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Obituary Notice

RALPH ST JOHN-BROOKS, 1884–1963

The death on 27 April 1963 of Ralph Terence St John-Brooks took from us a most friendly and likeable man, and recalled to many the exceptional services he had rendered to the development of microbiology, in particular in the foundation and early evolution of the Society for General Microbiology.

Ralph T. St John-Brooks was born on 27 October 1884, the third and youngest son of Professor Henry St John-Brooks, University Anatomist in the University of Dublin. On leaving Erasmus Smith's School, Dublin, Ralph St John-Brooks went to Trinity College, Dublin, where he graduated with first-class honours in Natural Science in 1904. He won the Haughton Medal and Prize in Medicine and Surgery in 1909 and the qualifications of M.B., B.Ch. and B.A.O., in 1910 D.P.H. and in 1911 D.T.M. & H. (Cantab.). On appointment as British Medical Association Research Scholar he spent two years in the West Indies (1911–13) as special sanitary investigator for the Government of the Windward and Leeward Islands. In 1914 he was appointed Secretary of the Commission for Plague Investigation in India and worked at the Lister Institute of Preventive Medicine until he resigned to join the Royal Army Medical Corps in 1915. He was Specialist in Bacteriology, with rank of Captain, at the County of London War Hospital, Epsom, from 1915 to 1919, and at the Royal Army Medical College, Millbank, 1919–20. In 1920 he was invited by Sir Charles Martin, F.R.S., to become Curator of the National Collection of Type Cultures at the Lister Institute. The Collection was formed mainly from strains collected by members of the Institute's staff, and many came from the Diagnostic Laboratory of the Institute under John Ledingham, the Chief Bacteriologist. The Collection was sponsored jointly by the Lister Institute (which supplied laboratory space and media) and the Medical Research Committee (later Council) which paid salaries. St John-Brooks remained in this post until his retirement in 1946.

Many of the older generation of microbiologists will remember the early days of the National Collection of Type Cultures in the Lister Institute in Chelsea Bridge Road, where St John-Brooks and his chief assistant Miss Mabel Rhodes (who was with him from the beginning until he retired) built up this collection from very small beginnings. The Lister Institute already had a collection of 100–200 cultures and St John-Brooks added to these by collecting cultures from personal acquaintances whom he visited in his off-duty hours while still in the Royal Army Medical Corps. At first only bacteria were collected; later some fungi were added, though the Collection was at first restricted to organisms of medical interest. Eventually the range of organisms was expanded to include bacteria, yeasts and fungi of medical, veterinary and economic importance. The Collection was evidently not run as an economically self-supporting venture, for initially a charge of only one shilling per culture was made; this was later increased to one shilling and six pence to cover the cost of postage!

During his Curatorship of the National Collection St John-Brooks made his major contribution to science. He was largely responsible, with R. E. Buchanan and

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R. S. Breed, for developing the International Code of Bacteriological Nomenclature (eventually approved in 1947 at Copenhagen) and he was certainly responsible for the name of his beloved Collection appearing as a footnote to the Code, as one of the two official Collections.

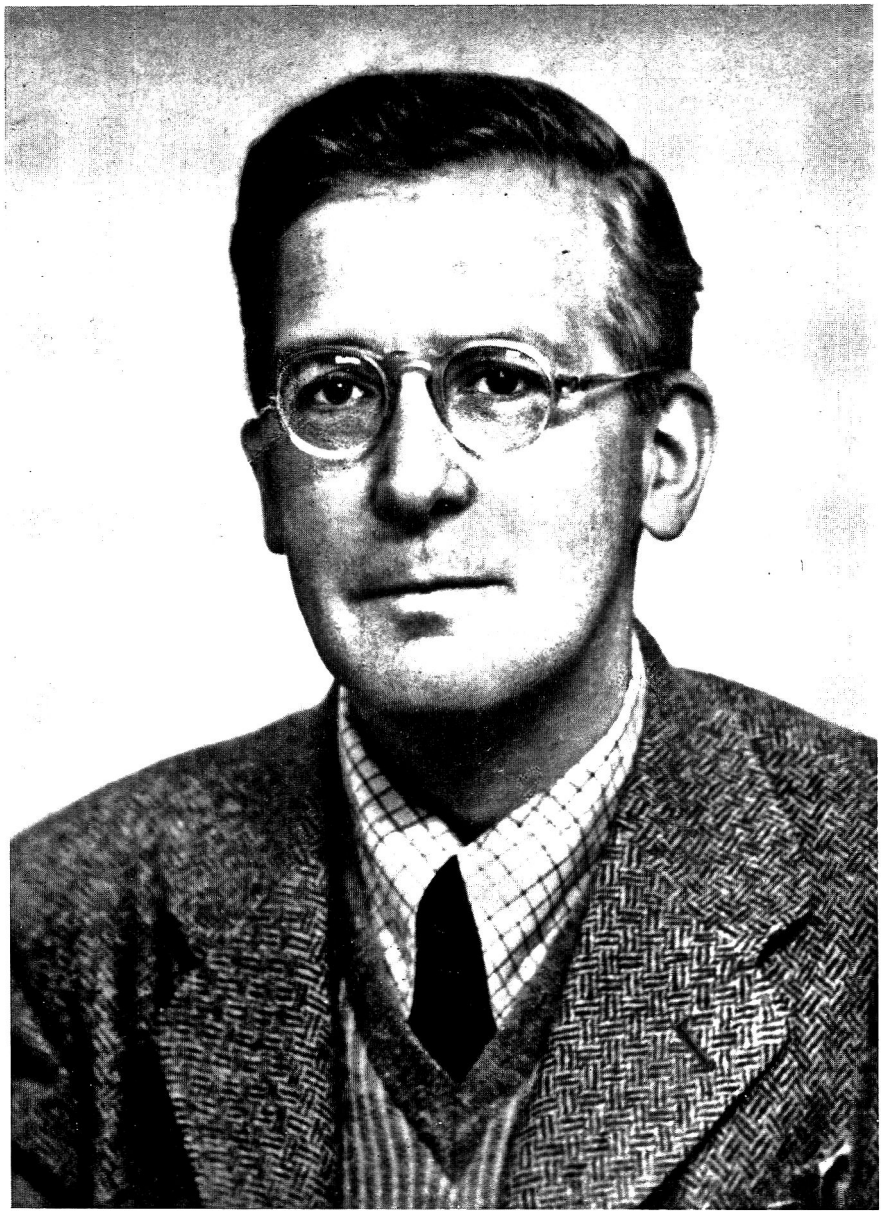
In 1934, on a visit to London, A. Sordelli showed a method he had developed for preserving cultures in a vacuum over phosphorus pentoxide; the principle had been used before, but the simplicity of Sordelli's method appealed to St John-Brooks who, from that time onward, started to dry two ampoules of each culture in his Collection. This was a long task but his foresight was rewarded when the Collection was hastily moved, lock, stock and barrel, to the Lister Institute's farm laboratories at Elstree in September 1939. Miss Rhodes used to describe the scene of packing cases of cultures, and boxes of files dumped in one room, and in the middle of it all St John-Brooks, supported by his pedigree chow-chow Rex (from whom many cultures were 'isolated by the Curator himself') surveying the scene with equanimity and even pleasure. Although many of the Collection's tube cultures became contaminated, the Sordelli-dried tubes saved most of them for posterity and they survived the war and another removal of the Collection.

In 1923 St John-Brooks was seriously ill with tularaemia which he and some of his colleagues contracted as a laboratory infection. This infection may have contributed to his break-down with pulmonary tuberculosis in 1925. He spent two years (1925–27) in the Schatzalp Sanatorium in Davos, Switzerland. After his return to work another relapse necessitated a further period at Davos, from 1932 to 1934. Although the disease was arrested he never made a complete recovery. It says much for his courage and his devotion to his subject that he continued for nearly twenty more years to play an active and inspiring part in national and international committees concerned with the development of microbiology.

At the First International Congress for Microbiology in 1930 St John-Brooks was appointed Secretary to represent medical bacteriology on the new Nomenclature Committee. For the Second International Congress, which was held in London in 1936, he undertook the exacting duties of General Secretary and spent many months of hard work in making arrangments for what proved to be a most successful week of paper-reading conferences, international committee meetings, and social occasions. In 1939 he was President of Section I of the Third International Congress in New York. He was Secretary-General of the International Association of Microbiologists from 1936 to 1953 and even after his retirement as Curator of the National Collection of Type Cultures in 1946 he continued to be active in this aspect of his work, first from Lausanne and then from Washington, D.C., where he worked from the office of the American Type Culture Collection.

In 1950 St John-Brooks returned to his native Dublin, where he lived for the remainder of his life and where, in 1952, an honorary Sc.D. of Trinity College, Dublin, was conferred upon him.

About his private life it may be said that in 1912 he married Julia Margaret Gordon; they had two sons and a daughter. St John-Brooks had very wide interests, from natural history to languages; his quiet humour was a great joy to his friends and family. Though always busy professionally he was devoted to his family; in his later years he was openly proud of being the youngest grandfather at the Lister Institute. Keenly observant, sympathetic and unassuming, he combined



RALPH ST JOHN-BROOKS

(*Facing p.* 167)

wide experience with the human qualities so necessary for welding unobtrusively the outspoken comments of academic individualists into the harmonious agreement of committees. So much for the outline of the life of Ralph St John-Brooks. But, as foreshadowed in the first paragraph of this notice, St John-Brooks played a crucial part in the foundation of the Society for General Microbiology. Some of this wider history will be recorded below.

With L. A. Allen, St John-Brooks became the first joint Honorary Secretary of the Society, but on medical advice, St John-Brooks relinquished these duties at the Annual General Meeting in 1946. St John-Brooks served as a member of the Committee of the Society until he retired in October 1946. In recognition of his valuable services he was nominated by the Committee for election as the Society's first Honorary Member. As a further token of esteem his fellow members of present and past committees of the Society presented him with a silver salver engraved with all their signatures.

L. A. ALLEN

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Obituary Notice

LESLIE ALFRED ALLEN, 1903–1964

With the death of L. A. Allen, D.Sc., Ph.D., D.I.C., F.R.I.C., F.I.Biol., on 30 December 1964 microbiology in general suffered a grievous loss, and the Society for General Microbiology in particular has cause to regret the death of an ex-officer who assisted so much in its formative years.

Leslie Alfred Allen was born in 1903 and received his education at Portsmouth Grammar School and the University of Reading, graduating with First Class Honours in Chemistry in 1925. He did postgraduate work at various places, including Imperial College, London, in Northern Ireland, at the Hannah Dairy Research Institute in Scotland, and at the National Institute for Research in Dairying at Shinfield. At these various research stations his investigations ranged from animal feeding-stuffs to milk, his work becoming more and more microbiological, particularly in relation to dairy products. He was for some nine years lecturer at Reading University, being appointed to an Independent Lecturership in Agricultural Bacteriology from September 1930. From this post he resigned in September 1939, being appointed to the staff of the Water Pollution Research Laboratory of the Department of Scientific and Industrial Research, then at Watford. There, as Chief Microbiologist, he was engaged for the next fourteen years on various aspects of water bacteriology, covering water treatment, sewage and trade wastes.

In 1953 Allen left the Government's scientific service and joined the Research Department of Messrs Tate and Lyle to take charge of a unit investigating certain aspects of molasses utilization, particularly the production in this country of a food yeast. At this time I was closely associated with Allen in this work and can testify to the efficient operation of the team under his leadership. Much valuable work was done and any results emanating from the unit were invariably reliable. Any work which Allen undertook could be counted upon as having been performed most conscientiously, for he was absolutely meticulous.

He was the author of well over 60 papers and contributions containing much original work. In the last few years Allen had become a consultant in microbiology, particularly in relation to hygiene, water and effluents. He was External Examiner in biology to Brunel College, about to become a university.

The Society for General Microbiology owed more, in its inception, to Allen and to Ralph St John-Brooks than to any other men. In the dark days of the Second World War Allen was President of the (then) Society of Agricultural Bacteriologists and the idea of a Society to cover all the various disciplines of microbiology was in the air; but progress had to be cautious if the good will of all those in the different branches of the science was to be achieved. This Allen and St John-Brooks by their meetings and talks were able to do, so that when the time came to enlist the support and foster the necessary enthusiasm among a wide range of microbiologists, their careful ground-work yielded good fruit. In the earliest days of the Society, Allen and the present writer concurrently held various offices in three microbiological associations—the Society for General Microbiology, the Society for Applied Bac-

teriology (as it subsequently metamorphosed from the Society for Agricultural Bacteriology) and the Microbiology Group of the Society of Chemical Industry (formerly the Microbiology Panel of the Food Group of that Society). Our activities in these three organizations brought us almost daily into close association and I would claim to be as entitled as anyone to appreciate the steadfastness of Allen's character and his unremitting efforts on behalf of any cause he took up. Actually he had a somewhat shy manner but was natural and easy to work with: he would never exploit a situation at the expense of his colleagues or acquaintances for the purpose of his personal advancement. He was a kind man and is now sadly missed by his many friends who so admired his character. His widow and two daughters have our sympathy in their loss.

H. J. BUNKER

LESLIE ALFRED ALLEN

(*Facing p.* 170)

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The Early History of the Society for General Microbiology

AN ACCOUNT WRITTEN BY L. A. ALLEN SHORTLY BEFORE HIS DEATH

The foundation of the Society for General Microbiology was laid at the Annual General Meeting on 9 September 1943 of the Society of Agricultural Bacteriologists. For several years this Society (SAB) had had under review the question of the best direction in which its activities should expand to cater for the needs of the increasing number of bacteriologists, and in 1938 it set up a subcommittee to make recommendations. The Committee of the SAB, of which L. A. Allen was then the Secretary, decided to devote part of the time at its Annual General Meeting at the Leeds meeting (in 1938) to discuss 'the future status and title of the Society', to give this item a prominent place in the agenda, and to notify in advance all its members of this intention. In the notice sent to members it was explained that the Committee felt that the scope of the Society should be widened and that the title should reflect this change. It had been suggested that the title should be altered to 'Society of Bacteriologists' and that the society's functions should be similar to those of the Society of American Bacteriologists.

St John-Brooks had become a member of the Society of Agricultural Bacteriologists and he received the notice of the 1943 Annual General Meeting. His reply was illuminating:

Lister Institute, Elstree, Herts.
30 August 1943

Dear Dr Allen,

It would have given me great pleasure to have been able to have attended the Annual Conference of the Society of Agricultural Bacteriologists at Leeds next week but I fear that on account of staff difficulties I cannot leave Elstree for four days at this time. I should have liked very much to have joined in the discussions, especially if the question had arisen of widening the scope of the Society and of altering its name to 'Society of Bacteriologists' with functions similar to those of the Society of American Bacteriologists.

I have discussed this very important matter with several of my colleagues here, and especially with Sir John Ledingham, our late Director, whom I expect you will remember as President of the Second International Congress for Microbiology and in which some thirty-five of your members took part. I had a long discussion with Sir John the other day and we both felt that the time was fast approaching when the kind of Society you suggest should be founded and in which bacteriologists working in all the various fields might find common ground. The question really is—how should this be brought about? We wondered if the action contemplated by your Society might not delay instead of hasten this happy event. The new Society, at its inception, would, I imagine, contain only one medical man, which would perhaps seem strange when one considers the large number of distinguished medical bacteriologists in this country, many of whom are Fellows of the Royal Society, and of which the University of Leeds alone contains outstanding examples.

As a concrete proposal I suggest that it might be a good idea to get together a small committee of your members and, say, members of the Pathological and Biochemical Societies to discuss the matter and perhaps draw up tentative proposals to form an association of which your Society might well be the nucleus. I should be very glad to know of any decisions your committee may make on this subject and I and my colleagues here will do anything in our power to forward co-operation on the lines suggested.

Yours sincerely,

[signed] R. St John-Brooks

At the 1943 Leeds meeting, this letter figured prominently in the discussion on the future of the Society of Agricultural Bacteriologists and it played an important part in helping to form the decisions that were finally made. The most significant of these decisions were (1) that feeling was strongly in favour of the formation of a more general bacteriological society, (2) that representatives should be appointed to explore with representatives of other societies or groups the possibility of forming such a society, and (3) that Dr L. A. Allen (who had been elected President of the Society of Agriculture Bacteriologists during the conference) should discuss with Dr R. St John-Brooks the best way to arrange a meeting of representatives of societies and groups who might be interested in the project. It happened that St John-Brooks and Allen were then living at Bushey, Hertfordshire, and it was fortunately easy, in spite of war conditions, for them to arrange informal meetings at their homes in the evenings. They quickly agreed that the best way to collect microbiologists who represented different fields of interest was to compose a letter explaining the objectives in mind and to send this with an invitation to attend a meeting to certain members of scientific societies, groups, research institutes and industries, chosen because of their known interest in microbiology. These nominees were selected to cover applied biology, biochemistry, mycology, pathology, agricultural bacteriology, virology, animal parasitology, dairying, type culture collections, veterinary research and industry.

The response to this letter was gratifying and the proposed meeting was held on Tuesday 17 November 1943 at the London School of Hygiene and Tropical Medicine.

The following people attended: L. A. Allen, C. H. Andrewes, H. J. Bunker, D. H. F. Clayson, C. E. Coulthard, J. T. Duncan, A. Fleming, H. B. Hutchinson, B. C. J. G. Knight, Sir John Ledingham, J. W. Macleod, E. W. Mason, A. T. R. Mattick, A. A. Miles, W. T. J. Morgan, Muriel Robertson, R. T. St John-Brooks, K. M. Smith, A. W. Stableforth, Marjory Stephenson, H. G. Thornton, S. P. Wiltshire, W. R. Wooldridge. Among those asked but who were unable to attend were S. P. Bedson, W. J. Dowson, and P. Fildes.

At this meeting, at which Sir John Ledingham was elected Chairman, the general feeling was clearly in favour of the formation of an entirely new society rather than the expansion of one already existing. Moreover, it was decided that, although such a society should include those working in all fields of applied microbiology, it should not be run on the same lines as the Society of American Bacteriologists. It was felt, for example, that if the projected society held meetings at which papers on the different applications of bacteriology were given this would inevitably involve the formation of panels. This system would tend to encourage the segregation of the members into cliques, and by so doing would defeat one of the main objectives in view, which was to provide a common ground where microbiologists from different fields would talk freely and get to know each other. The majority of those present considered, for this reason, that the new society should be concerned mainly with the more fundamental aspects of microbiology. These were defined tentatively as 'consisting of a study of systematics, taxonomics, dissociation, variation, physiology (including oxidation-reduction potentials, vitamins, etc.) and the study of germicides, nutrition and ecology'. After a lively discussion the following proposal was adopted unanimously:

'That a subcommittee should be set up to consider the formation of a society for

the establishment and extension of common ground between all forms of microbiology—a society for general microbiology.' Implicit in the views expressed at the meeting was the feeling that the new society should sponsor the publication of a journal. The following were elected members of this subcommittee: H. J. Bunker, B. C. J. G. Knight, Sir John Ledingham, A. T. R. Mattick, A. A. Miles, Muriel Robertson, A. W. Stableforth, Marjory Stephenson, S. P. Wiltshire; with R. St John-Brooks and L. A. Allen as joint Honorary Secretaries.

This subcommittee held three meetings between December 1943 and March 1944, in which the main recommendations of the full committee (known hereafter as the Inaugural Committee) were endorsed and plans were formulated for establishing the new society. Marjory Stephenson took the chair at the first of these meetings and A. A. Miles at the remaining two. It was decided that to form a nucleus from which the new society might expand its membership, the Inaugural Committee should invite a certain number of people to become Original Members. A letter suitable for this purpose was drafted and sent to the individuals chosen. This letter was drawn up by the Secretaries and B. C. J. G. Knight. Each member of the Inaugural Committee was invited to submit to the subcommittee a list of about 200 microbiologists whom he considered should be asked to become Original Members.

The Society for General Microbiology held the inaugural meeting of Original Members on 15 February 1945 and its first scientific meeting at Cambridge on 6 July 1945; its second scientific meeting was a symposium on culture collections, a subject dear to the heart of St John-Brooks. Although not so designated in the first place, the two Honorary Secretaries so shared the work that one (St John-Brooks) became meetings secretary mainly responsible for organizing scientific meetings and paper-reading sessions, while the other (Allen) became a general secretary concerned chiefly with committee meetings and the business of the Society.

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The genus *Mallomonopsis*

By KATHARINE HARRIS
Botany Department, University of Reading

(*Received* 20 *April* 1965)

SUMMARY

The genus *Mallomonopsis* (Chrysomonadales) is discussed and five taxa are described from observations with the electron microscope: *M. elliptica*, *M. elliptica* var. *salina*, *M. elliptica* var. *oviformis* (formerly *Mallomonas oviformis*), *M. peroneides* sp.nov. and *M. ouradion* (formerly *Mallomonas ouradion*). The two flagella, which distinguish *Mallomonopsis* from *Mallomonas*, comprise a stout one with mastigonemes and a slender one which is smooth and has a photoceptor at its base. The silica scales are compared in detail and are shown to differ in construction as well as in appearance in the different species and are considered to provide the only satisfactory basis for specific separation.

Mallomonopsis is a genus of free-swimming unicellular flagellates of the Chrysomonadales and found in fresh water. The living organisms look very like those of the long-known *Mallomonas* and were distinguished by Matvienko in 1941; Lund (1942) recognized it and described it under another name. Since then a few more species have been added to the genus, together with some more morphological information but until recently all the information was obtained with the light microscope.

Mallomonopsis proves to be widespread and must be fairly common and I feel sure was overlooked by many as it certainly was by me. The sole distinction is that it has two unequal flagella and not just one as in *Mallomonas*. *Mallomonopsis* has silica scales, usually bearing hinged silica bristles as in *Mallomonas*. In the present paper I supplement the light microscope observations with electron microscope studies of the scales which have elaborate structure and I have distinguished the different layers that compose them. These silica scales as in other Chrysomonads prove to be of the greatest taxonomic value and it is now realized that it is difficult to distinguish species with any certainty without electron microscope data.

METHODS

The free swimming organisms were concentrated by a low-speed centrifugation; spores were sometimes obtained by keeping the original collection in a bottle in the light. Concentrates were fixed on a glass slide for 2 min. in osmic tetroxide vapour after which they were dried. When the silica bristles were to be removed the fixed dried organisms of a nearly pure preparation were treated with 6 drops of concn hydrofluoric acid in 50 ml. of water for 30 min. on a Formvar-coated slide. The preparation was then mounted on grids for examination in the electron microscope. Crystal violet was used as the flagellar stain when the organisms were

examined in balsam with the light microscope. To prepare the cells for the electron microscope the fixed dried concentrate on a glass slide was coated with 400 Å carbon and mounted on copper grids in the usual way for direct examination. Very dilute hydrofluoric acid was used in preparing the replicas (3 drops in 50 ml. water for 15 min.).

History. Matvienko in 1941 based *Mallomonopsis* on *M. elliptica* and *M. clavata* both from Kharkov, U.S.S.R. Later he added *M. squamulaeperforata* (1949) and *M. robusta* (1952). Lund (1942) described what we now believe is *M. elliptica* as *Ochromallomonas pelophila* from England. Bourrelli (1960) described *M. elliptica* from the Ivory Coast and Ettl (1960) from Czechoslovakia. Asmund & Hilliard (1965) describe a variety of *M. elliptica* from Alaska, with electron micrographs. Takahashi (1959) figured (without names) isolated scales which closely resemble *M. elliptica* and *M. peroneides* and in 1960 he figured another under the name *Mallomonas oviformis* which looks exactly like the scale of *Mallomonopsis elliptica* var. *oviformis*. These are all from Japan. The only other species known to me is *M. deltoides* which I suspect may be a few-celled form of Synura.

Mallomonas elliptica Matvienko 1941, Figs. 1–6; Pl. 1, figs. 1–6

The living organism (Fig. 2) shows concave scales along its sides and most scales carry one bristle. However, there may be three bristles as in Fig. 6, a fixed and dried organism; the electron micrograph, Pl. 1, fig. 2, also shows three. The dried scales (Figs. 4, 5) often show an opaque area towards the distal end. All the scales, whether anterior or from the middle region or posterior end are alike in this species, as indeed they are in its varieties. The cyst (Fig. 3) is round and smooth as in Matvienko's material.

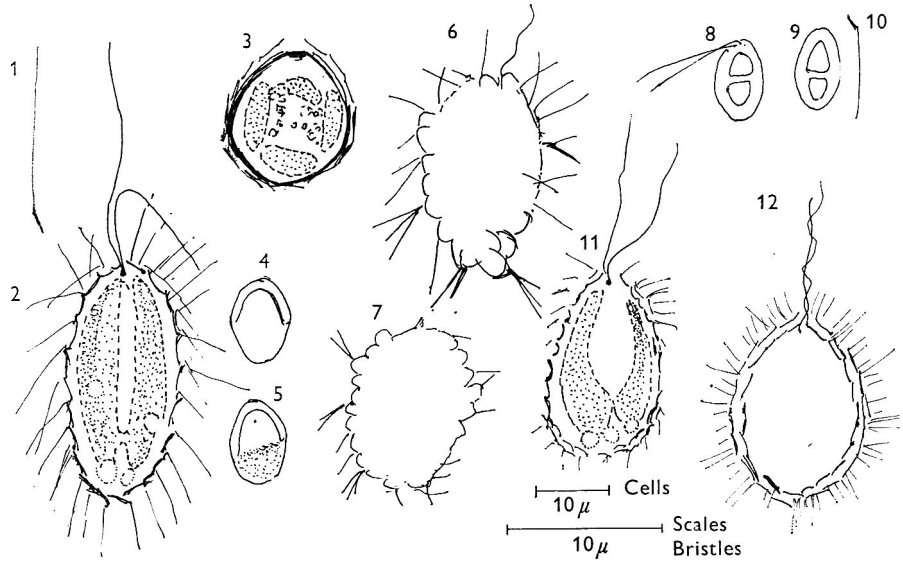
I give an electron micrograph of a nearly complete cell (Pl. 1, fig. 1) which shows the scales strongly overlapping as is usual in this species, but when the organisms swells up before forming a spore there is much less overlap. In direct electron micrographs (Pl. 1, figs. 2, 3) the scale shows a uniformly dense proximal border and most of the scale surface is occupied by a dark network. This network grows denser towards the distal end (in Pl. 1, fig. 2, but less markedly in fig. 3). These electron-dense parts are the opaque regions seen under the light microscope. There is a small hole near the proximal end surrounded by a closer network. In a replica (Fig. 6) the proximal border is now clear but there is a darker region where the border overlaps the scale. The dense network has largely disappeared, but instead each mesh is represented by a little papilla with a thin point at its middle. The proximal hole is conspicuous and it is surrounded by an area with no papillae.

Dimensions of my material: cell 14–34 $\mu \times 8$–17 μ; scales 4·5–5·5 $\times$ 3·0–4·5 μ; cyst 17–22 μ.

Occurrence. Mallomonas elliptica has been found in the neighbourhood of Reading, Berkshire, in a number of ponds and small lakes on a subsoil of shallow acid peat. The localities are usually surrounded by trees but sometimes only a few trees or bushes are present. I have found the species in each month except June but its appearance is always unpredictable by me. I have not found it in large numbers since I was able to avail myself of the electron microscope.

Mallomonas elliptica var. *salina* Asmund & Hilliard (Figs. 17–20; Pl. 2)

This variety is not described here in detail because Asmund & Hilliard (1965) do so, but I give some points not covered by them. The living organism of the variety is distinguishable by the lens-shaped scales seen along its sides (Fig. 17) and the dried scales (Fig. 18), as they remarked, shown an opaque inner area. Most scales carry one bristle, but I have seen up to three. The cyst (Fig. 20), which was unknown, is smooth, rounded and with a small anterior pore.



Figs. 1–6, *M. elliptica*. 1, Bristle; 2, living organism; 3, cyst in mother-cell armour; 4, 5, scales; 6, fixed dried organism showing some bristles in bunches.

Figs. 7–12, *M. peroneides*. 7, Fixed dried organism showing some bristles in bunches; 8, 9, scales, one with two bristles; 10, bristle; 11, living organism; 12, living organism showing what appear to be two layers of scales.

All complete organisms, × 1000; all scales and bristles, × 2000.

In the electron microscope the scales and bristles look just like their figures in direct view (Pl. 2, figs. 9–10) but replicas (Pl. 2, figs. 7, 8) are very different. The strong radiating struts along the proximal margin and the electron-dense network occupying most of the scale surface both disappear. Evidently both are internal features. For some reason the tops of the papillae often appear as open holes in the replica.

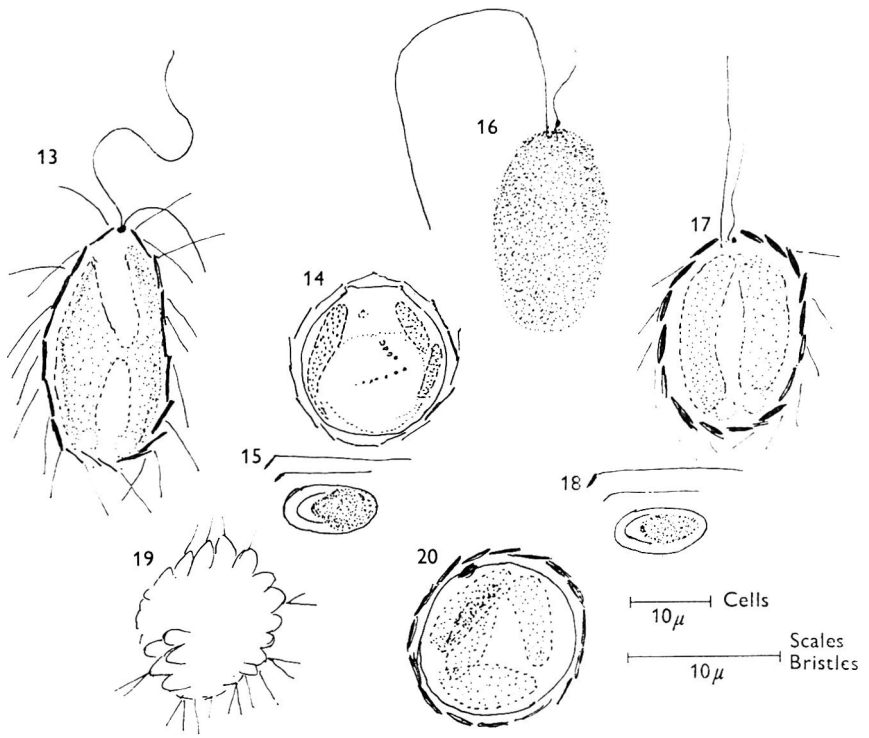
Dimensions of my specimens: cell 21–32 × 16–19 μ; scales 5·5–6·0 × 3·0–3·5 μ, bristles 7–12 μ; cyst 19–21 μ wide.

