Smithies, 1955), agar gel or polyvinyl chloride gel (Kunkel & Trautman, 1959) were ineffective: the enzyme remained at the starting line. The same difficulty was encountered with paper electrophoresis, although Purr, Hottenroth, Döring & Schneider (1957) successfully separated two polygalacturonases from a commercial pectolytic enzyme by this method.

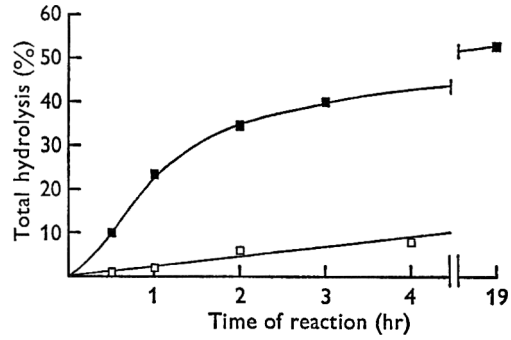

Fig. 3. *Sclerotinia fructigena*. Hydrolysis of 0·4 % solutions of sodium polypectate (■) and pectin (□) measured by the release of reducing groups at pH 4·7.

Properties of the enzymes

PG. The PG preparations used for these experiments were 0·1 M-NaCl eluates from Ecteola-cellulose showing maximum PG activity; these were dialysed overnight at 4°. Figure 3 shows the time-course of liberation of reducing groups by PG with sodium polypectate and pectin as substrates. The pattern of the curves is very similar to that reported for an endo-PG from *Aspergillus niger* by Mill & Tuttobello (1961) and shows that breakdown became very slow after 40 % hydrolysis. Polypectate was more readily attacked than pectin; this was also found by the viscosity method. The viscosity of sodium polypectate solution (0·5 %) was halved in about 1 min.

AF. The relationship between time and *p*-nitrophenol liberated from *p*-nitrophenyl-α-L-arabinofuranoside solution was linear only up to absorption values of approximately 0·1, corresponding to 15 % hydrolysis. This value was inevitably low because of the low concentration of substrate which was in short supply. By making estimations at a range of higher substrate concentrations, the Lineweaver-Burk plots obtained gave a K_m. value between 0·35 and 0·36 mM in each of two experiments. This compared with values of the order of 0·65 mM for the AF of *Phytophthora palmivora* (Akinrefon, 1968).

Breakdown products. When a purified PG preparation was incubated at 25° under toluene with sodium pectate or pectin (final concentration 16 mg./ml.) no spot corresponding to D-galacturonic acid was detected after 48 hr, although two spots of lower R_F value, which were probably oligouronides were observed, even after 8 hr; these

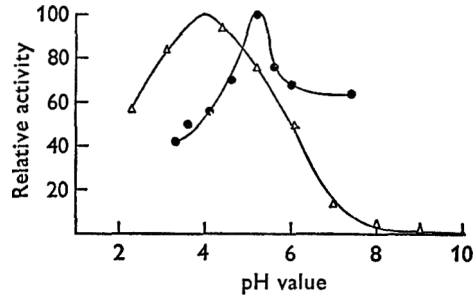

Fig. 4. *S. fructigena.* pH/activity relationships for polygalacturonase (●) and arabinofuranosidase (△).

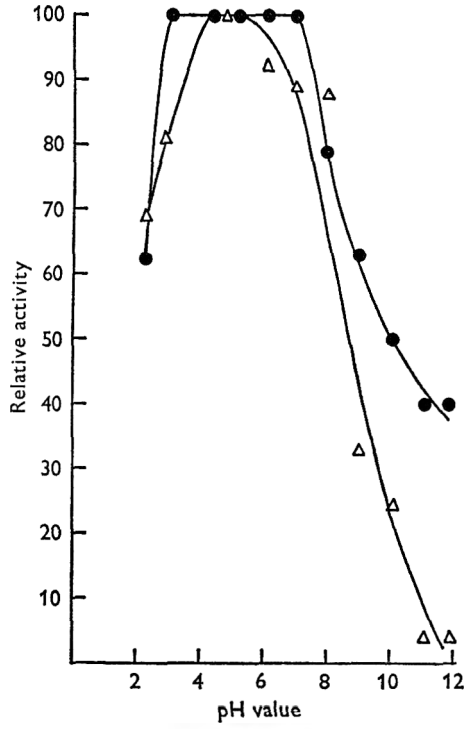

Fig. 5

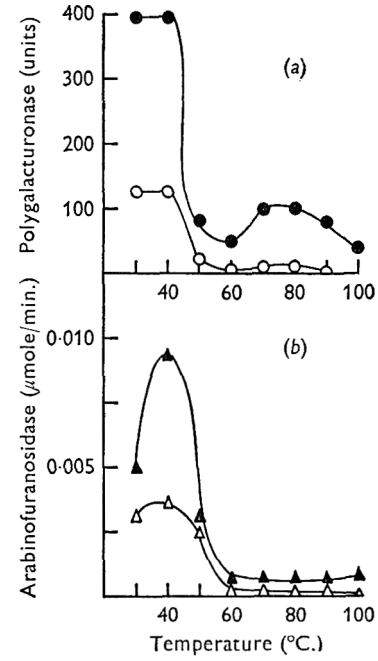

Fig. 6

Fig. 5. *Sclerotinia fructigena.* pH/stability relationships for polygalacturonase (●) and arabinofuranosidase (△) for 20 min. exposure.

Fig. 6. *S. fructigena.* Thermal stability (20 min. exposure) of: (*a*) polygalacturonase in culture filtrate (●) and purified preparation (○); (*b*) arabinofuranosidase in culture filtrate (▲) and purified preparation (△).

were more pronounced when sodium pectate was the substrate. Spots corresponding to glucose were also observed.

When a purified AF preparation was similarly incubated overnight with Araban or an apple fibre preparation, monomeric arabinose was detected, but no evidence of oligomers was obtained. A trace of arabinose was also detected when pectin was used as substrate.

pH relationships. PG activity at a range of pH values was estimated by the reducing group method with sodium polypectate as substrate and acetate and phosphate buffers (final concentration 0·2 M). The results plotted in Fig. 4 were comparable with those found by Cole (1956) for *Sclerotinia fructigena* by the viscosity method with sodium polypectate as substrate, though with the peak of activity about one pH unit lower. AF activities estimated in the presence of citrate + phosphate + borate buffer are also shown in Fig. 4.

The effect of pH value on PG stability was examined over the range pH 2 to 12 by mixing 1 vol. of the appropriate citrate + phosphate + borate buffer (Teorell & Stenhagen, 1938) with 9 vol. of enzyme solution. After 20 min. at room temperature an equal volume of 0·2 M-acetate buffer (pH 4·7) was added. The decrease in PG activity assessed by the agar plate method is shown in Fig. 5, relative to untreated enzyme. The enzyme showed remarkable stability at small pH values and also up to pH 11·8. Figure 5 also shows comparable data for AF activity.

Table 3. *Sclerotinia fructigena: molecular weight of enzyme components estimated by gel-filtration*

Enzyme	Elution vol. (ml.) Expt 1	Expt 2	Expt 3	Expt 4	Mean	Corresponding mol.wt.
PG	157	152	148	148	151	77,000
PG	199	205	202	193	200	38,500
AF	135	127	130	128	130	> 200,000
AF	174	177	182	173	176	53,700
AF	216	—	—	219	217	30,500

PG = Polygalacturonase. AF = α-L-Arabinofuranosidase.

Temperature stability and effect of enzyme inhibitors. Samples (2 ml.) in 20 ml. test-tubes were held at a range of temperatures for 20 min., then cooled rapidly to about 0° by immersion of the tube in acetone at −25°. Residual PG activities were estimated viscometrically, and residual AF by the usual method. Figure 6 summarizes the data for each enzyme using both crude (culture filtrate) and purified preparations.

The PG activity of each sample was also estimated by the agar plate method. The results by the two methods in three experiments showed a highly significant correlation ($P < 0{\cdot}001$). The equation relating the agar-plate value (y) to the viscosity estimate (x) was:

$$y = 2{\cdot}50\,x.$$

The inhibition of enzyme activity at 60° was not prevented by gelatin (1 mg./ml.), cysteine (10^{-3} M), ethylenediaminetetra-acetic acid (10^{-3} M) or potassium cyanide (10^{-4} M).

Tests for polyphenol oxidase activity at room temperature and at 60° were negative.

Heating to 80° for 20 min., however, resulted in a more active preparation after a further 20 min. at 60° than a control sample heated only to 60° for 20 min.

Less than 20% inhibition of PG activity in fractions eluted from Sephadex G.75 occurred in the presence of the following: KCN, 8-hydroxyquinoline, sodium diethyldithiocarbamate, sodium azide, iodoacetic acid, mepacrine, *o*-phenanthroline or 2,4-dinitrophenol (each at 10^{-3}M), diethyl p-nitrophenyl phosphate or di-*iso*propyl fluorophosphate (each at 10^{-4}M). Compounds were incubated for 1 hr with the enzyme before assay by the agar plate method at pH 4·7. Under these conditions, mercuric chloride inhibited activity by 50% at 10^{-3}M and by 34% at 10^{-4}M, p-chloromercuribenzoic acid by 57% at 10^{-3}M, and urea by 80% at 8M.

When the enzyme was exposed as indicated below to proteolytic enzymes before assay, the following gave less than 20% inhibition: pepsin (0·03 mg./ml., pH 3, 37°. 30 min.), papain (0·03 mg./ml., pH 6, 37°, 30 min.), trypsin or chymotrypsin (each at 0·1 mg./ml., pH 7·5, 37°, 2 hr). Exposure to 'Pronase' (secreted by *Streptomyces griseus*) (0·1 mg. ml., pH 7·4, 30°, 24 hr) resulted in 30% loss of activity.

Molecular weight determination from Sephadex gel filtration data. One ml. of a dialysed freeze-concentrated culture filtrate of *Sclerotinia fructigena* was mixed with 1 ml. eluant and applied to a calibrated Sephadex G75 column. Molecular weight determinations (Andrews, 1964) in four experiments are summarized in Table 3. The form of AF with the smallest molecular weight was detected in only two of the four experiments.

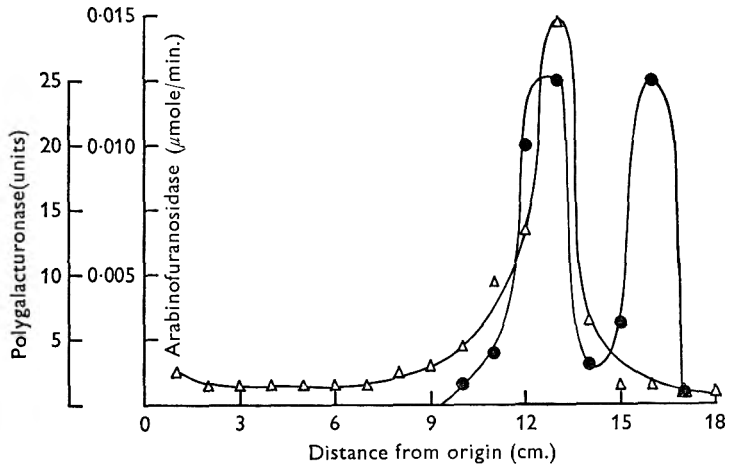

Fig. 7. *Sclerotinia fructigena*. Migration towards cathode of polygalacturonase (●) and arabinofuranosidase (△) on electrophoretogram on cellulose acetate strips.

Electrophoresis on cellulose acetate strips. In experiments with crude culture filtrates, or various preparations from them, both enzymes migrated consistently towards the cathode. Best results were obtained by using a tannic acid preparation (10 mg./ml.); when electrophoresis was done at pH 7·6, two PG peaks were detected, one being coincident with the single peak of AF activity (Fig. 7). A similar pattern was found in three other experiments at pH 7·6 and also at pH 8·5, although the faster running PG component was generally slightly less active than the slower running one. The peak of macerating activity of the fractions coincided with the joint peak of PG and AF.

DISCUSSION

The polygalacturonase of *Sclerotinia fructigena* is apparently an endo-polygalacturonase Type I according to the classification of Demain & Phaff (1957). Its ability to cause the rapid decrease in viscosity of sodium polypectate solutions with a slow release in reducing groups, together with the absence of D-galacturonic acid in incubation mixtures, is characteristic of this type of PG. The more rapid hydrolysis of pectate than of pectin indicates that the enzyme is a polygalacturonase rather than a polymethylgalacturonase. The pH/activity relationships of the enzyme, with sodium polypectate as substrate, characterize it as being of Type I (optimum at relatively small pH value); it is in many respects similar to the endo-PG of *Aspergillus niger* described by Mill & Tuttobello (1961).

The Sclerotinia enzyme showed unusual stability over a wide range of pH values and was in general little affected by classical enzyme inhibitors. This is in accord with general experience with pectolytic enzymes (e.g. Husain & Dimond, 1958). The enzyme is, however, readily inactivated by several polyphenolic compounds (Byrde, Fielding & Williams, 1960; Cole & Wood, 1961) probably in a non-specific manner. It also resists breakdown by the highly versatile proteolytic enzyme from *Streptomyces griseus* (Nomoto, Narahashi & Murakami, 1960).

McClendon (1964), who used a preparation from *Sclerotinia fructigena*, showed the existence of more than one component by ion-exchange chromatography. That such components may represent polymeric forms was indicated by the present results of the molecular weight estimation with a dialysed concentrated culture filtrate, which gave peaks corresponding to molecular weights of 38,500 and 77,000. It may be significant that Swinburne & Corden (1969) observed changes in the properties of a PG secreted by *Penicillium expansum* during purification by ion-exchange chromatography which they attributed to changes in the molecular configuration of the enzyme. Two forms of the *Sclerotinia fructigena* enzyme in the tannic acid preparation were also detected on strip electrophoresis; these were not further characterized, but both carried a strong overall basic charge at pH 7·6 and pH 8·5. This basic character is likely to assist in the adsorption of the enzyme on the acidic polyuronides of the plant cell wall.

The temperature inactivation pattern of the crude *Sclerotinia fructigena* PG shows an unusual bimodal curve, reminiscent of that obtained for a proteolytic enzyme by Virtanen (1934) and with the PG's of *Botrytis cinerea* and *Erwinia aroideae* by Jarvis (1953). Virtanen ascribed these effects to complexing with other proteins. It may, however, be significant that with the purified preparation from *S. fructigena* (which, by contrast with the crude PG, was very low in uronide content) the second peak of activity at higher temperatures was much decreased. Alternatively, depolymerization by heat of a component in the crude culture filtrate into two molecules of lower polymers might be involved.

The α-L-arabinofuranosidase appears to be a typical glycosidase attacking araban by a terminal mechanism to liberate the monomer as did the arabanases from Aspergillus species described by Kaji, Taki, Shimazaki & Shinkai (1963). By contrast, arabanase from *Sclerotinia libertiana* liberated small quantities of oligomers in addition to monomer (Kaji *et al.* 1965). The *S. fructigena* enzyme is specific for the furanoside configuration (Byrde & Fielding, 1965), and its properties are in general similar to

those of an AF from *Phytophthora palmivora* described by Akinrefon (1968), although the K_m value is appreciably lower.

By contrast with the PG from *Sclerotinia fructigena* the AF was appreciably inactivated at high pH values and showed a normal temperature inactivation pattern. Whilst one peak of activity was detected in a tannic acid preparation by strip electrophoresis, or in a culture filtrate by gel filtration with water elution, there was evidence of two and sometimes three peaks on gel-filtration of a freeze-concentrated preparation eluted with tris buffer containing 0·1 M-KCl. Although the estimated molecular weights of the two smaller components showed no obvious numerical relationship, and that of the largest component could not be assessed with the dextran gel used, it is clear that, as with PG, the enzyme can exist in multiple forms which are readily interconverted.

We wish to thank Miss Celia Slowley and Mrs M. Gooday for valuable technical assistance, and Mr G. M. Clarke for the correlation analysis.

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Lactic Dehydrogenases of Strains of the Genus *Leuconostoc*

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SUMMARY

The lactic dehydrogenases of 11 strains of the genus *Leuconostoc* were examined. All possessed a D(−) but no L(+) NAD-dependent lactic dehydrogenase, and three strains (1 *L. mesenteroides* and 2 *L. paramesenteroides*) also had D(−) and L(+) NAD-independent lactic dehydrogenases. The NAD-dependent enzyme of the seven strains belonging to the species *L. mesenteroides*, *L. dextranicum*, *L. paramesenteroides*, *L. lactis* and *L. cremoris* moved together during electrophoresis in acrylamide gel. The enzymes of the other four strains, all *L. oenos*, were close together but widely separated from the enzyme of the other species. One strain of *L. mesenteroides* oxidized NADH without added pyruvate. The enzyme responsible was located after electrophoresis.

INTRODUCTION

Lactic acid bacteria have two types of enzyme for which lactate is substrate. The most active system catalyses the frequently reversible reaction pyruvate⇌lactate and is NAD-dependent. This type of enzyme has been reported for a variety of species (Dennis & Kaplan, 1960; van den Hamer, 1960). A second system, which is relatively less active and which is not NAD linked (independent), has also been found in several species (Snoswell, 1963; van den Hamer, 1960; Kaufmann & Dikstein, 1961). The function of this second system is not yet clear; Snoswell (1963) reported the conversion lactate → pyruvate by the NAD-independent enzymes of a strain of *Lactobacillus* (*Lb*) *plantarum* but was unable to reverse the reaction. Another type of enzyme forming lactic acid was found by van den Hamer (1960) in *Lb casei*. In this case methyl glyoxal and not pyruvate was the substrate and the product was D(−) lactic acid. In the genus *Leuconostoc* the species are ill defined and appear to merge one into the other. A study of the electrophoretic pattern of the lactic dehydrogenase of strains of this genus was made in an attempt to assist in their classification.

METHODS

Strains. These were selected from the National Collection of Dairy Organisms (NCDO) as typical of their species; they were grown in the media and under the conditions described by Garvie (1967*a*). The identity of the strains used is given in Fig. 3.

Preparation of cell extracts for electrophoresis

Media. Two media were used, each prepared in two parts, A and B. Part A of Medium I consisted of (%, w/v): Bacteriological peptone (Oxoid), 1·0; Bacto yeast extract (Difco), 0·5; KH_2PO_4, 0·5; triammonium citrate, 0·5; sodium acetate, 0·25; $MgSO_4.7H_2O$, 0·02; $MnSO_4.4H_2O$, 0·005; Tween 80, 0·1 % (v/v). Amounts to make

1 l. of medium were dissolved in 900 ml. water adjusted to pH 6·5 and autoclaved 121° for 15 min. Part B consisted of 100 ml. of 10·0% glucose in water, autoclaved at 121° for 15 min. The glucose solution was added to the rest of the medium before inoculation. Cysteine hydrochloride was added to 0·05% for *Leuconostoc cremoris*. The aqueous cysteine solution was sterilized by Seitz filtration.

Medium II was modified from medium I to grow strains of *Leuconostoc oenos* (Garvie, 1967*b*). In part A, 0·5% citric acid replaced ammonium citrate and the pH value was 4·8. Part B consisted of 10 g. glucose; 0·5 g. cysteine hydrochloride in 100 ml. tomato juice. This solution was sterilized by Seitz filtration.

A few non-acidophilic strains were also examined after growth in medium II. However, since these strains will not grow in media at pH 4·8, it was adjusted to pH 6·5.

Harvesting bacteria. Bacteria were harvested by centrifugation from cultures in the early stationary phase and washed in 0·85% (w/v) NaCl, suspended in about 10 ml. 0·85% NaCl in 0·002 M-phosphate buffer (pH 7·0) and disrupted with a Soniprobe (Dawe, London) for 15 min. at 20 kcyc. The tube containing the bacteria was kept in an ethanol bath at −20° during ultrasonic treatment. The suspensions were then centrifuged at 84,000 *g* for 20 min. The supernatant fluid was tested at this stage for ability to oxidize NADH in the presence of pyruvate. It was then dialysed against 15·0% (w/v) carbowax (20 M, Union-Carbide Co.) dissolved in a solution of 0·425% (w/v) NaCl in 0·002 M-phosphate buffer (pH 7·0). The volume was decreased to about 1·0 ml. (for convenience in freeze-drying), divided into 0·1 ml. amounts, freeze-dried and the ampoules sealed under vacuum. They were stored at 4°. The lactic dehydrogenase in these preparations could still be detected easily after storage for 18 months to 2 years. When required the ampoule contents were taken up in distilled water to the required concentration.