Occurrence. *Mallomonas* var. *salina* occurs in winter, spring or summer. It has been found in an old overgrown gravel pit and an overgrown pond on gravel subsoil. I have also found scales in a small lake on old agricultural land and in a pool in woodland near acid peat with Sphagnum growing near. Asmund's original sample was found in brackish water, so the variety is adaptable.

Mallomonopsis elliptica var. *oviformis* (Nygaard) comb.nov.
(Figs. 13–16; Pl. 3. figs. 11–14)

Mallomonas oviformis Nygaard, 1949
Mallomonas oviformis Nygaard. Asmund, 1959
Mallomonas oviformis Nygaard. Harris & Bradley, 1960

The first two of the above descriptions relied on the light microscope alone, but in the third the electron microscope was also used. I have now found that there are two flagella; they can be seen in living organisms, in stained organisms mounted in Canada balsam and in electron micrographs.



Figs. 13–16, *M.* var. *oviformis*. 13, Living organism; 14, cyst in mother-cell armour; 15, two bristles and a scale; 16, cell stained and mounted in Canada balsam showing two flagella and flagellum-swelling.

Fig. 17–20, *M.* var. *salina*. 17, Living organism; 18, two bristles and a scale; 19, fixed dried organism showing bunches of bristles; 20, cyst in mother-cell armour.

All complete organisms, × 1000; all scales and bristles, × 2000.

In the variety *oviformis* the scales look like flat plates along the sides of the organism (Fig. 13). The dry scale (Fig. 15) has a proximal rim, then a small clear area and a large opaque area occupying the middle and distal parts. Each scale commonly bears one bristle, but these may be two or even three. The cyst, which has not been seen previously, is smooth and round with a small pore in a wide saucer-shaped rim.

In a direct electron microscope examination, a scale (Pl. 3, fig. 14) showed a dense proximal border not crossed by any darker struts. Next to this is a less dense area and then a large part of the scale shows a very complex pattern. I consider that this is made up of three superimposed patterns, each belonging to a different layer. First, there is an inner layer with tiny holes, regularly spaced over the whole surface; this layer alone is seen near the thick rim in Pl. 3, fig. 14. Then over most of the surface is a middle layer with a pattern of dark curved bars, but these are absent near the margins. Finally, on top of this is a layer with prominent dark papillae but these do not extend to the distal margin (Pl. 3, fig. 11).

A few scales are simpler and I believe immature and have fewer layers; Pl. 3, fig. 12, shows one of these. It must already be external because it bears two bristles but it consists of the margin and the basal perforated layer alone (Pl. 3, fig. 12). Another unusual scale is seen in Pl. 3, fig. 13, where just a small part of the outer layer is missing and the curved bars are exposed.

Dimensions of my material: cell 18–42 $\mu \times 11$–19 μ; scales 5–6·5 $\mu \times 3$–3·5 μ, bristles 8–12 μ; cyst typically 18×20 μ, diameter range 16–13 μ.

Occurrence. Variety *oviformis* is sometimes met in enormous numbers in ponds on agricultural land from November to March; I also found a few in one pond on a thin acid peat. In some years I found none at all, but in 1965 it grew very well and appeared in ponds where I had not previously seen it. As a rule it encysts when the weather becomes warm; but in one pond, while many encysted in late January, large numbers of motile organisms continued in the water until late March.

Discussion and comparison of the varieties. The three distinct taxa here described as *Mallomonopsis elliptica* and its two varieties all agree equally well with Matvienko's drawing, for this did not show the small differences which distinguish them under the light microscope. These differences are in the scales; what I take as type *elliptica* has scales which look concave in profile; variety *oviformis* has flat scales and variety *salina* convex lens-shaped scales. The isolated dried scales can often be distinguished by the position of the opaque area, in the *elliptica* it is small and limited to the distal end; in *salina* it is in the middle region, and in *oviformis* it is large and extends nearly to the proximal end. The electron microscope makes these differences more definite.

As I have said, there is nothing to show which should have the name *Mallomonopsis elliptica* but current practice has settled it. Bourrelly gave figures of what he identified as *M. elliptica* and these figures show the opaque area mentioned above; others have assumed he was right and have followed him in regarding his account as an amplified description of the type. I have included the three taxa in the one species because they are very similar indeed and even electron micrographs of the scales have many features in common. It is in the deeper layers of the scales that the main differences lie and these are clearly revealed by direct electron micrographs.

Mallomonopsis peroneides sp.nov. (Figs. 7–12; Pl. 4, figs. 15–21)

Latin diagnosis. Cellula rotundata ovoides, setis brevibus et tenerĭbus ubique contecta, nonnunquam setis absentibus. Squamae ellipticae, margine lato et crasso vallum fabricante per medium squamae et foveas duo formante. Pars

proximalis marginis laevis, tignis intus instructa; residuum squamae foraminibus multis et papillis dispersis ornatum, sed papillae paucae in cavo proximali. Pars distalis marginis in exteriore parte scrobiculum exiguum rotundatum ferens, prope commissuram setarum. Setae una vel duo vel tres per squamam, tenues laeves non attenuatae sed apex acumitatus ipse saepe alterum ferens apicem acuminatum subtiliorem. Cystum ignotum.

Dimensions. Cellula 11–13 × 11–26 μ, squamae *c.* 3 × 5 μ, setae 6–8 μ.

English diagnosis. Cell round ovoid covered all over with short slender bristles which may sometimes be absent. Scales oval with a broad thick margin an extension of which forms a ridge across the middle of the scale leaving two depressions. Proximal part of margin smooth with internal struts. Rest of scale covered with perforations and scattered papillae, the papillae, however, are infrequent in the proximal depression. Distal margin has small circular depression on the outer side near point of insertion of bristles. Bristles, one to three per scale, short, slender, smooth, not tapering but bearing at the tip a small needle-shaped point which may in turn bear a second finer needle-shaped point. Cyst not known.

The name *peroneides*, meaning like a buckle, refers to the appearance of the scale.

Description. This small and inconspicuous organism is usually covered with fine bristles (up to three to a scale) but is sometimes without bristles. Often the cell looks as though clothed in two or three layers of scales (Figs. 11, 12) but this may be an effect of strong overlap. The flagella were nearly equal in the few organisms where they were seen. The scales were studied as direct electron micrographs, as replicas of both sides, and as direct electron micrographs after some but not all of the silica had been removed.

Plate 4, fig. 20, shows a scale in a direct micrograph. The dense parts are too dark to show details but the pattern over the rest of the scale is seen. This consists of two superimposed patterns; the first a uniform layer showing regularly spaced minute pores or pits seen as pale spots, the second a pattern of dark spots which are papillae. Plate 4, fig. 21, shows a small misshapen scale with similar marks; it probably comes from one or other end of the cell. A replica of the inner surface (Pl. 4, fig. 15) is nearly smooth but shows the minute pores as inconspicuous pits. A replica of the outer surface (Pl. 4, fig. 16) shows the strong transverse bar and the papillae over the greater part of the surface. Plate 4, fig. 19, shows a scale which has been partly dissolved with dilute hydrofluoric acid; here some of the scale has vanished entirely and most of its surface has gone. The papillae are seen as black rings and the proximal flange is now transparent enough to show its radiating struts which evidently are internal.

Occurrence. Mallomonopsis peroneides occurred in the shallow, swampy end of an artificial lake in a forest Benyon's Enclosure (Grid ref. 628 E/633 N). It was only found in small numbers, in August 1959.

Mallomonopsis ouradion (Harris & Bradley) comb.nov. (Pls. 5–7)

Mallomonas ouradion Harris & Bradley, 1958

New observations showed that this species has two flagella and must therefore be transferred to *Mallomonopsis*. The new material agreed precisely with that previously described except that a larger range of size was noted, the motile

organism is 11–33 × 11–21 μ. Further observations were made on the scales. The living organism often looks as though it has more than one layer of scales, but as in other species I suppose that this is an optical effect caused by great overlap of one layer of scales. I have not seen signs of more than one layer in electron micrographs. The extent of overlap varies; in Pl. 5, fig. 24, about three-quarters of each scale was overlapped, but in a similar electron micrograph of another organism only a quarter of each scale was overlapped.

The structure of the scales is now better understood; this is of value for comparison with other species. The scale is here simpler. It consists of a single delicate layer of perforated silica on to which various ridges and ornamentations are later developed externally. There is no middle layer. In Pl. 6, fig. 27, the scale marked '*b*' (at the bottom right) has only the delicate basal layer. It is one of the incompletely silicified scales of the kind I call 'immature'. The scales on the left, particularly the one on the top left marked '*a*' shows the mature structure. Various electron-dense parts have been added, including the dark papillae over its surface. I have also seen bristles which I would describe as immature; their apices are composed of spongy silica and without a sharply defined margin. We do not yet have information how the scales of *Mallomonopsis* or *Mallomonas* are formed, but whatever the mechanism it is one which rather often ceases before the scale is fully silicified. A diffraction pattern of the scales of *M. ouradion* was examined, but it showed the pattern of amorphous silica only, so it would appear that all parts of the scale are made of this one material.

Flagella

Since the flagella characterise *Mallomonopsis* they merit further description. One flagellum, the stout one (with mastigonemes), is generally easy to see in the living organism. It usually points forwards. The other, which is smooth, may be rather longer or rather shorter but is always much more slender and it points in varied directions, but it does not seem to act as a trailing flagellum as in some flagellates. It is sometimes difficult to see because it is hidden among the fine silica bristles; this difficulty applies equally to the optical and the electron microscope. However, I have seen both flagella repeatedly in living organisms of four of the five kinds described here. The remaining one *Mallomonopsis ouradion* first showed the smooth flagellum under the electron microscope, but I did not see them both in the living organism, even though I examined large numbers.

Fully convincing views of both flagella can be seen in fixed organisms of all kinds when these are stained with a suitable stain and mounted in Canada balsam; then the silica bristles become nearly invisible and no longer confuse the picture (Pl. 5, figs. 22 and 23).

An electron micrograph of the flagella of a de-silicified cell of *Mallomonopsis ouradion* is shown in Pl. 7, fig. 29. It was taken chiefly to show the photoceptor or flagellum swelling at the base of the smooth flagellum. This has not been figured before with the electron microscope. Lying as it does within the top ring of scales it can only be seen on a de-silicified organism. The flagella resemble the flagella of Synura very closely and even have small points on the ends of the mastigonemes. These points are not shown here; but Mr D. E. Bradley informs me that he has seen them in Synura. The smooth flagellum, however, has certain differences. It

has the normal core at the base, but often tapers to a single strand at the free end. It is very fragile (unlike that of Synura) and easily breaks up on treatment.

CONCLUSIONS

It is likely that further species at present in *Mallomonas* may have to be transferred to *Mallomonopsis*. Once I began to search specifically for a second flagellum, I found it in two forms I had previously described as having one. One of these forms first showed the smooth flagellum in electron micrographs and then in stained preparations; but I never saw it in the live state. Then it is plain that while the species and varieties distinguished here can, with more or less reliability, be distinguished with the optical microscope, once their scales are examined with the electron microscope specific characters of another order of reliability emerge. It is indeed difficult to see how determinations based solely on the optical microscope will eventually be accepted alongside those with electron microscope details.

The taxonomic position of *Mallomonopsis* in relation to *Mallomonas* needs to be examined, but for this we need information not yet available. Bourrelly (1957) puts the two close together in his Synuraceae and the present work, as far as it goes, supports his conclusion, for it agrees with Synura very closely in its flagella. The position of *Mallomonopsis* close to *Mallomonas* is also supported since the two are similar in everything but their flagella.

I wish to thank Professor R. W. Ditchburn, F.R.S., and Dr T. Evans for allowing me to use the electron microscope in the J. J. Thomson Physical Laboratory, The University, Reading, Mr F. Robertson for translating my diagnosis into Latin, Dr R. W. A. Park for help with staining the preparations for the light micrographs, and Miss B. Asmund for allowing me to see her manuscript on *Mallomonas elliptica* var. *salina*.

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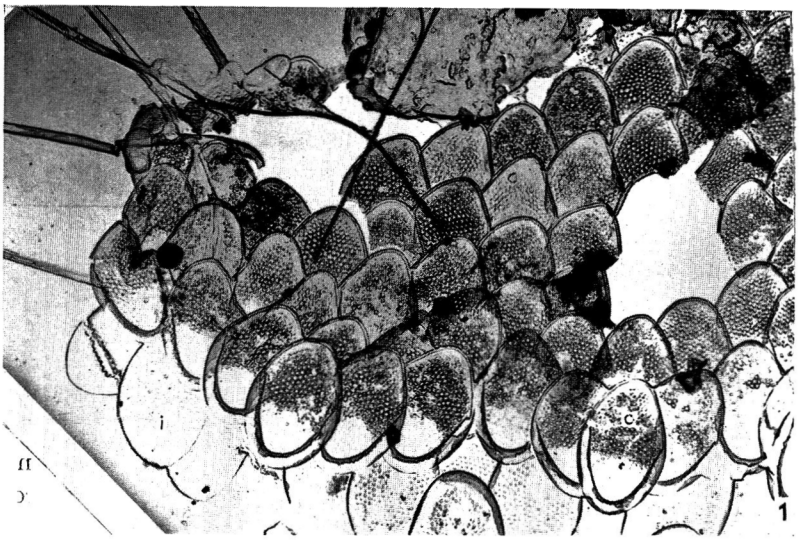

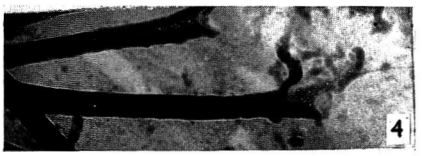

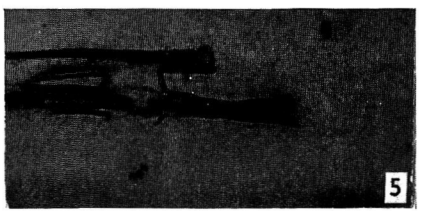

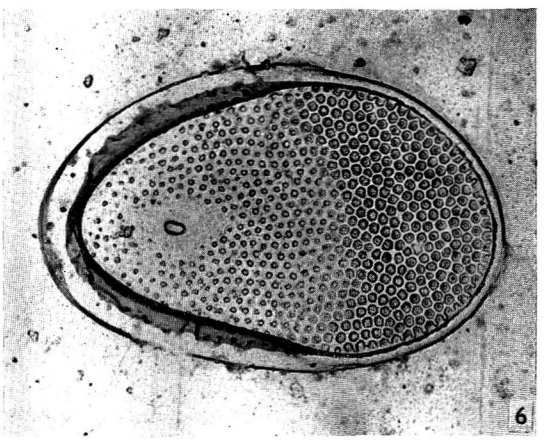

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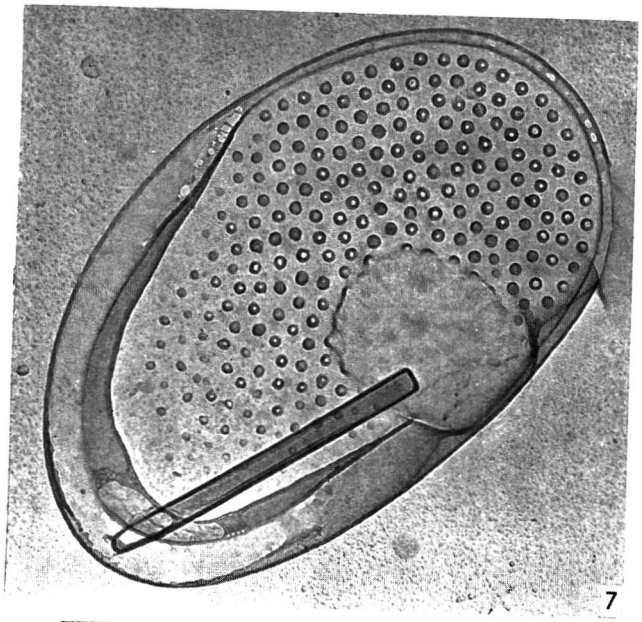

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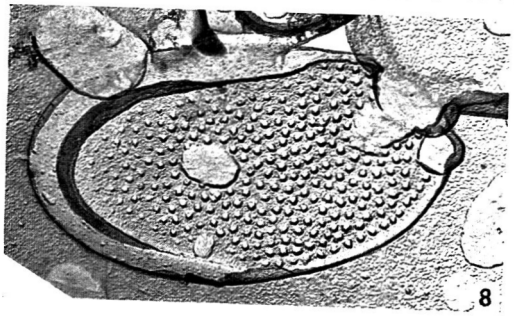

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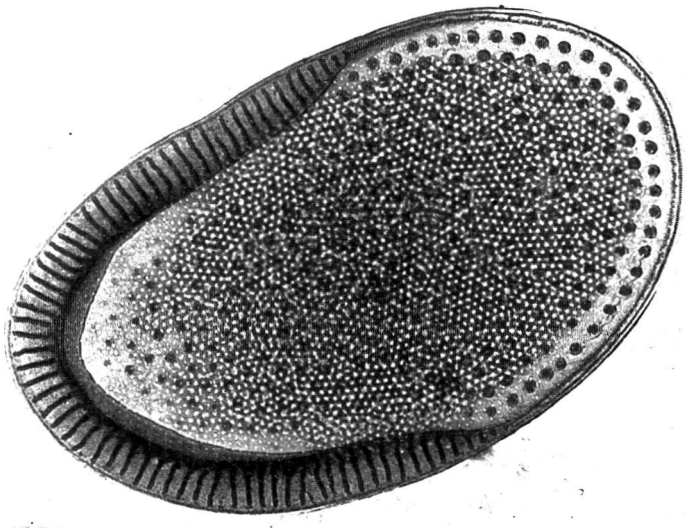

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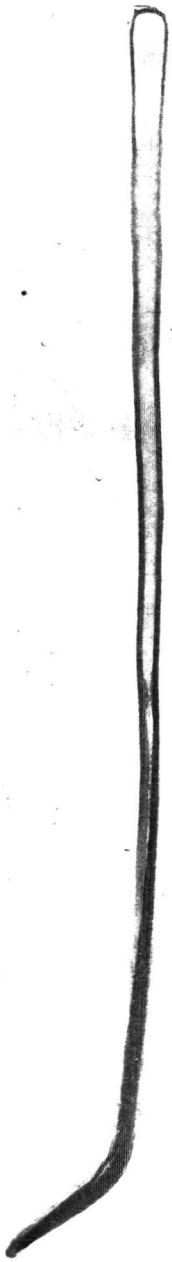

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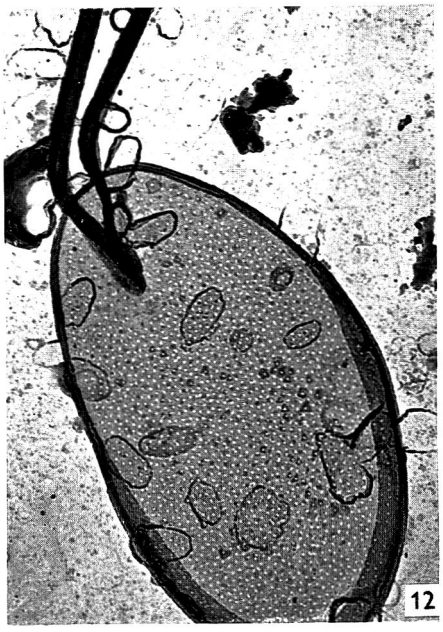
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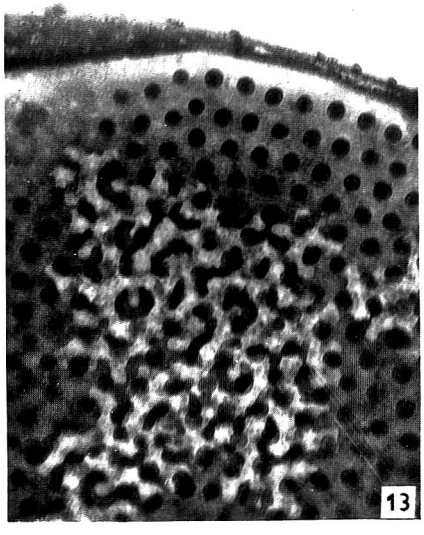
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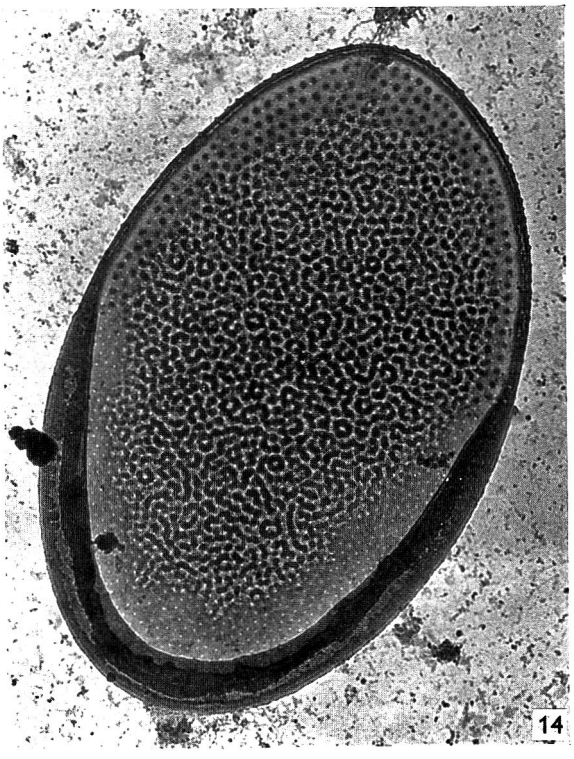
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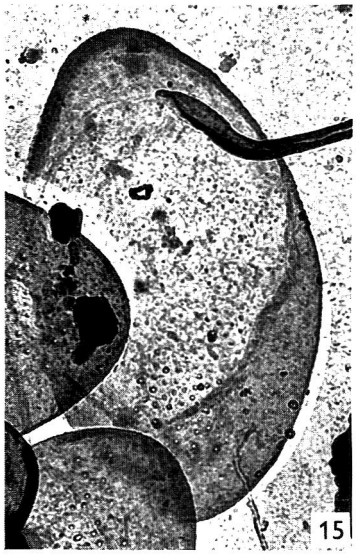
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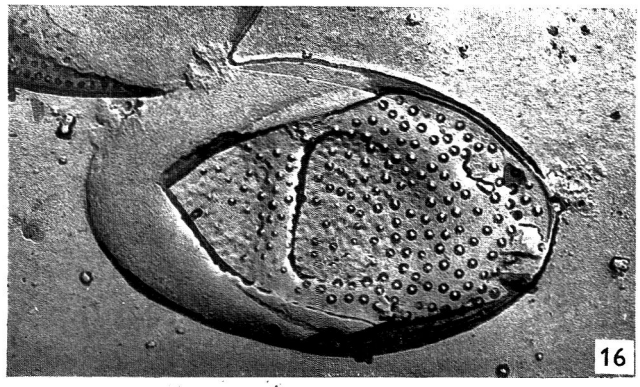
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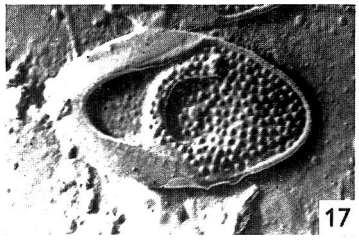
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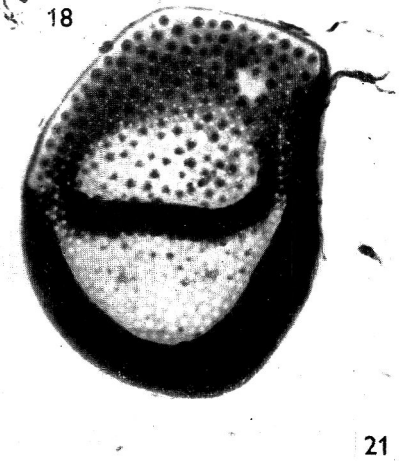
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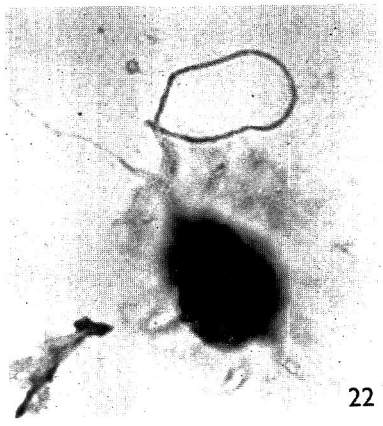

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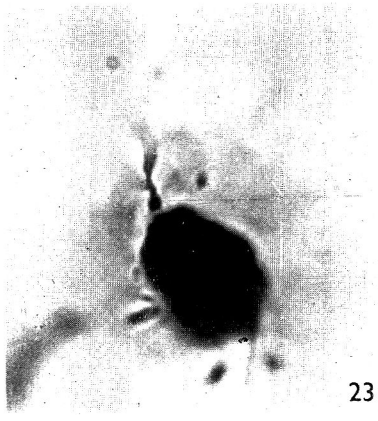

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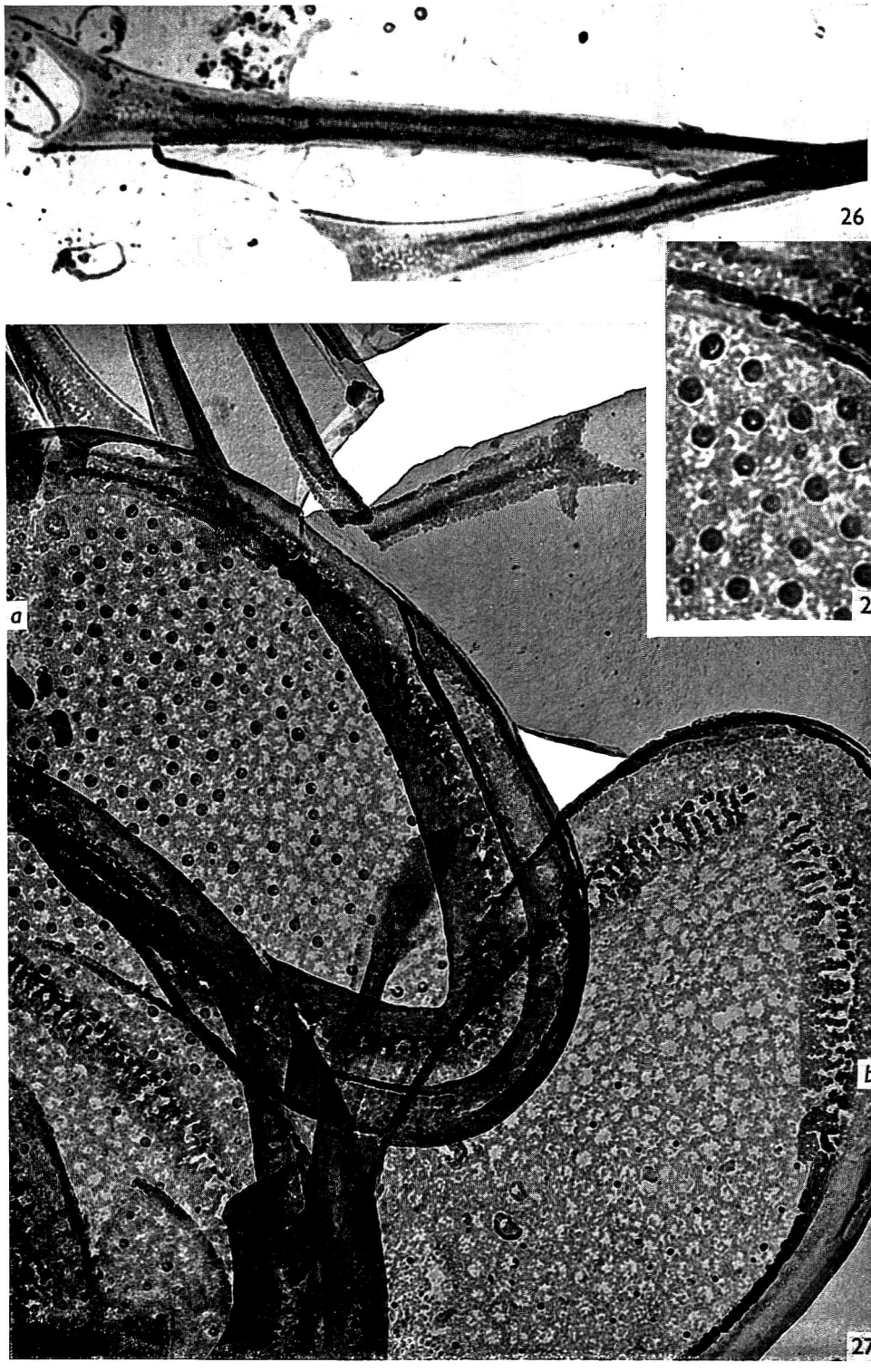

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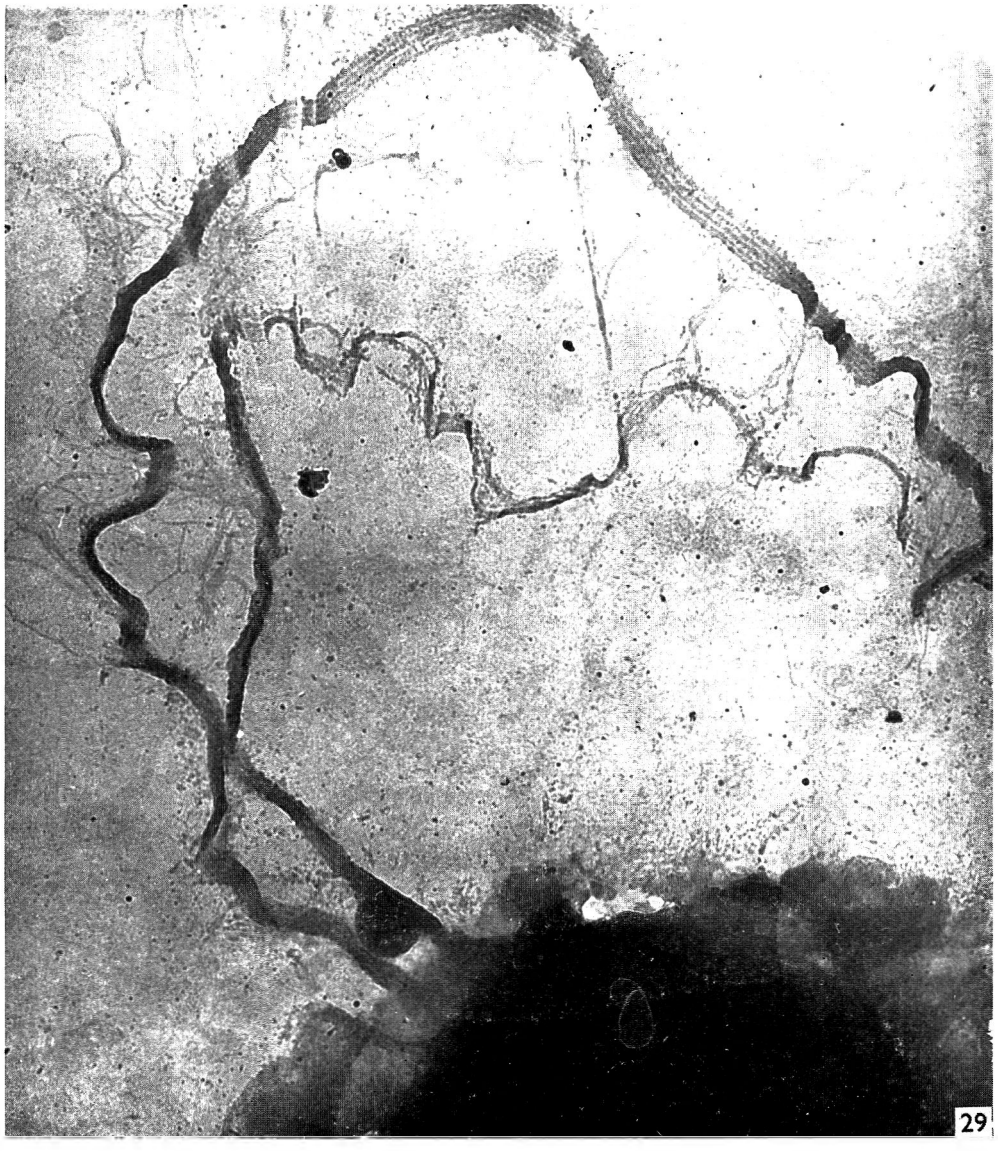
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EXPLANATION OF PLATES

PLATE 1

Mallomonopsis elliptica Matvienko

Fig. 1. Replica of part of the armour of an organism showing the inner and outer surfaces of the scales. × 4000. o, Outer surface; i, inner surface.

Fig. 2. Direct electron micrograph of a scale bearing three bristles. One complete unattached bristle below the scale. × 8000.

Fig. 3. Direct electron micrograph of a scale. × 16,000.

Fig. 4. Free end of a bristle showing a proliferating forked tip. Part of another bristle is above it. × 32,000.

Fig. 5. The free end of two bristles with truncated tips. × 32,000.

Fig. 6. Replica of scale. × 12,000.

PLATE 2

Mallomonas elliptica var. *salina*

Fig. 7. Replica of a scale. × 20,000. (The circular patch and the rod do not belong.) The apex of most papillae is intact.

Fig. 8. Replica of a scale. × 12,000. The apices of all the papillae are broken.

Fig. 9. Direct electron micrograph of a scale. × 20,000.

Fig. 10. Direct electron micrograph of a bristle. × 20,000.

PLATE 3

Mallomonopsis elliptica var. *oviformis*

Fig. 11. Replica of a group of scales with the outer side showing papillae and the inner side of one scale showing two bristle attachments. A forked bristle tip is seen on the left. × 16,000.

Fig. 12. Direct electron micrograph of an immature scale with the base of two bristles. × 12,000.

Fig. 13. Direct electron micrograph of part of a scale from which some of the outer surface has broken away, showing the interior structure. × 50,000.

Fig. 14. Direct electron micrograph of a scale. × 20,000.

PLATE 4

Mallomonopsis peroncides

Fig. 15. Replica of the inner surface of a scale and the foot of a bristle. × 16,000.

Fig. 16. Shadowed replica of a scale (outer side). × 16,000.

Fig. 17. Shadowed replica of a scale made by Miss D. Chessor (A.E.I.). Reversed print by Bradley's method. × 8000.

Fig. 18. Direct electron micrograph of bristle. × 16,000.

Fig. 19. Direct electron micrograph of a scale from which the surface silica had been removed. × 16,000.

Fig. 20. Direct electron micrograph of a scale with the base of three bristles. × 16,000.

Fig. 21. Direct electron micrograph of a scale from either the rear or anterior end of the organism. × 16,000.

PLATE 5

Mallomonopsis ouradion, figs. 22–24

Fig. 22. (Light microscope.) Organism stained with crystal violet, showing the two flagella × 1500.

Fig. 23. (Light microscope.) Same organism as fig. 21 in different focus showing flagellum swelling (photoceptor). × 1500.

Fig. 24. Replica of part of an organism with strongly overlapping scales. × 3000.

Fig. 25. *Mallomonas elliptica* Matvienko, electron micrograph of the flagella. × 4000.

PLATE 6

Mallomonopsis ouradion

Fig. 26. Direct electron micrographs of two bristle tips. × 26,000.

Fig. 27. Direct electron micrograph of group of scales. × 26,000. *a*, Mature scale; *b*, immature scale.

Fig. 28. Direct electron micrograph of part of scale. × 52,000.

PLATE 7

Mallomonopsis ouradion

Fig. 29. Direct electron micrograph of two flagella after removal of scales and bristles. × 12,000. A large flagellum swelling is seen near the base of the smooth flagellum. The distal part of the smooth flagellum has been damaged during the preparation of the material.

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Resistance to Inhibitors of Dihydrofolate Reductase in Strains of *Lactobacillus casei* and *Proteus vulgaris*

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SUMMARY

Strains of *Lactobacillus casei* and *Proteus vulgaris* resistant to small molecule inhibitors of dihydrofolate reductase were isolated under various nutritional conditions. When thymine and/or purines were available in the media, several of the isolated strains had nutritional requirements for these metabolites, but resistant lines could still be isolated in their absence. Thus, the biochemical alteration accompanying resistance could be predetermined to a major extent by the design of the experiment.

The wild-type strain of *Proteus vulgaris* did not incorporate exogenous thymine, and was insensitive to thymine antagonists, while the thymine-requiring strain was highly sensitive to 5-bromouracil, dithiothymine and 2-thiothymine. This suggested that resistance was accompanied by the appearance of a permeability or transport system for thymine.

INTRODUCTION

Replicate isolates of a number of strains of *Lactobacillus casei* have been consistent with respect to the biochemical lesion accompanying resistance. These lesions generally have taken the form of a deletion (or major alteration) of the enzyme system dealing with the incorporation of the analogue. Thus the diaminopurine-resistant *L. casei* was unable to incorporate adenine (Elion, Singer & Hitchings, 1953; Balis *et al.* 1952), the 8-azaguanine-resistant line failed to use guanine (Elion, Singer & Hitchings, 1956) and the 6-mercaptopurine-resistant clone was unable to utilize exogenously supplied hypoxanthine (Elion *et al.* 1953). In these instances the analogues affect biochemical pathways which are dispensable, 'salvage' mechanisms, and the organism can obtain its requirements for growth through alternate mechanisms involving synthesis *de novo*.

Inhibitors of dihydrofolate reductase are in another category in this respect since the product of the inhibited reaction, tetrahydrofolate, participates, in its coenzymic forms, in a variety of essential reactions (Friedkin, 1963; Huennekens & Osborne, 1959). Moreover, 5,10-methylenetetrahydrofolate, the coenzyme of thymidylate synthetase, is oxidized when its one-carbon fragment is transferred, and its renewal depends on the continued activity of dihydrofolate reductase.

The present paper deals with resistance to small molecular inhibitors of dihydrofolate reductase (Hitchings *et al.* 1952; Hitchings & Burchall, 1965). It will be shown that nutritional requirements for the end products of enzymic systems involving tetrahydrofolate-containing coenzymes appeared concurrently with re-

sistance. The nature of these requirements correlated closely with the composition of the medium from which the resistant isolate was obtained. Thus the outcome of the experiment designed to reveal the biochemical mechanism of resistance was predetermined to a considerable extent by the conditions of the experiment itself. Preliminary accounts of several portions of this study have been given (Singer, Elion & Hitchings, 1957; Singer, Elion & Hitchings, 1958; Hitchings, Singer & Elion, 1958).

METHODS

Media. Proteus vulgaris ATCC 9920 and the mutants derived from it were grown and maintained as described by Elion, Singer & Hitchings (1960). The basal medium (PVO) and a supplemented medium containing thymine (1 μg./ml.: PVT) were employed. Growth was estimated by measurements of turbidity with a Lumetron (model 403-E) photoelectric colorimeter, adjusted so that uninoculated medium gave a zero reading and opacity was 100.

The experiments with *Lactobacillus casei* (ATCC 7469) and the mutants derived from it were done as described by Hitchings *et al.* (1950) in basal medium O, plus the supplements of folic acid, thymine, adenine sulphate and drug described below. Growth after 3 days' incubation was estimated by titration of 10 ml. cultures with 0·1 N sodium hydroxide. The various media used were:

OFA = basal medium (O) + folic acid 0·05 mμg./ml. (sufficient for half-maximal growth),
FA+ = OFA + folic acid 0·625 mμg./ml. (sufficient for maximal growth),
PFA = OFA + adenine sulphate 10 μg./ml.,
TFA = OFA + thymine 1 μg./ml.,
PTFA = OFA + adenine sulphate 10 μg./ml. + thymine 1 μg./ml.,
PT = O + adenine sulphate 10 μg./ml. + thymine 1 μg./ml.

Isolation of the mutants. Strains of *Lactobacillus casei* resistant to pyrimethamine (2,4-diamino-5-*p*-chlorophenyl-6-ethylpyrimidine, B.W. 50–63) were isolated by the gradient plate technique (Szybalski & Bryson, 1952) in the presence of pyrimethamine and folic acid, with or without adenine sulphate and/or thymine. Strains of *Proteus vulgaris* resistant to diaveridine (2,4-diamino-5-(3′,4′-dimethoxybenzyl)-pyrimidine, B.W. 49–210: Roth *et al.* 1962) and to sulphadiazine or sulphadiazine + diaveridine were similarly obtained by means of the gradient plate technique. Except where Proteus strains resistant to various amounts of diaveridine were desired, the concentration of all the drugs used, both for the isolation and maintenance of the respective mutants, was 300 μg./ml. Once isolated the strains were maintained in the presence of the drug in the medium from which they were isolated. Some of the properties of the resistant strains are given in Table 1.

Thymineless death. Lactobacillus casei strain 50–63/II was inoculated into: (1) basal medium O, which contains all essentials except folic acid, thymine or adenine; (2) medium PTFA, a complete medium; and (3) medium PFA where folic acid and purine are supplied but thymine is omitted. Plating counts were made after 0, 6, 24, 30, 48, 64, and 72 hr.

Incorporation of [2-^{14}C]-thymine into bacterial DNA. The various strains of *Proteus vulgaris* were grown in replicate batches of 250 ml. of PVO medium con-

taining [2-^{14}C]-thymine (1·05 C/mole). *Lactobacillus casei* was grown similarly in OFA medium with added thymine. The bacteria were harvested, dried and defatted (cold TCA, ethanol, ethanol+ether (3+1), ether: Balis *et al.* 1952). The dried and defatted bacteria were digested directly with 70% perchloric acid for 1 hr in a boiling water bath. The perchloric acid digest was then neutralized with potassium hydroxide. The thymine present in the neutralized supernatants was determined in two ways, microbiologically, using the thymine-requiring strain 50–63/II, and spectrophotometrically. For the latter purpose, the hydrolysates were subjected to chromatography in a butanol+ammonia system, the radioactive spots were eluted with 0·1 N-hydrochloric acid and the absorption at 263 mμ measured. The radioactivity was determined by means of a Nuclear Chicago Model D 47 internal gas flow counter. All counts were carried out for a time sufficient to give a probable error of less than 10%. No coincidence correction was required. The results are reported in terms of relative specific activities (R.S.A.), i.e. the ratio of specific activities of the isolated and the proffered thymine.

Table 1. *Properties of resistant strains*

Strain	Medium for isolation	Drug	Concn. (μg./ml.)	End product requirement
Lactobacillus casei				
50–63/I	FA+	Pyrimethamine	300	None
50–63/II	PTFA			Thymine
50–63/II p*	PTFA			Thymine+adenine
50–63/III	PFA			None
50–63/IV	TFA			Thymine
Proteus vulgaris				
49–210/I	PVO	Diaveridine	300	None
49–210/II	PVT			Thymine
SD	PVO	Sulphadiazine	300	
SD/49-210†	PVO	Sulphadiazine	300	
		Diaveridine	300	
49–210/10	PVO	Diaveridine	10	None
49–210/30			30	
49–210/100			100	
49–210/300			300	

* Parent strain: 50–63/II. † Parent strain: SD.

RESULTS

The wild type of *Lactobacillus casei* can grow in the absence of purine and thymine when folic acid is present. This is seen in Table 2 which compares the growth of the wild and resistant strains of *L. casei* in various media. Strain 50–63/III grows in all of the media but somewhat more slowly than the wild strain. Strains 50–63/II and 50–63/IV, on the other hand, have absolute requirements for thymine. Nevertheless, they synthesize purines since they grow in TFA medium from which purine is absent. When strain 50–63/II was transferred repeatedly in PFTA medium containing drug, it apparently lost its ability to synthesize adenine. This new strain, 50–63/II p, could be grown only in the presence of both purine and thymine (PT or PTFA media). Subsequent experiments have shown that guanine, hypo-

xanthine or xanthine are able to substitute for adenine in satisfying the purine requirement of this strain.

The abilities of the wild type and strain 50–63/II to utilize thymine, its deoxyriboside and deoxyribotide, were compared (Fig. 1). The data for strains 50–63/II p and 50–63/IV are not shown, but their responses to exogenous thymine, thymidine and thymidylate, and their responses in the cross resistance experiments to be described, were identical to that of the 50–63/II strain. As can be seen, the mutant utilized thymine well but thymidine and thymidylic acid were used poorly. The wild strain utilized thymidine somewhat better but it, too, preferred free thymine.

Table 2. *Growth of wild type and mutant strains of Lactobacillus casei in various media*

Strain	Medium for isolation	Titre*					
		OFA	$FA_{\pm}$	PT	PFA	TFA	PTFA
Wild type	—	6·1	13·0	5·8	8·3	5·5	9·5
50–63/I	$FA_{\pm}$	1·4	6·1	1·2	3·6	1·5	1·8
50–63/II	PTFA	0·6	0·5	4·7	0·6	4·2	8·1
50–63/II p	PTFA	0·4	0·4	2·4	0·4	0·4	8·5
50–63/III	PFA	3·6	11·6	4·5	6·5	3·7	7·5
50–63/IV	TFA	0·4	0·5	5·0	0·4	9·2	10·7

* ml. of 0·1 N-acid/10 ml. culture.

The quantity of thymine required for growth in a folic acid-free medium (PT) was the same for the two strains.

The wild type and most of the drug-resistant strains of *Proteus vulgaris* were able to grow well in the absence of an exogenous source of folic acid, thymine and/or purines. These growth factors neither increased the growth of these strains when added nor reversed the inhibition caused by folic acid antagonists. However, strain 49–210/II possessed an absolute requirement for thymine. The utilization of thymine, thymidine and thymidylate by this strain are shown graphically in Fig. 2. Although this strain was developed in the presence of thymine, both thymidine and thymidylate are used more efficiently than thymine. This is in contrast to the thymine-requiring strains of *L. casei* which use the free base more efficiently than the deoxyribose derivatives. Neither uracil nor cytosine could replace the thymine requirement for 49–210/II.

The *Lactobacillus casei* and *Proteus vulgaris* mutants which required thymine exhibited the phenomenon of 'unbalanced growth' and 'thymineless death' (Cohen & Barner, 1954). This is shown by the data presented in Fig. 3. In the complete PFTA medium, the growth curve for 50–63/II reaches a plateau. In O medium the death rate was similar to that observed with the wild-type strain. But where only thymine was omitted (PFA medium) a precipitous death curve was observed. Microscopic examination of 72 hr cultures of this strain grown in PFA medium showed the classical picture of unbalanced growth with long filamentous cells in PFA medium but normal coccoid rods in PTFA medium. Similar results were obtained with the other thymine-requiring mutants.

Incorporation of [2-^{14}C]-thymine into the DNA of various strains of Proteus vulgaris. In view of the thymine requirement of strain 49–210/II, it was decided

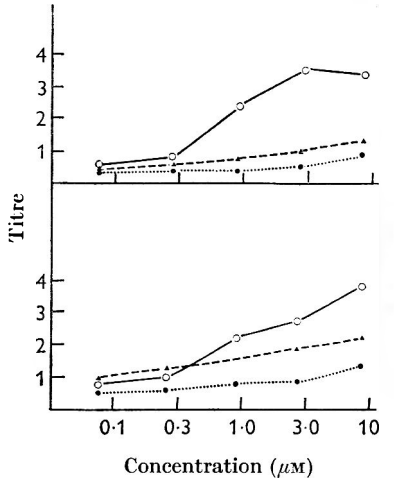

Fig. 1

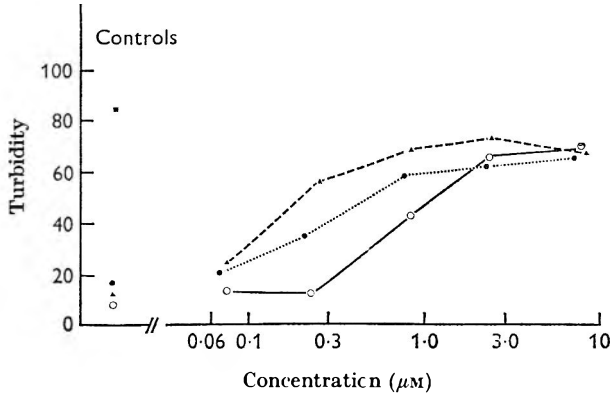

Fig. 2

Fig. 1. Growth of wild and thymineless strain of *L. casei* with thymine-containing metabolites in purine-containing medium. Upper figure, strain 50-63/II. Lower figure, wild type. ○——○, thymine; ▲– – –▲, thymidine; ●- - - - -●, thymidylate. Ordinate, ml. 0·1 N-acid/10 ml. culture.

Fig. 2. Growth of thymineless strain of *Proteus vulgaris* with thymine-containing metabolites. ○——○, thymine; ▲– – –▲, thymidine; ●- - - - -●, thymidylate. Controls for each experiment with strain 49-210/II indicated along ordinate; ■, control growth of wild type. Ordinate, turbidity (100 less the per cent transmission).

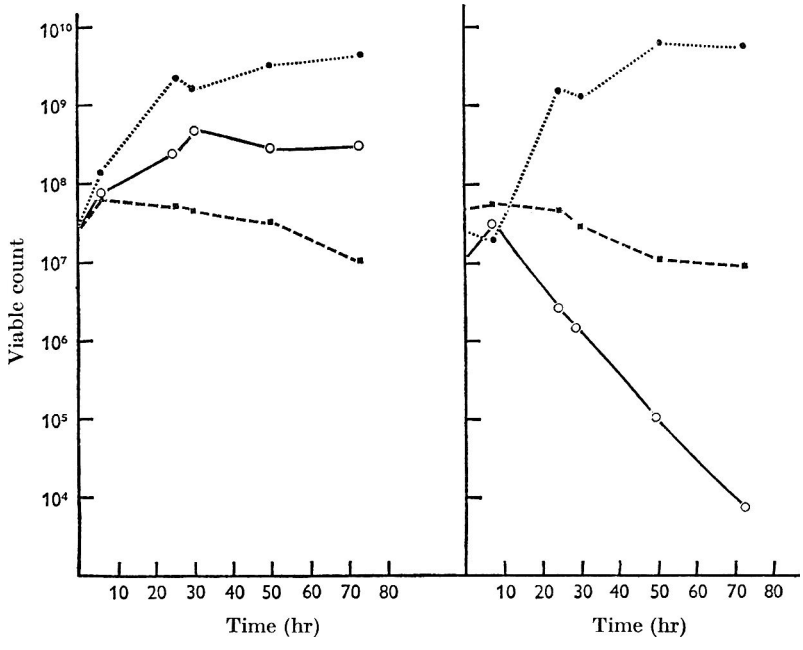

Fig. 3. Viability of wild and thymineless strains of *Lactobacillus casei* in various media. Figure on left, wild type; figure on right, strain 50-63/II. Media: ●- - - - -●, PTFA; ○——○, PFA; ■– – –■, basal. Ordinate, plate count/ml. culture.

to investigate possible alterations in the uptake of [2-^{14}C]-thymine by the Proteus strains (Table 3). Essentially no incorporation of [2-^{14}C]-thymine into the DNA of wild type or strain 49–210/I was found, whereas strain 49–210/II incorporated [2-^{14}C]-thymine without change in its specific activity. In one experiment, the wild type was grown in the presence of quantities of SD and diaveridine which limited but did not entirely suppress, growth, to investigate whether the incorporation of thymine might be an expression of stress. However, no incorporation of thymine resulted. The incorporation of exogenous thymine by the wild type was concentration dependent; but complete suppression of synthesis *de novo* was not attained and at the highest concentration of thymine, the results were erratic and sometimes, as shown in Table 3, a lower incorporation of proffered thymine was found at a concentration of 1 μg./ml. than at 0·3 μg./ml.

Table 3. *Incorporation of thymine during growth*

	Thymine	
Strain	Concn. (μg./ml.)	r.s.a. (%)
Proteus, wild type	0·12	0·09
	0·12	0·015
	0·10	0·036*
Proteus 49–210/I	0·12	0·08
49–210/II	0·12	96·7
Lactobacillus, wild type	0·03	10·3
	0·10	38·0
	0·30	78·0
	1·0	56·3
	0·3	5·3†

r.s.a. Relative specific activity of isolated thymine.
* Grown in the presence of SD and diaveridine each 0·4 μg./ml.
† Thymine added after growth.

Cross-resistance. The effects of antagonists of folic acid and of end products of one-carbon metabolism on wild-type and resistant strains of *Lactobacillus casei* and *Proteus vulgaris* are shown in Tables 4 and 5 respectively. Strains 50–63/I and 50–63/II were found to be resistant to all of the folic acid antagonists tested, including representative diaminoquinazolines, pyridopyrimidines, pteridines, benzyl- and phenylpyrimidines. In contrast, Proteus strains 49–210/I and 49–210/I were as sensitive as the wild strain to pyrimethamine and the quinazoline, although resistant to benzyl-, phenoxy- and pyridopyrimidines. In addition, both strains were hypersensitive to SD. Reciprocally, the SD-resistant Proteus strain SD had an increased sensitivity to the folic acid antagonists, diaveridine and the diaminoquinazoline.

While uracil antagonists such as 6-azauracil and purine antagonists such as 6-mercaptopurine, 8-azaguanine and diaminopurine were equally effective against the wild type and resistant strains of *Lactobacillus casei*, their effects against Proteus strains were more varied. Both diaveridine-resistant strains were more sensitive than the wild type to 8-azaguanine and 2,6-diaminopurine. However, strain 49–210/II was no more sensitive than the wild type to 6-mercaptopurine.

Both strain 49–210/I and 49–210/II were relatively resistant to dithiouracil, but as sensitive as the wild strain to 6-azauracil and isobarbituric acid. In contrast, strains SD and SD/49–210 were generally insensitive to all of the uracil and purine antagonists. The effects of a number of end product analogues on wild type have been reported earlier in studies designed to explore their potential for further potentiation of SD: diaveridine combinations (Elion, Singer & Hitchings 1960).

Table 4. *Cross-resistance studies with strains of Lactobacillus casei*

Strain / Compound	Wild type			50–63/I	50–63/II	50–63/II P
	OFA	TFA	ATFA*	OFA	TFA	ATFA*
	Inhibitory concentration†					
Folic acid antagonists						
Pyrimethamine	0.175	0.25	30	> 300	> 300	> 300
Diaveridine	0·008	0·02	—	> 100	> 100	—
Amethopterin	0·000013	0·028	—	0·001	0·0039	—
2,4-Diamino-6,7-dimethylpteridine	2·3	2·8	—	> 100	> 100	—
6,7-dimethylpyrido-(2,3-d)-pyrimidine	—	0·42	—	—	28	—
6-ethylquinazoline	—	0·10	—	—	100	—
Thymine antagonists						
5-Aminouracil	—	> 100	145	—	21	18
5-Bromouracil	—	> 300	> 300	—	30	60
6-Azathymine	—	> 300	> 300	—	180	> 300
Purine antagonists						
6-Mercaptopurine	23·5	22·0	165	22·6	25	135
8-Azaguanine	—	0·005	0·125	—	0·006	2·4
2,6-Diaminopurine	0·24	0·06	> 300	10·5	0·13	> 500
6-Methylpurine	2·15	—	7·4	7·0	—	245
Uracil antagonist						
6-Azauracil	1·25	1·6	0·33	1·45	2·8	0·28

* ATFA = TFA with addition of 3 μg./ml. adenine sulphate.
† Inhibitory concentration in μg./ml. giving 50 % inhibition.

The thymine-requiring mutants exhibited sensitivities to thymine antagonists greater than those of the wild types. The *Lactobacillus casei* mutants were more sensitive than the wild type to antagonists such as 5-aminouracil, 5-bromouracil and azathymine, while the Proteus mutant, 49–210/II, showed sensitivity to 5-bromouracil, 5-aminouracil, dithiothymine and 2-thiothymine. The effects of the thymine antagonists could be blocked by thymine in the thymineless strains of both *Proteus vulgaris* and *L. casei*.

The increased purine requirement of strain 50–63/II p did not give rise to increased sensitivity to purine antagonists. This is in contrast to the above results with the thymine-requiring strains.

Although the wild type and mutant strains of Proteus do not require purines or pyrimidines for growth (with the exception mentioned), purines (adenine, guanine, xanthine, hypoxanthine) and pyrimidines (uracil, cytosine) block the effects of their respective antagonists (6-mercaptopurine, 8-azaguanine, 6-methylpurine, azauracil and isobarbituric acid). Representative data are given in Table 6.

In order to determine whether sensitivity (or resistance) to diaveridine and SD were related, *Proteus vulgaris* was passaged repeatedly in diaveridine-containing

Table 5. *Cross-resistance with strains of Proteus vulgaris*

Strain	Wild type		49–210/I	49–210/II	SD	SD/49–210
Incubation period (hr)	18	42	18	18	42	42
Compound	Inhibitory concentration†					
Folic acid antagonists						
2,4-Diamino-5-(3′,4′-dimethoxybenzyl) pyrimidine	2·3	4·6	> 100	> 100	1·2	> 300
6-ethylquinazoline	1·4	3·8	6·3	4·7	0·43	14
5-(3′,4′-dimethoxyphenoxy) pyrimidine	4·8	—	> 300	> 300	—	—
5-(3′,4′-dimethoxy-5′-bromobenzyl) pyrimidine	3·9	—	150	400	—	—
5-(3′,4′,5′-trimethoxybenzyl) pyrimidine	0·48	—	210	330	—	—
7-*n*-propylpyrido (2,3-d) pyrimidine	14	—	100	250	—	—
5-*p*-chlorophenyl-6-ethylpyrimidine	40	—	60	41	—	—
Amethopterin	66	—	300	300	—	—
Sulphadiazine (SD)	8·2	30	4·0	4·0	390	150
Thymine antagonists						
5-Aminouracil	34	—	16	13·5	—	—
5-Bromouracil	> 300	—	> 300	11·0	—	—
Dithiothymine	> 300	—	> 300	10·0	—	—
2-Thiothymine	> 300	—	> 300	1·6	—	—
Purine antagonists						
6-Mercaptopurine	> 300	> 300	160	> 300	> 300	> 300
8-Azaguanine	30	100	7	15	300	> 300
2,6-Diaminopurine	> 500	> 500	500	35	> 300	> 300
Thioguanine	300	—	300	> 300	—	—
6-Methylpurine	11	> 300	13	12·5	50	—
Uracil antagonists						
6-Azauracil	1·3	> 100	1·0	2·4	> 100	> 100
Isobarbituric acid	3·7	> 300	3·7	4·0	> 300	—
Dithiouracil	30·0	—	100	> 100	—	—

† Inhibitory concentration in μg./ml. giving 50 % inhibition.

Table 6. *Reversal studies with Proteus vulgaris*

Metabolite (μg./ml.) Guanine	Antimetabolite (μg./ml.) 8-Azaguanine		
	0	100	1000
0	80	33	6
1	76	52	42
10	78	78	78

Uracil*	6-Azauracil				
	0	0·3	1	3	10
0	75	74	35	2	2
1	76	—	—	66	26
3	76	—	—	70	56
10	74	—	—	77	78

* Uridine, uridylic acid, cytosine, cytidine and cytidylic acid gave effects equivalent to those of uracil on a molar basis. The numerical values in the table refer to turbidity as described under Methods.

medium at several concentrations of the drug and the sensitivities of the derived strains to diaveridine and to sulphadiazine are compared with those of wild type Proteus and strains SD/49–210 (Table 7). The diaveridine-resistant strains were all more sensitive to sulphadiazine than the wild strain by a factor of 2 to 3. Conversely, the sulphadiazine-resistant strain was approximately 4 times as sensitive as the wild strain to diaveridine.

Table 7. *Sensitivities of strains of Proteus vulgaris to sulphadiazine and diaveridine*

Strain	Inhibitory concentration* Diaveridine	Sulphadiazine
Wild type	4·2	8·6
49–210/10	14·0	4·6
49–210/30	130·0	3·8
49–210/100	225·0	3·0
49–210/300	> 1000·0	4·7
SD	1·1	530·0
SD/49–210	300·0	300·0

* Inhibitory concentration in μg./ml. giving 50 % inhibition after 42 hr growth.