Testing for lactic dehydrogenase activity

NAD-dependent enzymes. Three methods were used. (1) A screening test, which was used when determining the effect of various factors on the rate of enzyme action, consisted of adding 0·02 ml. enzyme preparation to 0·5 ml. of the solution used to locate the enzymes after electrophoresis by direct staining (see below). The time of development of the reduced nitro-blue tetrazolium was observed. (2) Oxidation of NADH. The rate of oxidation of NADH was followed at 340 nm.: 0·02 ml. of enzyme preparation was added to 0·5 ml. of buffered substrate solution (0·4 mg. NADH + 1 μmole sodium pyruvate in 3 ml. of 0·03 M-phosphate buffer (pH 7·0)). (3) Reduction of NAD. The reduction of NAD was followed at 340 nm. when 0·02 ml. of enzyme preparation was added to 0·5 ml. of buffered substrate solution (0·5 mg. NAD, 100 μmole sodium DL-lactate or 50 μmole D or L lactic acid (Ca or Na salt) in 1·0 ml. of 0·1 M-tris + Cl buffer (pH 8·3)).

All these tests were done at room temperature (about 20°).

NAD-independent enzymes. The reduction of dichlorophenol indophenol was used to show the presence of the NAD-independent lactic dehydrogenase. The test solution consisted of 26 μg. dichlorophenol indophenol in 5·0 ml. of 0·1 M-tris + maleate buffer (pH 6·3) and lactate at the several concentrations given above; 0·02 ml. of enzyme preparation was added to 0·5 ml. of this solution and the reduction of the dichlorophenol indophenol followed at 600 nm.

ELECTROPHORESIS

The electrophoretic mobilities of enzymes were determined in acrylamide gel 1·5 mm. thick. The gel mixture consisted of 1 part tris+maleate buffer (0·05 M) at pH 7·0 or 8·3, 2 parts acrylamide, NN′-methylene bis-acrylamide (28·0 g. acrylamide, 735 mg. bis-acrylamide in 100 ml. water) 1 part 0·84% (v/v) N.N.N′.N′. tetramethylethylenediamine and 4 parts 0·175% (w/v) ammonium persulphate. The tank buffer consisted of 0·05 M-tris+maleate buffer at the same pH as the gel. Strips of 3 mm. filter paper (1 × 0·1 cm.) soaked in enzyme preparation to which a solution of bromphenol blue (1·0 mg./ml.) had been added were inserted while wet into slits in the gel and a current of 200 V. (13 mA) applied for 3·5 to 4 hr, during which time the bromphenol blue migrated 11 to 13 cm. Electrophoresis was done in a room at 4°.

After electrophoresis at pH 8·3 or pH 7·0 the NAD-dependent enzymes were located by direct staining by immersing the gel in a solution consisting of 50·0 mg. NAD, 10·0 mg. nitro-blue tetrazolium, 2·0 mg. phenazine methosulphate, 10 m-mole sodium DL-lactate (or 5·0 m-mole D- or L-lactic acid (Ca or Na salt)), in 100 ml. 0·1 M-tris+Cl buffer (pH 8·3). The gels were left for 60 min. at 22° in the dark and then examined.

Alternatively a modification of the 'reverse' staining method of Tarmy & Kaplan (1968) was used after electrophoresis at pH 7·0. The gels were placed in a solution containing 100 mg. NADH, 2·5 m-mole sodium pyruvate in 100 ml. 0·1 M-tris+maleate buffer (pH 7·0) for 30 min. at 22°, washed twice in water and then stained in a solution containing 25·0 mg. nitro-blue tetrazolium 2·0 mg. phenazine methosulphate in 100 ml. 0·1 M-tris+maleate buffer (pH 8·3).

The NAD-independent enzymes were located after electrophoresis at pH 7·0 by using a solution containing 5·2 mg. dichlorophenol indophenol in 100 ml. 0·1 M-tris+maleate buffer (pH 6·3) and lactate as above. The enzymes showed as white bands on a blue background. The distance of all the enzyme bands from the origin were calculated relative to a bromphenol blue migration of 10·0 cm.

Molecular weight of enzymes

The approximate molecular weight (mol. wt) of the enzymes was determined by filtration through G200, G150 and G100 Sephadex Superfine following the method of Andrews (1964). After swelling in 0·1 M-KCl dissolved in 0·01 M-tris+maleate buffer (pH 7·5) thin layer (0·5 mm.) 20 × 20 cm. glass plates were prepared. These were allowed to equilibrate for several hours in a flow of 0·1 M-KCl in 0·01 M-tris+maleate buffer (pH 7·5).

Samples (0·02 μl.) of enzyme preparation were applied to the plates and developed with the buffer for about 8 hr at room temperature. Blue dextran (mol. wt 2,000,000) was used to indicate the buffer front. A paper copy of the Sephadex was taken and dried in a current of cool air. The papers were sprayed with a solution consisting of 10·0 mg. NAD, 2·0 mg. nitro-blue tetrazolium, 0·8 ml. phenazine methosulphate (0·5 mg./ml.), 4·0 ml. sodium DL-lactate (0·25 M), 5·0 ml. tris+Cl buffer (pH 8·3; 0·2 M), water to 10·0 ml. Rabbit muscle lactic dehydrogenase (Boehringer, Mannheim) was used as reference and also enzyme preparations from *Lactobacillus plantarum* and *Leuconostoc mesenteroides*. The colour developed very quickly and the dye tended to diffuse. It was necessary to dry the papers in a current of warm air to stop the spots spreading.

RESULTS

NAD-dependent enzymes

While the NAD-dependent enzymes utilize either pyruvate or lactate as substrate, the reaction rate is faster with pyruvate and the concentrations of substrate coenzyme and enzyme required for adequate detection of enzyme activity in solution are less than those needed for the reverse reaction. The use of lactate, however, has the advantage of indicating the concentration of enzyme preparation to be used for electrophoresis when the enzymes are to be detected by the direct-staining method and also the relative activity of the preparation with L(+) and D(−) lactic acid. The use of pyruvate only indicates the overall activity of L(+) and D(−) lactic dehydrogenases and has the disadvantage that some preparations oxidize NADH without addition of substrate (Dolin, 1955; Kline & Mahler, 1965). This was particularly the case with *Leuconostoc mesenteroides* NCDO 523 which actively oxidized NADH irrespective of whether the strain was grown in medium I or medium II. The oxidation of NADH was observed in phosphate and tris + maleate buffer and both before and after dialysis of the cell extracts (Table 1). Because of this activity it was not possible to demonstrate a NADH-linked pyruvate reductase in NCDO 523. Preparations from other leuconostoc strains oxidized NADH very slowly or not at all in the absence of pyruvate, and NAD was not reduced by any strain unless lactate was present.

Table 1. *Oxidation of NADH by two strains of Leuconostoc mesenteroides (NCDO 768, 523)*

	Extinction at 340 nm.			
	With pyruvate		Without pyruvate	
Time (min.)	Medium I		Medium I	Medium I (enzyme conc. × 10)
NCDO 768				
0	0·862		0·900	0·900
1	0·050		0·900	0·875
2	—		—	0·875
3	—		0·900	—
4	—		—	—
5	—		0·900	—
	Medium I	Medium II (initial pH 6·5)	Medium I	Medium II (initial pH 6·5)
NCDO 523				
0	0·690	0·690	0·680	0·680
1	0·480	0·341	0·608	0·330
2	0·330	0·162	0·382	0·145
3	0·211	0·090	0·200	0·085
4	0·144	0·068	0·223	—
5	0·104	0·064	0·173	—

As expected NAD-dependent D(−) lactic dehydrogenase was active in all strains but some reduction of NAD was also generally found when L(+)-lactate was used as substrate (Fig. 1). This activity was not due to impurity in the isomeric lactate used, as both the D(−)- and L(+)-lactates used were examined for traces of the other isomer by the methods described by Garvie (1967*c*).

Effect of substrate on enzyme activity

Enzyme preparation from NCDO 523 reduced NAD more rapidly with Ca D(−)-lactate than with Na DL-lactate as substrate (Table 2); with other strains the difference between the two substrates was slight. The enzyme of NCDO 523 might be stimulated by Ca^{2+} or, on the other hand, L(+)-lactate might be acting as an inhibitor of the D(−) lactic dehydrogenase. The time of reduction of nitro-blue tetrazolium was 3 and 15 min. at pH 8·3 and 7·5 respectively when Ca D(−)-lactate was substrate but was increased to 7 and 23 min. when the same molar concentration of Na D(−)-lactate was used.

To test for inhibition of the D(−) lactic dehydrogenase by L(+)-lactate, an enzyme preparation from NCDO 523 was exposed to L(+)-lactate and NAD for 5 min. before D(−)-lactate was added. The results are shown in Fig. 2. No inhibition of enzyme activity by L(+)-lactate occurred. The difference between the results with Na DL-lactate and Ca D(−)-lactate were therefore apparently due to the effect of Ca ion.

Table 2. *Reduction of NAD by Leuconostoc mesenteroides NCDO 523 with different substrates*

Time (min.)	Extinction at 340 nm. 0·1 M-Na-DL-lactate	0·05 M-D(−) lactic acid (Ca salt)	0·05 M-L(+) lactic acid (Ca salt)
1	0·133	—	—
2	0·158	0·449	0·048
3	0·158	0·498	0·056
4	—	0·498	0·063
5	—	—	0·065

Effect of pH value on enzyme activity

Table 3 shows the time of reduction of nitro-blue tetrazolium in the presence of various substrates and at various pH values by the enzyme preparation from *Leuconostoc mesenteroides* NCDO 768. No reduction took place in the absence of NAD. The reaction with NAD was fastest at pH 8·3. It was found that dichlorophenol indophenol could be used in place of the nitro-blue tetrazolium + phenazine methosulphate system.

Electrophoresis. Only one area of lactic dehydrogenase activity was detected for any strain. However, on several occasions the area was seen as two merging adjacent bands. Strong reduction of nitro-blue tetrazolium was obtained with Ca D(−)-lactate or with Na DL-lactate; slight reduction was observed with Ca L(+)-lactate.

Figure 3 shows the relative positions of all the enzymes found. The enzyme of all the non-acidophilic leuconostocs moved together. Two areas of activity were found for the acidophilic *Leuconostoc oenos*; these were close together and migrated much more slowly than the enzyme of the non-acidophilic strains. The slowest moving enzyme belonged to the two strains NCDO 1674 and 1823 dependent for growth on a factor in tomato juice (Garvie & Mabbitt, 1967), while the faster moving lactic dehydrogenase of *L. oenos* was found in the strains which did not require tomato juice. To test whether the difference in enzyme pattern between the acidophilic and non-acidophilic strains was due to growth in different media *L. mesenteroides* NCDO 523 was grown in medium II and cell extracts examined. The lactic dehydrogenase was

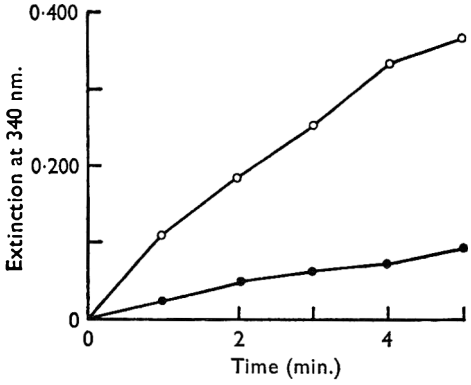

Fig. 1

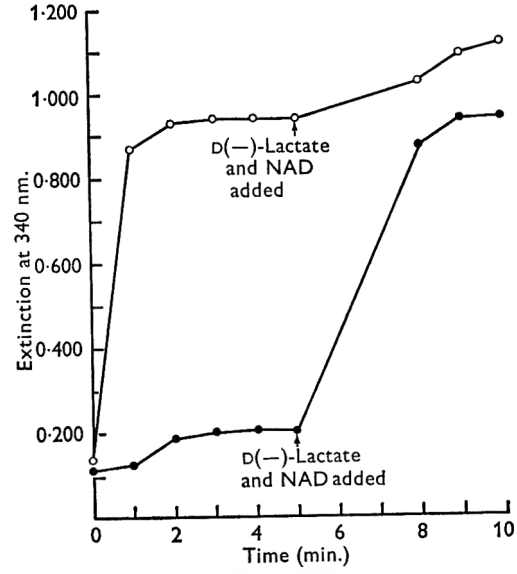

Fig. 2

Fig. 1. Reduction of NAD at pH 8·3 by enzyme preparations of *Leuconostoc mesenteroides* NCDO 768 with D(−) and L(+)-lactate as substrate: ○——○, calcium D(−)-lactate as substrate; ●——●, calcium L(+)-lactate as substrate.

Fig. 2. The effect of calcium L(+)-lactate on the oxidation of calcium D(−)-lactate by NAD-dependent lactic dehydrogenase at pH 8·3 by enzyme preparations of *Leuconostoc mesenteroides* NCDO 523; ○——○, initial substrate calcium D(−)-lactate; ●——●, initial substrate calcium L(+)-lactate.

Table 3. *Time of reduction of nitro-blue tetrazolium and dichlorophenol indophenol by cell extracts of Leuconostoc mesenteroides NCDO 768 under various test conditions*

	pH 6·3		pH 7·0		pH 7·5		pH 8·3	
Substrate	no NAD	+NAD	no NAD	+NAD	no NAD	+NAD	no NAD	+NAD
	Time (min.)							
H^+ acceptor-DCPIP*								
Ca D(−)-lactate	15	6	28	7	—	5	—	6
Ca L(+)-lactate	19	36	90	40	—	—	—	—
Na D(−)-lactate	13	6	60	6	—	8	nt	11
Na L(+)-lactate	40	40	—	—	—	—	nt	—
Water	—	—	—	—	—	—	nt	—
H^+ acceptor NBT†+PMS‡								
Ca D(−)-lactate	—	75	—	29	—	8	—	2
Ca L(+)-lactate	—	—	—	—	—	150	—	150
Na D(−)-lactate	—	60	—	21	—	8	—	3
Na L(+)-lactate	—	—	—	—	—	150	—	150
Water	—	—	—	—	—	—	—	—

* Dichlorophenol indophenol.
† NBT = Nitro-blue tetrazolium.
‡ Phenazine methosulphate.
nt = not tested.

identical with that present after growth in medium I. It was concluded that the differences between the acidophilic and non-acidophilic leuconostocs was not due to different growth conditions. The rate of migration of the enzymes was unaffected when the pH value of the electrophoresis buffer was decreased from pH 8·3 to 7·0.

Electrophoresis of NADH oxidase. After electrophoresis of enzyme preparations of *Leuconostoc mesenteroides* NCDO 523 two bands were found by using the 'reverse' staining technique when pyruvate + NADH were in the reaction mixture. The faster

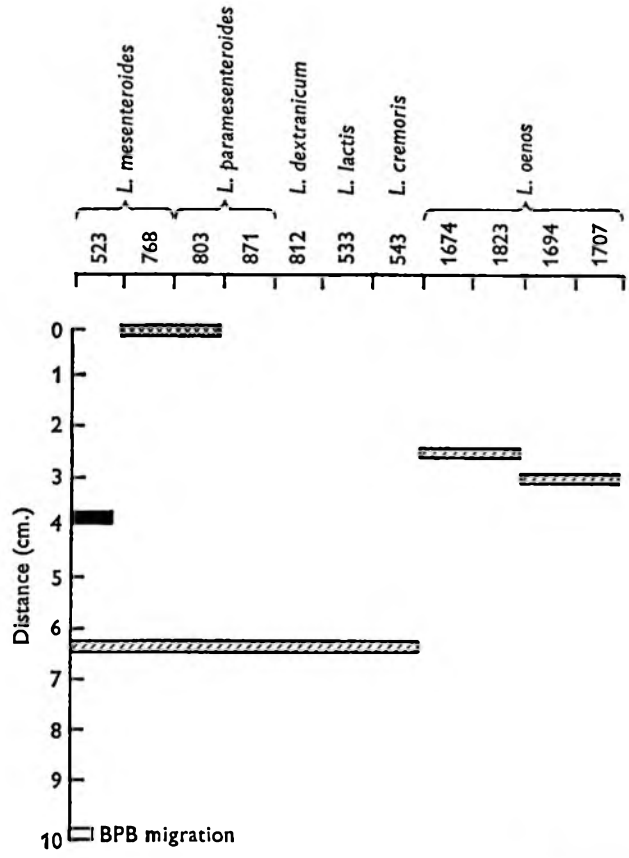

Fig. 3. The relative positions of lactic dehydrogenases and NADH oxidases of eleven strains of the genus *Leuconostoc* after electrophoresis in acrylamide gel; ■, NADH oxidase; ▨, NAD-dependent D(−)-lactic dehydrogenase; ▩, NAD-independent lactic dehydrogenase—reacting with both D(−)- and L(+)-lactate.

moving enzyme coincided with the D(−) lactic dehydrogenase. When pyruvate was absent this activity disappeared; however, the slower moving enzyme (NADH oxidase) was present and with undiminished intensity (Fig. 3). Other strains showed only one band i.e. the D(−) lactic dehydrogenase.

Molecular size of lactic dehydrogenase. Bacterial lactic dehydrogenases have been reported to be of different molecular weights. To see whether the different electrophoresis patterns were related to molecular size, crude extracts were examined by using thin-layer Sephadex plates. Several enzymes of known molecular weight were

used as markers. Tarmy & Kaplan (1968) gave a molecular weight of 80,000 for *Leuconostoc mesenteroides* lactic dehydrogenase, while Dennis, Reichlin & Kaplan (1965) gave a molecular weight of 68,000 for the D(−) lactic dehydrogenase of *Lactobacillus plantarum* and 155,000 for the L(+) enzyme of the same species. Mammalian lactic dehydrogenase was estimated at 140,000 by Jaenicke & Knof (1968).

Mammalian lactic dehydrogenase ran in advance of the L(+) enzyme of *Lactobacillus plantarum* (although the latter has the lower molecular weight) with the D(−) enzyme of the same strain hardly separated but behind the L(+) band. The D(−) lactic dehydrogenase of *Leuconostoc mesenteroides* and *L. oenos* was on a line with the D(−) enzyme of *Lb. plantarum*. This would suggest that the D(−) enzyme all had a molecular weight of 70,000 to 80,000.

NAD-independent enzymes

NAD-independent enzymes were not found in eight of the strains including *Leuconostoc mesenteroides* NCDO 523; *L. mesenteroides* NCDO 768 and both strains of *L. paramesenteroides* (NCDO 803, NCDO 871) had NAD-independent enzymes. Of these strains, NCDO 803 and NCDO 768 were more active than NCDO 871. Table 3 shows the time of reduction of dichlorophenol indophenol by enzyme preparation of *L. mesenteroides* NCDO 768 under different conditions. These enzymes were most active at pH 6·3 and inactive at alkaline pH values. The phenazine methosulphate + nitro-blue tetrazolium system cannot act as hydrogen acceptor for the NAD-independent enzymes of this strain. All three strains reduced dichlorophenol indophenol with either D(−)- or L(+)-lactate; the reaction was faster with the D(−) isomer in every strain.

Electrophoresis. The enzyme concentration in preparation from *Leuconostoc paramesenteroides* NCDO 871 was too low for any activity to be detected after electrophoresis. Both *L. mesenteroides* NCDO 768 and *L. paramesenteroides* NCDO 803 showed only one band, which did not move significantly from the point of origin, and which reacted strongly with D(−)-lactate but only weakly with L(+)-lactate. It is uncertain whether both NAD-independent enzymes were involved or whether the D(−) enzyme was reacting with both substrates, and the weaker L(+) enzyme was not located.