Table 8. *Effect of combinations of sulphadiazine and diaveridine on growth of Proteus vulgaris mutants*

Strain	Incubated (hr)	Inhibitory concentrations† Sulphadiazine	Diaveridine	Potentiative combinations§ Sulphadiazine f.i.c.	Diaveridine f.i.c.
Wild type	18	2·5	2·2	0·09	0·12
Wild type*	18	3·3	2·5	0·15	0·09
Wild type	42	4·5	5·4	0·18	0·03
49–210/I	18	1·3	300·0	0·27	0·15
49–210/II*	18	1·2	500·0	0·38	0·07
SD	42	105·0	0·82	0·18	0·05
SD/49–210	42	30·0	400·0	0·38	0·12

† Inhibitory concentration in μg./ml. giving 50 % inhibition.

§ f.i.c. fractional inhibitory concentration: = portion of inhibitory concentration required for 50 % inhibition when sulphadiazine and diaveridine are used in combination at optimum ratio.

* Grown in the presence of thymine, 1 μg./ml. of medium.

The potentiation in combinations of diaveridine and sulphadiazine with respect to the growth of wild-type Proteus has been reported previously (Elion *et al.* 1960). Table 8 shows the results of experiments designed to determine the magnitude of the potentiative effects in the resistant strains. When combinations of diaveridine and sulphadiazine were tested, potentiation (as measured by the graphical method previously reported, Elion, Singer & Hitchings, 1954) was still observed, but resistance was reflected in the higher concentrations of drug required to inhibit the growth of the mutants.

DISCUSSION

It is apparent that one can isolate a variety of strains of *Lactobacillus casei* and *Proteus vulgaris* which are resistant to folate antagonists but differ with respect to related biochemical alterations (cf. Hutchison, 1958). The character of the strain one finally obtains depends to a considerable extent on the nutrients which are

available in the medium. Thus the thymine-less mutants could not have been isolated from a medium which lacked thymine, but these prevail when this nutrilite is available. The biochemical changes which accompany resistance to a drug, therefore, reflect the opportunities available and are not determined solely by the nature of the drug.

The reciprocal (collateral) sensitivities between the sulphonamide- and pyrimidine-resistant strains were unexpected. However, this finding is not necessarily inconsistent with the view (Hitchings, 1960, 1961) that these two types of antimetabolite affect steps sequentially arranged on the identical metabolic pathway. When resistance involves altered enzymes, as appears frequently to be the case with sulphonamide resistance (Wolf & Hotchkiss, 1963; Pato & Brown, 1963), and recently has been reported in amethopterin resistance (Sirotnak *et al.* 1963), the reaction would be expected to be less efficient than that catalyzed by the normal enzyme. Such an enzyme might give rise to suboptimum quantities of products, and cells containing it would consequently show increased sensitivity to specific antimetabolites. Such strains might resemble those which were exposed to a metabolite:antimetabolite mixture containing the metabolite in a quantity just sufficient to restore growth. When a second, biochemically related, antimetabolite was introduced into such a system, the organisms were hypersensitive to it, and the effects of the first antimetabolite were nullified completely only when a very high metabolite:antimetabolite ratio was reached (Elion, Singer & Hitchings, 1958; Singer, Elion & Hitchings, 1959).

The end-product-requiring strains may derive some advantage through the sparing of folic acid-containing coenzymes. Whether this involves the deletion of one or more of the one-carbon transferases remains to be determined. In thymine-requiring strains, one would expect a reduction in the total tetrahydrofolate requirement by the amount necessary for thymidylate synthetase. Moreover, dihydrofolate reductase would be spared by the amount required for renewal of the 5,10-methylenetetrahydrofolate that is oxidized each time a molecule of thymidylate is synthesized.

With *Lactobacillus casei*, the mechanisms for the incorporation of exogenous thymine and purine exist in the wild type, since growth can be obtained in the absence of folic acid when these nutrilites are proffered. Its relative inability to use thymidine and thymidylate may reflect a dephosphorylation of thymidylate and a deficiency in thymidine kinase. The wild strain of *Proteus vulgaris*, however, is unable to incorporate exogenous thymine, and thus the drug-resistant mutant appears to have acquired one or more functions which are not present in the parent. Since this mutant uses thymine, thymidine and thymidylic acid essentially equally well, one might speculate that the enzymes necessary for their incorporation exist in the wild type, and that altered permeability permits their entrance into the mutant. With both organisms, the thymine heterotrophs are more sensitive to thymine antagonists than are the parent strains. However, the sensitivities of purine-requiring mutants to purine antagonists were essentially unaltered from those of the wild types.

Thymine-requiring strains of *Lactobacillus casei* (Anton & Nichol, 1959) and *Escherichia coli* (Okada, Yanagisawa & Ryan, 1960) have been obtained from amethopterin-inhibited cultures. However, the difficulties with regard to cellular transport of the structural analogues into folate auxotrophs, and the relative ease

with which the small molecule antagonists are assimilated (Wood, Ferone & Hitchings, 1961) make the latter more useful inhibitors for the majority of micro-organisms.

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Induction of an Anthranilate Oxidation System During the Metabolism of *ortho*-Nitrobenzoate by Certain Bacteria

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SUMMARY

Nocardia opaca, a flavobacterium and certain other bacteria, when grown on *o*-nitrobenzoate, accumulated anthranilate in the medium in the early stages of growth. The subsequent disappearance of this metabolite in growing cultures was always correlated with the appearance of an anthranilate oxidase system in the organisms. This phenomenon has the features of a typical enzyme adaptation except that the inducer is a by-product of the cells' own metabolism; hence it has been termed 'metabolite induction'. The results confirm previous suggestions that, in these micro-organisms, anthranilate is not an obligatory intermediate in the direct energy-producing pathway of *o*-nitrobenzoate breakdown but is produced in a side reaction.

INTRODUCTION

Previous work (Cain, 1958; Cartwright & Cain, 1959*a*, *b*) showed that the *p*- and *m*-aminobenzoic acids are not intermediates on the direct pathway of oxidative degradation of the corresponding nitrobenzoic acid isomers by nocardia species, but are produced by a reductive side reaction which operates concurrently (Cain & Cartwright, 1960*b*). At that time the situation in *Nocardia opaca* with regard to the *o*-isomer was not clear though Ke, Gee & Durham (1959) had found that growth of a flavobacterium on *o*-nitrobenzoate did not sequentially induce the enzymes to oxidize anthranilate. During the metabolism of *o*-nitrobenzoic acid by *N. opaca*, marked variations were often observed in the rates of oxidation of anthranilic (*o*-aminobenzoic) acid, a possible intermediate in the oxidative degradation of the nitro compound. Such variations appeared to depend on the time at which the organisms had been harvested from growing cultures. Closer examination of these changes showed that the bacteria developed the ability to oxidize anthranilic acid as it appeared in the culture medium from reduction of *o*-nitrobenzoic acid, and thereafter lost this ability progressively until it eventually almost disappeared as the anthranilic acid was used up. Oxidative attack on the *o*-nitrobenzoic acid substrate continued during these events. Since *N. opaca* oxidizes both *o*-nitrobenzoic acid and anthranilic acid, after growth on these respective substrates, through catechol (Cartwright & Cain, 1959*a*; Cain & Cartwright, 1960*a*; Cain, 1966), it was obvious that these variations in anthranilic-oxidizing activity would influence the interpretations of results obtained with the 'simultaneous adaptation' (sequential induction) technique (Stanier, 1947). The present paper describes the nature of

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these enzymic variations and adds further evidence for the original contention that in nocardia the aminobenzoates are not obligatory intermediates in the aerobic energy-yielding pathway of nitrobenzoate degradation.

METHODS

The organisms used in this study were strain 06 of *Nocardia opaca* (Cain, 1958); an organism which was isolated by elective methods on anthranilic acid from local soil in Oklahoma and later identified as a nocardia species; the flavobacterium used by Durham (1958); a pseudomonas isolated from soil by elective culture with *o*-nitrobenzoic acid.

All organisms grew equally well with *o*-nitrobenzoic acid or anthranilic acid as the sole source of carbon, nitrogen and energy. The Oklahoma organism differed from the other bacteria used in that it did not produce the deep reddish-brown coloration in the medium characteristic of growth of the other organisms on *o*-nitrobenzoate and anthranilate.

Cultures were grown at 30° with forced aeration (about 2 l. sterile air/min.) through a sintered glass distributor in 4 or 5 l. flasks fitted with a sterile sampling device. The flasks contained the following defined medium (g./l.): organic source, 1·0; KH_2PO_4, 0·5; $MgSO_4.7H_2O$, 0·1; trace elements solution (Barnett & Ingram, 1955) 10 ml./l. medium; in cases where the organic compound did not provide a source of nitrogen $(NH_4)_2SO_4$ (0·5 g./l.) was added to the defined medium. 'Marmite' (a British edible yeast extract preparation) or Difco yeast extract, was added to the medium (0·02 %, w/v) only where indicated. The medium was adjusted to pH 7·0–7·2.

Cultures (5 l.) were started with a 48-hr 100 ml. inoculum grown in Erlenmeyer flasks and after visible growth had begun, samples were removed aseptically at intervals, the organisms harvested by centrifugation at 4°, washed once with ice-cold distilled water and their activity towards anthranilic and *o*-nitrobenzoic acids examined without delay (usually within 1 hr from sampling) in the Warburg respirometer with air as the gas phase. Enzyme activities were expressed as Q_{O_2} (μl. O_2 uptake/hr/mg. dry wt. organism), oxygen uptakes being measured in air at 5 min. intervals from 0 to 30 min. at 30° in flasks containing 200 μmoles phosphate buffer (pH 7·0); 3 μmoles substrate; 0·5 ml. organism suspension (equiv. about 5 mg. dry-wt. organism); 0·2 ml. 20 % KOH in the centre well; total volume to 3·0 ml. with water. Duplicate experimental vessels and a control (no substrate) were run for each sample. The absence of continued enzyme induction in the presence of anthranilic acid in the Warburg vessels was shown by the linear oxygen uptakes observed during the first 20–30 min. In confirmatory experiments possible subsequent enzyme induction was prevented by irradiating the suspensions with ultraviolet radiation or by incubating the washed organisms for 30 min. with D-*threo*-chloramphenicol (10 μg./ml. final concentration) in the Warburg vessels before tipping the substrate. D-*threo*-chloramphenicol did not alter the existing degree of activity of the anthranilate-oxidizing system up to a concentration of 20 μg./ml. All three methods gave similar results. Dry-weights of organisms were the mean of duplicate 3 ml. samples of suspensions dried at 105° to constant weight on aluminium planchets. The manometric method was used to ensure a measure of 'anthranilic

oxidase' (Higashi & Sakamoto, 1960) in organisms where anthranilic acid may also be converted to other products not involved in the oxidative pathway (Cain & Cartwright, 1960*b*).

Anthranilic acid was estimated by diazotization (Glazko, Wolf & Dill, 1949) and *o*-nitrobenzoic acid by the quantitative chromatographic method used originally with the *p*-isomer (Cain, 1958). Where *o*-nitrobenzoic acid and anthranilic acid were present together in similar amounts, their respective concentrations could be calculated from their molecular extinctions at 268 and 240 mμ by solving the simultaneous equations provided by direct measurement, on suitably diluted samples, of the extinctions of the test solution at these wavelengths. At pH 7·0 in 0·1 M-phosphate buffer, the values are: *o*-Nitrobenzoic acid: at 240 mμ, $\epsilon = 2185$; at 268 mμ, $\epsilon = 3120$. Anthranilic acid: at 240 mμ, $\epsilon = 4505$; at 268 mμ, $\epsilon = 310$. *Nocardia opaca* did not reduce the aromatic nitro group of chloramphenicol (Cartwright & Cain, 1959*b*; and unpublished results) so the presence of this compound in culture media did not affect estimations of arylamines by the diazotization procedure.

The growth of cultures was measured turbidimetrically at 530 mμ with the Bausch and Lomb 'Spectronic 20' or the Beckman DU spectrophotometer; extinction readings were calibrated against dry-weight measurements for the appropriate organisms where required. Because the growth of *Nocardia opaca* and the flavobacterium results in the production of a deep reddish-brown coloration of the medium, it was necessary to observe the precautions detailed previously (Cain, 1958) in making extinction measurements in cultures of these organisms. The approximate generation times of these organisms in *o*-nitrobenzoic acid medium supplemented with yeast extract were: *N. opaca*, 3·5 hr; the flavobacterium, 1·5 hr; the Oklahoma isolate, 3·8 hr, the pseudomonad, 1·5 hr.

Ultraviolet irradiated organisms were obtained by suspending the organisms (in water) as a shallow layer in a flat-bottomed Petri dish and exposing at 15 cm. from a General Electric germicidal U.V. lamp (30 watts; predominant wavelength 254 mμ) for 5 min.

The fluctuation test of Luria & Delbrück (1943) was modified to suit the present system as follows. Fifteen test-tubes (15 × 230 mm.) were set up, each containing 1 ml. of the defined medium with *o*-nitrobenzoic acid as the only organic substrate. A large boiling tube and a 100 ml. Erlenmeyer flask each contained 15 ml. of the same medium, the tube and flask diameters being so chosen that the depth of medium therein was similar to that of the 1 ml. samples. After sterilization and cooling, all these tubes and flasks were inoculated with 5×10^3 *Nocardia opaca* organisms/ml. previously grown on *o*-nitrobenzoic acid medium, harvested in the log phase of growth and washed twice with sterile distilled water using aseptic precautions. The culture tubes were then incubated for 24–48 hr at 30° on a reciprocating shaker (throw 8 cm.; shake speed 94 strokes/min.) to increase aeration. Non-aerated cultures gave very erratic growth. At the end of the incubation period the contents of the 1 ml. test tubes were poured and spread on plates of anthranilic acid defined medium solidified with 2% (w/v) washed agar. The plates of anthranilic acid medium were incubated at 30°, the appearance of resulting colonies being observed at intervals thereafter. Additional 1 and 15 ml. cultures were always run for determination of anthranilate formation and for total viable count made on suitable dilutions on nutrient agar.

When the concentration of anthranilic acid, arising from *o*-nitrobenzoic acid reduction during growth of *Nocardia opaca* in the culture tubes, became appreciable before plating on anthranilic acid medium, however, the number of colonies which appeared on the plates was not a true measure of the possible anthranilic acid-utilizing mutants originally occurring in the *o*-nitrobenzoate culture. Such mutants, if they exist, would have already been undergoing selection and growth in the anthranilic acid-containing environment. Cultures were always plated, therefore, before appreciable amounts of anthranilic acid had appeared. In the experiments described, the anthranilic acid was always below 2 μg./ml. (14 μM) at which concentration development of an anthranilate oxidase was never observed in growing cultures. In the experiment illustrated in Table 4, this concentration was less than 0·5 μg./ml. (3·5 μM).

Replica plating from cultures on nitrobenzoate medium to anthranilate medium was done by the velvet pad method of Lederberg & Lederberg (1952). Replication was made from the master plate while the colonies were barely visible.

The chemicals used were of commercial origin. Technical grade anthranilic acid was decolourized with activated charcoal, and re-crystallized three times from ethanol + water before use in growth media or manometric experiments. The *N*-methylanthranilic acid and the *o*-aminobenzensulphonic acids (supplied by Eastman Organic Chemicals) were also recrystallized, after charcoal treatment, to yield off-white crystals. In spite of several recrystallizations from ethanol + water and ethyl acetate + benzene, *N*-methylanthranilic acid was not completely freed from anthranilic acid, as shown by chromatography and colorimetric analysis; 1 mg. of recrystallized *N*-methylanthranilic acid still contained 6·1 μg anthranilic acid as determined by the Bratton-Marshall procedure (Glazko *et al.*, 1949); since the primary amino group is alkylated, the *N*-methylanthranilic acid does not react in this test.

Chromatography of the nitro- and amino-benzoic acids was done by the ascending technique on Whatman No. 1 paper with the following solvent systems (all proportions by volume); (A) ethanol + ammonia (sp.gr. 0·88) + water (80 + 4 + 16); (B) *n*-butanol + glacial acetic acid + water (4 + 1 + 5). The compounds were detected under u.v. radiation, anthranilic acid and its derivatives by their blue fluorescence and *o*-nitrobenzoic acid by its quenching. D-*threo*-chloramphenicol and the biologically inactive L-*threo* isomer were the products of Parke Davis & Co. and were provided by Dr E. A. Grula.

Nomenclature. The anthranilate oxidizing system in intact organisms will be referred to in this paper as 'anthranilate oxidase'. The term oxidase is nowadays strictly used only for enzymes catalysing a reaction where oxygen is an acceptor of protons from the substrate. Taniuchi *et al.* (1964) have shown that the overall reaction in a pseudomonad is

$$\text{anthranilate} + O_2 + \text{NADH} + H^+ \rightarrow \text{catechol} + CO_2 + \text{NAD}^+ + NH_3$$

in which both oxygen atoms have actually entered the molecule. They have given their partially pure enzyme the trivial name anthranilic hydroxylase. For brevity and to conform with previous descriptions of impure systems (e.g. Higashi & Sakamoto, 1960) 'anthranilate oxidase' is used here to describe the system which metabolizes anthranilate with the simultaneous utilization of oxygen.

RESULTS

Variations in enzyme concentration in organisms grown with o-nitrobenzoic acid

Batches of yeast extract-enriched *o*-nitrobenzoate media (5 l.) at 30°, inoculated with 50 ml. of a log phase culture of *Nocardia opaca*, were sampled at 3-hr intervals after visible growth had appeared (12–15 hr), and the Q_{O_2} of the organisms with anthranilate as substrate and the concentration of this metabolite in the medium were measured. Figure 1 shows that after an initial increase in anthranilate concentration, the subsequent disappearance of anthranilate was correlated with the appearance of a marked ability of the organisms to oxidize this substrate and also that the anthraniliate oxidizing activity decreased as anthranilate disappeared. Such a pattern was repeatedly confirmed with this organism. A similar ready loss of the inducible enzyme system for *o*-nitrobenzoate oxidation in *N. opaca* (Cain, 1958) also occurred upon the exhaustion of the available substrate. It is obvious that in this particular case, the time of sampling would appreciably alter the conclusions to be drawn from sequential induction experiments regarding the significance of anthranilic acid as an intermediate in *o*-nitrobenzoate acid oxidation. In contrast, organisms grown on succinate showed a typical adaptive response to both anthranilate and *o*-nitrobenzoate, irrespective of the time of harvesting. Both the time of appearance and the maximum levels of the induced anthranilate oxidase varied in different experiments according to size of inoculum and aeration rate but anthranilate oxidase rarely appeared before 22–24 hr; the peak of the activity occurred between 33 and 40 hr after inoculation with *N. opaca*.

The variations in enzyme concentration in the four bacteria tested were similar. *Nocardia opaca* and the Oklahoma isolate generally showed a maximum Q_{O_2} of 35–40 for the metabolite-induced anthranilate oxidase but occasional maximum values as low as 15 were recorded. The flavobacterium also showed an adaptive response as a result of the very marked decrease in anthranilic acid concentration at 10 hr after inoculation; but the maximum Q_{O_2} values were somewhat lower (about 30–32; Fig. 2), and as the growth rate was much higher than that of the nocardia, these changes occurred earlier. The pseudomonad gave a similar result.

If the appearance of anthranilate oxidase in growing cultures is actually related to the presence of anthranilate in the medium, the corollary is that in the absence of this substrate, no anthranilate oxidase should develop. A possible reason for the decrease in the accumulation of anthranilate in the medium was given by observations that in shaking Warburg flasks where organisms were oxidising *o*-nitrobenzoate, and in shake cultures of *Nocardia opaca* growing on *o*-nitrobenzoate where aeration was very good, negligible accumulation of anthranilate occurred.

When the aeration rate of growing cultures of *Nocardia opaca* was raised from 2 to 5 and then to about 7 l. air/min. (anti-foam was necessary at these higher aeration rates when growth became appreciable) there was a substantial decrease in anthranilate accumulation and the enzyme did not form in such cultures (Table 1). The minimum concentration of anthranilate in the medium required to induce the enzyme was equivalent to about 5–6 μg. anthranilic acid/ml. At 2–5 μg./ml. no formation of the oxidase was ever observed; above 8 μg./ml. the enzyme always appeared. Between the values 5–8 μg./ml. the response was variable and the

degree of maximal enzyme activity, when present, was usually low (i.e. Q_{O_2} of less than 15). The growth rate was near maximal with aeration rates of about 2 l. air/min. and did not appreciably alter at higher rates; the variations in anthranilate production were thus not attributable to change in growth rate or yield.

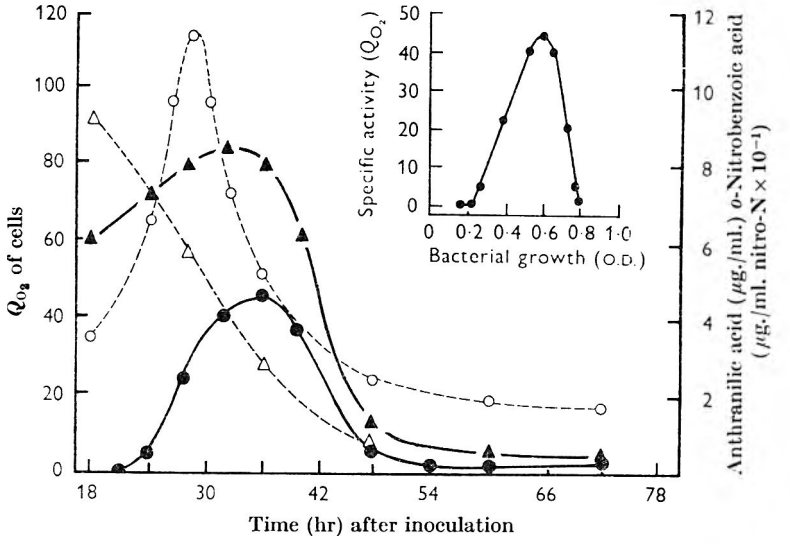

Fig. 1. Changes in enzyme activity with substrate concentration during growth of *Nocardia opaca* on *o*-nitrobenzoate. Enzyme activity represented by solid lines, substrate concentration by broken lines. △, *o*-nitrobenzoic acid; ○, anthranilic acid; ▲, *o*-nitrobenzoate oxidizing system; ●, anthranilate oxidase.

Inset. A plot of the 'differential rate of enzyme formation' (Monod, Pappenheimer & Cohen-Bazire, 1952) for anthranilate oxidase from the same experiment.

Table 1. *Effect of aeration rate on the anthranilic acid accumulation produced by Nocardia opaca in o-nitrobenzoate media*

Aeration rate* (air l./min.)	Mean observed maximum concentration of anthranilic acid (μg./ml.)	Anthranilate oxidase formed
1	12·3 (5)	Yes
2	11·7 (20)	Yes
3·5	5·5 (7)	Variable
5	3·8 (3)	No
7 (approx.)	2·3 (2)	No

* Aeration rates were measured with a precision bore flowrator tube calibrated by and loaned from the Research Division of Continental Oil Company, Ponca City, Oklahoma, U.S.A.

The figures in parentheses in column 2 are the number of experiments done at this aeration rate.

Some properties of the adaptive response

Experiments were done with *Nocardia opaca* to examine the development of the anthranilate oxidizing system after addition of exogenous anthranilic acid (30 μg./ml. final concentration) at 18 hr to cultures growing on *o*-nitrobenzoate. From

negligible amounts of the anthranilate oxidase at that time, there was a very rapid appearance of the enzyme system (sometimes up to 100-fold increase within 90 min.) but this appearance was completely prevented by the simultaneous addition of D-chloramphenicol (5 μg./ml. final concentration) with the inducer (Fig. 3). At this concentration of chloramphenicol, growth still continued, but at about 20% of the normal rate; that is, the chloramphenicol appeared to inhibit preferentially the synthesis of induced enzyme as compared with general cell synthetic mechanisms. Such preferential inhibition of induced enzyme synthesis by chloramphenicol and some other antibiotics has been shown with the β-galactosidase

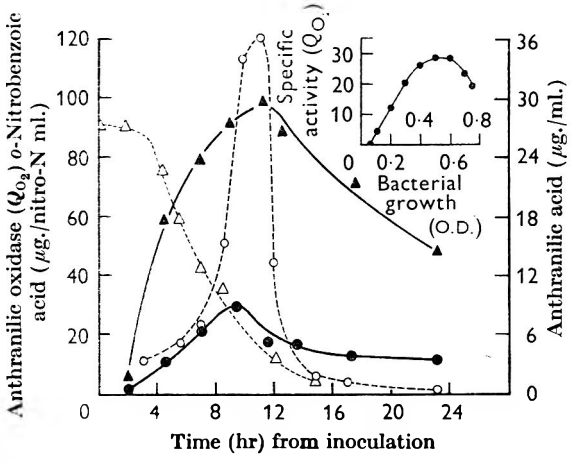

Fig. 2

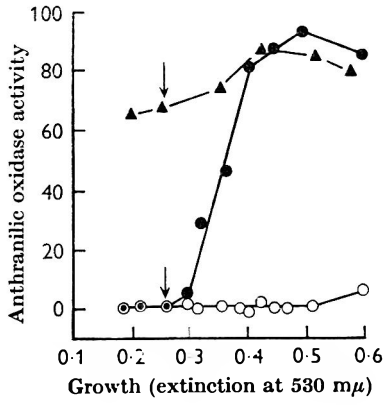

Fig. 3

Fig. 2. Changes in enzyme activity with substrate concentration during growth of a flavobacterium on *o*-nitrobenzoate. Symbols as in Fig. 1.

Inset. A plot of the 'differential rate of enzyme formation' for anthranilic oxidase in the flavobacterium from the same experiment.

Fig. 3. The induction of anthranilate oxidase in *Nocardia opaca* by exogenous anthranilate and its inhibition by chloramphenicol.

Three 5-l. cultures (two, A and B, of *o*-nitrobenzoate and the third, C, of anthranilate media) were incubated for 18 hr. Sodium anthranilate (inducer; 90 mg. in 25 ml.) was then added to all three cultures (zero time) followed immediately by 100 ml. chloramphenicol to cultures A and C and 100 ml. water to culture B (control). The final concentration of chloramphenicol in the media was 5 μg./ml. Growth and anthranilate oxidase activity was followed in all three cultures.

The differential rate of anthranilic oxidase formation after adding inducer and ●, no antibiotic to B; ○, +chloramphenicol to A; ▲, +chloramphenicol to C. Inducer added at (↓).

system (Sypherd, Strauss & Treffers, 1962), where the antibiotics act by inhibiting the inducer-promoted synthesis of messenger-RNA (Sypherd & Strauss, 1963). Aronson & Spiegelman (1961) showed, however, that D-chloramphenicol suppressed all protein synthesis when present in a sufficiently high concentration. Organisms previously grown in the presence of anthranilate, however, showed high values of anthranilate oxidase (Q_{O_2} of 66) even after addition of chloramphenicol (Fig. 3). The 'differential rate of enzyme synthesis' (Monod, Pappenheimer & Cohen-Bazire, 1952) was plotted, to exclude the possibility that the decreased rate of growth in

the presence of D-chloramphenicol was responsible for the non-appearance of anthranilate oxidase in growing cultures. Chloramphenicol produced a genuine suppression of enzyme formation (Fig. 3).

Metabolite induction

The adaptive nature of the appearance of the anthranilate oxidase was confirmed in growing cultures of *Nocardia opaca* where D-chloramphenicol (5 μg./ml.), but no exogenous anthranilic acid, was added at 24 hr. This concentration of chloramphenicol completely prevented the appearance of the self-induced anthranilate oxidase (Fig. 4) although the concentration of anthranilic acid (8·5 μg./ml.) at

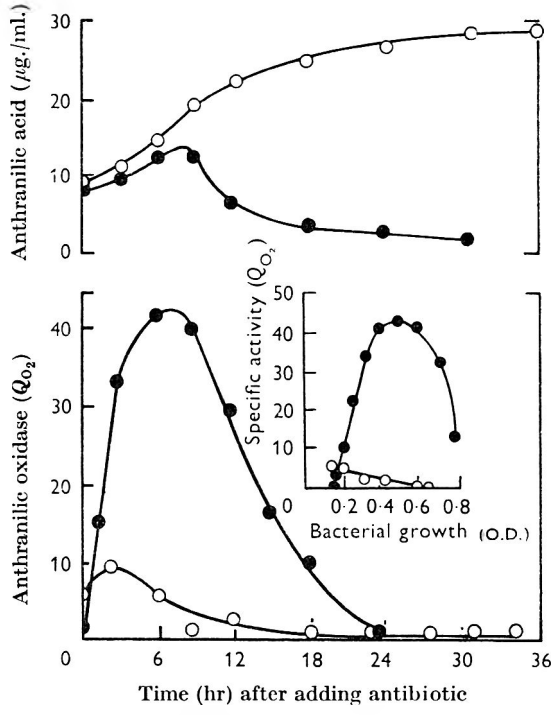

Fig. 4. Inhibition by chloramphenicol of the appearance of anthranilate oxidase induced by metabolically formed anthranilate. Chloramphenicol (final concentration 5 μg./ml.) was added to one of two similar 3-l. cultures of *Nocardia opaca*, incubated for 24 hr on *o*-nitrobenzoate medium. To the other culture was added water (control). Anthranilic acid concentrations in the medium, growth and anthranilate oxidase activities of the organisms were followed in both cultures.

The upper graph shows anthranilic acid concentrations in: ●, control culture; ○, with chloramphenicol. The lower graph shows anthranilate oxidase values in: ●, control culture; ○, with chloramphenicol, in the same experiment.