DISCUSSION

There is considerable variation in the electrophoretic mobilities of both D(−) and L(+) lactic dehydrogenases between different species of the lactic acid bacteria (E. I. Garvie, unpublished). The fact that strains of five species in the genus *Leuconostoc* possessed enzymes which moved together is therefore exceptional. In view of the variations in other species the identical mobility in the case of the Leuconostoc species suggests that they possess a common lactic dehydrogenase, and further that the relationship between species is close, probably closer than between species of other lactic acid bacteria. This may explain why the differentiation of the non-acidophilic leuconostocs into species is not as clear cut as with other lactic acid bacteria.

While this study of the lactic dehydrogenases has not detected differences between the non-acidophilic species it has given further evidence that *Leuconostoc oenos* is a separate species. Peynaud (1968) suggested that the acidophilic leuconostocs should be divided into two species on the basis of pentose fermentation. Of the four strains examined here NCDO 1674 (the type strain; Garvie, 1967*b*) and NCDO 1823 having the

same lactic dehydrogenase, belong to the non-pentose fermenting group, while the two strains with the other lactic dehydrogenase both ferment arabinose. The lactic dehydrogenase pattern therefore gives support to the division of *L. oenos* suggested by Peynaud.

Genetically different isoenzymes of lactic dehydrogenase have not been recorded in bacteria. In other organisms where these isoenzymes occur they are usually widely separated on electrophoresis. Conformational isoenzymes (Markert, 1968; Epstein & Schechter, 1968) on the other hand occur as adjacent bands and it is possible that the double band observed on some occasions with the leuconostoc enzyme preparations was of this nature.

When both L(+)- and D(−)-lactates were used separately as substrates in the gel stain all NAD-dependent lactic dehydrogenase showed with both isomers. The reaction would be strong with one lactate but weak with the other. This was observed with rabbit muscle lactic dehydrogenase, as well as with the bacterial enzymes of a variety of strains of lactic acid bacteria. With those strains of bacteria having both D(−) and L(+) lactic dehydrogenase these observations could be explained by assuming that in every instance a trace of one enzyme was mixed in the bulk of the other; this would not be applicable to the enzymes of those bacteria having only one lactic dehydrogenase or with the mammalian lactic dehydrogenase. It appears therefore that lactic dehydrogenases are able to reduce some NAD in the presence of the unnatural isomer. In the case of the leuconostocs this was also apparent when testing for enzyme activity at 340 nm. with L(+)-lactate.

The function of the NAD-independent enzymes is unknown. The isomeric lactic acid formed by lactic acid bacteria from the fermentation of glucose during normal growth is no indication of the NAD-independent lactic dehydrogenase possessed by the bacteria. *Pediococcus cerevisiae* forming DL-lactic acid possess only an L(+) enzyme, while *Lactobacillus casei* forming only L(+) lactic acid has both a D(−) and L(+) NAD-independent enzyme (E. I. Garvie, unpublished). Similarly leuconostocs forming lactic D(−) acid also have both L(+) and D(−) NAD-independent lactic dehydrogenase.

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The Serological Identity of Sabin's Murine type C Mycoplasma and *Mycoplasma pulmonis*

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SUMMARY

Serological evidence is presented that Sabin's murine type C mycoplasma belongs to the species *Mycoplasma pulmonis* and not to a separate species, *M. histotropicum.*

Between 1938 and 1941, several apparently distinct mycoplasmas were isolated from mice by Sabin (1938, 1939*a*, *b*, 1941). These organisms were designated types A, B, C, D and E and assigned to three species, '*Musculomyces neurolyticus*' (type A), '*Musculomyces arthrotropicus*' (type B) and '*Musculomyces histotropicus*' (types C, D, E) (Sabin, 1941). The toxinogenic type A was evidently the same as the organism first isolated by Klieneberger from mice with rolling disease (Findlay, Klieneberger, MacCallum & Mackenzie, 1938; Klieneberger, 1940) and later classified as *Mycoplasma neurolyticum* (Edward & Freundt, 1956). However, the relationship of types B, C, D and E to the other recognized rodent mycoplasmas, *M. pulmonis* and *M. arthritidis* (Edward & Freundt, 1956) could not be determined from Sabin's descriptions. In 1963, types A and C were recovered from cultures which had been lyophilized in 1943 (Tully & Ruchman, 1964): type A proved to belong to the species *M. neurolyticum*, while type C was provisionally designated, on the results of indirect fluorescent antibody tests, as *M. histotropicus* (Tully, 1965), a species distinct from the other rodent mycoplasmas. Since *Mycoplasma* is a neuter noun, with which the specific epithet must agree, the name of this organism was corrected by Edward & Freundt (1969) to *M. histotropicum.* More recently, the electrophoretic pattern of the cell proteins of type C (*M. histotropicum*, PG40, obtained from Dr D. G. ff. Edward) was found to resemble that of *M. pulmonis* strains (Razin, 1968). We show here that two lines of type C are antigenically indistinguishable from *M. pulmonis.*

A culture of type C, which had undergone 60 passages since recovery from Sabin's lyophilized material, was received from Dr J. G. Tully (National Institutes of Health, Bethesda, Md., U.S.A.) and examined at the Lister Institute in 1964. The culture was cloned by selecting a single colony at each of two successive subcultures, and tested by complement-fixation and gel-diffusion (Lemcke, 1964, 1965) and by an agar growth-inhibition technique (Clyde, 1964) against antisera to 17 serologically distinct mycoplasmas (Lemcke, 1964). It reacted only with antisera to two strains of *Mycoplasma*

pulmonis, KON from the lung of a rat and M I from the lung of a mouse (Lemcke, 1961). No reaction occurred with antisera to *M. hominis* (strain H34), *M. pneumoniae* (FH), *M. salivarium* (B3), *M. orale*, type I (823), *M. fermentans* (G2), *M. arthritidis* (CAMPO, PG27), *M. neurolyticum* (KSA), *M. gallinarum* (FOWL), *M. gallisepticum* (T), *M. laidlawii* (type A), *M. agalactiae* (agalactia), *M. bovigenitalium* (PG11), *M. mycoides* var. *mycoides* (GLADYSDALE), *M. mycoides* var. *capri* (pp. goat) or two strains, NAVEL and A36, not yet accorded species rank.

More recently, antiserum was prepared against the same cloned line of type C, and further complement-fixation, gel-diffusion and growth-inhibition tests were made with this antiserum and antisera to KON and M I. The gel-diffusion antigens were mycoplasma suspensions lysed with the non-ionic detergent Triton X-100 and not the sonicated suspensions used previously (Lemcke, 1965). The complement-fixation and growth-inhibition tests confirmed the close relation of the three organisms (Table 1). In gel-diffusion tests, reactions with homologous antisera tended to be more complex than those with heterologous antisera, but the majority of the antigens was shared by all three strains (Table 1).

Table 1. *Reactions of type C and strains M1 and KON of Mycoplasma pulmonis in complement-fixation (CF), growth-inhibition and gel-diffusion tests*

Complement-fixation cited as reciprocal of titre, growth-inhibition as width of inhibition zone. For gel-diffusion tests, figures in parentheses indicate number of precipitin lines shared with homologous strain. Homologous reactions in heavy type.

		Antiserum to		
	Antigen	Type C	M I	KON
Type C	CF titre	**1280–2560**	2560–5120	1280
	Growth inhibition (mm.)	**5**	5	3
	Gel diffusion (no. of lines)	**6**	3 (2)	4 (4)
M I	CF titre	5120	**5120**	2560
	Growth inhibition (mm.)	3	**5–6**	3
	Gel diffusion (no. of lines)	6 (5)	**4**	5 (5)
KON	CF titre	1280	2560	**640–1280**
	Growth inhibition (mm.)	3	5	**5**
	Gel diffusion (no. of lines)	6 (4)	3 (3)	**6**

Since it could be argued that this line of type C had been cloned and so might have been selected from a mixed culture, another culture of type C was examined in Glasgow by a growth-inhibition technique (Clyde, 1964), and by polyacrylamide gel electrophoresis, according to the method of Razin & Rottem (1967). This was a lyophilized culture, received from Dr Tully at the 24th passage. In tests on part of the reconstituted culture removed directly from the original ampoule, its growth was inhibited by antisera to the *Mycoplasma pulmonis* strains ASH (PG34), M50, CHENG, NEGRONI, 880 and KON, but not by antiserum to the GDL strain of *M. hyorhinis*. In polyacrylamide gel electrophoresis, the cell proteins of type C gave a similar electrophoretic pattern to those of the prototype *M. pulmonis* strain ASH (PG34).

Thus, the cultures of type C which we have examined appear to be strains of *Mycoplasma pulmonis*. Considering that cultures of type C received and examined independently in London and Glasgow were both closely related to *M. pulmonis*, it is unlikely that our results are due to accidental contamination with *M. pulmonis*,

particularly since the growth-inhibition tests on type C before subcultivation gave the same result. Moreover, since Razin (1968) obtained evidence of a similarity between the cell proteins of another culture of type C and *M. pulmonis*, it is improbable that the relationship between type C and *M. pulmonis* is confined to the cultures we examined. Manchee & Taylor-Robinson (1968) have also found a similarity between M. *histotropicum* and *M. pulmonis* in that colonies of both species adsorb rodent and guinea pig erythrocytes.

It is impossible, after nearly 30 years, to determine what relation the cultures now known as type C bear to that originally isolated by Sabin. They differ in one respect; type C originally produced arthritis in mice after intravenous inoculation, but the culture recovered from Sabin's lyophilized ampoules was not mouse-pathogenic by the intraperitoneal, intravenous or intracerebral routes (Tully & Ruchman, 1964). Nevertheless, current evidence suggests that type C belongs to the species *Mycoplasma pulmonis* and should not be classified as a separate species *M. histotropicum*. The extent to which strains differ within the species *M. pulmonis* is at present unknown, but the results of the gel-diffusion tests with type C, KON and M I, like those obtained by Fallon & Jackson (1967), suggest that serological subtypes may exist.

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Note added in proof

Since the completion of this paper, two earlier subcultures of type C, at the 4th and 7th passages from Sabin's lyophilized culture, were obtained from Dr G. J. Tully. After 4 and 2 passages, respectively, the cultures were tested by the agar growth-inhibition technique against antisera to the cloned line of type C and the 17 serologically distinct mycoplasmas cited in the text. Growth was inhibited only by antisera to type C and the *Mycoplasma pulmonis* strains KON and M1. Thus, cultures of type C passaged 8 and 9 times since recovery from Sabin's lyophilized material showed the same serological relationship to *M. pulmonis* as the later subcultures.

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Bacteriological and Serological Studies of Fast Growing Mycobacteria Identified as *Mycobacterium friedmannii*

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SUMMARY

Three recently isolated strains of a fast growing non-pigmented mycobacterium, two of which were from a series of injection abscesses and one which was recovered from a water tank, have been compared bacteriologically and serologically in gel-diffusion tests with a number of mycobacterial type strains. The three strains and type strains of *M. abscessus*, *M. runyonii*, *M. borstelense* and *M. friedmannii* were of one species. This species differed in several respects from *M. ranae* (*fortuitum*), *M. smegmatis* and *M. thamnopheos*. In view of the chronological priority of *M. friedmannii* over the other names, we recommend that this name be adopted as the correct designation for this species. The origin of the type strain of *M. friedmannii* is discussed and reasons are given for its acceptance as representative of Friedmann's original strains.

INTRODUCTION

In 1967 two strains of a fast growing non-pigmented mycobacterium isolated from a series of injection abscesses occurring in Hartlepool were sent to us for identification. During our investigation of the outbreak, reported elsewhere (Inman, Brown, Beck & Stanford, 1969) we found that these strains were identical with a type strain of *Mycobacterium abscessus*. Further studies revealed that this identity extended to the type strains of several other allegedly different species. This paper records our investigations into the identity of all these strains, and a comparison between this group of strains (the 'test' strains) and representatives of three well established species of fast growing non-pigmented mycobacteria.

METHODS

Bacteria. The test strains were I.W. and B.B. from the Hartlepool outbreak of injection abscesses; W.T., isolated from a cold water tank supplying the laboratory of one of the authors (A.B.); *Mycobacterium abscessus* ATCC 19977; *M. borstelense* and *M. borstelense* var. *niacinogenes*, received from Dr R. Muser (Heidelberg); *M. runyonii* NCTC 10269; *M. fortuitum* NCTC 946 (this was originally the type strain of *M. friedmannii* but Gordon & Smith (1955) reclassified it as *M. fortuitum*).

The strains used for comparison were *M. fortuitum* NCTC 8573; C.U. and J.C.,

isolated before from abscesses (Beck, 1965); *M. smegmatis* NCTC 333; *M. thamnopheos* ATCC 4445.

Stanford & Gunthorpe (1969) have shown *M. ranae* to be identical with *M. fortuitum* and to have chronological priority over it. Therefore the name *M. ranae* (*fortuitum*) will be adopted in the present paper to avoid confusion.

Bacteriological tests. Bacterial morphology was studied in Ziehl-Neelsen stained films of organisms taken from one-week-old cultures on Löwenstein-Jensen medium. Colonial appearances were examined on the same medium and cultures were incubated at 4, 20, 32, 37 and 45° for studies of growth at different temperatures.

The cultures were examined for: production of catalase (Middlebrook, 1954); nitrate reductase (Virtanen, 1960); arylsulphatase within 3 days (Whitehead, Wildy & Engbaek, 1953); Tween 80 degradation within 5 days (Wayne, 1962); growth on Löwenstein-Jensen medium containing 10 μg. thiosemicarbazone/ml.; growth on semi-solid agar (Marks & Richards, 1962); amidase activity (Bönicke, 1962); utilization of sugars and acids (Gordon & Smith, 1953). The amidase and acid utilization tests were done several times because of difficulties of interpretation; the results reported are those in which a majority of agreement was obtained. Sensitivity tests to isoniazid, streptomycin, *p*-aminosalicylic acid (PAS), cycloserine and ethionamide were done as described by Beck (1965). Sensitivity to ethambutol was tested on Löwenstein-Jensen medium containing 0·5, 1, 2, 4, 8 and 16 μg. drug/ml.

Serological tests. The serological methods were basically those of Stanford & Beck (1968). An extract was produced from each strain by freezing and disintegrating in an 'X-press' the harvested pellicles of cultures in a modified Sauton medium (Boyden & Sorkin, 1955). Antisera were produced in pairs of rabbits to *Mycobacterium abscessus* strain ATCC 19977, to *M. runyonii* strain NCTC 10269, and to strains I.W. and B.B., by a series of intramuscular injections of bacterial extract suspended in an equal volume of a mixture of 8·5 parts Bayol F and 1·5 parts Arlacel. Each of the extracts was examined in double gel-diffusion tests in agar against each of the antisera. Representative extracts of our strains were also examined against antisera to *M. smegmatis*, *M. ranae* (*fortuitum*) and *M. thamnopheos* available from other studies.

Absorption of antisera was performed in some cases. Samples of antisera were mixed with equal volumes of bacterial extract and incubated at 4° for 24 to 48 hr. They were then examined in gel-diffusion tests against the bacterial extracts which had been used for immunization and those used for absorption. The antiserum to strain I.W. was absorbed with bacterial extracts of strains I.W., ATCC 19977, NCTC 10269 and C.U. The antiserum to ATCC 19977 was absorbed with its autologous extract and strain NCTC 10269.

RESULTS

Bacteriological examinations. The majority of strains showed a smooth shiny growth, but the cultures of *Mycobacterium friedmannii* NCTC 946, *M. runyonii* NCTC 10269, *M. ranae* (*fortuitum*) J.C. and *M. thamnopheos* ATCC 4445 were rough and dull. No pigment was formed in the dark or after exposure to light.

The strains consisted of bacilli of medium length, except for the *Mycobacterium ranae* (*fortuitum*) strains J.C. and NCTC 8573 which were short bacilli. Strains which produced smooth colonies were easily emulsified and showed a fairly homogeneous distribution of organisms in stained smears, whereas the rough strains showed a

tendency to clump or form thin loose strands. The degree of fastness to acid ethanol varied. A few strains showed a preponderance of bacilli not fast to acid ethanol; of these *M. thamnopheos* was most notable.

Heavy growth was obtained in 2 to 3 days at 20°, 32° and, with the exception of *Mycobacterium thamnopheos*, at 37°. Only *M. smegmatis* grew at 45°; contrary to Friedmann's findings none of the strains grew at 4°. The recently isolated strains

Table 1. *Biochemical reactions of mycobacterial strains*

	I.W.	B.B.	W.T.	*M. abscessus*	*M. borstelense*	*M. borstelense* var. *niacinogenes*	*M. runyonii*	NCTC 946	*M. ranae* (*fortuitum*) NCTC 8573	*M. ranae* (*fortuitum*) C.U.	*M. ranae* (*fortuitum*) J.C.	*M. smegmatis*	*M. thamnopheos*
Fermentation of sugars													
Arabinose, dulcitol, xylose, erythritol, rhamnose	−	−	−	−	−	−	−	−	−	−	−	+	−
Galactose	−	−	−	−	−	−	−	−	−	−	−	−	+
Lactose, raffinose, α-methyl-D-glucoside	−	−	−	−	−	−	−	−	−	−	−	−	−
Inositol	−	−	−	−	−	−	−	−	−	+	−	+	−
Mannitol, sorbitol	−	−	−	−	−	−	−	−	−	+	−	+	+
Glucose, mannose, trehalose	+	+	+	+	+	+	+	+	+	+	+	+	+
Acid utilization													
Benzoate, mucate	−	−	−	−	−	−	−	−	−	−	−	−	−
Citrate	+	+	+	−	+	+	−	+	+	+	+	+	+
Lactate	−	−	−	−	−	−	−	−	+	+	+	+	+
Malate	+	+	+	+	±	+	+	+	+	+	+	+	+
Oxalate	−	−	−	−	−	−	−	−	−	−	−	+	−
Succinate	+	+	+	+	+	+	+	+	+	+	+	+	+
Amidases													
Acetamide	+	+	+	+	+	+	+	+	+	+	+	+	±
Benzamide	−	−	−	−	−	−	−	−	−	−	−	±	−
Urea	+	+	+	+	+	+	+	+	+	+	+	+	+
Isonicotinamide	−	−	−	−	−	−	−	−	−	−	−	+	−
Nicotinamide	+	+	+	±	±	±	±	±	±	−	−	+	±
Pyrazinamide	±	+	+	+	+	+	+	+	−	±	−	±	+
Salicylamide	−	−	−	−	−	−	−	−	−	−	−	±	−
Allantoin	±	+	+	±	±	±	±	±	+	+	+	−	+
Succinamide	−	−	−	−	−	−	−	−	−	−	−	+	−
Malonamide	−	−	−	−	−	−	−	−	−	−	−	−	±
Nitrate reduction	−	−	−	−	−	−	−	−	+	+	+	+	−

(I.W., B.B., W.T.) initially grew better at temperatures slightly below 37°. The organisms grew on Löwenstein-Jensen medium, nutrient agar, blood agar, Sabouraud glucose agar, and on the surface of semi-solid agars. The *M. ranae* (*fortuitum*) strains grew vigorously on MacConkey agar in which they produced strong acid; the other strains produced little or no acid. With the exception of the *M. borstelense* var. *niacinogenes* strain none of the strains produced nicotinic acid. They all gave a strong catalase reaction and all, except *M. thamnopheos* and *M. smegmatis*, a positive arylsulphatase test after incubation for 3 days. Only strains of *M. thamnopheos* and

M. smegmatis hydrolysed Tween 80 within 5 days; weak hydrolysis of this substance occurred on prolonged incubation with some of the other strains.

The results of the nitrate reductase, amidase, fermentation of sugars and utilization of organic acids tests are given in Table 1 and those of the drug sensitivity tests in Table 2.