The inset to the lower graph shows the differential rate of anthranilate oxidase formation: ●, in the absence and ○, in the presence of chloramphenicol, from the same experiment.

the time of antibiotic addition was quite sufficient to induce the enzyme (compare Fig. 1). A third culture containing L-chloramphenicol showed a response similar to that of the control experiment. In the absence of anthranilate oxidase in the D-

chloramphenicol-treated culture there was no removal of accumulating anthranilic acid; the concentration of this metabolite therefore continued to increase to high values (25–30 μg.ml.) never attained with the nocardia under normal conditions. Calculation of the differential rate of enzyme formation showed that this was a true effect and not due to changes in growth rate. When bacteria from such a culture were now harvested aseptically, washed free from chloramphenicol and re-inoculated to fresh chloramphenicol-free medium at 30° into which an amount of anthranilic acid equivalent to that existing in the original culture had been introduced, the anthranilate oxidase began to reappear within 2 hr and the concentration of anthranilic acid then decreased rapidly. When D-chloramphenicol (to 5 μg./ml.) was now again added to this fresh culture some 90 min. after induced anthranilate oxidase had appeared, further production of the enzyme ceased but the existing degree of activity was sufficient to prevent more accumulation of anthranilic acid.

Table 2. *The increase in the amounts of anthranilate oxidase in washed Nocardia opaca after the addition of various nitrogen sources*

Oxygen uptake per 15 min. was measured at intervals from zero time to 3 hr in duplicate experimental and one control flask for each of the additions, in the presence of excess sodium anthranilate. Control flasks contained no sodium anthranilate. During the 15 min. period, the uptake was recorded every 3 min. to compute Q_{O_2} values. Flasks contained: phosphate buffer (pH 7·0) 200 μmoles; sodium anthranilate (side-arm), 100 μmoles; washed suspension of organisms grown with *o*-nitrobenzoate, equiv. to 16·2 mg. dry-wt.; nitrogen source, 6 μmoles, or in the case of Marmite, 12 mg. Chloramphenicol was added to 5 μg./ml. Incubation temp. 30°.

There was a very small increase in cell mass (about 3 %) during the experiment as shown by extinction measurements at zero time, 90 min. and 3 hr.

Additions	Increase in anthranilate oxidase above zero time value* at times shown (hr)		
	1	2	3
None	0	3·5	10·9
D-Chloramphenicol	0	0·3	0·5
Ammonium sulphate	0·7	5·2	11·9
L-Asparagine	3·0	10·0	20·2
L-Asparagine + D-chloramphenicol	0	0·6	0·3
L-Glutamic acid	5·2	13·5	35·6
L-Glutamic acid + D-chloramphenicol	0	0·5	0·3
L-Glutamic acid + L-chloramphenicol	5·0	12·0	33·0
Marmite (a yeast extract preparation)	0	4·7	21·9

* The zero time value for anthranilate oxidase in the eight experimental conditions was $0·6 \pm 0·3$.

Similar experiments were done with washed suspensions of *Nocardia opaca* harvested at times when the anthranilate-oxidizing activity was absent or minimal. The adaptation of these suspensions to added anthranilic acid again occurred in 90–120 min. but the rate of appearance and final amounts of enzyme were markedly increased in the presence of a nitrogen source (Table 2); L-glutamic acid gave the best results. There was only a very small increase in cell mass during these experiments. The induced anthranilate oxidase had an optimum in intact organisms at pH 7·5.

The growth medium for these experiments contained yeast extract; ammonia also appeared as a metabolic product (Cain, 1958; Cartwright & Cain, 1959*a*). Both of these N-sources might be held responsible for the rapid appearance of the anthranilate oxidase and the high concentrations of this enzyme which developed as the result of the metabolic production of anthranilic acid in growing cultures

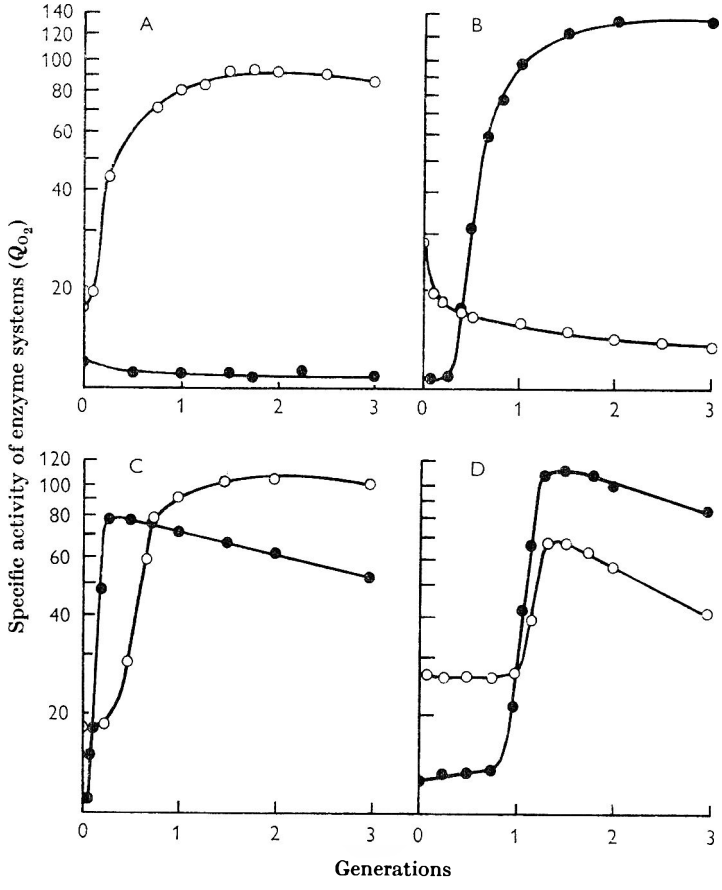

Fig. 5. Changes in enzyme content when *Nocardia opaca* was grown in different environments. Organisms were transferred aseptieally from an *o*-nitrobenzoate culture (entering the stationary phase) to fresh medium containing: A: 10 mM-sodium *o*-nitrobenzoate; B: 10 mM-sodium anthranilate; C: 5 mM-sodium anthranilate. Immediately after adaptation to anthranilate, sodium *o*-nitrobenzoate, to 5 mM, was added; D: 2 mM-sodium *o*-nitrobenzoate +8 mM-sodium anthranilate. Growth and enzyme activity were followed as described in methods. ○, *o*-nitrobenzoate oxidizing system; ●, anthranilate oxidase.

(see Fig. 1). An almost identical response, however, was given in minimal medium where both the Q_{O_2} of organisms oxidizing anthranilic acid and the concentrations of this metabolite formed from *o*-nitrobenzoic acid were similar to those found in Marmite-enriched media.

Variations in enzyme content with change of substrate

The most convincing evidence for the truly inducible nature of the utilization of anthranilic acid was provided by experiments in which changes of enzyme content were measured in *Nocardia opaca* organisms transferred from *o*-nitrobenzoate medium to different regimens (compare Kornberg, Collins & Bigley, 1960; Kornberg & Morris, 1962). The results are shown in Fig. 5. When organisms from an *o*-nitrobenzoate culture just entering the stationary phase was transferred to fresh *o*-nitrobenzoate (10 mM) medium, the specific activity of the *o*-nitrobenzoate-oxidizing system increased some fourfold before one cell division, after a brief growth lag of less than 30 min. and continued at this high level (Fig. 5A), whereas the anthranilate oxidase remained uniformly negligible throughout three generations. In contrast, transfer of *o*-nitrobenzoate-grown organisms to anthranilate (10 mM) medium (containing no yeast extract) resulted in a growth lag of some 8–10 hr during which time there was a 50-fold increase of anthranilate oxidase in the organisms before growth began again. The amounts of the existing *o*-nitrobenzoate-oxidizing system decreased concomitantly (Fig. 5B).

In a third experiment (Fig. 5C) *o*-nitrobenzoate-grown organisms were transferred to 5 mM-anthranilate medium. After adaptation to anthranilate had occurred (a 40-fold increase in anthranilate oxidase before one cell division) sodium *o*-nitrobenzoate was added to the culture to 5 mM. There was an immediate increase in the *o*-nitrobenzoate-oxidizing system from the previously stationary value and concurrently with this increase a slow decrease in the amount of anthranilate oxidase.

When organisms were transferred from *o*-nitrobenzoate medium to fresh medium containing 2 mM-*o*-nitrobenzoate+8 mM-anthranilate, the existing amounts of the *o*-nitrobenzoate-oxidizing system were maintained for the first generation while the nitrobenzoate substrate was being consumed (Fig. 5D). The anthranilate oxidase value also remained unchanged until near the end of this period but was followed by a rapid increase (17-fold) within half a generation. Chromatography of culture fluids showed that this enzyme increase occurred before the *o*-nitrobenzoate content of the medium was exhausted (at about 1·5 generations). Previous work has shown that *o*-nitrobenzoic acid and anthranilic acid are mutual competitive inhibitors of both growth and substrate oxidation in *Nocardia opaca* (Cain, 1966); growth and hence *o*-nitrobenzoic acid utilization in this experiment, where the initial ratio of *o*-nitrobenzoate to anthranilate concentrations was 1 to 4, was consequently slow. The much smaller (two-fold) increase in the *o*-nitrobenzoate-oxidizing system occurred as the growth rate began to increase near the exhaustion of the *o*-nitrobenzoate in this system and immediately after the organisms had adapted to anthranilate. The reason for this small increase is not known but the sequence of events was consistent in media with and without yeast extract. The experiment provides additional evidence for the capacity of *N. opaca* to form adaptive enzymes to a new substrate even while an existing system is functioning; such a phenomenon is uncommon (Oda, Yamomoto & Suda, 1951; Midgeley & Hinshelwood, 1962) if not hitherto unknown (Spiegelman & Dunn, 1947).

Inability of analogues to induce anthranilate oxidase

The anthranilate oxidase of *Nocardia opaca* was induced in organisms grown with *o*-nitrobenzoate only by the metabolic formation of anthranilate and its accumulation in the medium, or by adding this substrate to growing cultures or washed suspensions. The enzyme was not induced in washed organisms within 8 hr by *N*-methylanthranilic acid, *o*-aminobenzenesulphonic acid, 3- or 5-hydroxyanthranilic acids, *o*-aminophenol or benzoic acid, nor by *o*-nitrobenzoic acid itself. In washed suspensions derived from cultures grown on succinate, an identical picture was obtained.

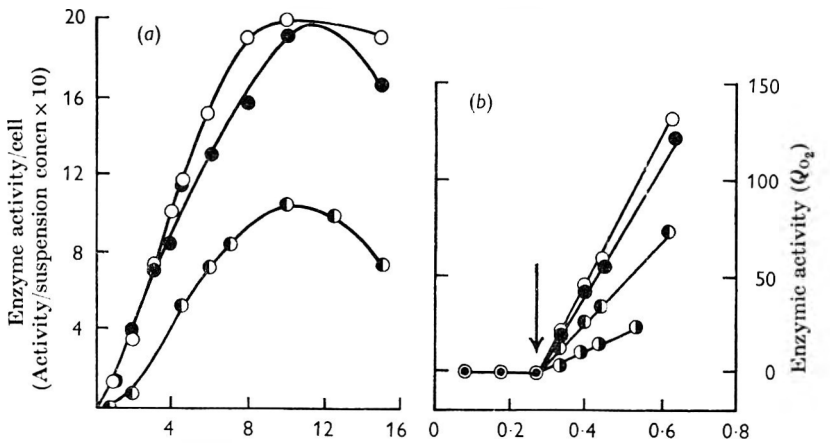

Fig. 6. Annullment by L-phenylalanine of the *p*-fluorophenylalanine inhibition of anthranilate oxidase formation in cultures of *Nocardia opaca* growing on *o*-nitrobenzoate.

(*a*) Three similar 5 l. cultures were incubated for 12 hr. Sodium anthranilate (to 5 mM) was added to all three cultures and then *p*-fluorophenylalanine (to 0·2 mM) was added to one, *p*-fluorophenylalanine (0·2 mM) + L-phenylalanine (1 mM) to the second, and water (equivalent volume) to the third. Growth and anthranilate oxidase activity was then followed in each of the cultures. ○, control culture (water added); ●, *p*-fluorophenylalanine + L-phenylalanine added; ◐, *p*-fluorophenylalanine only added.

(*b*) Differential rate of anthranilate oxidase synthesis in four 5 l. cultures of *Nocardia opaca* growing on *o*-nitrobenzoate. Anthranilic acid (5 mM) was added where indicated by the arrow, and ○, no additions; ●, *p*-fluorophenylalanine (0·2 mM) + L-phenylalanine (1 mM); ◐, *p*-fluorophenylalanine (0·2 mM) only; ◑, *p*-fluorophenylalanine (0·5 mM) only. In this experiment, the activity is recorded only during its increase; as in (*a*), amounts eventually fall. All concentrations represent final values in the culture medium.

The effect of some inhibitors on induced enzyme formation

As in growing cultures (Fig. 3) D-chloramphenicol at 5 μg./ml. or higher prevented the appearance of the anthranilate oxidizing system in washed suspensions of *Nocardia opaca* harvested from *o*-nitrobenzoate media, even in the presence of a stimulatory N-source (Table 2); L-chloramphenicol had no such inhibitory effect. A complete failure to develop the enzyme in washed suspensions under such conditions was also caused by 1 or 5 mM-*p*-fluorophenylalanine and by 5 mM- (but not 1 mM), *β*-2-thienylalanine. The fluorophenylalanine inhibition at 1 mM in these experiments was annulled by 2 mM-L-phenylalanine when added simultaneously

with the inhibitor (compare Halvorson & Spiegelman, 1952). The suppression of anthranilate oxidase induction by 0·2 mM-*p*-fluorophenylalanine and its annullment by added L-phenylalanine (1·0 mM) was particularly evident in growing cultures of *N. opaca* (Fig. 6). Above 1·0 mM-*p*-fluorophenylalanine, growth was so slow that variations of enzyme activity with cell mass were difficult to measure.

Because synthesis of new enzyme protein is an energy-requiring process some general inhibitors of energy-producing systems (dinitrophenols, azide, arsenate; Monod, 1944; Reiner, 1946; Spiegelman, 1947; Monod & Cohn, 1952; Cohen & Monod, 1957) were examined for their effects upon anthranilate oxidase induction.

Table 3. *Inhibition by 2,4-dinitrophenol and sodium azide of the induction of anthranilate oxidase in washed Nocardia opaca grown on o-nitrobenzoate*

Incubation flasks contained: phosphate buffer (pH 7·0) 1 m-mole; bacterial suspension (equiv. to 184 mg. dry-wt.); anthranilic acid, added at zero time, 50 μmoles; inhibitors to final concentrations as shown; water to total volume of 100 ml. These flasks were incubated at 30° with shaking; 2 ml. samples were taken at intervals for determination of anthranilic oxidase and respiratory activity on *o*-nitrobenzoate (after centrifuging and washing the cells) as described in Methods.

Expt.	Inhibitor added	Concentration (mM)	Amounts of anthranilate oxidase and respiration rate (μl./hr on *o*-nitrobenzoate) after addition of inducer at zero time time (hr) 0	2	4
1	None	—	1·5 (190)	4·0 (130)	20·5 (105)
	2,4-Dinitrophenol	0·01	1·5 (195)	5·0 (130)	22·0 (110)
		0·1	1·5 (195)	3·9 (135)	20·0 (100)
		0·2	1·5 (180)	3·7 (120)	14·3 (94)
		0·5	1·5 (160)	3·2 (122)	7·0 (85)
		0·8	1·5 (130)	2·5 (94)	4·0 (60)
		1·0	1·5 (80)	2·5 (32)	4·0 (20)
		5·0	1·5 (45)	0 (10)	0 (0)
2	None	—	2·3 (210)	7·0 (165)	20·5 (100)
	Sodium azide	0·1	2·3 (213)	9·3 (160)	20·8 (88)
		0·5	2·3 (200)	4·5 (150)	12·8 (90)
		2·0	2·3 (173)	3·8 (158)	7·8 (80)
		3·0	2·3 (190)	2·1 (143)	3·0 (75)

The figures in parentheses are the initial oxygen uptakes on o-nitrobenzoate (3 μmoles) corrected for endogenous respiration at the same inhibitor concentration.

2,4-Dinitrophenol at 0·1 mM was without effect on the induction of anthranilate oxidase in washed suspensions of *Nocardia opaca*, but at 5 mM it completely suppressed such induction and markedly decreased the respiration of washed organisms on *o*-nitrobenzoate. At intermediate concentrations of 2,4-dinitrophenol (0·2–0·8 mM), the adaptive response was suppressed without significantly depressing the respiration on *o*-nitrobenzoate; a similar result was given by 2 and 3 mM-sodium azide (Table 3). The decrease in respiration rate of the samples after increasing incubation times in the absence of *o*-nitrobenzoate was examined in the experiments on 'de-adaptation' (see Fig. 8 and later).

The anthranilic acid analogues *N*-methylanthranilic acid and *o*-aminobenzenesulphonic acid also decreased growth upon, and inhibited oxidation of, anthranilic and *o*-nitrobenzoic acids by *Nocardia opaca* and the flavobacterium (Cain &

Durham, unpublished). *N*-Methylanthranilate, although without appreciable effect on the amounts of anthranilate which accumulated in 5 l. cultures of *N. opaca* growing on *o*-nitrobenzoate, strongly suppressed the induction of anthranilate oxidase by this metabolite (Fig. 7).

Induction of the anthranilate oxidase in washed organisms was prevented by u.v. irradiation of the suspension and no induction took place when organisms were incubated with the inducer in anaerobic (N_2 or H_2) atmospheres.

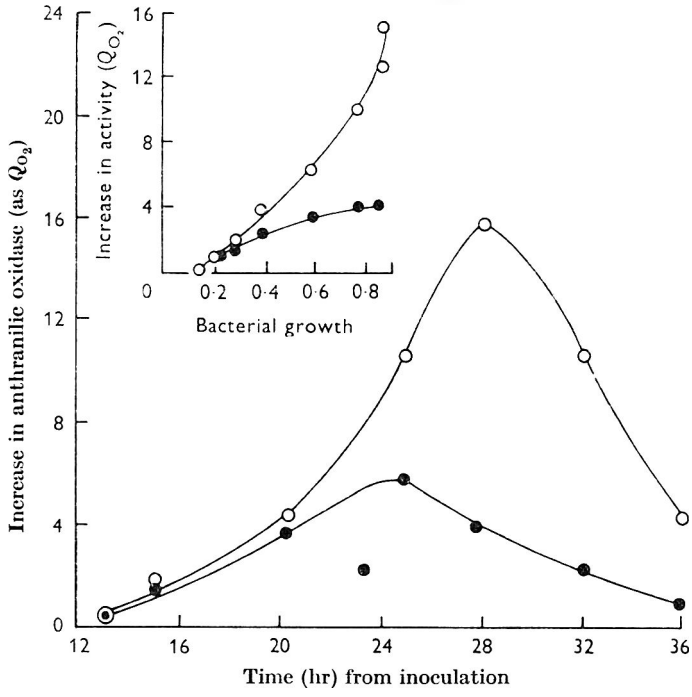

Fig. 7. The effect of *N*-methylanthranilic acid on induction of anthranilate oxidase in *Nocardia opaca* growing on *o*-nitrobenzoate.

Two similar 3 l. cultures of *N. opaca* were incubated at 30° for 12·75 hr after inoculation, and samples of each taken for measurement of growth and anthranilate oxidase activity. To one culture was added *N*-methylanthranilic acid solution (adjusted to pH 7·0) to final concentration 4 mM; an equivalent volume of distilled water was simultaneously added to the other (control). Growth and anthranilate oxidase activity measurements were continued at intervals thereafter. ○, Anthranilate oxidase increase in control culture; ●, anthranilate oxidase increase in culture treated with *N*-methylanthranilate.

Inset. Increase in enzyme activity plotted as a function of growth. (Increases in enzyme activity above that at 12·75 hr were used here to show the effect of the inhibitor more clearly. At this time, just before the addition of inhibitor, the actual values of existing anthranilate oxidase (Q_{O_2}) were 7·8 in the control culture and 10·3 in the experimental culture.)

Additional evidence for the inducible nature of the anthranilate oxidase in Nocardia opaca

The features of the appearance of anthranilate oxidase so far described are those characteristic of induced enzyme synthesis in response to the presence of an inducer but the possibility remained that some of the observations made with growing cultures were the result of the rapid selection of existing mutants. The

ability of organisms grown on *o*-nitrobenzoate to utilize anthranilate was therefore examined by the fluctuation test and by a replica plating technique (see Methods).

In the fluctuation test, the contents of the 1 ml. tubes and 1 ml. samples of the 15 ml. cultures were plated on anthranilate medium (containing no yeast extract) incubated at 30° and inspected at regular intervals. There was no growth at 24 hr or 36 hr but at 48 hr, and subsequently, generalized background growth occurred

Table 4. *Anthranilate-utilizing colonies developing from samples of a single 15 ml. culture and from samples taken from a series of independent 1 ml. cultures of Nocardia opaca growing for 18 hr on o-nitrobenzoate defined medium and containing 94·* $\times 10^4$ *organisms/ml.*

Number of colonies developing on anthranilic acid defined medium

Samples from single 15 ml. culture

Sample no.	No. of colonies	Sample no.	No. of colonies
1	131	8	152
2	123	9	145
3	130	10	129
4	129	11	83
5	112	12	114
6	120	13	127
7	225	14	124
	Mean	131·7	
	Variance	905·6	
	χ-square	6·87	
	P (approx.)	0·91	

Samples from independent 1 ml. cultures

Culture	No. of colonies	Culture	No. of colonies
1	117	8	123
2	120	9	161
3	102	10	116
4	153	11	80
5	180	12	97
6	115	13	105
7	127	14	65*
	Mean	119·8	
	Variance	478·0	
	χ-square	4·06	
	P (approx.)	0·98	

* This sample was splashed with condensed water from the lid of the Petri dish and was not used in the calculations.

on the anthranilate medium . This was the response to be expected if organisms grown with *o*-nitrobenzoate acid were uniformly adapting to utilize anthranilate. In a second experiment where the tube contents were plated before visible growth was evident (18-hr incubation at 30°), measurable numbers of small colonies were observable after incubation for 40 hr on the anthranilate medium; the results of this experiment, read at 48 hr, are shown in Table 4. All the colonies which eventually appeared, developed at approximately the same time after plating. The similarity of the means and the high values for P of 0·98, and 0·91, for independent cultures and samples of a single culture, respectively, showed that mutational

effects were unlikely to have been responsible for the appearance of mutants which oxidized anthranilate.

Replicas (Lederberg & Lederberg, 1952) from master plates of about 150 micro-colonies of *Nocardia opaca*, growing on *o*-nitrobenzoate medium, were made on plates of anthranilate, salicylate, benzoate and *p*-hydroxybenzoate media and on a control plate of *o*-nitrobenzoate medium. There was a lag of some 36 hr before any

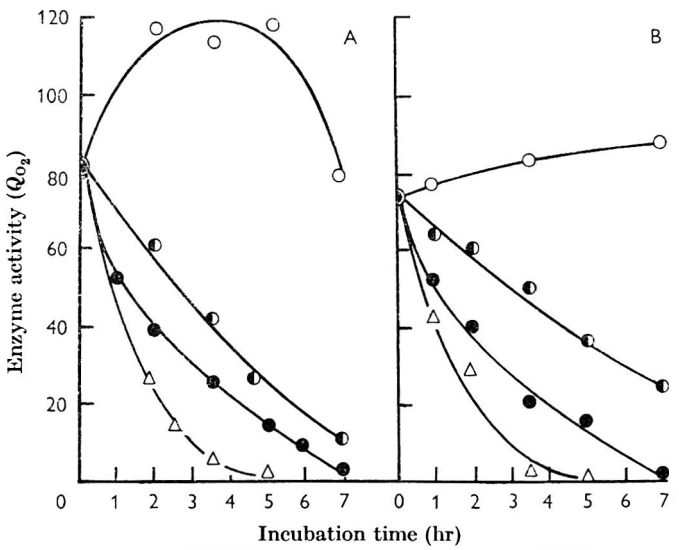

Fig. 8. Loss of enzyme activity by *Nocardia opaca* in the absence of substrate. Incubation flasks contained: phosphate buffer (pH 7·0) 2·5 m-moles; suspension of organisms originally grown on either *o*-nitrobenzoate (A) or anthranilate (B) medium; additions as shown. Total volume, 50 ml. Flasks were incubated at 30° and 37° in a metabolic shaking bath (throw, 3 cm.; 136 strokes/min.) and 5 ml. samples withdrawn at hourly intervals for manometric determinations of enzyme activity in the organisms (see Methods). For (A), Warburg flasks contained equiv. 1·40 mg. dry-wt. organisms; for (B), 1·30 mg. dry-wt. organisms.

A. Loss of the *o*-nitrobenzoate oxidizing system in the presence of: ○, sodium o-nitrobenzoate (10 mM) at 30°; ◐, sodium anthranilate (10 mM) at 30°; ●, no addition at 30°; △, no addition at 37°.

B. Loss of anthranilate oxidase in the presence of: ○, sodium anthranilate at 30°; ◐, sodium *o*-nitrobenzoate at 30°; ●, no addition at 30°; △, no addition at 37°.

growth appeared on these media (except on *o*-nitrobenzoate where visible colonies were evident at 24 hr, and on salicylate where no growth took place). After the growth lag, every colony on the master-plate was reproduced in the other media which supported growth, suggesting that such growth resulted from adaptation to the new substrates. In only 150 original organisms per master plate, the presence of mutants is unlikely if it be assumed that true mutation to anthranilate utilization occurred in the usual proportion of 1 in 10^7 to 1 in 10^8. When the master plate inoculum was increased to give between 10^8 and 10^9 organisms, general background growth took place after a 36-hr lag on the replica plates. There was no indication in any of many repeated experiments of the development of anthranilate utilizing mutants which should have shown preferential growth when plated on anthranilate medium.

De-adaptation

The loss of an enzyme after removal or disappearance of the inducer has been termed 'enzymic de-adaptation'; such loss of enzyme activity, upon exhaustion of the specific substrate, was consistently observed in *Nocardia opaca*. The rates of disappearance of both the *o*-nitrobenzoate-oxidizing system and the self-induced anthranilate oxidase in *N. opaca* were measured under different conditions in the absence of a readily metabolized nitrogen source. Figs. 8A and B show the marked loss in enzyme activity observed in the absence of the inducer, particularly at 37°. Addition of chloramphenicol did not appreciably decrease the rate of loss of enzyme, but addition of the specific inducer maintained or increased the amounts of enzyme. An unusual feature observed in experiments with the *o*-nitrobenzoate-oxidizing system and with the induced anthranilate oxidase was the slower rate of enzyme loss when an analogue of the inducer was present. Anthranilate decreased the rate of loss of the *o*-nitrobenzoate system by about 40% over the first 2–3 hr of the experiments; *o*-nitrobenzoate inhibited the loss of the anthranilate oxidase by some 30% over the same period. This increased maintenance of amounts of enzyme in the presence of analogues as distinct from true substrates recalls the inducing ability of fluorobenzene analogues in benzoic acid metabolism by *Pseudomonas fluorescens* (Hughes, 1953) where it was suspected that the analogue combined with a receptor to form an inducing complex, similar to that formed with the natural substrate; such a reaction was envisaged by Pollock (1953).

DISCUSSION

Adaptive enzyme formation (enzyme induction) is the term generally used to describe the synthesis of new enzyme under the influence of a specific exogenous inducer. A special case of enzymic induction is the phenomenon of 'sequential induction' (Stanier, 1947) which includes the enzyme induced by, and acting on, the added substrate together with all the enzymes induced by the successive intermediary metabolites along a metabolic route. In sequential induction it is usual to regard the inducer-metabolite as remaining within the organism, but in the present instance the inducer has to leave the cell and accumulate in the medium to above a threshold value before enzyme induction occurs. The expression 'metabolite-induced' has therefore been used to distinguish between anthranilate oxidase induction by anthranilate metabolically formed from *o*-nitrobenzoate, and induction by added (exogenous) anthranilate. A similar phenomenon has been observed in the 'aromatic-less' mutant Y7655 of *Neurospora crassa* which is blocked after dehydroshikimic acid; this mutant accumulates dehydroshikimic acid and converts it to protocatechuic acid which can be detected in the medium. Such mutants always show high values of protocatechuic acid oxidase (Gross, Gafford & Tatum, 1956) induced by this by-product of their own metabolism, whereas the wild type requires added protocatechuic acid or vanillic acid to induce this enzyme. The accumulation of enzyme inducer in this case with neurospora, shunts the products of a biosynthetic pathway into the constitutive pathway of energy-yielding reactions via two catabolic sequences previously not used to a significant extent (Gross, 1959). Similarly, in the present study with *Nocardia opaca*, the by-product of a secondary

reaction (reduction of *o*-nitrobenzoate to anthranilate) may be utilized in an energy-yielding process.

Another feature in which the present system resembles the classical sequential induction response is the ease of 'de-adaptation' on exhaustion or removal of the specific inducer. Enzyme values of *o*-nitrobenzoate and anthranilate oxidation in nocardia began to decrease almost immediately on removal of the inducer substrates. For the experiment recorded in Fig. 8, the time between harvesting the organisms and their suspension in the buffer systems (zero time) was about 20 min.; loss of enzyme was rapid from zero time onwards. Because these changes took place under conditions of non-proliferation of the organisms, it may be assumed that loss of activity was due to actual breakdown of the adaptive enzymes (Spiegelman, 1950; Robertson & Halvorson, 1957) rather than by diluting out (Wainwright & Pollock, 1949; Rickenberg, Yanofsky & Bonner, 1953). It is unlikely that cell death or enzyme inactivation were responsible for the losses in activity because recovery of activity could be shown, after the usual time lag, simply by adding more of the specific inducer.

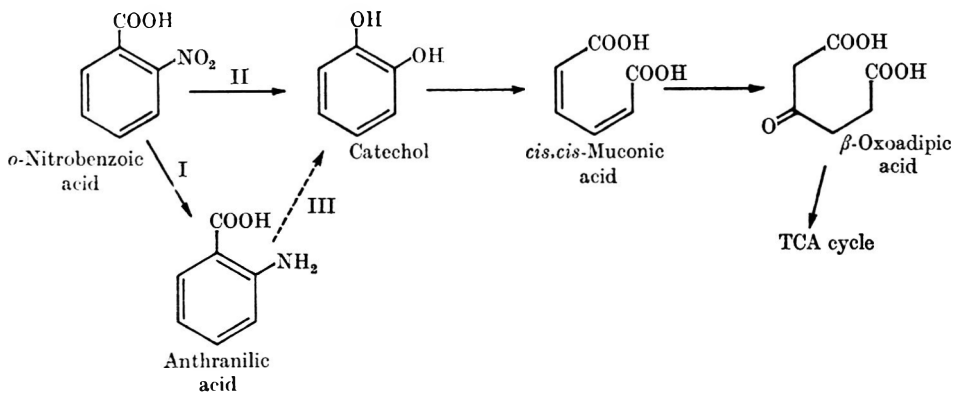

Fig. 9. Initial steps in the metabolism of *o*-nitrobenzoic acid and anthranilic acid by *Nocardia opaca*.