Serological examinations. Extracts of the eight test strains produced the same pattern of twelve precipitation lines with antisera to two of them (I.W. and B.B.). Similar patterns of lines, though of slightly fewer number, were obtained with antisera to strains NCTC 10269 and ATCC 19977. Extracts prepared from the strains of the other

Table 2. *Drug sensitivity of mycobacterial strains expressed as resistance ratio (ratio of m.i.c. of test strain to m.i.c. of strain H37Rv)*

	I.W.	B.B.	W.T.	*M. abscessus*	*M. borstelense*	*M. borstelense* var. *niacinogenes*	*M. runyonii*	NCTC 946	*M. ranae* (*fortuitum*) NCTC 8573	*M. ranae* (*fortuitum*) C.U.	*M. ranae* (*fortuitum*) J.C.	*M. smegmatis*	*M. thamnopheos*
Isoniazide	> 32	> 32	> 32	> 32	> 32	> 32	> 32	> 32	> 32	> 32	> 32	> 32	> 32
Streptomycin	4	> 8	> 8	> 8	> 8	> 8	> 8	> 8	> 8	> 8	> 8	2	2
PAS*	> 8	> 8	> 8	> 8	> 8	> 8	> 8	> 8	> 8	> 8	> 8	8	> 8
Cycloserine	> 16	4	4	> 16	4	4	> 16	4	> 16	> 16	> 16	> 16	8
Ethionamide	1	0·5	2	> 8	1	1	4	0·5	4	> 8	8	4	> 8
Ethambutol	> 32	> 32	> 32	> 32	> 32	> 32	> 32	> 32	> 32	> 32	> 32	1	> 32

* *p*-aminosalicylic acid.

three species selected for comparison shared only six lines of precipitation with the test strains. The same six lines alone were seen when extracts of representative test strains were examined with antisera to strains of *Mycobacterium ranae*, *M. smegmatis* and *M. thamnopheos*. When tested against extracts of the homologous strains each of these sera produced some additional lines. Absorption of the antisera to strains I.W. and ATCC 19977 with extracts of the test strains removed all precipitins, whereas absorption with the *M. ranae* (*fortuitum*) extract left intact those antibodies which were specific to the test strains.

DISCUSSION

The results of the bacteriological and serological examinations showed that the strains from the Hartlepool outbreak of injection abscesses, the strain isolated from a water tank and the type strains of *Mycobacterium abscessus*, *M. borstelense*, *M. runyonii* and strain NCTC 946 were identical in most respects, and that they differed from *M. ranae* (*fortuitum*), *M. smegmatis* and *M. thamnopheos*. There were no consistent differences of bacterial or colonial morphology to distinguish between any of the strains tested. Nor did growth at different temperatures allow any distinction, with the exceptions of *M. thamnopheos*, which would not grow at 37°, and *M. smegmatis*, which alone grew at 45°. Of the biochemical tests the sugar fermentation, organic acid utilization and amidase tests readily distinguished *M. smegmatis* from the other strains by its greater enzymic activity. It was, however, more difficult to distinguish between

the other species. The most consistent properties by which the eight test strains differed from *M. ranae* (*fortuitum*) were their inability to reduce nitrate and to utilize lactate. Less consistent were the minor differences of the amidase spectra of the two groups. *M. thamnopheos* could be distinguished from all the other strains by its ability to ferment galactose; it shared with *M. smegmatis* the ability to hydrolyse Tween 80 within 5 days, and the lack of arylsulphatase activity.

In the sensitivity tests all strains except *Mycobacterium smegmatis* and *M. thamnopheos* were resistant to streptomycin, *p*-aminosalicylic acid (PAS) and isoniazid and, with the exception of *M. smegmatis*, also to ethambutol. All strains were fully or doubtfully resistant to cycloserine. Most strains were sensitive or only doubtfully resistant to ethionamide, but one of the test strains (*M. abscessus*) and three of the strains used for comparison were completely resistant to this compound. All strains were resistant to 10 μg. thiosemicarbazone/ml.

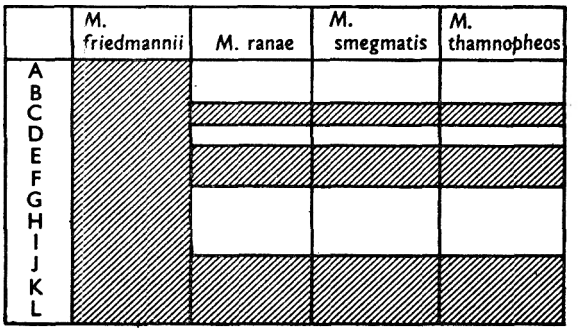

Fig. 1. Results of double diffusion precipitation tests with antisera to strains of *Mycobacterium friedmannii* and antigen extracts of *M. friedmannii*, *M. ranae* (*fortuitum*), *M. smegmatis* and *M. thamnopheos*. The letters A to L represent the immunoprecipitate lines in the order in which they fell between antigen and antiserum wells in the majority of tests.

Serologically the group of eight test strains behaved identically, in gel diffusion producing with the antisera to four of them up to six lines of precipitation specific to themselves, and another six lines which they shared with *Mycobacterium ranae* (*fortuitum*), *M. smegmatis* and *M. thamnopheos* (Fig. 1). Four of the latter lines corresponded to the common mycobacterial antigens described by Stanford & Beck (1968) and Beck & Stanford (1968). The results obtained with unabsorbed antisera were confirmed by absorption tests.

On the basis of the evidence presented it is concluded that all the test strains were of one species, to members of which the names *Mycobacterium friedmannii*, *M. abscessus*, *M. runyonii* and *M. borstelense* have been applied.

Mycobacterium friedmannii was the name given by Holland (1920) to strains isolated from extensive lesions of two turtles by Friedmann (1903*a*, *b*) which had previously been known as 'turtle tubercle bacilli'. Moore & Frerichs (1953) described as *M. abscessus* a strain isolated from the knee joint of a patient with chronic arthritis thought at first to have been tuberculous, and from injection abscesses following treatment with streptomycin in the same patient. *M. runyonii* was the designation given by Bojalil, Cerbón & Trujillo (1962) to strains isolated from sputum and from an hepatic abscess. *M. borstelense* and its nicotinic acid producing variant were

described by Bönicke (1964) and Bönicke & Ewoldt (1965) who applied this name to strains isolated from sputum and gastric washings of patients with pulmonary lesions.

Mycobacterium friedmannii is undoubtedly the oldest of the names but there has been some controversy about the authenticity of the available strains now bearing this name. Until fairly recently the ATCC and the NCTC both listed strains of *M. friedmannii*. Following the work of Gordon & Smith (1953, 1955) who showed that the two strains in the ATCC were *M. smegmatis* and the strain 946 in the NCTC was *M. fortuitum*, the ATCC strains have been withdrawn and the NCTC strain is now listed as *M. fortuitum*. Nevertheless, there is a considerable body of published evidence, both serological (Wilson, 1925; Mudd, 1925; Furth, 1926; Furth & Aronson, 1926; Griffith, 1930) and biochemical (Gordon, 1937; Gordon & Hagan, 1938), that *M. friedmannii* differs from *M. ranae* (*fortuitum*), *M. smegmatis* and other mycobacteria. These findings and our results with the strain NCTC 946, strongly suggest that this strain, and not those identified by others as *M. smegmatis*, is the true representative of Friedmann's strains.

We therefore consider that the appropriate designation for the species represented by our eight test strains is *Mycobacterium friedmannii* Holland, and that the term *niacinogenes* should be retained for the variant which produces nicotinic acid. The names of *M. abscessus*, *M. borstelense* and *M. runyonii* are synonyms for the species and should be discarded.

Our results agree with those of Bönicke & Stottmeier (1965) who found *Mycobacterium borstelense* to differ in phage susceptibility and in a number of biochemical tests from *M. ranae* (*fortuitum*), and with those of Takeya, Nakayama & Nakayama (1967) who distinguished between *M. runyonii* and *M. ranae* (*fortuitum*) by biochemical and cross-sensitivity skin tests. Tsukamura (1967), on a basis of numerical taxonomy, also concluded that *M. runyonii* and *M. ranae* (*fortuitum*) were different. He considered, however, that these differences justified the classification of *M. runyonii* only as a subspecies of *M. ranae* (*fortuitum*), not a separate species. Tsukamura, Tsukamura & Mizuno (1967) also found *M. abscessus* and the strain NCTC 946 (which they received under the designation of *M. fortuitum*) to be the same as *M. runyonii*. The strain NCTC 946 was also found by Marks & Szulga (1965), who analysed the lipid chromatography pattern of mycobacteria, to differ in this respect from other strains of *M. ranae* (*fortuitum*) that they examined. Our conclusion that *M. borstelense* is identical with *M. abscessus* and *M. runyonii* is, however, at variance with the views of Tsukamura, Mizuno & Tsukamura (1968) who consider it to be a separate species. Although *M. friedmannii* differs taxonomically from *M. ranae* (*fortuitum*) the two species are similar in habitat and in their significance as potential pathogens. The finding of *M. friedmannii* in a water tank shows that like *M. ranae* (*fortuitum*), this organism can occur elsewhere than in animals. Both species are opportunist organisms which under certain circumstances give rise to lesions in poikilothermic animals and in man.

We thank Dr P. Inman and Mr A. E. Brown for permission to report on the two strains isolated from injection abscesses in Hartlepool, Dr R. Muser (Tierhygienisches Institut in Heidelberg), for the supply of the two strains of *M. borstelense*, and Dr E. H. Runyon of the Veterans Administration, Salt Lake City, Utah, for arranging for us to receive the type strain of *M. abscessus*. Mrs S. Hunnes and Mr W. J. Gunthorpe gave valuable technical assistance.

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Note added in proof

Since the submission of this paper Dr E. H. Runyon has drawn our attention to the fact that this designation *Mycobacterium friedmannia* does not comply with the International Code of Nomenclature of Bacteria and Viruses. The Index Bergeyana lists this organism as *Mycobacterium chelonei*. This designation was used by Bergey *et al.* in the first edition of their Manual (1923).

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Stable Coexistence of Three Resistance Factors (*fi*⁻) in *Salmonella panama* and *Escherichia coli* K12

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SUMMARY

During an epidemic, a strain of *Salmonella panama* resistant to ampicillin, kanamycin, tetracycline and which was colicinogenic was isolated. The three drug-resistance determinants were transferred singly, in pairs or *en bloc* to sensitive *Escherichia coli* K 12 strains. When the recipient was an Hfr strain, it remained sensitive to F-specific phage f 2, so the transferred resistance factors can be classified as *fi*⁻. The three resistance characters were always independently transduced by phage P 1-b, and remained transferable in subsequent conjugations. In contrast to closely related R factors or Col factors which cannot coexist stably in the same cell, the three R factors described here do not specifically interfere with one another. They are maintained together in the naturally occurring strain of *S. panama* and after successive transfer to *E. coli* K 12. These results support the hypothesis that three independent *fi*⁻ resistance factors, R (Am), R (Km), R (Tc) and a Col factor coexist in *S. panama* and *E. coli* K 12 strains.

INTRODUCTION

Resistance factors (R factors) have been classified in two groups: *fi*⁺ (or *i*⁺) factors which inhibit the properties of the sex factor F, and *fi*⁻ (or *i*⁻) which do not inhibit these properties (Egawa & Hirota, 1962; Watanabe, Nishida, Ogata, Arai & Sato, 1964). When two R factors are harboured by a single bacterium, both elements coexist stably in the progeny when they do not belong to the same *fi*⁺ or *fi*⁻ class. When both R factors are *fi*⁺ or *fi*⁻, mutual exclusion occurs, and it has been assumed that the two compete for a single replication site (Watanabe *et al.* 1964).

During an epidemic in Paris, a *Salmonella panama* strain (LA 46.R 11), resistant to ampicillin (Am), kanamycin (Km), tetracycline (Tc) and harbouring a Col factor was isolated and this seems to behave differently from the previously described systems. Transfer of drug-resistance to sensitive *Escherichia coli* K12 was easily obtained (Chabbert, Baudens & Bouanchaud, 1967). The recipient bacteria rarely became resistant to all three drugs (Am, Km, Tc), but generally to only one or two of them. When any or all resistance characters were transferred to an *E. coli* K12 Hfr strain, the recipients remained sensitive to F-specific RNA phage f 2, thus the transferred resistance factors can be classified as *fi*⁻ (Chabbert, Baudens & Bouanchaud, 1969).

Spontaneous loss of resistance occurred at low frequency in *Salmonella panama* LA 46.R 11: 0·1 % for Am and Tc, and 2 % for the Km character. Growth at a sub-inhibitory concentration of acriflavine increased the percentage of Km sensitive bacteria to 41 % (Baudens & Chabbert, 1967). Similar results were obtained with

ethidium bromide, another 'curing' agent (Bouanchaud, Scavizzi & Chabbert, 1968). In contrast, the percentages of Am or Tc sensitive bacteria were not increased by these treatments. The present work is an attempt to determine whether the three resistance characters (Am, Km, Tc) belong to a single plasmid (R 11), or whether there are several independent *fi*$^-$ factors, R 11-1 (Am), R 11-2 (Km), R 11-3 (Tc) and a Col factor which can stably coexist in *S. panama* LA 46 or in *Escherichia coli* K 12.

METHODS

Bacterial strains. Salmonella panama LA 46.R 11 (Am Km Tc) (ColIb), kindly supplied by Professor L. Le Minor (Centre National des Salmonelles, Institut Pasteur), was isolated during an epidemic in Paris. Colicin type was determined by Dr Y. Hamon (Institut Pasteur). *Escherichia coli* K 12 54117 F$^-$ *lac*$^+$ was also obtained from

Table 1. *Origin of strains*

Escherichia coli K 12	Drug-resistance pattern	Transfers donor × recipient		Transductions by P 1-b donor × recipient	
LA 117	Am Km Tc	LA 46	LA 115	—	—
LA 118	Am Tc	LA 46	54117	—	—
LA 119	Sm Cm Tc Su	LA 49	LA 116	—	—
LA 120	Am	LA 46	LA 115	—	—
LA 121	Tc	LA 117	LA 116	—	—
LA 122	Tc	LA 46	LA 115	—	—
LA 123	Am	LA 117	LA 116	—	—
LA 124	Km	—	—	LA 117	54117
LA 125	Km	—	—	LA 117	LA 116
LA 126	Tc	LA 141	LA 116	—	—
LA 129	Am	LA 139	LA 116	—	—
LA 139	Am	—	—	LA 117	54117
LA 141	Tc	—	—	LA 117	54117
LA 142	Km Tc	LA 126	LA 124	—	—
LA 143	Am Km Tc	LA 129	LA 142	—	—

Professor Le Minor. *E. coli* K 12 C 600 F$^-$ *lac*$^-$ *thr*$^-$ *leu*$^-$ *bio*$^-$ was kindly supplied by Dr E. L. Wollman (Institut Pasteur). *E. coli* K 12 LA 115, mutant resistant to sodium azide, derived from *E. coli* K 12 54117 and selected on gradient plates (Szybalski, 1952). *E. coli* K 12 LA 116, mutant resistant to rifampicin derived from *E. coli* K 12 C 600. *E. coli* LA 49.R 15 (Sm Cm Tc Su)* *fi*$^+$, serotype 111.B 4, was isolated from clinical material. Other *E. coli* K 12 strains were obtained in transfer or transduction experiments and are listed in Table 1. All resistant strains derived from *S. panama* LA 46.R 11 harboured a *fi*$^-$ resistance factor.

Culture media and drugs. Tryptic soy broth (0370 Difco) and Mueller Hinton solid medium (0252-01 Difco) were routinely used. Fermentation of lactose was observed on Drigalski medium. Phage P 1-b was propagated on bacteria grown in pancreatic peptone broth (Peptone P.T.V. obtained from Ets. Réactifs, 75-Gentilly, France).

Ampicillin was obtained from Laboratoires Delagrange, Paris; kanamycin sulphate from Laboratories Bristol, Paris; sodium azide from Laboratoires Rhône-Poulenc, Paris; tetracycline hydrochloride from Laboratoires Pfizer-Clin, Paris. Rifampicin

* Sm, streptomycin; Cm, chloramphenicol; Su, sulphonamides; Tc, tetracycline.

was a gift from Laboratoires Lepetit, Paris. Antibiotic resistance was determined with sensitivity discs from the Institut Pasteur.

Conjugation. Multiply resistant donor strains (*Salmonella panama* LA 46.R 11 and *Escherichia coli* LA 143) were cultivated for 4 hr on cellophan membranes lying on nutrient agar plates supplemented with the following concentrations (μg./ml.) of antibiotics: ampicillin, 20; kanamycin, 12·5; tetracycline, 16; to obtain entirely multi-resistant populations (Chabbert & Patte, 1960). The donor bacteria were suspended in broth ($2{\cdot}5 \times 10^8$ bacteria/ml.) and mixed with the recipient strain. In other cases, conjugation conditions were as described by Watanabe & Fukasawa (1961*a*). Twenty minutes later, the mixture was blended and diluted in broth containing methionine (20 μg./ml.) (Mitsuhashi, 1965); 0·2 ml. of each dilution were spread on Drigalski medium containing sodium azide (300 μg./ml.) or rifampicin (100 μg./ml.), and the antibiotic selecting for the R factor (μg./ml.): ampicillin, 20; kanamycin, 12·5; tetracycline, 12. Frequencies of transfers were expressed per donor bacterium in the mixture.

Transduction. Phage P 1-b, a mutant of phage P 1-kc was kindly supplied by Professor F. Jacob (Institut Pasteur; originally from Dr S. Brenner). Phage P 1-b was propagated in resistant donor bacteria. Preparations containing about 10^8 p.f.u./ml. were obtained and sterilized with chloroform. The recipients were infected (multiplicity of infection = 1) and transductants were selected on gradient plates containing the selecting antibiotic (Szybalski, 1952). Trypsin (200 μg./ml.) was added to the phage suspension when the donor bacteria were colicinogenic.

RESULTS

Transfers in 20 min. from Salmonella panama LA 46.R 11 to Escherichia coli K 12 LA 115 and from E. coli K 12 LA 143 to E. coli K 12 LA 116.

Salmonella panama LA46.R 11 (Am Km Tc) (colIb) was grown on agar supplemented with the three antibiotics to obtain an entirely multi-resistant population. Before conjugation, 396 colonies were isolated and tested for drug-resistance: no sensitive segregant was observed on replica plates. The same results were obtained with *E. coli* K 12 LA 143 (Am Km Tc) (*col*⁻). The transfers observed in 20 min. are reported in Table 2. It can be seen that some recipients became resistant to all three drugs, but transfer of single or pairs of resistance characters (Am Km, Am Tc, Km Tc) was also obtained, and that all combinations of transfer were possible. The frequencies of transfer from *S. panama* to *E. coli* K 12 LA 115 varied from 4×10^{-4} to $1{\cdot}2 \times 10^{-6}$. The isolated characters Am and Tc were transferred at the highest frequency, and the character Km at the lowest. Each pair of characters was transferred at about the same frequency, and the complete combination of three at lower frequency. Similar results were obtained in transfers from *E. coli* K 12 LA 143 to *E. coli* K 12 LA 116, and the frequencies of transfers varied from $3{\cdot}1 \times 10^{-6}$ to 2×10^{-7}. Transfer of colicin factor was frequently observed, but not with all the drug-resistant recipient bacteria. This transfer from *S. panama* was always associated with transfer of Km character (alone or with other characters). In contrast, colicin production was not always observed in recipients resistant to Am, Tc or Am Tc.

Superinfection immunity and stability. Conjugal transfers were carried out between *Escherichia coli* K 12 donor strains, resistant to one drug and non-colicinogenic, and

recipient *E. coli* K 12 strains, resistant to one of the other drugs or sensitive. It can be seen in Table 3 that the frequencies of transfer in 20 min. were the same to sensitive or resistant recipients. In mixtures tested after overnight incubation, the frequencies of transfer were as usual increased, but again did not differ with sensitive or resistant recipients.

Table 2. *Frequencies of transfers of drug-resistance in 20 minutes*

Selecting antibiotic	Transferred characters	Frequencies of transfer/donor bacterium: *Salmonella panama* LA46 × *E. coli* K12 LA115	Frequencies of transfer/donor bacterium: *Escherichia coli* K12 LA143* × *E. coli* K12 LA116
Am	Am	$8{\cdot}4 \times 10^{-5}$	2×10^{-6}
	Am Km	$2{\cdot}4 \times 10^{-5}$	$3{\cdot}1 \times 10^{-6}$
	Am Tc	1×10^{-5}	2×10^{-7}
	Am Km Tc	6×10^{-6}	$2{\cdot}9 \times 10^{-8}$
Km	Km	$1{\cdot}2 \times 10^{-6}$	$1{\cdot}3 \times 10^{-6}$
	Am Km	$1{\cdot}2 \times 10^{-5}$	2×10^{-7}
	Am Km Tc	2×10^{-6}	—
Tc	Tc	$1{\cdot}1 \times 10^{-4}$	7×10^{-7}
	Km Tc	4×10^{-4}	—
	Am Km Tc	8×10^{-6}	—

* Origin of this strain can be seen in Table 1.

Table 3. *Frequencies of transfers in superinfection experiments*

Donor strains	Recipient strains	Selecting antibiotics	Frequencies of transfer/donor bacterium in 20 min.
LA 120 (Am)	LA 121 (Tc)	Am	$1{\cdot}4 \times 10^{-6}$
	LA 116		$1{\cdot}5 \times 10^{-6}$
LA 121 (Tc)	LA 120 (Am)	Tc	2×10^{-4}
	LA 115		1×10^{-4}
LA 122 (Tc)	LA 123 (Am)	Tc	1×10^{-6}
	LA 116		8×10^{-7}
LA 124 (Km)	LA 121 (Tc)	Km	8×10^{-6}
	LA 123 (Am)		$1{\cdot}2 \times 10^{-6}$
	LA 116		4×10^{-7}

Twenty-three recipient colonies resistant to Am and Tc obtained in these transfers were picked at random, grown for 24 hr in antibiotic-free medium and streaked on nutrient agar; the colonies were then tested for drug-resistance. All colonies (2300) on replica plates were still resistant to both antibiotics. Five were used as donors in transfers, and recipient bacteria resistant to one or two drugs were obtained. Similar stability and separate transfers were observed with bacteria resistant to Am and Km, or to Km and Tc obtained in superinfection experiments.

Transduction by phage P 1-b. Phage P 1-b was propagated on two resistant *Escherichia coli* K 12 strains obtained as described in Table 1: *E. coli* K 12 LA 117 (Am Km Tc) and (colIb), and *E. coli* K 12 LA 118 (Am Tc). As a control, phage P 1-b was also propagated on *E. coli* K 12 LA 119 carrying a *fi*$^+$ R factor usually transferred *en bloc*

(Bouanchaud, Scavizzi & Chabbert, 1968). The results of the transduction in *E. coli* K 12 54117 and *E. coli* K 12 LA 116 can be seen in Table 4 (*a*, *b*). The entire *fi*+ R factor was transduced by phage P 1-b and remained transferable in subsequent transfers. In contrast, phage P 1-b transduced only isolated resistance characters from *E. coli* K 12 LA 117 to *E. coli* K 12 54117. The single Tc character was transduced from *E. coli* K 12 LA 118. The Km and Tc characters were separately transduced from *E. coli* K 12 LA 117 to *E. coli* K 12 LA 116. All the tested transductants were effective donors at the usual frequencies in subsequent transfer experiments. No transduction of groups of resistance characters or of colicinogeny was observed in these experiments.

Table 4.

(a) Transduction by phage P 1-b from Eschericha coli K 12 LA 117, LA 118 and LA 119 to E. coli K 1254117

Donor strains	Resistance pattern	Selecting antibiotics	Drug-resistance of transductants	No. of transductants obtained and tested	*Frequency of transduction
LA 117	Am Km Tc	Am	Am	6/6	1×10^{-7}
		Km	Km	3/3	1×10^{-8}
		Tc	Tc	40/40	7×10^{-6}
LA 118	Am Tc	Am	—	—	—
		Tc	Tc	8/8	$1{\cdot}3 \times 10^{-7}$
LA 119	Sm Cm Tc Su	Cm	Sm Cm Tc Su	11/11	1×10^{-6}
		Tc	Sm Cm Tc Su	31/31	3×10^{-5}

(b) Transduction by phage P 1-b from Escherichia coli K 12 LA 117 to E. coli K 12 LA 116

LA 117	Am Km Tc	Am	—	—	—
		Km	Km	2/2	3×10^{-8}
		Tc	Tc	14/14	2×10^{-7}

* Frequency of transduction as no. transductants/p.f.u. phage.

DISCUSSION

Transferable resistance in a *Salmonella panama* strain isolated in Paris is different from previously described R+ strains. Instead of being transferred *en bloc*, the three resistance determinants (Am, Km, Tc) are usually transferred either singly or in pairs such as Am Km, Am Tc or Km Tc. The complete combination can be transferred but generally at a lower frequency to sensitive *Escherichia coli* K 12. This might be explained by frequent spontaneous segregation, but such segregation has been mainly observed when contacts between donor and recipient bacteria have been maintained for a long time (Walton & Fulton, 1967). During growth of *S. panama* LA 46.R 11 spontaneous segregants appeared rarely: 0·1 % for Am and Tc and 2 % for the Km character in 18 hr. Resistant recipients grown in drug-free medium also showed only a low frequency of spontaneous segregation. Thus it can be assumed that those recipients resistant to one or two drugs are not segregants of bacteria originally resistant to all three drugs.

In superinfection experiments performed with a donor carrying an R factor conferring resistance to one of the drugs, the frequencies of transfer were not decreased

when the recipient bacteria harboured an R factor conferring resistance to one of the other drugs. After transfer the recipients remained resistant to both drugs for at least 10 generations in antibiotic-free medium. Nevertheless, the two resistance characters were still independently transferred in subsequent conjugations.

The observed transfers with *Salmonella panama* LA 46.R 11 might be compared with those described by Anderson and colleagues with *S. typhimurium* phage-type 29. But in the *S. typhimurium* system, some resistance characters, when present singly, are not in themselves transferable, and their transfer has to be mediated by an independent transfer factor (Anderson & Lewis, 1965; Anderson, 1966; Anderson, 1967; Anderson & Perret, 1967). In other systems, a non-transferable colicin factor can be transferred when the host bacterium is superinfected with a transferable extrachromosomal element (Fredericq, 1956; Ozeki, Stocker & Smith, 1962). The *S. panama* system seems to be different, each element was in itself transferable, and remained transferable after transduction.

Transduction experiments were made with phage P1-b, a mutant of phage P1-kc which has been assumed to transduce entire resistance factors between *Escherichia coli* K12 strains (Watanabe & Fukasawa, 1961*b*). Phage P1-b was shown to be able to transduce the entire R15 (*fi*$^+$) factor, and all the transductants which were tested were effective donors in subsequent transfers. In contrast, when phage P1-b was propagated on *E. coli* K 12 LA 117 or on *E. coli* K 12 LA 118, only single resistance characters were transduced. Transductants resistant to two or three drugs were never observed. All the transductants which were tested were effective donors at usual frequencies in subsequent transfers. Colicinogeny was not co-transduced with drug-resistance in these experiments, although the two properties were frequently co-transferred; so it can be suggested that the colicin determinant and the drug-resistance determinants are carried by independent plasmids. R factors governing the production of colicin I have been described, but in these strains, drug-resistance and colicinogeny were co-transduced (Siccardi, 1966; Meynell, Meynell & Datta, 1968; Romero & Meynell, 1969).

The results reported here suggest that several discrete genetic elements responsible for drug-resistance can stably coexist with a Col factor in *Salmonella panama* and *Escherichia coli* K 12. It follows that there is no competition for a unique site of replication between these elements: R 11-1 (Am), R 11-2 (Km), R 11-3 (Tc) and ColIb. Stable coexistence of different plasmids have been often reported, but not of 'homologous' *fi*$^-$ resistance factors. Coexistence of these three replicons supports the hypothesis that genetic replication and pili formation (and type) are wholly dissimilar processes (Meynell, Meynell & Datta, 1968).

The fact that the plasmids harboured by *Salmonella panama* are independent is not incompatible with frequent simultaneous transfers, and such types of transfers have been previously reported (Dubnau & Stocker, 1964; Hardy & Nell, 1967). It should be of interest to investigate whether the fact that the plasmids described here have closely related sex factors can explain their frequent co-transfer.

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Correlation of Bacterial Sensitivities to Ionizing Radiation and Mild Heating

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SUMMARY

DNA, isolated from bacteria which had been heated to 52° for several minutes, sedimented in an alkaline sucrose gradient more rapidly than DNA from untreated bacteria, in a similar manner to DNA from bacteria exposed to ionizing radiation. There is a general correlation between the sensitivities to γ- radiation and to incubation at 52° of various strains of *Escherichia coli*. Heated bacteria were more sensitive to subsequent exposure to γ-radiation, indicating that recovery capacity was itself heat-sensitive. The normal function of some of the cellular systems conferring radiation resistance might therefore be the mitigation of DNA damage due to mild thermal stress at elevated and perhaps also at normal temperatures.

INTRODUCTION

There is now a very considerable amount of evidence that the sensitivity of bacteria to radiation is under the control of distinct genes, many of which have been identified and mapped (see Adler (1966) for a review of the situation in *Escherichia coli*). It is also clear that most, if not all of these genes are concerned with the ability of the bacterium to recover from the radiation-induced damage, either by repairing damaged structures or by permitting the organisms to bypass or tolerate them (Haynes, 1964; Witkin, 1967; Moseley, 1968; Howard-Flanders, 1968).

Ionizing radiation is not found in natural environments at a value high enough to constitute a hazard to living organisms and there is thus speculation about the normal function of these recovery systems. One possibility is that they may be involved in normal metabolism, for example the process of genetic recombination (Howard-Flanders & Theriot, 1966). Alternatively they may counter the effect of other environmental hazards which produce damage not unlike that produced by ionizing radiation. The most common lesion known to be produced by ionizing radiation is breakage of one of the two strands of the bacterial DNA (Freifelder, 1965; McGrath & Williams, 1966; Hagen & Wellstein, 1965). Single strand breaks can also be produced by thermal stress. Spontaneous hydrolysis is known to be temperature dependent in the region between 80 and 100° (Eigner, Boedtker & Michaels, 1961). Depurination also occurs spontaneously at elevated temperatures (Greer & Zamenhof, 1962) and apurinic sites might well be attacked by specific endonucleases (B.S. Strauss, personal communication). Moreover, endonucleases might be released into the cytoplasm by heat treatment.

The purpose of the experiments described here was to test the hypothesis that mild

heating, at near-physiological temperatures, produces a type of damage similar to that produced by ionizing radiation. This hypothesis was suggested by the observation that single-strand DNA breaks are formed at near physiological temperatures. We have examined specifically the possibility that, at a temperature just above the maximum growth temperature, the rate of loss of viability of different strains might be determined largely by the presence of recovery systems and might therefore show a correlation with the sensitivity of the strains to ionizing radiation.

METHODS

Organisms. The *Escherichia coli* strains used, together with their sources and phenotypes with respect to radiation sensitivity, are given in Table 1. For characterization of most of these phenotypes the reader is referred to Witkin (1967). Also used were *Bacillus stearothermophilus* strains and *Micrococcus radiodurans*.

Table 1. *Strains of Escherichia coli, their sources, and radiation sensitivity phenotypes*

		Phenotype				
Strain	Parent	rec	exr	lon	hcr	Source
B/r	B	+	+	Su	+	Witkin
B_3r	(B)	+	+	Su	+	Alper & Moore
B	.	+	+	−	+	.
B_{s-1}	B	+	−	−	−	Hill
B_{s-2}	B	+	−	−	+	Hill
WP 2	B/r	+	+	Su	+	Witkin
WP 2 hcr⁻	WP 2	+	+	Su	−	Hill
IV-5	(B_{s-1})	+	−	Su	−	Witkin
V-5	IV-5	+	+	Su	−	Witkin
H/r 30-R	(B)	+	+	Su	+	Kondo
R I 5	H/r 30-R	− ?	− ?	Su	+	Kondo
NG 30	H/r 30-R	−	+	Su	+	Kondo
AB 1157	.	+	+	+	+	Howard-Flanders
AB 2463	AB 1157	−	+	+	+	Howard-Flanders

Su indicates that the lon⁻ phenotype is suppressed extragenically. This has been demonstrated for B/r (Greenberg, personal communication) and is assumed to be so for other radiation-resistant derivatives of B. Parent strains in parentheses are more than one isolation step removed from their derivatives.

Cultural details. Exponential phase bacteria, grown at 37° to about $1{\cdot}5 \times 10^8$ cm.$^{-3}$, were used for all experiments, being heated or irradiated in their growth medium. Bacteria grown in glucose+salts medium (Haas & Doudney, 1957) were diluted after treatment in salts medium without glucose and plated on to Oxoid Brain Heart Infusion solidified with 1·5 % Difco Purified Agar at 37°. Bacteria grown in Oxoid Nutrient Broth no. 2 were diluted in M/15 phosphate buffer (pH 7) and plated on to the same medium solidified with 1·5 % Difco Purified Agar. In some experiments plating was also done on glucose salts medium with 1·5 % Difco Purified Agar. Three independent dose-survival curves were obtained with each strain. *Micrococcus radiodurans* was grown in Oxoid Dextrose Peptone Broth and plated on Oxoid Dextrose Peptone Agar at 30°.

Treatments. (*a*) *Heat.* Bacteria in 5 cm.^{3} growth medium in a thin walled glass tube were placed in a water bath at 52° for the required length of time. The temperature in the tube rose to within 0·5° of the final temperature in 50 sec. (*b*) *Radiation.* Bacteria in 5 cm.^{3} growth medium were chilled from 0 to 5° to halt growth, which treatment produced no detectable loss of viability, and exposed to γ-radiation from two ^{60}Co sources at a dose rate of approximately 3·6 krad. min.$^{-1}$. Air was bubbled through the suspension during irradiation.

Sedimentation of DNA. Spheroplasts were produced from *Escherichia coli* B_3r Thy$^-$ which had been grown in glucose+salts medium in the presence of ^{3}H-thymine and lysed on the top of a sucrose gradient at pH 12. The techniques described by McGrath & Williams (1966) were followed closely. The 5 cm.^{3} gradients were centrifuged at 30,000 rev./min. in an M.S.E. Superspeed 65 ultracentrifuge for 90 min. Tubes were pierced with an M.S.E. tube piercer and samples collected on to filter paper. The papers were washed twice in cold 5 % trichloracetic acid, once in cold 95 % ethanol, twice in cold acetone, and the activity counted in a Beckman scintillation counter.

RESULTS

Sedimentation of heated DNA

The alkaline sucrose sedimentation profile of DNA from *Escherichia coli* B_3r which had been heated at 70° for 20 min. indicated that the average molecular weight was very considerably decreased compared with DNA from unheated bacteria, i.e. much strand breakage had occurred. The same effect was observed, although to a lesser degree, when bacteria were heated at 52°, only a few degrees above their maximum temperature for growth (Fig. 1). Similar results were obtained with *E. coli* B_{s-1} Thy$^-$. The lowering of molecular weight produced by 20 min. at 52° was roughly the same as that produced by 20 krads. of γ-radiation. We therefore conclude that mild heating may directly or indirectly give rise to DNA strand breakage or alkali-labile sites.

Correlation of heat and radiation sensitivities

Figure 2 shows heat and radiation survival curves for strains B/r and B_{s-1} of *Escherichia coli*. Strain B_{s-1} clearly has a greater sensitivity than B/r to both mild heating and γ-radiation. A similar ordering of heating and radiation sensitivities was found (Fig. 3) with *E. coli* H/r-30 R, which is resistant at the known radiation sensitivity loci, and two sensitive mutants, NG 30 which behaves as a 'reckless' Rec$^-$ and R 15 which behaves as a 'cautious' Rec$^-$ or possibly Exr$^-$ or Lex$^-$ (for a discussion of these terms see Howard-Flanders, 1968).

Values of LD 90 (dose or time to decreased initial viable population to 10 %) for heating at 52° have been plotted against those for γ-irradiation for a number of strains (Fig. 4) and the correlation between the two is good. The point which lies most markedly off the general trend is that for *E. coli* B plated on nutrient agar and there is a ready explanation for this. The sensitivity to ionizing radiation of B relative to B/r on rich media is due to an allele (*lon*) responsible for a defect in cell division probably caused by DNA synthesis becoming out of phase with RNA and protein synthesis (Alper & Gillies, 1958; Witkin, 1966; Kantor & Deering, 1968). This sensitivity may be abolished by heating the bacteria to 45° (Harm & Stein, 1952; Alper, 1961). Therefore, even if

heating to 52° caused damage to DNA, its effects on the cell division system would not be expressed and the resistance to heat would appear higher than expected. When *E. coli* B is plated on minimal agar where the defect in cell division is suppressed (Alper & Gillies, 1960), the LD90 for mild heat has the expected value relative to that for radiation.

Occasionally the heat sensitivity of radiation sensitive strains was extreme, which accounts for the large variation about the mean in these cases.

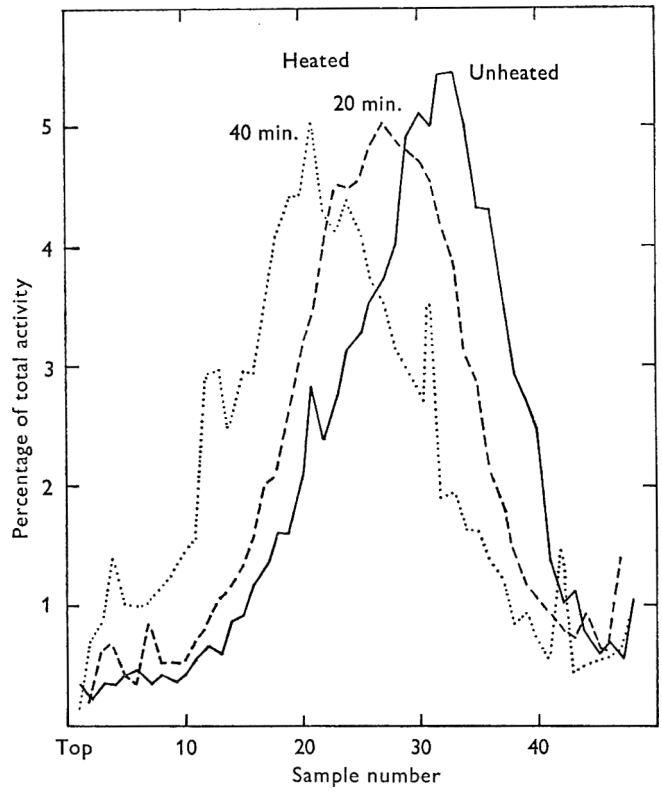

Fig. 1. Sedimentation profiles in a sucrose gradient at pH 12 of ^{3}H-thymine-labelled DNA from *E. coli* B_3r heated for 0, 20 and 40 min. at 52° and lysed on top of the gradient using the technique of McGrath & Williams (1966). The means of the curves are 29·2, 26·6 and 22·9, and the second moments about the means 78·7, 79·8 and 97·9, respectively.