Metabolite induction in *Nocardia opaca* provides further evidence that anthranilic acid is not an obligatory intermediate on the pathway of the aerobic metabolism of *o*-nitrobenzoic acid by this organism. A similar situation also appears to exist in the flavobacterium strain used where the amounts of anthranilate oxidase were drastically decreased by chloramphenicol and also varied with the marked changes in anthranilate concentration which occurred during its growth in *o*-nitrobenzoate medium. If anthranilic acid were an obligatory direct intermediate in *o*-nitrobenzoic acid degradation, the absence of anthranilate oxidase should lead to a complete block in the ability of the organism to grow, because this enzyme is responsible for conversion of its substrate to catechol, the intermediate which is ultimately degraded to the aliphatic products utilized for energy production and cell carbon (Hayaishi & Stanier, 1951; Higashi & Sakamoto, 1960). It is known that in *Nocardia opaca* both anthranilic and *o*-nitrobenzoic acids are separately metabolized through catechol (steps II and III, Fig. 9; Cain & Cartwright, 1960*a*; Cain, 1966); a route for *o*-nitrobenzoate breakdown through catechol is thus followed

whether anthranilic acid is regarded as an intermediate in *o*-nitrobenzoic acid metabolism or not. In the absence of anthranilate oxidase, the reduction step (I) is the only known reaction which could occur when *o*-nitrobenzoic acid is the substrate. Energy considerations (reduction of the nitro group is NADH-mediated Saz & Slie, 1954; Cartwright & Cain, 1959*b*; formation of NADH usually requires, not yields, energy) and molar growth yield experiments with anthranilic and *o*-nitrobenzoic acids as substrates (Cain, 1966) suggest that no energy is available from this reduction step. Since growth does occur in the normal absence of the anthranilate oxidase enzyme (Fig. 5A) and when its formation is inhibited (Figs. 3, 6 and 7), the conclusion must be that anthranilate cannot be an obligatory intermediate in the energy-producing pathway of *o*-nitrobenzoate metabolism and that, for energy requirements, *o*-nitrobenzoate is degraded to catechol either directly or via some alternate route not involving initial reduction.

The use of whole organisms in these experiments is open to the criticism that permeability effects may have operated, i.e. the response measured may have been that of the induction of a specific transport mechanism for anthranilate rather than of a new intracellular enzyme. Experiments with organisms slowly dried in air (compare Sleeper, Tsuchida & Stanier, 1950) and with detergent-treated organisms where the permeability barrier has been destroyed (Cain, 1966) indicate that this is not the case. Experiments with (carboxyl-^{14}C)-anthranilic acid have shown that whole organisms grown on *o*-nitrobenzoate or succinate media, only accumulate anthranilate very slowly before adaptation occurs, whereas organisms originally grown on anthranilate rapidly take up and oxidize added anthranilate; both these processes are inhibited by *o*-nitrobenzoic acid (Cain, 1966). Organisms from nitrobenzoate cultures, metabolite-induced by anthranilate, or from such cultures to which anthranilate has been added, have similar rates of anthranilate accumulation and oxidation as those originally grown on anthranilate. If a transport system is involved in anthranilate metabolism therefore, it must be metabolite-induced and lost under the same conditions as is the 'oxidase'.

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Utilization of Anthranilic and Nitrobenzoic Acids by *Nocardia opaca* and a Flavobacterium

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SUMMARY

Anthranilic and *o*-nitrobenzoic acids act as mutual inhibitors of both growth and substrate oxidation for *Nocardia opaca* and a flavobacterium which can utilize either substance as sole source of carbon, nitrogen and energy. Growth of the former bacterium on anthranilate induced, apparently simultaneously, both the transport system for anthranilate uptake and the enzymic mechanism necessary for its complete oxidation to CO_2 and NH_3. Among the enzymes induced by anthranilate was the complete sequence that oxidizes catechol to β-oxoadipate; this was absent from organisms grown in fumarate or glucose media. The properties of the first enzyme in this sequence, a catechol-1,2-oxygenase, differ in several features from those of the same enzyme induced in this bacterium by growth on *o*-nitrobenzoic acid.

INTRODUCTION

Anthranilic (*o*-aminobenzoic) acid is an important intermediary metabolite in both biosynthetic and catabolic pathways in micro-organisms. It serves for instance as a precursor for tryptophan in *Aerobacter aerogenes* and *Escherichia coli* (Doy & Gibson, 1961), in *Neurospora crassa* (Yanofsky, 1956; Yanofsky & Rachmeler, 1958) and in saccharomyces mutants (Lingens, Hildinger & Hellman, 1958). Hydroxylation of anthranilic acid, in the 3-position, has been observed with rat liver preparations (Wiss & Hellman, 1953) but no hydroxylation of anthranilic acid has been conclusively demonstrated in micro-organisms. The finding, by sequential induction methods, that 5-hydroxyanthranilic acid may be a product of anthranilic acid metabolism by an achromobacter (Ladd, 1962) remains the only indication at present that bacteria might hydroxylate the ring of this substrate during metabolism. Anthranilic acid has also been known for some time as an intermediate in the aerobic dissimilation of L-tryptophan by micro-organisms, especially pseudomonas species which use the 'aromatic pathway' (Suda, Hayaishi & Oda, 1950; Knox, 1959); it occupies a similar position in degradation of the D-isomer (Behrman 1962; Martin & Durham, 1962). Reduction of *o*-nitrobenzoic acid by a flavobacterium (Cain, 1966) and *Nocardia opaca* (Cain, 1958; Cartwright & Cain, 1959*b*) yields anthranilic acid, but the amino compound is not an obligatory intermediate on the direct energy-yielding pathway of degradation of *o*-nitrobenzoic

* Present address.

acid by these organisms (Ke, Gee & Durham, 1959; Cain, 1966). Both species, nevertheless, will utilize anthranilic acid separately as sole source of carbon, nitrogen and energy as does the achromobacter isolated by Ladd (1962), but the pathways of utilization by these three organisms appear to be different.

The present paper describes some observations made on the growth patterns of *Nocardia opaca* and a flavobacterium which can utilize anthranilic and *o*-nitrobenzoic acids, and the unusual mutually antagonistic effects of these substrates when present together in the medium.

METHODS

Organisms. The two bacteria used in this study were strain 06 of *Nocardia opaca* (Cain, 1958) and a flavobacterium isolated by Durham & Hubbard (1959). They were maintained on slopes of nutrient agar and on the defined medium solidified with 2% (w/v) New Zealand agar. The ELH$^-$ mutant of *Pseudomonas putida*, provided by Dr N. Ornston, was grown upon the defined medium containing succinate and 0·1% (w/v) benzoic acid to induce muconolactone isomerase (Ornston & Stanier, 1964).

Media. The medium for bulk growth of organisms consisted of (g./l.): organic source, 1·0; K_2HPO_4, 1·0; $MgSO_4.7H_2O$, 0·1; trace elements solution (Barnett & Ingram, 1955) 10 ml./l. medium; adjusted to pH 7·0–7·2. Where the organic substrate did not provide a source of nitrogen $(NH_4)_2SO_4$ (0·5 g./l.) was included. Yields of organism were improved by adding 0·02% (w/v) Difco yeast extract or 'Marmite' (a British commercial yeast-extract preparation).

Growth experiments. These were done in new stout-walled Pyrex tubes selected for the best optical matching after rigorous cleaning. The defined medium (GM) was dispensed in 5 ml. volumes and consisted of: substrate, 0–10 mM; K_2HPO_4, 0·1 M; $MgSO_4.7H_2O$, 1 mM; inhibitor (or alternative substrate), 0–10 mM; adjusted to pH 7·2 before autoclaving. The tubes were inoculated with 0·1 ml. of a suspension of either organism grown on the appropriate substrate, harvested, washed twice and resuspended in sterile distilled water to an extinction value of 0·4 at the appropriate wavelength. All cultures were incubated at 30° with the tubes racked at 45° from the vertical and shaken in a reciprocating shaker (96 strokes/min.; throw, 5·5 cm.). Uniform turbid growth was ensured only by agitation; stationary cultures of *Nocardia opaca* developed a surface pellicle and granular growth. Growth was measured turbidimetrically at 530 mμ.

Growth yield coefficients. Determinations of yield coefficients were made in 250 ml. Erlenmeyer flasks fitted with a side-arm suitable for determination of turbidity in the Bausch-Lomb 'Spectronic 20' spectrophotometer.

Triplicate flasks contained 30 ml. of the defined (GM) medium containing up to 300 μmoles of substrate. Because in such cases nitrogen might have been the limited factor, excess NH_4Cl (0·1%, w/v) was added in other experiments because it is known (Cain, 1958; Cartwright & Cain, 1959*a*) that the nitrogen from nitro- and amino-benzoates appears in the medium largely as ammonia. The flasks were inoculated with 0·1 ml. (about equiv. 4 μg. dry-wt. organism) of aseptically washed *Nocardia opaca*, originally grown on the appropriate substrate, and then incubated at 25° in air on a New Brunswick 'Gyrotory' shaker. Growth was followed turbidi-

metrically and when complete the flask contents and washings were filtered through a tared 'Millipore' filter. The organisms on the filter disc were washed several times to remove dissolved materials derived from the medium; the disc with its organisms was then dried on an aluminium planchet to constant weight in an oven at 105° and the dry-wt. of the organisms determined. In earlier experiments, 20 ml. samples from each flask were centrifuged, the packed organisms washed twice and recentrifuged, made up to the original volume in water and then a 10 ml. sample dried to constant weight.

Manometry. Respirometric studies were done by the usual manometric methods (Umbreit, Burris & Stauffer, 1957) at 30° with air as gas phase. Duplicate manometer flasks contained (unless otherwise recorded): phosphate buffer (pH 7·2), 150 μmoles; substrate, 0·5–10 μmoles; inhibitors, where required, 0–50 μmoles; cell suspension, 0·5 ml.; 20% KOH in the centre well, 0·2 ml.; total volume made to 3·0 ml. with distilled water. Oxygen uptakes were determined at intervals for 60 min., or longer when required; rates of uptake were calculated over the initial linear part of the curves.

Uptake of radioactive anthranilic acid. Anthranilic acid- (carboxyl-^{14}C) was the gift of Dr L. M. Henderson; on assay it was found to have an activity of 102,250 c.p.m./mg. The very small quantity available was mixed with unlabelled carrier anthranilic acid to give a final specific activity of 10,600 c.p.m./μmole. The incubation mixture for uptake experiments contained: phosphate buffer (pH 7·0), 500 μmoles; anthranilic acid, 200 μmoles; thick cell suspension, 10 ml.; distilled water to a final volume of 20 ml. In one experiment 200 μmoles *o*-nitrobenzoic acid were also added to the incubation mixture. Incubation was at 30° in a Dubnoff metabolic shaker bath under aerobic conditions. 1 ml. samples (equiv. about 3–4 mg. dry-wt. organisms in different experiments) from the incubation mixtures were taken at frequent intervals and the organisms rapidly collected by filtration on a small Millipore filter disc (1 cm. diam.) where they were washed with 2 × 2 ml. volumes of distilled water. The disc was then transferred to an aluminium planchet where it was fixed with cement to prevent curling and dried under an ultraviolet lamp. The dried planchets were counted under an automatic Picker gas-flow counter (Picker X-ray Co., Oklahoma City) for 200 min. and corrected for the low background count. Results for organisms originally grown with anthranilic, *o*-nitrobenzoic or succinic acids were corrected to μmoles uptake/g. dry-wt. organism. The dry weights of organisms in 1 ml. samples of each incubation mixture were determined separately by drying samples of twice-washed organisms to constant weight at 105° on aluminium planchets.

Cell-free preparations. Dried cell preparations were made by spreading the wet paste of organisms thinly over a Petri dish and drying in a vacuum desiccator over P_2O_5 or granular anhydrous $CaCl_2$ for 5 days in the cold room at 4°. The dried preparation was ground to a fine powder with a pestle and mortar and stored at −20° until required. Cell-free extracts were made by ultrasonic disintegration for 15 min. at 20 kcyc./sec. with the M.S.E.-Mullard instrument or with a Hughes press (Hughes, 1951) without abrasive; in the latter case the frozen crush was thawed, taken up in the minimal quantity of distilled water and centrifuged at 10,000*g* for 30 min. to remove debris and larger particles. The concentration of soluble protein in the extracts was usually about 15 mg./ml. Cell-free preparations able to convert

catechol to β-oxoadipic acid were prepared by extracting freeze-dried organisms with 0·01 M-tris-HCl buffer in the cold for 30 min.

Analytical methods. Anthranilic acid was estimated by the Bratton-Marshall procedure (Glazko, Wolf & Dill, 1949) with twice-recrystallized anthranilic acid as a standard. Nitrite was estimated by the Griess-Ilosvay diazotization method modified by the use of *N*,*N*-dimethyl-α-naphthylamine as coupling reagent (Wallace & Neave, 1927). Ammonia was determined by nesslerization after distillation into dilute H_2SO_4 in Conway micro-diffusion units; and protein by the biuret method of Gornall, Bardawill & David (1949).

Chemicals. *o*-Nitrobenzoic acid, anthranilic acid and 3-hydroxyanthranilic acid were recrystallized commercial samples. Anthranilamide, 3,4-dihydroxyanthranilic acid and 5-hydroxyanthranilic acid were the gift of Dr L. M. Henderson. 2-Nitro-3-hydroxybenzoic acid (m.p. 174°) was synthesized by the method of D'Angeli, Koski & Henderson (1955). Commercial gentisic acid was recrystallized several times from water and then contained only traces of other dihydroxybenzoic acids as shown by chromatography. *cis,cis*-Muconic acid, m.p. 197°, was prepared by peracetic acid oxidation of redistilled phenol according to the directions of Elvidge *et al.* (1950). The corresponding *cis,trans*-(m.p. 189°) and *trans,trans*- (m.p. 301°) isomers were then prepared from it (Elvidge *et al.* (1950). (+)-Muconolactone was prepared enzymically from protocatechuic acid by using heat-treated extracts of *Moraxella lwoffii* NCIB 8250 ('Vibrio 01'; Sebald & Veron, 1963) which had been grown on *p*-hydroxybenzoate (Cain, 1961). The synthetic (±) isomer was the gift of Professor W. C. Evans. β-Carboxymuconolactone and β-carboxymuconic acid were prepared enzymically as described previously (Cain, 1961). β-Oxoadipic enol lactone was prepared from (+)-muconolactone *in situ* when required (but not isolated) by using extracts of the ELH⁻ mutant of *Pseudomonas putida* grown upon succinate in the presence of benzoate (Ornston & Stanier, 1964). This mutant lacks the enzyme enol lactone hydrolase which converts β-oxoadipic enol lactone to β-oxoadipic acid. All other chemicals were commercial samples and were used without further purification.

Chromatography. Keto acids were run as their 2,4-dinitrophenylhydrazones in the following solvent systems (all quantities by vol.): (A) *tert*-amyl alcohol + *n*-propanol + aq. NH_3 soln. (sp.gr. 0·880), (13 + 1 + 6); (B) *n*-propanol + aq. NH_3 soln. (sp.gr. 0·880) + water (6 + 3 + 1). Anthranilic acid and phenolic compounds were run in (C) *n*-butanol + acetic acid + water (4 + 1 + 5); (D) ethanol + aq. NH_3 soln. (sp.gr. 0·880) + water (40 + 1 + 6). Arylamine compounds were detected by Ehrlich's reagent [1% (w/v) *p*-dimethylaminobenzaldehyde in 200 ml. *n*-butanol + ethanol (30:70, v/v) + con. HCl (30 ml.)]. The keto acids were detected by spraying with N-NaOH and the phenolic compounds by their fluorescence under u.v. radiation.

Enzymic assays. Conversion of catechol to *cis,cis*-muconic acid by catechol 1,2-oxygenase was followed by increase in extinction at 260 mμ with the Unicam SP 800 recording spectrophotometer fitted with the SP 820 constant wavelength attachments; at this wavelength, ε for catechol is 2000 and for *cis,cis*-muconic acid is 17,200. Cuvettes contained: tris-HCl buffer (pH 7·0) 300 μmoles; catechol, 0·5 μmole; enzyme equivalent to about 0·1 mg. protein; inhibitor or metal ions at concentrations shown in the text; total volume made to 3·0 ml. with water. The

enzyme activity was measured at 30° in a constant temperature cuvette holder. Under these conditions, the rate of reaction was linear for at least 5 min. and strictly proportional to enzyme concentration.

Lactonizing enzyme in extracts of *Nocardia opaca* was measured by the decrease in absorption at 260 mμ in the presence of 0·5 μmole *cis,cis*-muconic acid; muconolactone isomerase by the decrease in the absorption at 225 mμ in the presence of 0·5 μmole (+)-muconolactone or, in some cases, 1·0 μmole of its synthetic (±) isomer; β-oxoadipic enol lactone hydrolase by the decrease in absorption at 225mμ in the presence of 0·5 μmole (+)-muconolactone and excess of crude extract of the benzoate-induced ELH$^-$ mutant of *Pseudomonas putida* sufficient to ensure that the muconolactone isomerase of this organism was not rate-limiting (Ornston & Stanier, 1964; Dr L. N. Ornston, personal communication). Other cuvette contents for these assays were identical with those for catechol-1,2-oxygenase.

RESULTS

Growth of Nocardia opaca

Nocardia opaca subcultured from nutrient agar or succinate medium grew equally well in either *o*-nitrobenzoate or anthranilate media (with or without added yeast extract) after a lag of about 20 hr. There was, however, a longer lag before growth began in anthranilate medium when the inoculum organisms had been derived from a culture grown with *o*-nitrobenzoate, and the time lag was similarly increased when bacteria growing in *o*-nitrobenzoate medium were derived from an inoculum grown on anthranilate. Addition of anthranilate to cultures of *N. opaca* growing in *o*-nitrobenzoate media produced very marked differences. When anthranilate was added at the time of inoculation or in the lag phase of growth, the onset of logarithmic growth was delayed by some 15 hr or more. When the anthranilate was added at the beginning of, or during, the log phase, there was no such delay, nor was the growth rate altered as compared with control tubes with no added anthranilate (Fig. 1). A similar effect was found when *o*-nitrobenzoate was added to cultures of *N. opaca* growing on anthranilate; thus, growth which in 10 mM anthranilate began at 32 hr after inoculation, was delayed for a further 10–12 hr when 5 mM-nitrobenzoate was present in the lag phase, but a similar addition at the beginning of the exponential phase did not affect growth. The extent of the delay in logarithmic growth was also related to the ratio of the concentration of substrate and inhibitor added (Fig. 2), although the lag effect was more pronounced at the same ratio with lower substrate concentrations. *o*-Nitrobenzoate and anthranilate (20 mM) both strongly inhibited growth of *N. opaca* on succinate, whereas a nitrobenzoate concentration some fivefold higher was required for comparable inhibition with the flavobacterium strain tested (Kirkland & Durham, 1963).

Total growth on mixed media

Although the presence of either anthranilate or *o*-nitrobenzoate increased the time lag in growth on the other substrate, there was no decrease in the final growth yields in the defined medium (Fig. 3). Excellent correlation between available nutrient concentration and final growth yields were found irrespective of whether the two nutrient sources were (i) present together in the medium or (ii) whether

the second source was added to cultures after growth on the first substrate had ceased. In the former case, there was no evidence of a diauxic response; growth experiments (repeated many times) with anthranilate and *o*-nitrobenzoate present in various proportions always gave an uninterrupted growth curve, the final yield of organism being proportional to the concentration of added substrate. The good correlation between substrate concentration and final growth measured turbidimetrically prompted estimations of the actual yield coefficients (μg. dry-wt. organism formed/μmole substrate utilized; Bauchop & Elsden, 1960) obtained with

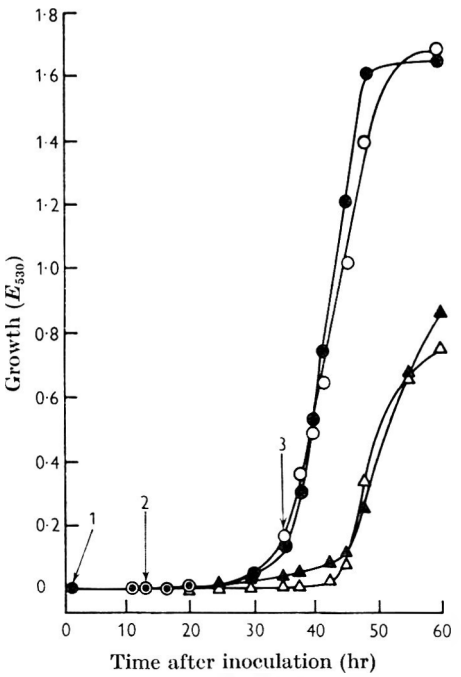

Fig. 1

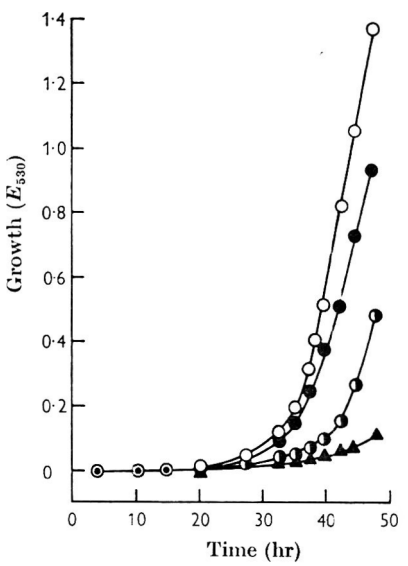

Fig. 2

Fig. 1. The effect of anthranilic acid on the growth of *Nocardia opaca* with *o*-nitrobenzoic acid as nutrient source. Anthranilic acid (adjusted to pH 7·0) was added in different cultures at: 1, inoculation; 2, in lag phase of growth; or 3, at the beginning of the log phase of growth as indicated by the three arrows. ●, *o*-Nitrobenzoate (10 mM) only (control); ○, *o*-nitrobenzoate (10 mM) with anthranilic acid (5 mM) added at the beginning of logarithmic growth; ▲, *o*-nitrobenzoate (10 mM) with anthranilic acid (5 mM) added in the lag phase; △, *o*-nitrobenzoate (10 mM) with anthranilic acid (5 mM) added at inoculation. Inoculum organisms were grown on *o*-nitrobenzoate.

Fig. 2. The effect of varying the *o*-nitrobenzoate:anthranilate ratio on growth of *Nocardia opaca*. Two substrate (*o*-nitrobenzoate) concentrations were used, 10 and 5 mM. ○, ratio 5:1 (10); ●, ratio 2·5:1 (5); ◑, ratio 2:1 (10); ▲, ratio 1:1 (10). The figures in parentheses give the substrate concentrations (mM) used. The inoculum organisms were originally grown upon *o*-nitrobenzoic acid. Inoculation was made into media containing *o*-nitrobenzoate and anthranilate together at the indicated ratios.

o-nitrobenzoate and anthranilate. When these two substrates provided the sole source of carbon, nitrogen and energy, the coefficients were $20{\cdot}2 \pm 0{\cdot}2$ and $23{\cdot}7 \pm 0{\cdot}2$, respectively; in the presence of excess nitrogen provided as NH_4Cl, these coefficients

were raised to $28{\cdot}3 \pm 0{\cdot}7$ and $28{\cdot}3 \pm 0{\cdot}3$, respectively. In some ten experiments the growth yields with anthranilic acid as substrate were always slightly higher than those from the nitro compound.

Inhibition of oxidation by o-nitrobenzoic acid and anthranilic acid

The inhibition by *o*-nitrobenzoate of the growth of *Nocardia opaca* utilizing anthranilate suggested that the inhibition might be at the stage of oxidation of the substrate where the nitro compound, because of its similar chemical structure, could compete for the enzyme sites normally occupied by anthranilate. The effects

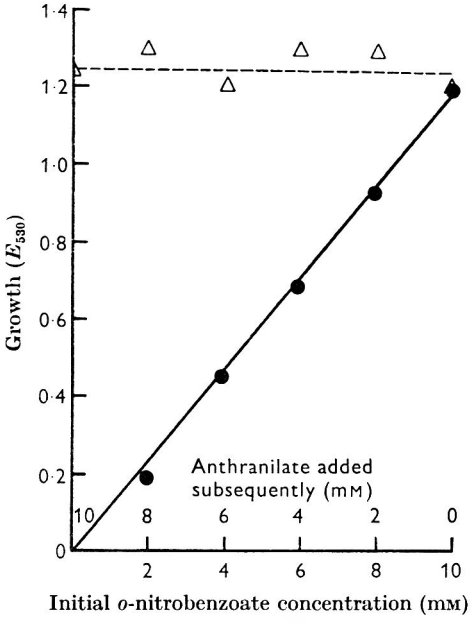

Fig. 3

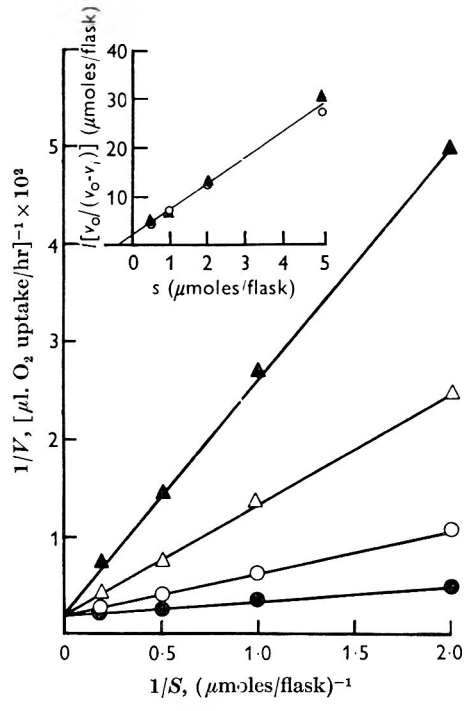

Fig. 4

Fig. 3. The relationship between growth and added substrate concentration. Duplicate cultures of *Nocardia opaca* were grown with various concentrations of *o*-nitrobenzoate until growth was complete, ●. Sodium anthranilate (pH 7·0) was then added to bring the final aromatic substrate concentration to 10 mM and incubation was continued until growth had again ceased, △.

Fig. 4. Competitive inhibition by *o*-nitrobenzoic acid of anthranilic acid oxidation in *Nocardia opaca*. Manometer flask contents as described in Methods with suspension of organisms equivalent to 8·8 mg. dry-wt. ●. No inhibitor; ○, 10 μmoles *o*-nitrobenzoate; △, 20 μmoles *o*-nitrobenzoate; ▲, 50 μmoles *o*-nitrobenzoate. V (the reaction velocity) was calculated as μl. O_2 uptake/hr. over the first 30 min., corrected for endogenous respiration. Inset: the results at the 10 μmoles/flask (○) and 50 μmoles/flask (▲) inhibitor concentrations plotted by the method of Hunter & Downs (1945). v_0 reaction velocity in absence of inhibitor; v_i, velocity at inhibitor concentration, I.

of *o*-nitrobenzoate on anthranilate oxidation were followed manometrically. It was found that a marked inhibition of substrate oxidation occurred; the inhibitory effects were partially annulled by increasing the anthranilate concentration. A

Lineweaver & Burk (1934) plot of the results (Fig. 4) showed that the inhibition was competitive. Calculation of the enzyme constants from this plot and by the Hunter & Downs (1945) method gave mean values of K_m for the anthranilate system of 0·12 mM; K_I for *o*-nitrobenzoate inhibition of this system was 1·2 mM, indicating some tenfold preference of the enzyme for its natural substrate. A similar result was found when anthranilate was added to organisms oxidizing *o*-nitrobenzoate after growth with this substrate; K_m for the *o*-nitrobenzoate system was 0·07 mM, and K_I for the anthranilate inhibition of this system was 2·2 mM. Although these compounds are mutual competitive inhibitors of each other's oxidation, however, the maximum concentrations of anthranilic acid accumulating in *o*-nitrobenzoate medium during growth of *N. opaca* and the flavobacterium (approximately 0·08 and 0·12 mM, respectively) would be insufficient to have any appreciable effect on oxidation of the nitrobenzoate substrate present at some 30-fold higher concentration.

Permeability effects

Anthranilic acid uptake by Nocardia opaca. The rate of uptake of anthranilic acid-(carboxyl-^{14}C) by *N. opaca* was investigated with batches of organisms grown on *o*-nitrobenzoate, anthranilate or succinate as substrates. Organisms originally grown with anthranilate rapidly accumulated labelled anthranilic acid from the incubation mixture, but the accumulated material disappeared equally rapidly because of further metabolism (Fig. 5). (An early step in anthranilate metabolism is conversion to catechol during which the labelled carboxyl group is lost; rapid disappearance of label was, therefore, anticipated.) Anthranilic acid was much more slowly accumulated, however, by organisms grown with *o*-nitrobenzoate or succinate. Simultaneous determinations of the rate of oxidation of anthranilate by these same organisms grown in *o*-nitrobenzoate or in succinate media gave Q_{O_2} values of 3·3 and 0, respectively, whereas that of the organisms originally grown with anthranilate was 47·0. It would seem, therefore, that growth on anthranilate was necessary to induce not only the enzyme system capable of oxidizing this substrate but also a transport system for its rapid uptake. A separate experiment in which unlabelled *o*-nitrobenzoate was included in the incubation mixture resulted not only in a significant decrease of the rate of anthranilic acid uptake (19·0 μmoles/min./g. dry-wt. organisms decreased to 6·2 μmoles/min./g.; 67 % inhibition) but also in increased values of total label accumulated. That is, the subsequent metabolism of anthranilate was being inhibited, a result which confirmed the inhibition by *o*-nitrobenzoate observed in respirometric experiments.