Heat inactivation of recovery

Examination of the survival curves in Fig. 2 and 3 reveals that the heat resistance of the resistant strains is in large measure due to the presence of a shoulder; at the longer heating times the slopes of the survival curves approach those of the sensitive strains. This suggests that the ability of a cell to repair or bypass thermal damage is itself sensitive to heating. This being so it would be expected that resistant bacteria surviving a heat treatment should be more sensitive to ionizing radiation. Figure 5 shows that the sensitivity of *Escherichia coli* B/r to γ-radiation was increased by a factor of 2·7 after 15 min. incubation at 52° which decreased the viable fraction to about 7 %. The sensitive strain B_{s-1} showed only a slight increase in sensitivity after a heat treatment

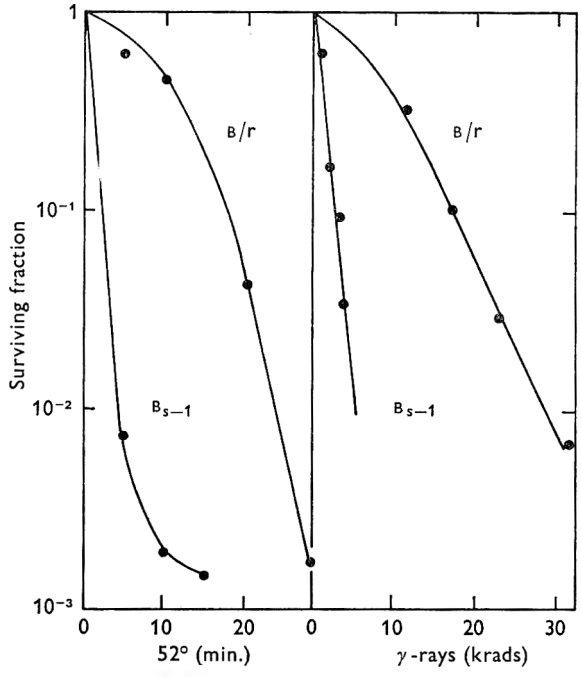

Fig. 2. Survival of *E. coli* B/r and B_{s-1} from exposure to γ-radiation under aerobic conditions, or to a temperature of 52°. Bacteria were grown in glucose+salts medium.

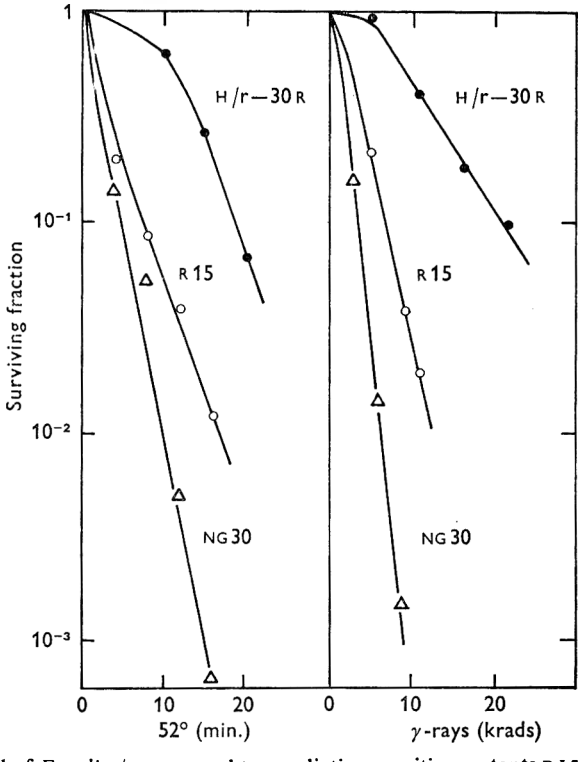

Fig. 3. Survival of *E. coli* H/r—30R and two radiation-sensitive mutants R15 and NG30 from exposure to γ-radiation under aerobic conditions, or to a temperature of 52°. Bacteria were grown in nutrient broth.

which decreased the viability to a comparable extent. This interaction between heat and ionizing radiation resembles, at least superficially, that between u.v. and ionizing radiation studied by Haynes (1964).

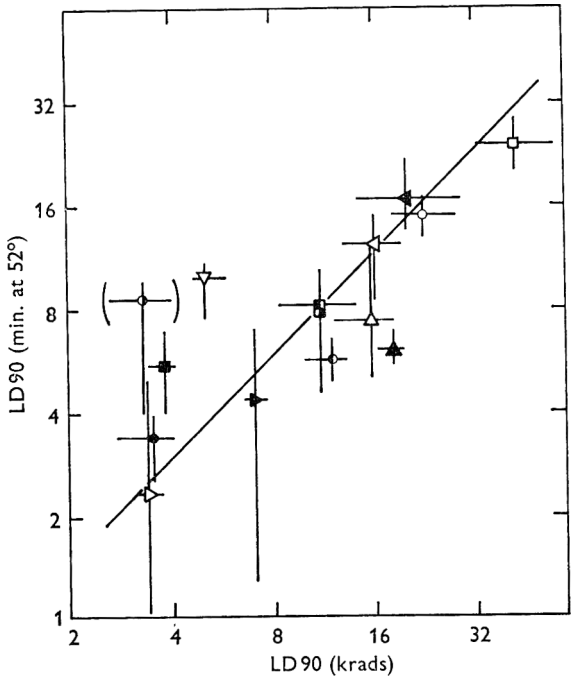

Fig. 4. Plot of sensitivity to a temperature of 52° against sensitivity to γ-radiation of various strains of *E. coli* grown in nutrient broth: ○, B/r; ◑, B; ◐, B on glucose-salts agar; ●, B_{s-1}; ▽, B_{s-2}; △, WP2; □, AB1157; ■, AB2463; ◁, H/3r-30R; ▶, R15; ▷, NG30; ◀, B/r; ▲, WP2 *hcr*⁻; ◧, IV-5; ◨, V-5. LD90 is the dose required to reduce the initial viable population to 10 % and is taken from three independent survival curves. The lines represent the range of experimental values.

Involvement of known loci in heat sensitivity

At the present time we know of several well defined phenotypes with regard to the response of *Escherichia coli* strains to ionizing and ultraviolet radiations (see, for example, Witkin, 1967; Howard-Flanders, 1968) and many of the genetic loci involved have been mapped (e.g. Adler, 1966).

The effect of known loci on heat sensitivity can be determined only by the comparison of pairs of strains which are believed to be otherwise isogenic, one having been derived from the other.

There are two pairs of strains differing in the 'reckless' Rec⁻ phenotype: AB 1157 and AB 2463, and H/r30-R and NG 30. In both cases the Rec⁻ phenotype confers extreme heat sensitivity.

The Exr⁻ phenotype sometimes has an insignificant effect on radiation sensitivity (*cf.* for example, VI-5 with V-5). Heat sensitivity is also identical in these strains. In combination with the Lon⁻ and Hcr⁻ phenotypes, however, Exr⁻ may have a large effect on both heat and γ-radiation sensitivity (*cf.* B_{s-1} with B/r).

The Hcr⁻ phenotype usually has a large effect on sensitivity to u.v. radiation

(WP 2 Hcr$^-$ is about 16 times more sensitive than WP 2) but has no effect on sensitivity to heat and ionizing radiation (as judged by comparing WP 2 and WP 2 Hcr$^-$) except possibly when combined with Lon$^-$ and Exr$^-$ (as in B_{s-1}).

The Lon$^-$ phenotype increases sensitivity to both heat and ionizing radiation as may be seen by comparing B with its suppressed derivative B/r. This is manifest even though the expression of the Lon$^-$ phenotype itself is known to be partially inhibited by heating as mentioned above.

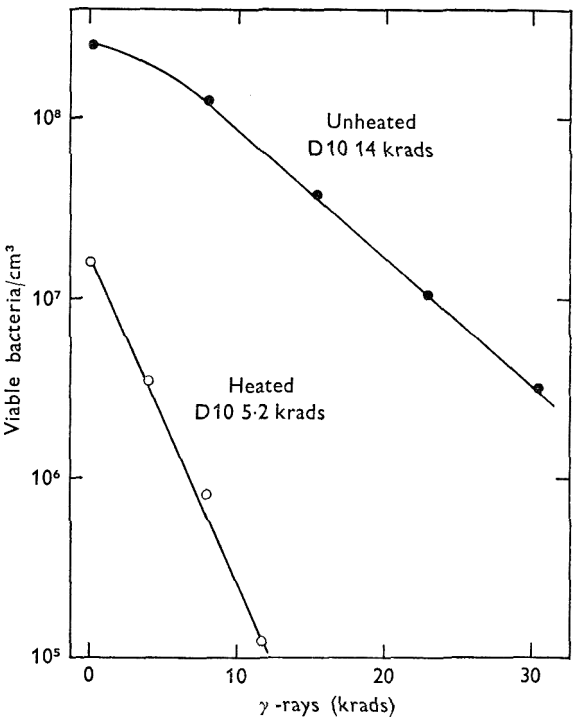

Fig. 5. Sensitivity to aerobic γ-radiation of *E. coli* B/r with and without preliminary exposure to 52° for 15 min. Bacteria were grown in glucose-salts medium. D 10 is the dose necessary to decrease the viable population by a factor of 10 on the straight portion of the curve.

Extension of hypothesis to other organisms

A further possibility that arises from our hypothesis is that organisms which grow well at high temperatures might be expected to show greater than average resistance to ionizing radiation. We have tested vegetative forms of several thermophilic strains of *Bacillus stearothermophilus* and obtained LD 90 values of 40 to 50 krads when the incubation temperature was 52°, a temperature at which the recovery systems presumably have to cope with both thermal and radiation damage. They thus exhibit rather more resistance to γ-radiation than any of the *Escherichia coli* strains or non-thermophilic strains of Bacillus (Freeman & Bridges, 1960). These thermophiles did not grow, however, at 37° where thermal damage to DNA would be further decreased and where one might expect even greater resistance to ionizing radiation.

One cannot predict with any certainty that extremely radiation-resistant organisms should be heat-resistant since factors other than DNA damage, e.g. protein denatura-

tion, are expected to be involved in heat inactivation and the importance of these factors will tend to be greater at longer heating times. It is nevertheless noteworthy that the extremely radiation resistant *Micrococcus radiodurans* with an LD90 value of 68 min at 52° is considerably more resistant to heat than our most resistant *Escherichia coli* strain (Fig. 6), and that the survival curve has the long shoulder typical of that exhibited to ultraviolet and X-radiation (Moseley & Laser, 1965).

Obviously much more information will be required before it is clear how general is the correlation we have shown for *Escherichia coli.*

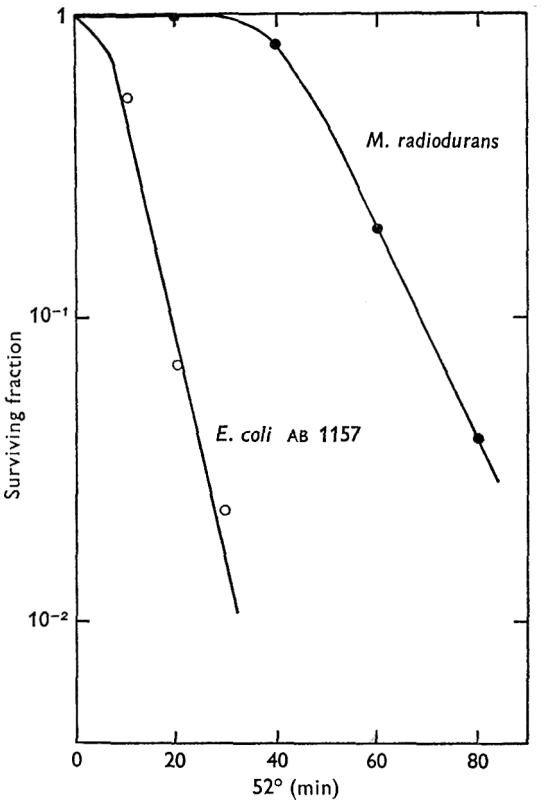

Fig. 6. Survival of *Micrococcus radiodurans* and *E. coli* K12 AB1157 grown in nutrient broth from exposure to a temperature of 52°.

DISCUSSION

Haynes (1964) previously examined the sensitivity to incubation at 60° of three strains of *Escherichia coli*, B/r, B and B_{s-1}. All three strains were equally sensitive and Haynes was probably correct in assuming that death was due to generalized protein denaturation and enzyme destruction. Our present results show clearly that there is a general correlation between sensitivity to ionizing radiation and sensitivity to incubation at 52° among various strains of *E. coli*, although the scatter around the line in Fig. 4 suggests that there are factors influencing heat sensitivity alone or radiation sensitivity alone.

The correlation implies that, as far as the *Escherichia coli* cell is concerned, mild thermal damage and ionizing radiation damage appear very similar. There is strong evidence that inactivation by ionizing radiation is due to damage to the DNA (Haynes, 1964; Moseley, 1968; Strauss, 1968; Bridges & Munson, 1968), so that it is a logical step to suppose that at 52°, in contrast to 60°, protein damage (at least at short heating times) is relatively unimportant and that the effective lethal damage is also located in the DNA.

Our results further imply that *Escherichia coli* bacteria use the same processes to repair or mitigate the effects of both types of damage. The fact that mild thermal stress is much more common in nature than ionizing radiation suggests that the repair and bypass systems may have evolved to cope with the former rather than the latter. However attractive this might be as an explanation, it must be pointed out that the ability of cells to cope with a certain amount of both types of damage may be merely a bonus conferred on the cell by systems whose real function is much more important, for example in effecting genetic recombination or in controlling cell and nuclear division. A similar suggestion has been made to explain the widespread occurrence of photoreactivating enzyme (Cook & McGrath, 1967).

Among the strains we examined, every mutation affecting sensitivity to ionizing radiation also affected heat sensitivity, but a mutation (*hcr*$^-$) which altered sensitivity to u.v. radiation by a factor of 16 or more, was without effect on heat or gamma ray sensitivity.

The ability of the more resistant bacteria to cope with mild thermal and gamma radiation damage is itself heat-sensitive. In this respect it is similar to the system involved in repair of X-ray and ultraviolet damage in yeast (Patrick & Haynes, 1964).

Although our observation of the change in sedimentation pattern of bacterial DNA after heating prompted us to perform the biological experiments, we have not established that the thermal damage involved in inactivation is single-strand breakage of DNA. We have not, for example, been able to demonstrate any repair of thermal DNA damage during subsequent incubation of resistant bacteria as was done for X-ray damage by McGrath & Williams (1966). Nevertheless, the correlation both of sensitivity and of DNA profile in the alkaline sucrose gradient is highly suggestive.

We acknowledge with thanks the technical assistance of Miss Rachel Dennis and Mr John Law.

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Linear Growth Kinetics of Plaque-forming Streptococci in the Presence of Sucrose

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SUMMARY

Growth of intact plaques of dextran-forming strain SL-1 was linear both on a dry weight and DNA basis. Growth of broth cultures of dextran-forming strains SL-1 and E-49 was linear with sucrose but exponential with other sugars; non dextran-forming strains 4M4 and JR8LG grew exponentially with all sugars tested. When dextranase was added to slowly growing cultures of dextran-forming strains SL-1 and E-49, growth became rapid. Linear growth of strains SL-1 and E-49 with sucrose is ascribable to extracellular dextran synthesized by the organisms.

INTRODUCTION

Certain streptococci cause dental caries and dental plaque formation in experimental animals (Fitzgerald & Keyes, 1960). These cocci can be isolated, often in high numbers, from the plaques of experimental animals (Krasse & Edwardsson, 1966) and humans (Carlsson, 1967; Krasse, Jordan, Edwardsson, Svensson & Trell, 1968). The growth characteristics of plaque-forming streptococci have not been described, either as adhesive aggregates (plaque) or in broth culture.

Stralfors (1950) assumed that conditions in the mouth would favour rapid bacterial proliferation. From the data of Krasse (1954), Gibbons (1964) deduced that caries-conducive microbial plaque has only two or three growth cycles per day *in vivo*. Measurements of steady-state rates of faecal excretion of micro-organisms by conventional and gnotobiotic animals (Gibbons & Kapsimalis, 1967) indirectly supported this deduction.

This report describes the *in vitro* growth of intact streptococcal plaque and of streptococci suspended in broth under conditions favourable for growth. Sucrose restricts these cells from growing exponentially. This effect is ascribable to dextran formation, a characteristic property of these organisms when grown with sucrose.

METHODS

Micro-organisms. Two strains of plaque-forming caries-conducive streptococci, SL-1 and E-49, and two strains of non plaque-forming streptococci, 4M4 and JR8LG were studied. Implantation of SL-1, a human plaque isolate, or E-49, a streptomycin resistant variant of hamster plaque isolate HS-6 (Fitzgerald & Keyes, 1960), results in

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heavy plaque formation and caries in the hamster. The colonial characteristics of these strains resemble those described by Krasse (1966) for other caries-conducive streptococci of hamster or human origin. SL-1 is similar antigenically (Kennedy, Sklair & Bahn, 1967) and biochemically (R. J. Fitzgerald, personal communication) to strain HS-6 described by Fitzgerald & Keyes (1960). The *in vitro* plaque-forming characteristics of SL-1 and E-49 have been described (McCabe, Keyes & Howell, 1967; Tanzer & McCabe, 1968). Both strains form extracellular dextrans from sucrose (Gibbons, Berman, Knoettner & Kapsimalis, 1966; Fitzgerald, Spinell & Stoudt, 1968) and produce dextran sucrase constitutively (J. M. Tanzer & S. A. Robrish, unpublished data; R. J. Gibbons, personal communication). Both strains agglutinate in the presence of high molecular weight dextran and agglutinated cocci are dispersed by dextranase (Gibbons & Fitzgerald, 1969). These strains are similar to those named *Streptococcus mutans* by Guggenheim & Schroeder (1967) and Edwardsson (1968). Strain 4M4, isolated from the hamster, and strain JR8LG, isolated from the rat, are non plaque-forming, and presumably non dextran-forming. Strain 4M4 does not agglutinate in the presence of high molecular weight dextran (Gibbons & Fitzgerald, 1969). Tests of the pathogenicity of 4M4 and JR8LG have been negative (Jordan & Keyes, 1966).

Growth of intact plaque in vitro. Cultures were maintained by passage at 10 to 14 day intervals in fluid thioglycollate medium (Baltimore Biological Laboratories, Baltimore, Md., U.S.A.) containing 20 % (w/v) horse meat infusion and excess $CaCO_3$. Intact *in vitro* bacterial plaques were grown at 37° as described by Tanzer & McCabe (1968) using 10 ml. broth (Jordan, Fitzgerald & Bowler, 1960) supplemented with 50 mg. Na_2CO_3/l. and 5 % (w/v) sucrose. Tubes of broth containing a cylindrical tared sterile glass rod (5 mm. dia.) immersed to a depth of 45 mm. were inoculated with 0·2 ml. of SL-1 and then stoppered with cotton. Each rod with adhering plaque was transferred at daily intervals to tubes of uninoculated broth for up to 7 days. Some of the glass rods were removed daily from the broth and dried to constant weight at 95°. The difference between the weight of the rod covered by dried plaque and that of the tared rod was taken as the dry weight of plaque. After weighing, the DNA content of the dried plaques was measured (see below). The DNA content of the broth tubes used for plaque growth also was assessed.

Growth of cells in broth culture. Cells were grown initially in broth (Jordan *et al.* 1960) supplemented with 50 mg. Na_2CO_3/l. and containing an appropriate concentration of filter-sterilized sugar. After growth to stationary phase, 1 ml. was transferred to 40 ml. of the same broth contained in 200 × 25 mm. cotton-stoppered tubes. Growth was followed at 37°. After vigorous agitation, 1·0 ml. samples were withdrawn periodically, their turbidity and pH measured, and DNA extracted and analysed.

In some experiments, 50 units of filter-sterilized dextranase were added/ml. broth; control tubes received boiled dextranase. Dextranase (Fitzgerald *et al.* 1968) was kindly supplied by Dr R. J. Fitzgerald (Dental Research Unit, V.A. Hospital, Miami, Florida, U.S.A.).