Experiments with organisms with the permeability barrier destroyed. Destruction of the cell membrane of *Nocardia opaca* organisms by cetyltrimethylammonium bromide (CTAB) at 80 μg./ml. was virtually complete as measured by the release of u.v.-absorbing material. The permeability barrier was also destroyed in dried-cell preparations as indicated by the rapid oxidation of citrate, in contrast to the initial lag period obtained before its oxidation with intact washed organisms (Barrett, Larsen & Kallio, 1953; Clarke & Meadow, 1959). *Nocardia opaca* grown either on *o*-nitrobenzoate or succinate and then dried or treated with CTAB did not oxidize anthranilate whereas organisms grown with anthranilate and similarly treated, very rapidly oxidized this substrate.

Effect of an energy source on anthranilate oxidation by unadapted organisms

Clarke & Meadow (1959) reasoned that as the induction of new enzyme (protein) synthesis requires energy the addition of small amounts of oxidizable substrates to which organisms were already permeable, should accelerate the induction process and thus decrease the time lag before the inducer was oxidized. In their investigations, induction of a permease was accelerated, but a similar effect would be anticipated for the synthesis of an intracellular enzyme. In the present work *Nocardia opaca* grown upon *o*-nitrobenzoate for 24 hr did not oxidize anthranilate for some

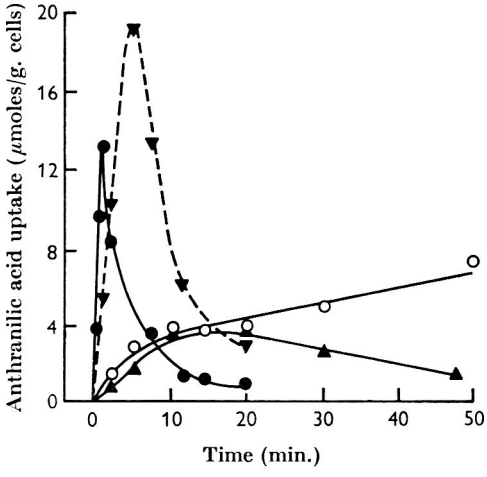

Fig. 5

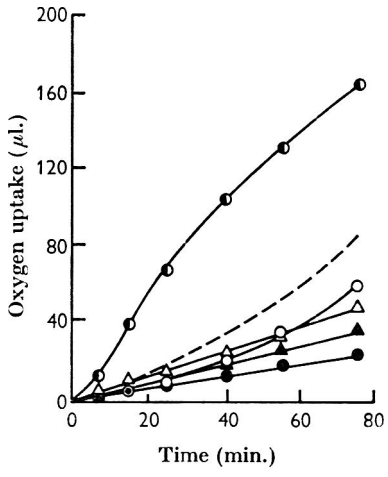

Fig. 6

Fig. 5. Uptake of anthranilic acid-(carboxyl-^{14}C) by *Nocardia opaca*. Uptake of labelled anthranilic acid was followed in organisms originally grown upon ●, anthranilic acid; ○, *o*-nitrobenzoic acid; ▲, succinic acid; (solid lines). Uptake of anthranilic acid was also followed in organisms originally grown on anthranilic acid, but the incubation medium contained an equimolar concentration of (unlabelled) *o*-nitrobenzoic acid, ▼ (dotted line).

Fig. 6. The effect of an energy source (fumarate) upon anthranilic acid oxidation by *Nocardia opaca* grown on *o*-nitrobenzoic acid. ◐, anthranilic acid + fumaric acid; ○, anthranilic acid alone; △, fumaric acid alone; ------, sum of ○ and △; ▲, anthranilic acid + fumaric acid + chloramphenicol; ●, no substrate and no substrate + chloramphenicol. Duplicate flasks contained: anthranilic acid, 5 μmoles; fumaric acid (where required), 0·5 μmole; chloramphenicol (where required) to 60 μg./ml. Other flask contents as described in Methods.

20–60 min., and then only slowly, but did oxidize fumarate from zero time. In the presence of 0·5 μmole fumarate, however, anthranilate was rapidly oxidized without a perceptible lag at a rate considerably in excess of the sum of the two components separately (Fig. 6). This adaptation was completely inhibited by chloramphenicol 60 μg./ml, as would be expected of a reaction involving protein synthesis. When a small amount of *o*-nitrobenzoate (0·5 μmole) was substituted for the fumarate as an energy source in this experiment an identical result was obtained. After incubation for 24 hr *o*-nitrobenzoate-grown organisms occasionally show low amounts of the anthranilate oxidation system (Cain, 1966). The usual sigmoid-shaped curve characteristic of new enzyme synthesis was, in such experiments, barely perceptible.

Intermediary metabolism

Anthranilic acid production from o-nitrobenzoic acid. Determinations were made of the yields of the major *N*-containing end-products which appeared in the defined medium during growth of *Nocardia opaca* and the flavobacterium when *o*-nitrobenzoic acid was the sole source of carbon, nitrogen and energy. The characteristic feature during the growth of both organisms was an accumulation of anthranilate during the log phase of growth, followed by an equally rapid decrease in concentration of anthranilate as the culture entered the stationary phase; with the flavobacterium this decrease was always precipitous, some 80% or more of the accumulated anthranilate disappearing within 2–3 hr (Fig. 7). The arylamine was identified as anthranilic acid by isolation, chromatography and chemical characterization as

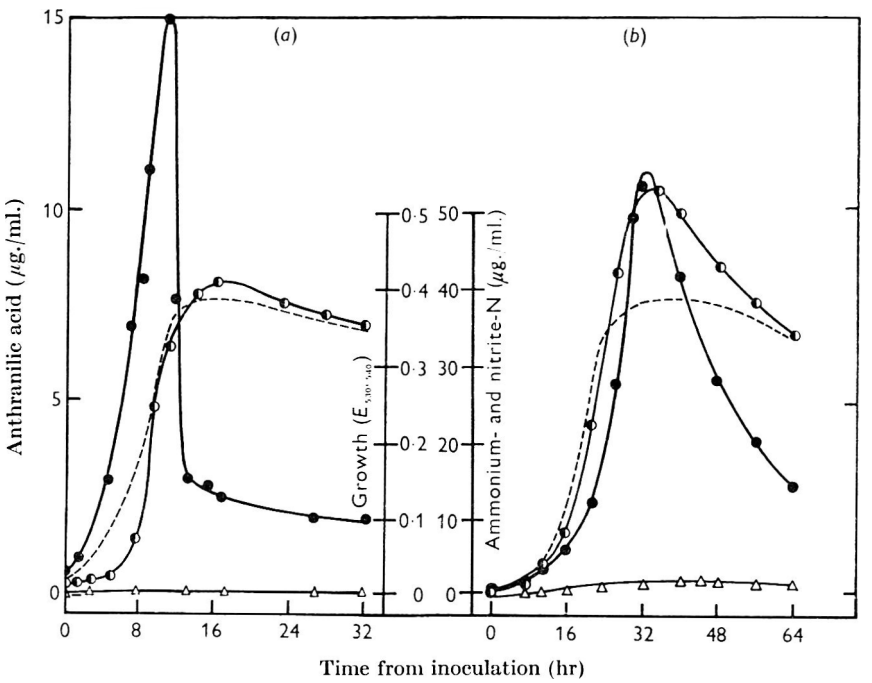

Fig. 7. Metabolic products of *o*-nitrobenzoic acid degradation by (*a*) flavobacterium; (*b*) *Nocardia opaca*. ●, anthranilic acid; ◐, ammonium-N; △, nitrite-N; ----, growth. Measurements were made on samples from 5 l. cultures of each bacterium growing in minimal medium with *o*-nitrobenzoic acid (0·1%, w/v) supplying the sole source of carbon, nitrogen and energy.

described by Cartwright & Cain (1959*b*). The concentration of anthranilate accumulated by both organisms was 12–14 μg./ml.; occasionally values as high as 35 μg./ml. were recorded with the flavobacterium. The decrease in anthranilate concentration with both bacteria was always correlated with the appearance of an anthranilate oxidation system (Cain, 1966).

The other important nitrogenous product was ammonia, the production of which in cultures of *Nocardia opaca* and the flavobacterium closely followed growth.

Maximum concentrations of ammonia in the medium which supported the growth of the flavobacterium accounted for about 45% of the total original nitro-N, a considerably larger proportion than the 25% or so recoverable as this metabolite during degradation of *p*-nitrobenzoic acid by *N. erythropolis* (Cartwright & Cain, 1959*a*). Small amounts of nitrite were also found in cultures of *N. opaca* (up to 0·8 μg. nitrite-N/ml.) but the formation of this metabolite by the flavobacterium was negligible (less than 0·05 μg. nitrite-N/ml.). Only traces of nitrite were formed in cultures of either organism when anthranilate was the organic source, although ammonia yields in these instances paralleled those found in *o*-nitrobenzoate media.

Anthranilate oxidation by washed organisms and extracts. Washed *Nocardia opaca* harvested from 24 to 30 hr cultures oxidized anthranilate rapidly (Q_{O_2} varied from 45 to 72) but consumed only about half the theoretical amount of oxygen and produced only about half the expected CO_2 necessary for complete oxidation according to the equation: $C_7H_7NO_2 + 7O_2 \rightarrow 7CO_2 + 2H_2O + NH_3$. Sodium azide prevented the considerable oxidative metabolism (Clifton, 1946) observed with washed suspensions; in the presence of 7 mM azide the theoretical figures for oxidation according to the above equation were obtained (Table 1). The nitrogen of the anthranilate substrate appeared solely as ammonia; the quantity, unaffected by azide, varied between 70 and 100% of that required by theory.

Table 1. *Oxidation of anthranilic acid by washed Nocardia opaca*

Azide added (mM)	Moles consumed or produced/mole substrate oxidized		
	O_2	CO_2	NH_3
None	3·7 (10)*	3·9 (6)	0·82 (10)
3	5·6 (1)	6·5 (1)	0·72 (1)
5	6·4 (1)	6·7 (1)	0·77 (1)
7	6·9 (2)	7·0 (2)	0·80 (2)
Theoretical value (equation, see above)	7·0	7·0	1·0

* Figures in parentheses represent the number of experiments carried out under those conditions, and the results are the means of data obtained from all such experiments.

Extracts, prepared in 0·01 M-phosphate buffer (pH 7·0) by ultrasonic disintegration or by the Hughes press, did not oxidize anthranilic acid even after supplementation with substrate quantities of NADH, NADPH or catalytic amounts of NAD or NADP and the requisite reducing systems (alcohol dehydrogenase + ethanol and isocitric dehydrogenase + isocitrate, respectively). The anthranilic oxidizing system in *Nocardia opaca* thus appears much more labile than that of the pseudomonas examined by Higashi & Sakamoto (1960), Hosokawa, Nakagawa & Takeda (1961) and Taniuchi *et al.* (1964) in which a NADH-mediated anthranilic hydroxylase system was detected.

Oxidation of probable intermediates by washed suspensions. Washed suspensions of *Nocardia opaca* harvested at 24 hr from yeast extract-enriched *o*-nitrobenzoate and anthranilate cultures were tested for ability to oxidize a wide variety of related compounds and probable intermediates. Catechol was the only compound oxidized at a rate comparable with that of *o*-nitrobenzoate or anthranilate by such organisms.

Salicyclic and gentisic acids, the most likely intermediates (Ke *et al.*, 1959; Ladd, 1962), were oxidized at low rates or not at all. 2-Nitro-3-hydroxybenzoic acid, anthranilamide, 3,4-dihydroxyanthranilic acid (after correction for non-enzymic oxidation), kynurenine, kynurenic and 2,3-dihydroxybenzoic acids also did not stimulate oxygen uptake above the endogenous value.

3-Hydroxyanthranilate was also oxidized without lag by *Nocardia opaca* grown on *o*-nitrobenzoate or anthranilate. Oxidation ceased at 1·5 and 2 moles O_2 consumed/mole substrate oxidized, respectively; these were well below the figures required for complete oxidation even allowing for 10–30% assimilation as found with *o*-nitrobenzoate itself. The corresponding 5-isomer was also oxidized but this oxidation, though not that of anthranilate or catechol, was almost entirely abolished by previous incubation with chloramphenicol (30 μg./ml. final concentration). The evidence suggests, therefore, that catechol is an intermediate common to the pathways for *o*-nitrobenzoic and anthranilic acids. Catechol oxidation was also observed with dried preparations of organisms harvested in the log phase from anthranilate but not from succinate media.

Catechol-1,2-oxygenase activity in extracts of Nocardia opaca. Cain & Cartwright (1960*a*) found that growth of *Nocardia opaca* on *o*-nitrobenzoic acid induced catechol-1,2-oxygenase activity, and they described some of the properties of the enzyme in this organism. Growth of this organism upon anthranilic acid also induced catechol-1,2-oxygenase; the enzyme was completely absent, however, from extracts of organisms grown in nutrient broth or fumarate medium (as measured manometrically or by the more sensitive spectrophotometric method).

Extracts of anthranilate-grown *Nocardia opaca* prepared by the Hughes press or by ultrasonic disintegration resembled those derived from *o*-nitrobenzoate cultures in being unable to metabolize catechol to β-oxoadipate, although dried organism preparations effected this change. The extracts converted catechol quantitatively to *cis,cis*-muconic acid which was not further metabolized, although the enzymes for converting (+)-muconolactone or its synthetic (±) isomer and β-oxoadipic enol lactone to β-oxoadipate were present. The amounts of the enzymes present in extracts of dried organisms grown on anthranilate and fumarate, respectively (Table 2), showed clearly that the complete enzyme sequence for converting catechol to β-oxoadipate was induced by growth on anthranilate. The same extracts also had activity towards some related metabolites such as protocatechuate (0·36 μmole degraded/hr/mg. enzyme protein) and β-carboxymuconate (10·0 μmoles/hr/mg. protein); but β-carboxymuconolactone was not attacked. The inability of crude extracts to lactonize *cis,cis*-muconate enabled some properties of the catechol-1,2-oxygenase to be studied conveniently. Extracts which converted catechol to *cis,cis*-muconate were fractionated with ammonium sulphate, all the activity being precipitated in the 33–45% saturation range. This fraction, after dialysis against water for 12 hr, was used as enzyme source; it was very active only for catechol. 3,4-Dihydroxybenzoate (protocatechuate) was slowly attacked by the same preparation but the corresponding 2,3-, 2,5- and 3,5-isomers were not utilized nor did they act as inhibitors of catechol oxidation.

The product of catechol oxidation by extracts was identified as follows. Catechol was added in 1 m-mole quantities to 150 ml. 0·1 M-phosphate buffer (pH 7·0) at 25° containing cell-extract equivalent to 40 mg. protein and 0·1 mM-Fe^{2+}. Aeration was

done by a magnetic stirrer sufficiently rapid to form a vortex in the liquid to the base of the 500 ml. reaction vessel. The reaction was followed manometrically in a pilot vessel, further catechol, to a total of 5 m-moles, being added to the main reaction flask when the oxygen uptake in the pilot vessel had ceased. The solution was maintained at pH 7·0 by adding 0·1 N-NaOH. At the completion of the reaction the contents were deproteinized with 10% metaphosphoric acid and the acidified solution ether extracted five times with 0·25 vol. of ether. After drying over anhydrous Na_2SO_4, the combined ether fractions were evaporated to dryness and

Table 2. *The amounts of enzymes catalyzing the conversion of catechol to β-oxoadipic acid in extracts of Nocardia opaca*

Enzyme	Reaction catalysed		Rate of substrate decomposition (μmoles/hr./mg. protein) Anthranilate-grown	Fumarate-grown
Catechol-1,2-oxygenase	catechol to *cis,cis*-muconate		7·9	0
Lactonizing enzyme	*cis,cis*-muconate to (+)-muconolactone		0·6	0
Muconolactone isomerase	(+)- or (±)-muconolactone to β-oxoadipic enol lactone	(*a*)*	4·4	0·07
		(*b*)	4·3	—
β-Ketoadipic enol lactone hydrolase	β-oxoadipic enol lactone to β-oxoadipate		6·0	0·1

*(*a*) is (+)-isomer; (*b*) is (±)-isomer.

the residue recrystallized from hot absolute ethanol. *cis,cis*-Muconic acid (300 mg.) crystallized as off-white needles, m.p. 195°. The mixed m.p. with authentic material (m.p. 197°) was 196°; with the authentic *cis,trans*-isomer (m.p. 189°) the mixed m.p. was 167°. The enzymically-prepared material had two titratable carboxylic acid groups and an equivalent of 70 from titration data; *cis,cis*-muconic acid requires 71. The prepared material was identical with synthetic *cis,cis*-muconic acid when chromatographed in solvents (C) and (D). After reduction of this material in water with hydrogen and Adam's catalyst (platinic oxide) the reduction product was extracted into ether, the ether evaporated off and the residue recrystallized from ethyl acetate+benzene. This product did not depress the m.p. of adipic acid (152°).

The effects of some inhibitors on the enzyme are shown in Table 3; from this it is clear that only metal-chelating agents and sulphydryl reagents brought about significant inhibition. The inhibition by EDTA, 2,2′-dipyridyl and 1,10-phenanthroline was almost completely annulled by Fe^{2+}, and that due to mercury compounds by L-cysteine or reduced glutathione. The inhibitory effect of 0·01 mM-$HgCl_2$ was not so severe when added after enzyme and substrate had already reacted (53% inhibition) as when pre-incubated with enzyme for 10 min. at 30° (90% inhibition). Both these inhibitions were annulled by 1 mM-L-cysteine, but not by 0·1 mM-Fe^{2+} to any degree. This suggests that sulphydryl groups are probably involved at the active site of the enzyme, a conclusion which Suda & Tokuyama (1958) reached about a purified pyrocatechase (catechol-1,2-oxygenase) from a pseudomonad.

Added divalent metal ions (0·1 mM) were uniformly without effect except for

Fe^{2+} which produced a more than twofold stimulation of enzyme activity of the ammonium sulphate fractionated enzyme preparations, and Cu^{2+} which caused some 42% inhibition. The copper inhibition was progressive and not relieved by subsequent addition of equimolar Fe^{2+} or by reduced glutathione (compare Hayaishi, Katagiri & Rothberg, 1957). Crude extracts showed no decrease in activity after dialysis for 24 hr against 1 mM 2,2′-dipyridyl or EDTA.

Table 3. *The effect of inhibitors on catechol-1,2-oxygenase of Nocardia opaca*

Substance added	Concentration (mM)	ΔE_2 * (1)	ΔE_2 * (2)	ΔE_2 * (3)	μl. O_2 uptake† /hr	Percent inhibition‡
None	—	0·198	0·170	0·166	—	—
EDTA	1	0·154	—	—	—	22
EDTA	3	—	—	0·098	—	41
Sodium azide	1	0·196	—	—	—	1
Sodium fluoride	1	0·172	—	—	—	13
Sodium arsenite	1	0·150	—	—	—	24
Sodium arsenate	1	0·158	—	—	—	20
Mercuric chloride	0·01	—	0·016	—	—	90
Mercuric chloride + reduced glutathione	0·01 1	—	0·220	—	—	(+) 29
p-Chloromercuribenzoic acid	0·1	0·043	—	—	—	77
	0·01	0·144	—	—	—	27
p-Chloromercuribenzoic acid + reduced glutathione	0·1 1	0·134	—	—	—	21
p-Chloromercuribenzoic acid + reduced glutathione	0·01 1	—	0·170	—	—	0
Reduced glutathione	10	0·142	—	—	—	28
L-Cysteine	1	—	—	0·320	—	(+) 93·0
None	—	—	—	—	189	—
2,2′-Dipyridyl	1	—	—	—	87	54
1,10-Phenanthroline	1	—	—	—	96	49
8-Hydroxyquinoline	1	—	—	—	72	62
2,2′-Dipyridyl + Fe^{2+}	1 1	—	—	—	180	5

* Under (1), (2) and (3) in this column are results for separate experiments.

† Manometric determinations were necessary with these chelating agents because of their high extinction at 260 mμ. Flasks contained 5 μmoles catechol and the enzyme protein (1·2 mg.) was incubated with the inhibitors for 10 min. before tipping substrate. Uptake figures are corrected for endogenous respiration.

‡ (+) sign in this column means stimulation.

The Michaelis constant determined for the ammonium sulphate fractionated extract from organisms grown on anthranilate gave a $K_m = 8$ μM from a double reciprocal plot. This compares favourably with the $K_m = 11$ μM found for the same enzyme in this *Nocardia opaca* when induced by growth on *o*-nitrobenzoate, but is higher than that of a pseudomonas pyrocatechase (0·5 μM) induced by growth on tryptophan (Hayaishi *et al.* 1957).

DISCUSSION

Utilization of nitro- and amino-phenyl compounds as sources of microbial carbon and nitrogen are well known, actinomycetes and pseudomonads being particularly efficient in this respect (Simpson & Evans, 1953; Durham, 1956; Gundersen & Jensen, 1956; Cain, 1958; Cartwright & Cain, 1959*a*; Ladd, 1962; Taniuchi *et al.* 1964). Although in some cases reduction of the nitro group precedes assimilation (Durham, 1958) in others the nitro group is released as nitrite ion (Gundersen & Jensen, 1956; Cain & Cartwright, 1960*b*). Corresponding nitro- and amino-phenyl derivatives may be degraded in the early stages by quite independent metabolic routes, though in most cases these pathways converge at common hydroxylated intermediates. It might be expected, therefore, that the molar growth yields from carbocyclic aromatic compounds and their nitro- and amino-derivatives would be comparable so long as the nitrogen content of the derivatives was not limiting growth. In mononitro- and monoamino-substituted aromatic compounds, the nitrogen content is probably always limiting since it comprises less than 10% of the total molecular weight. The increased yield coefficients of *Nocardia opaca* upon addition of a source of ammonium-N (the form in which nitrobenzoate- and aminobenzoate-N ultimately appears in cultures of this organism) reported here, substantiate this suggestion. Even with supplementary nitrogen, however, the molar growth yields of *N. opaca* upon anthranilate were always equal to, or slightly greater than, those given upon *o*-nitrobenzoate; this suggests that the organism can obtain no energy from a reduction of the nitro group in this substrate, which it is known to effect (Cartwright & Cain, 1959*b*).

Cain (1966) produced evidence that anthranilic acid is not a direct intermediate in the metabolism of *o*-nitrobenzoate by *Nocardia opaca*, although it appears transiently in the culture medium. These conclusions are reinforced by several observations reported here: (i) the mutual inhibitory effects of *o*-nitrobenzoic and anthranilic acids in growth and in oxidation, confirmed in the latter case by the isotopic experiments; (ii) the lag of some 28 hr before an inoculum of *o*-nitrobenzoate-grown organisms began to produce measurable growth in anthranilate media; (iii) the long adaptation time for *o*-nitrobenzoate-grown washed organisms to begin to oxidize anthranilic acid and the low degree of activity even after induction; (iv) *N. opaca* grown upon *o*-nitrobenzoate accumulated anthranilate only very slowly.

Numerous differences in the properties of the catechol-1,2-oxygenases induced in *Nocardia opaca* by growth on anthranilate and *o*-nitrobenzoate were also observed although it should be emphasized that only partially purified enzyme preparations were used. In particular, the nitrobenzoate-induced enzyme was active only when prepared and reacted in the presence of L-cysteine (Cain & Cartwright, 1960*a*), whereas the anthranilate-induced enzyme was quite stable in the absence of sulphydryl compounds; the former was rapidly inactivated by dialysis against water or 2,2′-dipyridyl, whereas the latter was unaffected by dialysis for 48 hr against these materials. The effects of chelating agents on the two enzymes were also different. There is little doubt from the accumulated evidence that the energy-yielding pathway of *o*-nitrobenzoate metabolism does not involve anthranilic acid and that the pathways for these two substrates are distinct.

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Spore Swelling and Germination in *Fusarium culmorum*

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SUMMARY

Carbon and nitrogen sources were found to be necessary both for the germination and the associated swelling of the macroconidia of *Fusarium culmorum*. The swelling of the spores took place before the emergence of the germ tube and was measured by two methods. The spores were shown to have a mucilaginous covering, which affected the uptake of nutrients and into which glucose was absorbed by a physical process rather than by an enzymic one. Tritiated water was used to demonstrate that the swelling of the spores in the presence of a nitrogen source was due largely to water uptake and only in a small part to an increase in dry weight.

INTRODUCTION

The germination of fungal spores has received a great deal of attention but in general the available data cover many species with few examples of detailed studies. Activation and germination of *Neurospora tetrasperma* ascospores (Goddard, 1935; Sussman, 1954) and germination of basidiospores of *Schizophyllum commune* (Hafiz & Niederpruem, 1963; Ratts, Hafiz, Niederpruem & Egbert, 1964) have both received extensive investigation.

Fusarium culmorum exists parasitically and saprophytically on cereal crops, producing asexual fusiform to falcate macroconidia only, which are 3- to 5-septate and 25–50 μ long by 5–7 μ wide. The mycelial and conidial chlamydospores which are produced by this fungus were not investigated in the present study. The macroconidia germinate in liquid culture in 4–6 hr, after their swelling. Ekundayo & Carlile (1964) studied the similar swelling which occurs before the germination of sporangiospores of *Rhizopus arrhizus*. They postulated a system of active water uptake and a permeability change in the cell wall, although they did not investigate relative changes in dry weight and water content of the spores. The present study was designed to investigate the swelling of the macroconidia of *F. culmorum* and the relative contributions of water uptake and dry weight changes to this swelling.

METHODS

Organism. The strain of *Fusarium culmorum* used was obtained from the Commonwealth Mycological Institute (IMI. 96283) and was a typical isolate in all respects. The organism was grown on potato sucrose agar in large culture bottles at 25° in an incubator which had glass doors and was exposed to normal daylight.

Culture medium. The standard culture medium was potato sucrose agar, containing the filtered extract of 250 g. potato in 1 l. medium, supplemented with 2%

(w/v) sucrose. The culture bottles were normally inoculated with a conidial suspension in sterile water.

Preparation of spore suspensions. The conidial suspensions used in the experiments were prepared by washing conidia from the surface of a culture with a small quantity of sterile distilled water. Mycelial fragments were removed by filtration through glass wool and the conidia then washed three times with water. Conidial suspensions for use were adjusted to a turbidity reading of 2·0 at 610 mμ on a Hilger colorimeter, which represented a dry weight of about 2 mg./ml. Washing the conidia was shown to have no effect on their germinative capacity, in agreement with the results of Cochrane, Cochrane, Simon & Spaeth (1962).

The assessment of germination. Germination experiments were done at 25° in media buffered with 0·03 M-phosphate buffer (pH 6·5) and germination was recorded as having occurred when a visible germ tube had been produced. Germination counts were made after shaking for 6 hr. Optical measurements of maximum conidial width were made with a micrometer eyepiece. The media for conidial swelling and dry-weight determinations contained 50 μmoles glucose/ml. and 20 μmoles ammonium sulphate/ml. in 0·03 M-phosphate buffer (pH 6·5).

Dry-weight determinations. Measurements of dry weight were done in glass tubes which had been previously dried and weighed. Samples of conidia suspended in medium were placed in these tubes and centrifuged. The supernatant fluid was discarded and the tube containing the pellet of conidia was dried to a constant weight at 80°. In experiments in which the conidia were washed after sampling the pellet was resuspended in 5 ml. distilled water and recentrifuged before drying.

Manometric methods. Oxygen uptake was measured in the usual Warburg apparatus at 25°, with direct absorption of carbon dioxide by 10% (w/v) KOH.

Tritium labelling. Estimations of relative water uptake were made after 4·5 hr at 25°, before germ-tube emergence, by using tritiated water. The conidia were spun down from the tritium-labelled medium containing 1 mC. tritium, and were resuspended in 1 ml. of unlabelled water. Samples (0·01 ml.) of the resuspended conidia were counted in a liquid scintillation counter, with an NE 220 scintillator liquid.

Mucilage staining. The mucilaginous covering of the macroconidia was revealed by staining with 0·25% aqueous thionine for 10–15 min., after fixation in acetic acid + ethanol (1 + 3) saturated with mercuric chloride. The conidia were fixed to a slide with Haupt's adhesive and mounted in 10% aqueous nigrosin. The mucilaginous coat was then visible as a thin faintly stained layer.

Inhibitor methods. Cyanide inhibition was achieved by supplying 10^{-2} M-KCN in shake cultures.

RESULTS

Requirements for spore germination

Cochrane, J. C. *et al.* (1963) found that macroconidia of *Fusarium solani* f. *phaseoli* required an exogenous supply of carbon and nitrogen sources for germination, and also a factor present in yeast extract which could be completely replaced by ethanol or acetoin and partially replaced by acetaldehyde or several amino acids. Sisler & Cox (1954) showed that washed conidia of *Fusarium roseum* required only carbon and nitrogen sources for germination. The requirements of *F. culmorum*

macroconidia are similar to those reported by Sisler & Cox, as can be seen from Table 1. The conidia were capable of about 14% germination in distilled water, but the maximum values were only achieved in the presence of carbon + nitrogen sources. Ammonium sulphate was the most efficient source of nitrogen in comparison with potassium nitrate and potassium nitrite; asparagine was capable of serving as carbon + nitrogen source. Various sugars, including fructose, xylose, arabinose, galactose, glucose and lactose, were tested as carbon sources; all supported germination and were utilized to some extent (Fig. 1). Acetate did not support germination, although measurements of oxygen uptake showed that it was utilized. This result is in agreement with Cochrane, Cochrane, Vogel & Coles (1963), who found that acetate did not support germination in *F. solani* f. *phaseoli* without the addition of the yeast extract factor.

Table 1. *Percentage germination of macroconidia of Fusarium culmorum in various media at 25°*

Germination in distilled water varied between 11 and 14% in different experiments and therefore the results were standardized by expressing germinations as percentages above the value in distilled water.

Medium	% germination above the value in distilled water
0·03 M-phosphate buffer (pH 6·5)	7·2
0·01 M-glucose	3·7
0·008 M-ammonium sulphate	34·4
0·008 M-ammonium sulphate + 0·01 M-glucose	68·9
0·004 M-potassium nitrate + 0·01 M-glucose	27·8

Oxygen uptake during germination

Oxygen uptake by germinating conidia was followed in media containing carbon and nitrogen sources. Carbon was supplied as D-glucose (10 μmoles/flask) and nitrogen as ammonium sulphate (25 μmoles/flask) in media buffered at pH 6·5 with 0·03 M-phosphate buffer. The graphs for oxygen uptake are given in Fig. 2. The graphs for glucose utilization show an initial period of about 1 hr when the rate of oxygen uptake gradually increased until linearity was reached. This period is unlikely to represent an enzyme induction, partly because of the short time involved and partly because the organism had been grown on a medium containing glucose. The delay in reaching maximum oxygen uptake would thus appear to be due to some barrier, physical or metabolic, to the entry of glucose into the cells.