Analytical methods. The dry weight of intact plaques was evaluated using a semi-micro analytical balance, maximum variation ± 0·05 mg. DNA was extracted by a slight modification of the method of Ogur & Rosen (1950). Dried plaques were extracted for 30 min. in 0·5 N-$HClO_4$ at 70° (longer periods of extraction solubilized

no additional DNA). To the culture tubes from which the plaque-coated glass rods were removed, $HClO_4$ was added to 0·5 N and DNA was extracted by the same procedure. In the broth culture experiments, DNA was extracted by 0·5 N-$HClO_4$ from the material trapped on 0·45 μ pore diameter Millipore discs (Millipore Corporation, Bedford, Mass., U.S.A.) when samples of culture were filtered. DNA was analysed by the method of Burton (1956), using 2-deoxy-D-ribose standards (Sigma Chemical Co., St. Louis, Mo., U.S.A.) and expressed as deoxyribose. Turbidities of culture samples were assessed at 600 mμ with a Model 300 microsample spectrophotometer (Gilford Instrument Laboratories, Oberlin, Ohio, U.S.A.), 1 cm. light path. pH values were measured using a standard laboratory combination electrode and pH meter. In all experiments Gram stains of smears were made as a check of purity.

RESULTS

Growth of intact plaque in vitro. Growth of plaque on glass rods was linear, both on a dry weight and deoxyribose content basis (Fig. 1). Regression analysis of individual values of dry weight and deoxyribose shows that 98·3 and 96·7 % of the respective variations are accounted for by the linear growth model. The mean rate of plaque growth on the rods was 6·8 μg. dry weight/mm.^{2}/day.

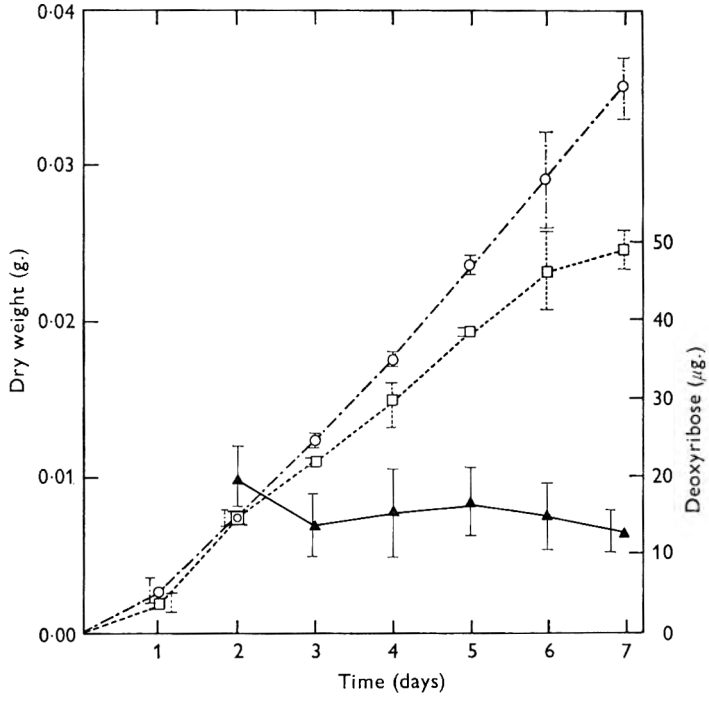

Fig. 1. Growth of intact plaque of SL-1 *in vitro*. Twenty-eight plaques were grown on glass rods in broth containing 5 % sucrose. At daily intervals all but four rods were transferred to tubes of fresh broth. The dry weight (○) and deoxyribose (□) content of the four plaques for each day were determined. Deoxyribose was determined in the spent broth tubes (▲) used for each day's plaque growth after removal of the plaque-coated glass rod. Mean values ± S.D. are plotted.

The daily mean values of deoxyribose in the culture tubes, after the removal of the plaque-coated rods, were not different from each other except on day 2 ($p < 0.05$), when tested by analysis of variance. Possibly this difference was due to carry-over of non-adhering cells from the initial inoculum, since it was not seen on subsequent days.

Growth of cells suspended in broth culture. Linear rather than exponential growth of intact plaque was unexpected. To determine whether this character was due to geometrical limitation imposed by the glass rod supporting the plaque or whether it was due to some other factor, growth without the glass rod was studied, employing various sugars in the broth.

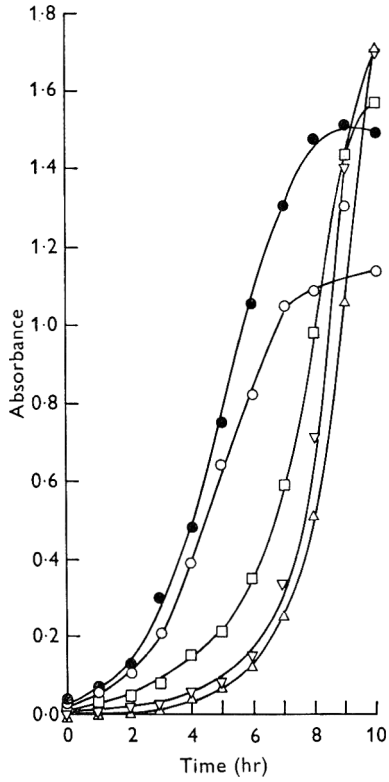

Fig. 2

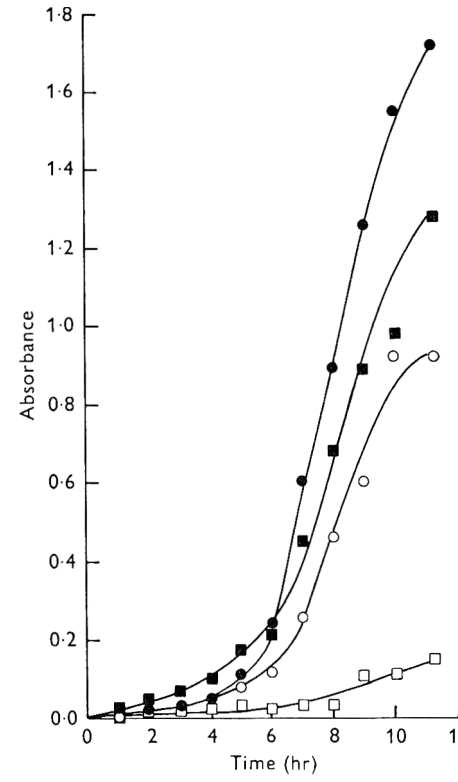

Fig. 3

Fig. 2. Plot of growth of SL-1 in broth culture. Medium contained either 0·01 M-sucrose (●), 0·02 M-sucrose (○), 0·02 M-fructose (□), 0·02 M-glucose (△), or 0·01 M-glucose and 0·01 M-fructose (▽).

Fig. 3. Effect of dextranase upon the growth of SL-1 or E-49 in broth containing 0·02 M-sucrose. Dextranase was added to the broth with the inocula as follows: SL-1 without dextranase (○), with dextranase (●); E-49 without dextranase (□), with dextranase (■).

Gram stained smears of cultures from sucrose broth revealed clumping of cocci while smears of cultures from other sugars revealed discretely separated chains of cocci. Thus it was necessary to show that absorbance was a valid index of microbial mass, irrespective of the nature of sugar employed and over the wide range of absorbance values observed. When SL-1 was grown in broth cultures containing either 0·02 M-sucrose, fructose, glucose or 0·01 M-fructose + 0·01 M-glucose, the absorbance of the

culture at 600 mμ was highly correlated with the deoxyribose content of the culture ($r = 0{\cdot}982$), over the range 0 to 1·8 absorbance and 0·2 to 5 μg. deoxyribose/ml. Accordingly, turbidity was used as an index of growth.

Growth of SL-1 in broth containing 0·02 M-fructose, glucose, or fructose + glucose was exponential and gave high growth yields. Growth in sucrose, however, deviated from exponential behaviour (Fig. 2). Growth in 0·01 M-sucrose tended to be more rapid and gave higher yield than in 0·02 M-sucrose. Both appeared linear over most of

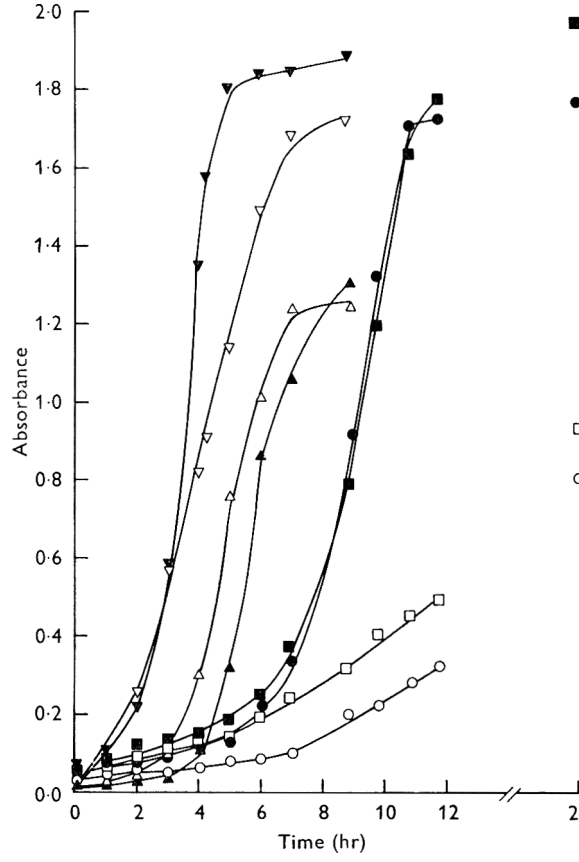

Fig. 4. Growth of dextran-forming strains SL-1 or E-49 and non dextran-forming strains JR8LG or 4M4 in broths containing various sugars. All sugars were at a concentration of 0·02 M. SL-1 grown in broth containing glucose + fructose (●) or sucrose (○); E-49 grown in broth containing glucose + fructose (■) or sucrose (□); 4M4 grown in broth containing glucose + fructose (▲) or sucrose (△); JR8LG grown in broth containing glucose + fructose (▼) or sucrose (▽).

their range. The pH always fell more slowly in the sucrose-containing tubes than in others. In other experiments, cultures with less sucrose (e.g. < 0·005 M) showed a tendency to exponential growth while more sucrose (e.g. > 0·02 M) also showed growth restriction. Comparable concentrations of other sugars did not reveal any deviation from exponential growth or unexpected limitation of growth yield.

Throughout this paper linear plots will be presented. In all cases semilogarithmic plots revealed non-exponential growth whenever sucrose was in the culture medium.

Formation of dextrans by plaque-forming caries-conducive streptococci depends on sucrose (Donahue, Kestenbaum & King, 1966; Gibbons *et al.* 1966; Wood & Critchely, 1966; Dahlquist, Krasse, Olsson & Gardell, 1967; Gibbons & Banghart, 1967; Gibbons & Banghart, 1968; Guggenheim & Schroeder, 1967; Fitzgerald & Jordan, 1968). Formation of extracellular dextran might therefore restrict the growth of plaque-forming streptococci.

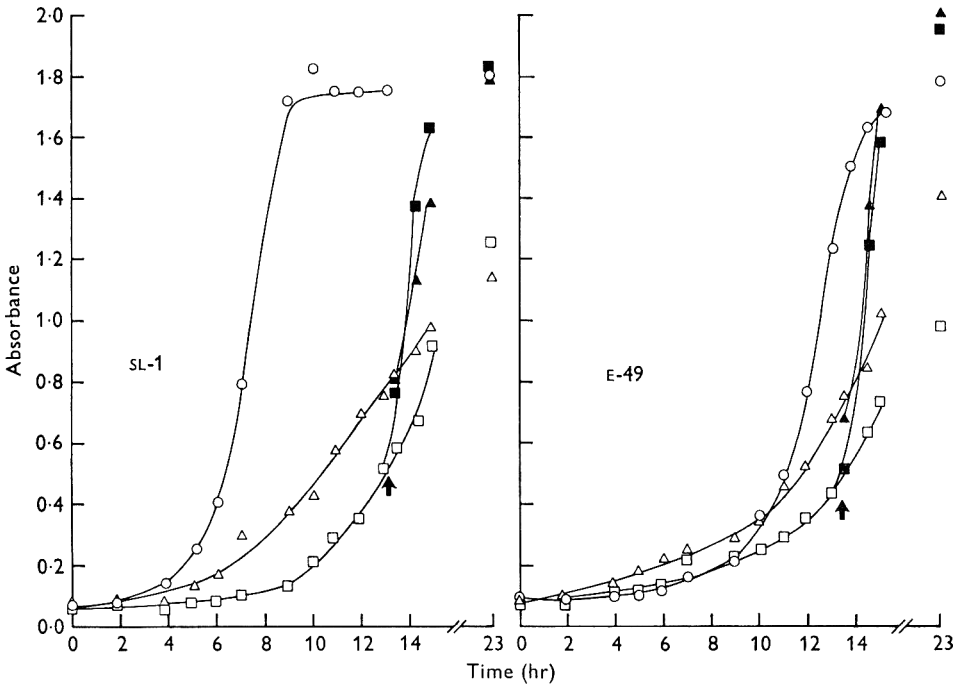

Fig. 5. Growth of either SL-1 or E-49 before and after dextranase addition to broths containing various sugars. Arrows indicate the point of addition of either dextranase (closed symbols) or boiled dextranase (open symbols) to broths containing sucrose. The following symbols are employed: glucose+fructose (○); glucose, fructose, sucrose and boiled dextranase (□); glucose, fructose, sucrose and active dextranase (■); sucrose and boiled dextranase (△); or sucrose and active dextranase (▲). All sugars were initially present at 0·02 M. At 15 hr the culture of SL-1 in broth containing glucose, fructose+sucrose (□) was shown by gramme staining to be impure. In all other growth experiments reported here cultures were judged by this criterion to be uncontaminated.

To test this hypothesis, SL-1 or E-49 was grown in sucrose-containing broth to which dextranase preparation was added simultaneously. Growth was restricted when inactive dextranase was present (Fig. 3); both SL-1 and E-49 grew poorly, with SL-1 the better of the two. Growth of both strains was exponential with dextranase preparation and growth yields were higher. We later learned that the dextranase preparation also had substantial invertase activity (R. J. Fitzgerald, personal communication), so exponential growth in sucrose-containing broth might either be the result of a true dextranase effect or the result of presenting the cells with glucose and fructose produced by invertase activity.

To resolve this ambiguity two types of experiments were carried out. In the first, non dextran-forming strains 4M4 and JR8LG were compared to dextran-forming

strains SL-1 and E-49 in either sucrose-containing broth or in broth containing equimolar amounts of glucose + fructose. Sucrose restricted growth only for the dextran-forming strains; the other strains grew exponentially (Fig. 4). All strains grew exponentially with glucose + fructose.

The second experiment took advantage of the observation that the dextran-forming system is constitutive: this enzyme should function as long as sucrose is present, irrespective of the presence of other sugars. Strains SL-1 and E-49 were grown in broths containing (*a*) glucose + fructose, (*b*) sucrose, or (*c*) a mixture of all three sugars. Dextranase was added later during growth. Growth was restricted with the combination of sucrose, glucose and fructose as well as sucrose alone (Fig. 5). Thus the presence of substantial amounts of glucose and fructose, which would be produced by invertase activity, did not permit exponential growth so long as sucrose was present. When the sucrose-containing cultures were divided and dextranase preparation was added to half of each slowly growing culture, a period of rapid growth followed. Addition of boiled dextranase preparation to the remaining half of each culture produced no apparent increase of growth rate. Gram stained smears of cultures containing sucrose revealed clumps of cocci. After dextranase addition streptococci were seen in discrete chains.

DISCUSSION

Plaque-forming streptococcus SL-1 adhering to a solid surface *in vitro* grew in a linear fashion. The proportion of DNA to the total dry mass of the plaque did not change, indicating that intact *in vitro* plaque probably does not change its composition grossly with time. The experiment did not test directly whether intact plaque growth within a 24 hr period was linear or exponential. The restricted growth of plaque-forming streptococci in sucrose-containing broth without the presence of a glass rod, strongly indicates that growth on a solid surface during any part of a 24 hr period is not exponential.

Growth of intact plaque in complex bacterial growth medium was slow: it required about 1·9 days for the dry mass of plaque to double. This contradicts the suggestion of Stralfors (1950) that plaque grows rapidly in the oral fluids and indicates that Gibbons's (1964) estimation of the rate of proliferation of bacterial plaques *in vivo* may be too high. In the medium employed in the present study the conditions for streptococcal proliferation are probably much better than those existing in the mouth at the surface of the tooth. Therefore, streptococcal plaque growth *in vivo* should be even slower than the rate *in vitro*. Unfortunately, direct determinations *in vivo* of the rate of plaque growth in bacterially well-defined plaques have not been reported.

Sucrose led to linear growth of plaque-forming strains SL-1 and E-49 in broth culture. Hence, the linear growth observed for intact plaques was not due to geometrical limitations imposed by the solid surface on which plaque was growing. Formation of extracellular dextrans by SL-1 and E-49 in the presence of sucrose appears to limit their growth because (1) non plaque-forming strains JR8LG and 4M4, which do not form dextrans from sucrose, grew exponentially with sucrose, (2) plaque-forming strains grew rapidly with sucrose after addition of dextranase. The invertase activity of the dextranase preparation was not relevant.

Growth restriction may result from a diffusion barrier imposed by extracellular dextran. However, linear growth cannot be explained by postulating a diffusion

barrier surrounding individual cocci, since binary fission of such cells should produce exponential growth kinetics, although with a slower generation time. Because growth in the presence of sucrose is linear, the data suggest that the diffusion barrier exists around clumps of cells, i.e. microcolonies.

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The Problem of the Similarity of Objects in Numerical Taxonomy

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SUMMARY

The existing definitions of coefficients of association in numerical taxonomy are not sufficiently well based theoretically. The present paper aims to make more objective the definition of the coefficient of association. It proceeds from the fact that the main source of difficulty is the third value (NC) used in scoring characters, and eliminates it. An improved coefficient of association between objects is then defined as the mean value of the coefficients of association of the individual pairs of forms of objects that are constructed by the method proposed below.

For interpretation we use the example in Table A-10 from Sokal & Sneath (1963 p. 301) which we reproduce here as Table 1.

For the cases of those objects (OTU) which are described by characters with only two values '+' and '−' (in Table 1 objects B, D, F) we shall accept the definition of the coefficient of association of Sokal & Michener (1958), the Simple Matching Coefficient:

$$S(M, N) = m/n, \tag{1}$$

where m is the number of characters, both positive and negative, in which objects M and N agree, while n is the total number of characters (see Table 2). This definition has two advantages. Firstly it interprets the coefficient of association as a probability that the objects agree on a randomly chosen character of the set under study. This definition, unlike others, is thus not based on a merely empirical approach, but proceeds from a theoretical basis of probability theory. Secondly, the definition presupposes the same value for the positive or negative form of the characters. This is a conception which consistently respects Adanson's principle of equal weight for every character and which is also currently used in logic. Just as we cannot quote any objective criterion that would generally distinguish the importance of two randomly chosen characters, so we cannot quote a criterion that would generally distinguish whether the positive state of character, (+Q) or its negation (−Q) is of greater importance. We should thus try to apply Adanson's principle so that it does not matter which state we score '+' or '−'.

We also try to respect this definition in those cases where the characters have, besides the value '+' and '−', yet a third value 'NC', expressing indefinite value. We assume that 'NC' denotes those cases where +Q or its logical negation −Q cannot be assigned unambiguously. This means that our further consideration refers only to those cases where the value 'NC' means that the object must in theory have the

character +Q or −Q, but where the assignment of +Q or −Q cannot for some reason be decided. We thus consider the value 'NC' in the sense of 'missing data' (*cf.* Sokal & Sneath, 1963 pp. 162–163) and not in the sense of 'missing characters' or 'missing organs'. The value 'NC' in the last two cases can be eliminated by the syntactic method (see Jičín, Pilous & Vašíček, 1969).

Table 1. *The examples of OTU's and characters from Sokal & Sneath (1963) used for illustration*

	Objects					
Characters	A	B	C	D	E	F
1	−	+	−	+	−	+
2	−	+	−	+	−	+
3	+	−	+	−	+	−
4	+	−	+	−	+	+
5	NC	+	−	+	NC	−
6	NC	+	NC	+	−	−
7	+	−	+	−	+	+
8	−	+	−	+	−	+
9	−	+	−	+	−	−
10	+	−	+	−	+	−

If we do not know whether 'object *M* has the character +Q' or 'object *M* has the character −Q' (the latter is equivalent to 'object *M* has not the character +Q') we simply conclude that 'object *M* has the character +Q or −Q'. We thus interpret the value 'NC' of the character Q as an alternative to the values '+' and '−' in the description of object *M*. If there is only this one character Q with the value 'NC' we treat it as the description of two hypothetical forms, M_1 and M_2, of the object. These two forms agree in all characters except Q. We therefore assume that object *M* may at least in theory occur either in the form M_1 (with +Q) or in the form M_2 (with −Q). We can illustrate this by object *C* (from Table 1), whose character 6 has the value 'NC', as follows:

	Objects			Objects	
Characters	C_1	C_2	Characters	C_1	C_2
1	−	−	6	+	−
2	−	−	7	+	+
3	+	+	8	−	−
4	+	+	9	−	−
5	−	−	10	+	+

Generally, this means that the value 'NC' can be excluded from consideration in that an object with *p* characters scored 'NC' is now described by 2^p columns, expressing all the forms the object could adopt.

Let us now establish the coefficient of association between object *C* and, let us say, object *F* (from Table 1). If we determine the values of the coefficient of association, we find for *F* and C_1 that $S(F, C_1) = 0{\cdot}5$. For *F* and C_2, $S(F,C_2) = 0{\cdot}6$. We assume that object *C* adopts both forms C_1 and C_2 with the same probability (we know nothing about the likelihood that character 6 in object *C* is positive or negative and cannot prefer one over the other). We must therefore take the resultant coefficient of associa-

tion as the mean value of $S(F,C_1)$ and $S(F,C_2)$. The new coefficient of association is then

$$S(F,C) = \frac{0{\cdot}6+0{\cdot}5}{2} = 0{\cdot}55.$$

Generally, this means that if object M has p characters with the value 'NC' (described by 2^p columns, so that it may appear in the forms $M_1, M_2, \ldots, M_{2^p}$) and if object N has q characters with the value NC (occurring in 2^q forms, $N_1, N_2, \ldots, N_{2^q}$), our suggested coefficient of association $S(M,N)$ is given by the mean value of all the coefficients of association $S(M_i, N_j)$, where M_i is any one of the forms $M_1, M_2, \ldots, M_{2^p}$, and N_j any one of the forms $N_1, N_2, \ldots, N_{2^q}$. Hence:

$$S(M,N) = \frac{\sum_{i=1}^{2^p}\sum_{j=1}^{2^q} S(M_i,N_j)}{2^{p+q}}. \tag{2}$$

We can simplify equation (2) to the form

$$S(M,N) = \frac{m+r/2}{n}, \tag{3}$$

where m is the number of agreements between the '+' and '−' values of objects M and N, r the sum of agreements and disagreements involving the 'NC' values, and n the total number of characters (see Table 2). This definition permits us to calculate the coefficient of association without having to construct all the 2^p and 2^q alternatives. Hence it is of importance especially for those cases where there are many 'NC' values.

Table 2. *The possible combinations of character states on comparing objects M and N*

	Object M		
Object N	(+)	(−)	NC
+	$n_{+,+}$	$n_{+,-}$	$n_{+,\mathrm{NC}}$
−	$n_{-,+}$	$n_{-,-}$	$n_{-,\mathrm{NC}}$
NC	$n_{\mathrm{NC},+}$	$n_{\mathrm{NC},-}$	$n_{\mathrm{NC},\mathrm{NC}}$

$n_{+,+}$ = the number of character in which both objects are positive (*cf.* Sokal & Sneath, 1963 p. 126), and similarly for the other symbols; $m = n_{+,+}+n_{-,-}$; $r = n_{\mathrm{NC},+}+n_{\mathrm{NC},-}+n_{+,\mathrm{NC}}+n_{-,\mathrm{NC}}+n_{\mathrm{NC},\mathrm{NC}}$; $n = r+n_{+,+}+n_{+,-}+n_{-,+}+n_{-,-}$.

In Table 3 are presented all the similarities for all objects and their hypothetical forms, respectively, while Table 4 shows the mutual similarity of the objects using equation 3.

This Table can be compared with the results of other formulae for coefficients of association, as given by Sokal & Sneath (1963, p. 304) for these examples (Table 5).

This comparison shows that the new definition expresses more adequately the relationship between the pairs of objects. For example, of the previous ones, only the coefficient S^{RR} distinguishes between $S(B,D)$ and $S(C,E)$, although in the first case identical objects are involved, while in the second case evidently not. It also makes a finer distinction in the similarities of the individual pairs (thus according to the

existing formulae there are only 4 or 5 different S values, but we obtain 7 different values according to the new formula). The fundamental difference resides, of course, in the fact that the new definition employs the value 'NC'. Let us consider the extreme case where for the objects M, N and the characters 1 to 10 all values are 'NC'. This means that we do not know anything at all about the assignment of any character

Table 3. *The similarity values between all objects in Table 1 and the hypothetical forms of them, using equation 1*

	Objects										
Objects	A_1	A_2	A_3	A_4	B	C_1	C_2	D	E_1	E_2	F
A_1	—	—	—	—	0·2	0·9	0·8	0·2	0·9	0·8	0·4
A_2	—	—	—	—	0·1	0·8	0·9	0·1	1·0	0·9	0·5
A_3	—	—	—	—	0·1	1·0	0·9	0·1	0·8	0·9	0·5
A_4	—	—	—	—	0·0	0·9	1·0	0·0	0·9	1·0	0·6
B	—	—	—	—	1·0	0·1	0·0	1·0	0·1	0·0	0·4
C_1	—	—	—	—	—	—	—	0·1	0·8	0·9	0·5
C_2	—	—	—	—	—	—	—	0·0	0·9	1·0	0·6
D	—	—	—	—	—	—	—	1·0	0·1	0·0	0·4
E_1	—	—	—	—	—	—	—	—	—	—	0·5
E_2	—	—	—	—	—	—	—	—	—	—	0·6
F	—	—	—	—	—	—	—	—	—	—	1·0

Table 4. *The similarity values between the objects in Table 1 using equation 3*

	Objects					
Objects	A	B	C	D	E	F
A	0·9	0·1	0·9	0·1	0·9	0·5
B	—	1·0	0·05	1·0	0·05	0·4
C	—	—	0·95	0·05	0·9	0·55
D	—	—	—	1·0	0·05	0·4
E	—	—	—	—	0·95	0·55
F	—	—	—	—	—	1·0

Table 5. *Values of the other coefficients described by Sokal & Sneath (1963) for all pairs of the objects A-F*

Coefficient	A,B	A,C	A,D	A,E	A,F	B,C	B,D	B,E	B,F	C,D	C,E	C,F	D,E	D,F	E,F
SM	0·0	1·0	0·0	1·0	0·5	0·0	1·0	0·0	0·4	0·0	1·0	0·56	0·0	0·4	0·56
J	0·0	1·0	0·0	1·0	0·33	0·0	1·0	0·0	0·25	0·0	1·0	0·33	0·0	0·25	0·33
RR	0·0	0·5	0·0	0·5	0·25	0·0	0·6	0·0	0·2	0·0	0·5	0·22	0·0	0·2	0·22
D	0·0	1·0	0·0	1·0	0·5	0·0	1·0	0·0	0·4	0·0	1·0	0·5	0·0	0·4	0·5
un_1	0·0	1·0	0·0	1·0	0·67	0·0	1·0	0·0	0·57	0·0	1·0	0·71	0·0	0·57	0·71
un_2	0·0	1·0	0·0	1·0	0·2	0·0	1·0	0·0	0·14	0·0	1·0	0·2	0·0	0·14	0·2
RT	0·0	1·0	0·0	1·0	0·33	0·0	1·0	0·0	0·25	0·0	1·0	0·38	0·0	0·25	0·38
$K1$	0·0	∞	0·0	∞	0·5	0·0	∞	0·0	0·33	0·0	∞	0·5	0·0	0·33	0·5
un_3	0·0	∞	0·0	∞	1·0	0·0	∞	0·0	0·67	0·0	∞	1·25	0·0	0·67	1·25
$K2$	0·0	1·0	0·0	1·0	0·5	0·0	1·0	0·0	0·42	0·0	1·0	0·5	0·0	0·42	0·5
un_4	0·0	1·0	0·0	1·0	0·5	0·0	1·0	0·0	0·42	0·0	1·0	0·55	0·0	0·42	0·55
0	0·0	1·0	0·0	1·0	0·5	0·0	1·0	0·0	0·41	0·0	1·0	0·5	0·0	0·41	0·5
un_5	0·0	1·0	0·0	1·0	0·25	0·0	1·0	0·0	0·17	0·0	1·0	0·3	0·0	0·17	0·3
H	−1·0	1·0	−1·0	1·0	0·0	−1·0	1·0	−1·0	−0·2	−1·0	1·0	0·11	−1·0	−0·2	0·11
Y	−1·0	1·0	−1·0	1·0	0·0	−1·0	1·0	−1·0	−0·33	−1·0	1·0	0·2	−1·0	−0·33	0·2
ϕ	−1·0	1·0	−1·0	1·0	0·0	−1·0	1·0	−1·0	−0·17	−1·0	1·0	0·1	−1·0	−0·17	0·1

state to the two objects. With most of the existing definitions, $S\ (M,N) = 0$. This result, however, is not logical, because the finding of complete dissimilarity requires a decision on the assignment of character states to the objects. If we do not know anything about this we ought to assume for each character that the objects M and N may agree or disagree with the same probability and that, therefore, $S\ (M,N)$ must equal $\frac{1}{2}$. This is the actual result obtained by the new definition. We may also note, though this ordinarily has little practical importance, that comparison of an object with itself yields a value less than 1 if it has characters scored 'NC', and this can be seen in Table 4. This is an expression of the uncertainty about the states of these characters.

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Generation Times of Bacteria: Real and Artificial Distributions

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Under the subheading 'Real and artificial distributions' I gave in a previous paper (Powell, 1964) a brief sketch of the relationships between the various distributions which come to be considered in any discussion of the pattern of bacterial generation times. The present note is an elaboration of this sketch, prompted by Painter & Marr's (1967) imputation of error in my treatment. It will be seen that the supposed discrepancies arise from nothing more than a misunderstanding of symbols and terminology.

If individual generation times in a growing culture were independent of one another there would be no problem; in fact growth is affected by internal correlations. Here I shall deal with the only manageable case which is at all realistic: significant correlation between generation times of mother and daughter organisms, other correlations being irrelevant or quantitatively negligible. It will be convenient to think of the culture as maintained in a steady state in a chemostat (exactly the same relationships are obtained by considering exponential growth in batch culture, but the argument is more tedious). Those organisms which remain in the culture until their termination, i.e. those whose generation times compose the carrier distribution, I shall call 'successful' organisms.

The artificial distributions

If we have a hypothetical mechanism from which precise inferences to the pattern of generation times (τ) can be made, we are naturally led to construct, for comparison with experiment, a frequency function (say $f(\tau)$) applicable in some sense to all organisms whether considered as mothers or daughters; and a bivariate function (say $h(\xi, \tau)$) for the joint distribution of mother (ξ) and daughter (τ) generation times, with the property that its border distributions are alike of the form $f(\tau)$:

$$\int_0^\infty h(\xi, \tau)\, d\xi = f(\tau); \quad \int_0^\infty h(\xi, \tau)\, d\tau = f(\xi).$$

This construction implies, then, the assumption that mothers and daughters of the same generation time have the same frequency. This is not true of actual growth and so it must be admitted at once that $f(\tau)$ and $h(\xi, \tau)$ are artificial in the sense that they are not directly represented in the growing culture. If in the chemostat the population were maintained constant by selecting organisms for removal in a manner independent of their generation times, $f(\tau)$ and $h(\xi, \tau)$ would be realized; in fact, the probability that an organism of generation time τ is successful is $e^{-\nu\tau}$ (where ν is the growth rate) and so the probability is greater the smaller is τ. Suppose that during a short interval in the life of the culture the newly-formed organisms had the distribution $f(\tau)$; of these the successful organisms would have predominantly short generation times. Because of the association between successive generation times, the progeny of the successful organisms would not (in general) have the distribution $f(\tau)$.

Thus the composition of the population would alter. If we write $\mathscr{P}(\tau)$ for the 'population distribution', i.e. the generation time distribution of all those organisms, whether successful or not, whose inception occurs in the culture vessel, the carrier distribution ($\mathscr{C}(\tau)$, corresponding to the successful organisms) must be such that both $\mathscr{C}$ and $\mathscr{P}$ constantly reproduce themselves:

$$\mathscr{C}(\tau) \equiv 2e^{-\nu\tau}\mathscr{P}(\tau), \tag{1}$$

the factor 2 arising because on average only one of each pair of sisters is successful. The distribution $f(\tau)$ cannot reproduce itself under the actual circumstances of growth, but in fact it determines $\mathscr{P}(\tau)$ through the integral equation (Powell, 1964):

$$\mathscr{P}(\tau) = 2\int_0 e^{-\nu\xi}\frac{h(\xi, \tau)}{f(\xi)}\mathscr{P}(\xi)\,d\xi. \tag{2}$$

This equation at the same time determines the growth rate because its only solution is $\mathscr{P}(\tau) \equiv 0$ unless ν is such that 2 is a characteristic number of the kernel

$$e^{-\nu\xi}h(\xi, \tau)/f(\xi).$$

Equation (2) may be reached by an approach rather different from my earlier one. Given a successful organism of generation time ξ, we can say that the probability of some one of its daughters having generation time τ is unaffected by the circumstances of growth; that is, the distribution of τ for any fixed ξ is unaffected. To determine $\mathscr{P}$ and $\mathscr{C}$ we therefore seek a variable multiplier $\lambda(\xi)$ such that

$$\mathscr{C}(\xi) = \lambda(\xi)f(\xi) = \lambda(\xi)\int_0^\infty h(\xi, \tau)\,d\tau,$$

$$\mathscr{P}(\tau) = \int_0^\infty \lambda(\xi)\,h(\xi, \tau)\,d\xi,$$

together with the condition (1) imposed on $\mathscr{C}$ and $\mathscr{P}$ by the washing out of organisms from the culture.

Then

$$\lambda(\xi) \equiv \mathscr{C}(\xi)/f(\xi) = 2e^{-\nu\xi}\mathscr{P}(\xi)/f(\xi)$$

and

$$\mathscr{P}(\tau) = 2\int_0^\infty e^{-\nu\xi}\frac{h(\xi, \tau)}{f(\xi)}\mathscr{P}(\xi)d\xi$$

as before.

The construction of the frequency functions f and h has some slight historical sanction and mathematically is usually relatively easy to do. In experimental work, care has to be taken to obtain a sample of generation times which is unbiased with respect to f or h (Powell, 1964); an attempt to sample for $\mathscr{C}$ or $\mathscr{P}$ needs equal care, however, and for a given sample size is more time-consuming.

It is the function $f(\tau)$ which I call 'the generation time distribution' *tout court*.

The real distributions

If it were conveniently possible to measure the generation times associated with a random selection of fissions occurring in a steady-state continuous culture, they would provide a sample from the realized joint distribution of mother and daughter generation times, say $\mathscr{H}(\xi, \tau)$, with borders $\mathscr{C}(\xi)$ (corresponding to mothers) and $\mathscr{P}(\tau)$ (corresponding to daughters).

We now approach from the opposite direction the relation between f and h on the one hand and $\mathscr{C}$, $\mathscr{P}$ and $\mathscr{H}$ on the other. We ask if it is possible to construct from $\mathscr{H}(\xi, \tau)$ an associated bivariate distribution whose borders are alike. As before, we note that for any fixed ξ, the distribution of τ is not altered by the circumstances of growth, and in fact we have for every ξ

$$\frac{h(\xi, \tau)}{f(\xi)} \equiv \frac{\mathscr{H}(\xi,\tau)}{\mathscr{C}(\xi)}, \tag{3}$$

identically in τ; each member of (3) is the frequency function of the τ associated with a particular ξ. We therefore seek a variable multiplier $\kappa(\xi)$ such that

$$\kappa(\xi) \int_0^\infty \mathscr{H}(\xi, \tau)\, d\tau = \kappa(\xi)\, \mathscr{C}(\xi) = f_a(\xi), \text{ say:}$$

$$\int_0^\infty \kappa(\xi)\, \mathscr{H}(\xi, \tau)\, d\xi = f_a(\tau)$$

with the further condition

$$\int_0^\infty f_a(\tau) d\tau = 1.$$

That is, we try to satisfy the integral equation

$$\kappa(\tau)\, \mathscr{C}(\tau) = \int_0^\infty \kappa(\xi)\, \mathscr{H}(\xi, \tau)\, d\xi$$

or

$$f_a(\tau) = \int_0^\infty f_a(\xi) \frac{\mathscr{H}(\xi, \tau)}{\mathscr{C}(\xi)}\, d\xi. \tag{4}$$

It is easy to verify that the Fredholm determinant of the kernel in (4) vanishes in virtue of the relation

$$\mathscr{C}(\xi) = \int_0^\infty \mathscr{H}(\xi, \tau)\, d\tau$$

(see, e.g. Aitken, 1942), and so (4) always has a non-trivial solution (see, e.g. Smithies, 1958). We readily identify this solution by substituting (3) into (4):

$$f_a(\tau) \equiv \int_0^\infty f_a(\xi) \frac{h(\xi, \tau)}{f(\xi)}\, d\xi$$

which is satisfied by

$$f_a(\tau) \equiv f(\tau)$$

because

$$\int_0^\infty h(\xi, \tau) d\xi = f(\tau).$$

Conversely, if in (2) we substitute (3) we obtain

$$\mathscr{P}(\tau) = \int_0^\infty \mathscr{H}(\xi, \tau)\, d\xi$$

which is also true.

The frequency functions f and h can thus be completely reconciled with $\mathscr{C}$, $\mathscr{P}$ and $\mathscr{H}$.

Painter and Marr's analysis

Painter & Marr (1967) showed that there is associated with a culture in steady growth a frequency function of generation times (say, temporarily, $f_{pm}(\tau)$) which does reproduce itself under the actual circumstances of growth, that

$$\mathscr{C}(\tau) = 2e^{-\nu\tau}f_{pm}(\tau)$$

and that

$$2\int_0^\infty e^{-\nu\tau}f_{pm}(\tau) = 1.$$

They show further that the mean of this distribution is greater than $(\log 2)/\nu$. Their reasoning is so far correct. But unfortunately they use the symbol $f(\tau)$ for the distribution in question, and then identify it with the $f(\tau)$ discussed above and used by me elsewhere in cases in which mother and daughter generation times are not independent. They infer that $f(\tau)$ in the latter sense cannot be symmetrical and that its mean cannot be $(\log 2)/\nu$ (as it is under the restricted Koch & Schaechter (1962) hypothesis; Powell, 1964).

It should now be clear that Painter & Marr's distribution is identical with $\mathscr{P}(\tau)$, which I have called the 'population distribution' and which is to be carefully distinguished from $f(\tau)$. There is, then, no real discrepancy. $\mathscr{P}$ relates to all the organisms in the culture, past, present and to come. The distribution for those organisms extant at any one time is

$$\mathscr{I}(\tau) = 2(1-e^{-\nu\tau})\,\mathscr{P}(\tau).$$

When mother and daughter generation times are not independent, '$\mathscr{P}(\tau)$ replaces $f(\tau)$ in expressions for the carrier and age distributions and for $\mathscr{I}(\tau)$' (Powell, 1964).

Conclusion

The difference between $\mathscr{P}(\tau)$ and $f(\tau)$ is usually not great, and as the extreme examples in Powell (1956) show, an error of less than 3 % is incurred if ν is calculated from the τ as if they were independent. Although I have written of $f(\tau)$ as 'artificial', an effective hypothesis about the distribution of τ leads directly to it; yet there is something adventitious about $\mathscr{P}(\tau)$ in that it comes to differ from $f(\tau)$ only as a secondary result of multiplicative growth.

I think therefore that both distributions still have a place in the theory of the pattern of generation times. But their precise meanings must be kept clearly in mind.

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