When a limited amount of glucose was supplied to the spores it was exhausted at about the same time in the presence or absence of ammonium sulphate, although more was oxidized in the presence of the nitrogen source. Ammonium sulphate supplied in the absence of a carbon source had no effect on the basal respiration rate. The ammonium sulphate must have a profound effect on the fate of carbon in the germinating spore, as is borne out by the failure to germinate in its absence.

Conidial swelling

When conidia of *Fusarium culmorum* were germinated in a medium containing glucose and a nitrogen source the majority showed a marked swelling, leading to

distension of the wall between the septa (Plate). Conidia germinated in a medium lacking nitrogen showed no such swelling. The swelling of the conidia was measured directly during the period preceding germ-tube emergence by optically following the maximum conidial width. The results in terms of % increase in mean maximum conidial width for samples of 50 conidia are shown in Fig. 3 together with the standard errors of the points. It can be seen that, while the conidia in glucose + ammonium sulphate showed a near-linear increase in width, those in glucose alone did not significantly differ from the initial sample or from the buffer control.

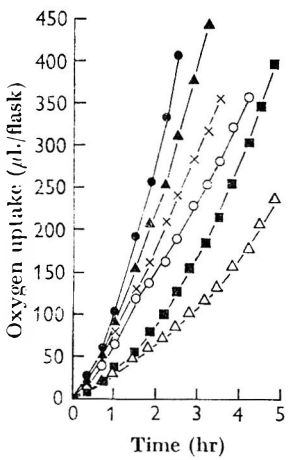

Fig. 1

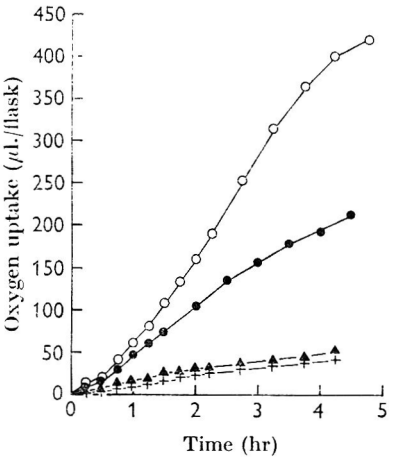

Fig. 2

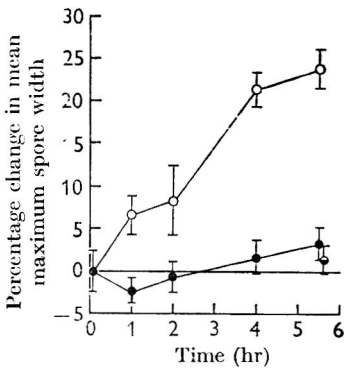

Fig. 3

Fig. 1. Utilization of various sugars by germinating macroconidia of *Fusarium culmorum*, as measured by oxygen uptake at 25°. △—△, Lactose; ■—■, arabinose; ○—○, xylose; × — ×, galactose; ▲ — ▲, fructose; ● — ●, glucose.

Fig. 2. The oxidation of glucose (10 μmoles) in the presence or absence of ammonium sulphate, and the effect of ammonium sulphate on the basal respiration rate of, maeroconidia of *Fusarium culmorum*. ● — ●, Glucose; ○—○, glucose + ammonium sulphate; + —+, ammonium sulphate; ▲— ▲, control. Oxygen uptake was measured at 25°.

Fig. 3. Percentage change in mean maximum conidial width for macroconidia of *Fusarium culmorum* shaken in: ● — ●, glucose; ○—○, glucose + ammonium sulphate; ◒, buffer (pH.) The optical measurements were made on samples of 50 conidia and the standard errors are shown. The experiment was done at 25°.

As a complementary method a precipitation technique was devised to estimate total changes in conidial volume. The method depends on the fact that the conidia of *Fusarium culmorum* precipitate easily and because of their shape pack down in a regular layered fashion. Samples of conidia in liquid medium were put into sealed lengths of 2 mm. bore glass tubing. The conidia were then centrifuged for 2 min. at constant speed (1400g) and the depth of the precipitate expressed as a proportion of the total height of the column of liquid. The method was applied to the same set of conditions that had been examined by the optical method. The % changes in the volume parameter are expressed graphically in Fig. 4. From the results obtained with the optical measurement method it was to be expected that any increase would be linear, but in the presence of glucose, whether ammonium sulphate was present or not, there was a sudden rise during the first hour followed by the predicted linear

increase. The rate of increase over the linear portion of the graph agrees well with the rate found by optical measurement for conidia in glucose + ammonium sulphate. The buffer control showed no rapid initial increase. The similarity of time scales suggested that increasing oxygen uptake and overall conidial swelling might be linked phenomena. As optical measurement does not reveal the initial increase it is implied that the cause of this swelling was a layer outside the conidial wall which was not visible in unstained preparations. The nature of this layer is suggested by the work of Drs J. M. Hirst and H. L. Nixon (personal communication 1965), who

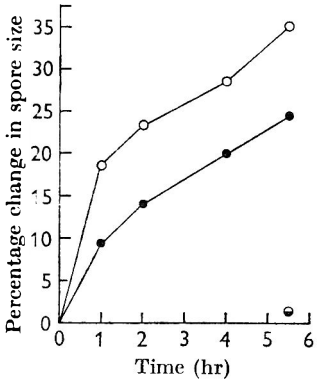

Fig. 4

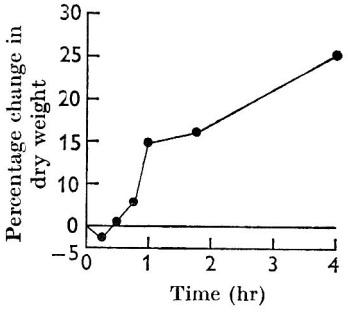

Fig. 5

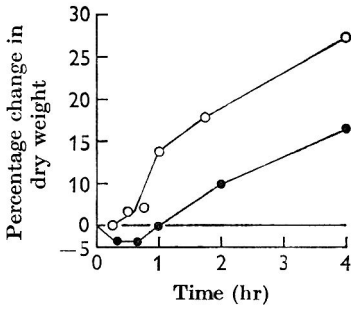

Fig. 6

Fig. 4. Percentage change in conidial size, as measured by the precipitation method, for macroconidia of *Fusarium culmorum* shaken in: ●—●, glucose; ○—○, glucose + ammonium sulphate; ◒, buffer (pH 6·5). The experiment was done at 25°.

Fig. 5. Percentage change in dry weight for macroconidia of *Fusarium culmorum* shaken in glucose medium at 25° and unwashed before drying.

Fig. 6. Percentage change in dry weight for macroconidia of *Fusarium culmorum* shaken in glucose + ammonium sulphate at 25°: ○—○, unwashed before drying; ●—●, washed before drying.

showed a mucilaginous covering on the wettable microconidia of *Fusarium oxysporum* by an electron microscope replica technique. The conidia of *F. culmorum* were examined for any mucilaginous layer by a staining technique with thionine and nigrosin. This showed that conidia shaken in any medium containing glucose had a distinct faintly-staining layer, 0·5–1 μ thick, which surrounded the conidia. This layer was not detectable on conidia freshly sampled or on conidia shaken in buffer. Although this layer did not show the normal intense staining reaction of mucilage (Wilson, 1965), it is obviously responsible for the rapid overall increase in size of the conidia in glucose-containing media.

Dry-weight changes

Associated with the respiration of glucose is its oxidative assimilation, with a consequent increase in dry weight. Experiments were therefore designed to investigate the changes in dry weight of conidia during germination, especially during the first hour, and the role of the mucilaginous sheath in nutrient uptake. The increase in dry weight was determined in conidial samples shaken in the presence of glucose and glucose + ammonium sulphate, without washing the samples before

drying. The results are shown in Figs. 5 and 6; once again there was the rapid increase over the first hour, followed by a period of linear increase up to the time of germination, when the increase became exponential. To determine whether the uptake of glucose into the mucilaginous sheath was a physical process or an enzymic one, samples of conidia were washed before drying; the results of this experiment are shown in Fig. 6. If the binding of glucose into the sheath were enzymic, washing ought not to remove it; but the graph shows that there was no increase in dry weight over the first hour, indicating that the glucose in the sheath had been removed. The dry weight does show the normal linear increase after the first hour. The observation that various sugars, pentoses and hexoses, were taken through the mucilage and reached a maximum rate of oxidation after the same period of time appears to reinforce the view that the process was not enzymic and hence non-specific. The uptake of glucose was not inhibited by cyanide, although respiration was completely inhibited for several hours.

Table 2. *Estimation of water uptake by tritium labelling in media containing glucose in the presence and absence of ammonium sulphate*

Corresponding spore swelling and dry-weight increases are also shown.

	Increase over control (%)	
Water uptake		
Glucose	8·9	
Glucose + ammonium sulphate	31·7	
Dry weight		
Glucose	11·5	
Glucose + ammonium sulphate	19·6	
	Mean max. conidial width (μ)	Standard error
Spore width		
Glucose	6·43	± 0·05
Glucose + ammonium sulphate	7·55	± 0·08
Buffer control	6·41	± 0·06

The extent of water uptake

The extent of water uptake was estimated comparatively by using tritiated water with samples of conidia shaken in glucose medium, with and without ammonium sulphate. The amount of water taken up was calculated as the excess over a buffer control shaken for the same period of time. The results of this experiment together with dry-weight changes and conidial size changes are given in Table 2. The results show that the conidia shaken in glucose alone, while showing no increase in width, did show an increase in dry weight and free-water content. The conidia shaken in glucose + ammonium sulphate, however, showed a marked swelling and a larger increase in free water; therefore the swelling may at least in part be attributed to water uptake. Conidia shaken in glucose + ammonium sulphate displayed a 17·4% increase in width over conidia shaken in glucose alone; coupled with this was a 6·2% increase in dry weight and a 21·1% increase in water content of the organisms. It is therefore reasonable to say that the main part of the swelling which took place

in the presence of ammonium sulphate was due to an enhanced capacity to take up water, although the difference in dry weight may also contribute something to the swelling.

DISCUSSION

The macroconidia of *Fusarium culmorum* are only able to germinate satisfactorily when supplied with carbon and nitrogen sources. The basal respiration value of the conidia is very low and the necessity for an external carbon source undoubtedly arises from the need for carbon skeletons and high respiratory activity. The way in which a nitrogen source is able to cause the swelling of the conidia by water uptake is not clear. It may be that a nitrogen source, in its role as a wall component, permits the walls to become more elastic, or it may directly affect the permeability properties of the wall. The swelling process, as observed optically, is a roughly linear process and therefore the necessity for a nitrogen source as a precursor for enzyme protein synthesis seems to be eliminated. The stimulation of oxygen uptake during the oxidation of glucose in the presence of a nitrogen source does suggest that the latter is playing a major role in switching the metabolic path of carbon in a germinating conidium. As a limited amount of carbon source is exhausted at the same time in the presence or absence of a nitrogen source it seems that the nitrogen effect is not simply one of rate of utilization.

Ekundayo & Carlile (1964) suggested that in *Rhizopus arrhizus* the uptake of water by conidia in the presence of glucose was an active process. In *Fusarium culmorum* water uptake is only allowed in the presence of a nitrogen source and therefore the process could be termed active in that there is some process other than, or complementary to, osmosis which controls water uptake. Yarwood (1950) suggested that fungal conidia have to attain a degree of hydration comparable to that of powdery mildews before germ-tube emergence can occur. But Ekundayo & Carlile (1964), in the light of their work, suggested that this hypothesis was untenable, and the results of the present work indicate that some modification is necessary. In view of the several roles of a nitrogen source in germination it seems that the uptake of water may be only subsidiary to other changes in the conidia, which culminate in germination.

The authors thank the Science Research Council for a grant held by R.M. during the completion of this work.

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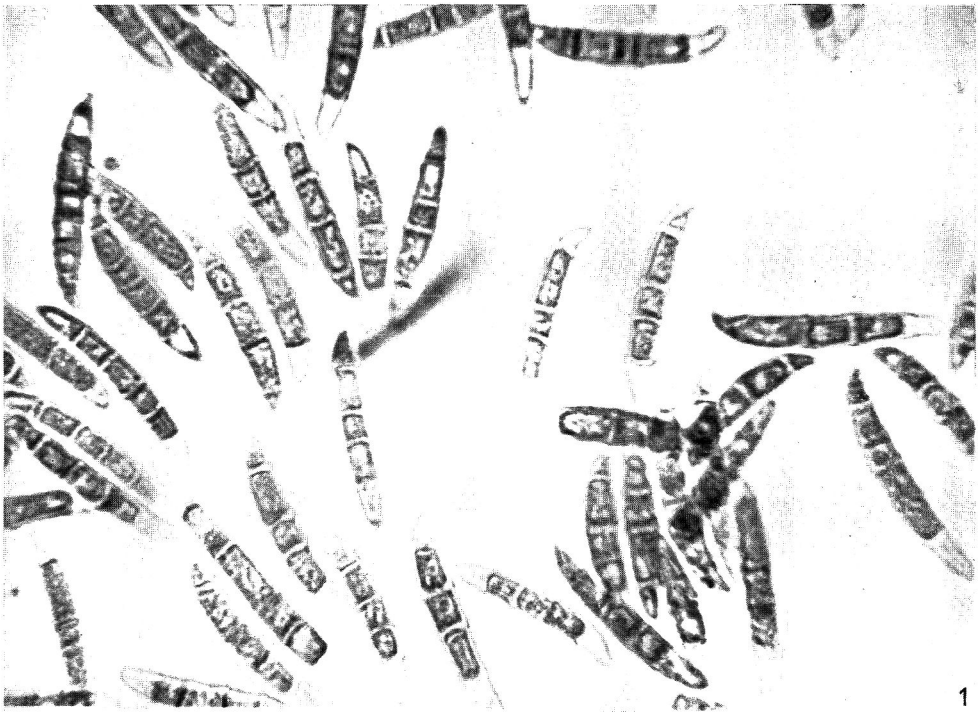
EXPLANATION OF PLATE

Photomicrographs of macroconidia of *Fusarium culmorum* stained in Sudan Black B to show the spore swelling associated with germination.

Fig. 1. Freshly harvested conidia. × 1000.

Fig. 2. Conidia shaken for 4 hr in a medium containing glucose and ammonium sulphate. × 1000.



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Carbon dioxide: Signal for Excystment of *Naegleria gruberi*

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SUMMARY

Exposure of cysts of *Naegleria gruberi* to slightly increased environmental CO_2 caused excystment. Excystment was also induced by addition of proline or by an increase in the cyst population density, but both did so by increasing the amount of CO_2 produced by the bacteria (*Aerobacter aerogenes*) which contaminated the cyst suspensions. Molecular CO_2 would seem to be an excellent signal to induce excystment of a phagotrophic soil amoeba since the presence of CO_2 would indicate an environment favourable for growth of the amoebae. Once excystment is initiated, it can proceed to completion in atmospheric CO_2.

INTRODUCTION

An adaptive environmental signal for excystment should lead to emergence of the organism into an environment in which there would be a high probability of survival and growth. In the laboratory numerous stimuli can induce the excystment of protozoan cysts. These stimuli include shifts in temperature (Johnson & Evans, 1940) and pH value (Darby, 1929), the addition of water (Garnjobst, 1928) or of chemicals such as carbohydrates (Barker & Taylor, 1933), glycine (Beers, 1945), dicarboxylic acids and acetate (Haagen-Smit & Thimann, 1938), or viable bacteria (Crump, 1950). This diversity of stimuli might reflect the variety of organisms studied, each with its specific stimulus, a variety of stimuli for a given organism or laboratory artifacts. Certain observations are particularly suggestive. Colpoda excysts in response to all the chemicals listed above, to bacteria (Singh, 1941), and even to appropriate agitation (Barker & Taylor, 1933). One frequent stimulus, the presence of viable bacteria, causes numerous changes in the environment, only one of which may be the signal for excystment. In addition, viable bacteria, often of unknown type and unknown quantity, are usually present with the other agents studied, and possibly alter the added agent in an unknown way. The effect of bacteria on chemically induced excystment has usually been ignored or disposed of with a statement such as 'in all crucial experiments the cysts become active long before the bacteria could possibly produce an effect' (Beers, 1945).

Naegleria gruberi is a small phagotrophic soil amoeba which under appropriate conditions forms cysts or transforms into flagellates. Though the stimulus for naegleria excystment has not been studied, excystment has been described (Wilson, 1916; Rafalko, 1947; Schuster, 1963). One of the most dramatic cytological

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changes during excystment is the reappearance of contractile vacuoles, which are not present in the uninduced cyst. Active amoeboid motion precedes the emergence of the amoeba through one of several pores in the cyst wall. For our purposes a cyst is considered to be 'full' as long as the cytoplasm is entirely within the cyst wall, but once emergence begins and the cytoplasm is partially within and partially without, the cyst is 'transitional'. The emerging amoeba leaves behind an empty cyst wall. The excysted amoebae develop flagella, but this is without apparent effect on excystment.

This paper describes a study of three environmental variables—proline, CO_2, cyst population density—which induce the excystment of *Naegleria gruberi*, and of the role of bacteria in the process.

METHODS

Growth and preparation of cysts. *Naegleria gruberi* NB-1 and the details of procedures used for cultivating the amoebae and performing counts will be described by Fulton & Dingle (to be published). Amoebae were grown in association with *Aerobacter aerogenes* on autoclaved agar medium, NM, containing (g./l. distilled water): Difco Bacto-peptone, 2·0; glucose, 2·0; K_2HPO_4, 1·5; KH_2PO_4, 1·0; Difco Bacto-agar, 20. About 10^5 cysts and 0·1 ml. of an overnight broth culture of *Aerobacter aerogenes* were spread over the surface of NM agar in a 100 mm. diam. Petri plate, and the plate incubated at 33–34°. Under these conditions the cysts excysted, and the resulting amoebae grew exponentially until they reached stationary phase at $2–3 \times 10^7$ amoebae/plate. Some amoebae began to encyst after about 20 hr and all encysted within 48 hr. The plates of cysts were stored at 34° and used 4–6 days after plating.

Cysts were prepared for excystment experiments by harvesting each plate into 10 ml. demineralized water and centrifuging the suspension at 10° for 105 sec. at 500 *g* in a swinging bucket rotor. The supernatant fluid was discarded and the pellet washed twice more in demineralized water. The final pellet was resuspended under conditions suitable to the experiment. The cyst concentration was determined by a Coulter counting apparatus (Coulter Electronics, Hialeah, Florida, U.S.A.) and the bacteria by colony count.

Excystment on agar. Agar excystment experiments were done on autoclaved agar media containing 2 % (w/v) Bacto agar and supplements as indicated for each experiment. A sample of a washed counted cyst suspension was spread over the surface of a plate, and the plate placed, agar upward, without its cover, in a desiccator of about 2 l. capacity which contained 50 ml. of a 10 % (w/v) NaCl solution to maintain constant humidity. The gas phases were adjusted (see below), and the desiccators incubated at 34°. The beginning of incubation was the zero time for each experiment.

To evaluate excystment on agar, the organisms were examined *in situ*. The plates were removed from the desiccators and a circular coverslip 18 mm. in diameter was placed directly on the agar, with care taken to allow a film of liquid to flow between the agar and the coverslip. The organisms were examined with phase contrast optics at a magnification of ×400. When the plates were to be re-incubated they were returned to the desiccators and the gas phases readjusted. For each time point a fresh area of a plate was examined.

Excystment in liquid. Liquid excystment experiments were normally done in demineralized water containing either 2×10^{-3}M tris buffer (pH 7·4) or 10^{-3}M potassium phosphate buffer (pH 7·7). Unless otherwise stated, a sample of a washed and counted cyst suspension was diluted into 10 ml. of buffer in a 125 ml. Erlenmeyer flask. The flask was sealed with a serum vial stopper, its gas phase adjusted, and then shaken at 30° in a water bath at the rate of seventy 1-inch strokes/min. The beginning of incubation was taken as the zero time of the experiment.

Evaluation of excystment in liquid. At intervals 0·2 ml. samples of the cyst suspension were removed from the flasks by using a 1 ml. syringe with a 5-inch 20-gauge needle. The cysts in the samples were fixed and stained by injecting the samples into tubes containing one drop of a 1/10 dilution of stock Lugol's iodine (Fulton & Dingle, to be published) in distilled water. The fixed samples were examined with phase contrast optics at × 400, and classified as full, transitional and empty cysts (amoebae being ignored). The process of excystment can be measured as either percent full or percent transitional and empty cysts; full cysts were used. Each determination represented a count of a sample of 100 cysts. Measurements of reconstructed populations of cysts and amoebae indicated that clumping or other events capable of yielding inaccurate counts did not occur during incubation, sampling and fixing, or slide preparation.

Adjustment of the pCO_2. Desiccators or flasks were evacuated by aspiration, a measured volume of CO_2 gas (Matheson 'Bone Dry' grade, minimum purity 99·8 % CO_2) injected from a syringe, and water-washed air allowed to enter the vessel until atmospheric pressure was reached (see Loomis, 1959). The pCO_2 was calculated as the percent of the total volume of the vessel occupied by the volume of CO_2 injected. The pCO_2 of the laboratory atmosphere was assumed to be 0·03 %.

To compare excystment in the presence and absence of CO_2, cysts were incubated in absorption flasks fitted with centre wells containing paper wicks and either 1 ml. of distilled water or 1 ml. of 20 % (w/v) KOH. Warburg vessels shaken at 140 strokes per minute were used in some experiments to improve gas exchange. To guard against spillage of KOH, the pH of the excystment solution was measured at the end of each experiment.

RESULTS

Inducers of naegleria excystment

Proline. In the course of other work, proline was observed to induce the excystment of *Naegleria gruberi*. Liquid suspensions of cysts excysted when L-proline was added at concentrations greater than 10^{-5}M; the rate of excystment was similar and maximal at 10^{-4}, 10^{-3}, and 10^{-2}M. The excysted amoebae immediately transformed into flagellates, and to avoid this we attempted to obtain excystment on agar plates where transformation does not occur. Contrary to the effect of proline in liquid, cysts placed on proline agar did not excyst, regardless of the concentration of proline. Extending the time of incubation, washing the agar, or varying the number of cysts plated did not result in excystment. The disparity between the results in liquid and on agar was resolved by a chance observation. On proline agar plates, uncovered cysts remained dormant while those under a coverslip excysted after an hour (Fig. 1). It seemed likely that the coverslip

created an environment stimulating excystment by interfering with gas exchange. One possibility was an increase in CO_2 concentration under the coverslip.

Carbon dioxide. Proline agar plates spread with cysts were incubated in atmospheres of different CO_2 concentrations (Table 1). Increased pCO_2 induced excystment, and the amount of excystment was a function of the pCO_2. Furthermore, when the pCO_2 was increased, excystment occurred independently of the presence of proline in the agar.

In liquid, where measurement of degree of excystment is much more precise, either proline or CO_2 induced excystment (Fig. 2). Under similar conditions of temperature and aqueous environment and with saturating concentrations of

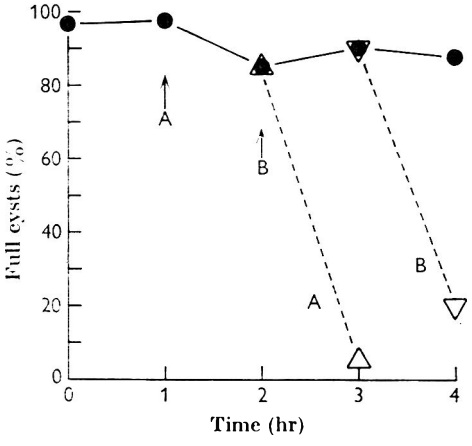

Fig. 1

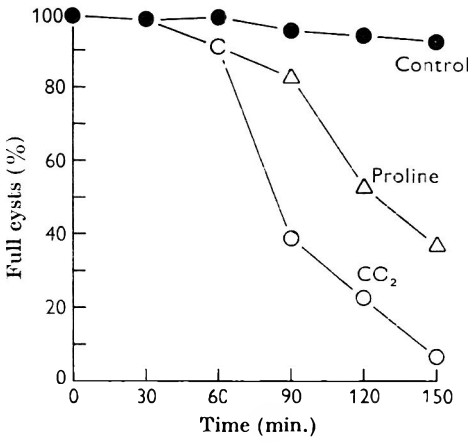

Fig. 2

Fig. 1. The coverslip experiment. An agar plate buffered at pH 7·4 with 0·02 M-tris and containing 10^{-3} M-L-proline was spread with 2×10^6 cysts and incubated at 34° under standard conditions. A sample of the initial cyst suspension was fixed and counted. At hourly intervals a coverslip was applied to a new area of the plate and excystment evaluated *in situ*; at the same time the areas under the coverslips applied at one (A) and at 2 hr (B) were re-examined.

Fig. 2. Induction of excystment in liquid by proline and by CO_2. Three flasks were prepared each containing 5 ml. of phosphate buffer, pH 6·8, with 8×10^5 cysts per ml. One flask also contained 10^{-3} M-proline, and another flask was capped and its atmosphere adjusted to 0·95 % pCO_2. The flasks were shaken at 25° with 100 strokes per minute. At the end of the experiment the pH in the flasks ranged from 6·85 for the control to 6·7 for the flask with increased pCO_2.

Table 1. *Excystment on proline agar in varying CO_2 atmospheres*

Washed cysts were spread at 2×10^6 cysts per plate on agar plates buffered at pH 6·8 with 0·02 M-potassium phosphate and containing 10^{-3} M-L-proline. The plates were placed in desiccators, the atmospheres adjusted to various CO_2 concentrations, and the desiccators incubated at 34°. Excystment was evaluated after 4 hr incubation.

	pCO_2 (%)			
	0·03	0·33	1·0	2·0
Full cysts	90	50	5	0
Transitional cysts	3	15	5	7
Empty cysts	7	35	90	93

CO_2 or proline, excystment regularly occurred sooner in response to CO_2 than to proline.

Measurement of the response to CO_2 depended on having a measure of the relative rate of excystment. If excystment of a population were synchronous, there would be an abrupt transition from full cysts to transitional cysts and then to empty cysts. Excystment under the conditions we used was asynchronous, and a plot of the % full cysts against time of incubation gave a sigmoid curve. If such a curve represented a summed normal distribution of excystment times, the probit transformation would convert it to a linear curve (Finney, 1962). Probability curves for naegleria excystment approximate linearity (Fig. 3). The mean time of

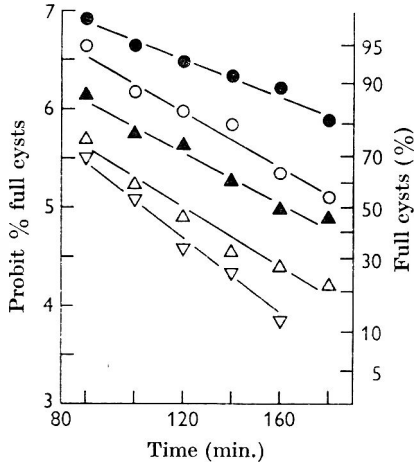

Fig. 3

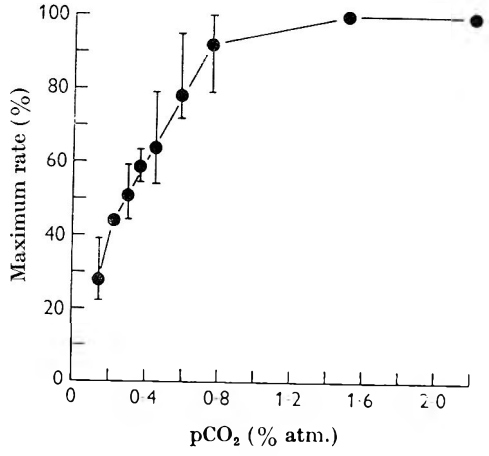

Fig. 4

Fig. 3. Excystment at various CO_2 concentrations. Washed cysts were suspended at a concentration of 1×10^5 per ml. in 10 ml. of phosphate buffer in 125 ml. flasks, the atmosphere of the flasks adjusted, and the flasks shaken at 25° at 70 strokes per minute. The CO_2 concentrations of the atmospheres were: ●, 0·15; ○, 0·30; ▲, 0·44; △, 0·59; ▽, 0·74 %.

Fig. 4. Excystment as a function of CO_2 concentration. Conditions for excystment were described in Fig. 3, and the relative rate of excystment was the reciprocal of the time required for 50 % excystment (obtained from probability curves). Where several curves were obtained at the same pCO_2, the mean value with the range is plotted.

excystment (the time for 50% excystment) varied with the pCO_2 (Fig. 3). The reciprocal of the mean excystment time was used as a measure of the relative rate of excystment (this approach, used with a similar morphological change, will be discussed in Fulton & Dingle, to be published). Though the mean excystment time varied with pCO_2, the slopes of the probability curves, which represent the standard deviation did not vary greatly, so the heterogeneity of the response of the cyst populations, to increased pCO_2 was not markedly affected by the relative rate of excystment.

The effect of CO_2 concentration on the relative rate of excystment was determined by multiple measurements under standardized conditions (Fig. 4). The response of cysts was a function of pCO_2 in the range from 0·15% to about 1%. Excystment at any pCO_2 less than 0·15% was too slow and variable to obtain valid

measurements of rate. Thus we do not know whether a threshold level of pCO_2 (i.e. above atmospheric) is necessary to induce excystment. Cysts can be stored on agar plates in atmospheric pCO_2 for prolonged periods without significant excystment; this suggests that excystment does not occur unless pCO_2 exceeds 0·03%.

A portion of the CO_2 dissolving in an aqueous solution ionizes to increase the H^+, HCO_3^-, and $CO_3^=$ content of the solution. Is molecular CO_2 the stimulus for excystment, or is one of its ionic products the effective agent? Acid pH values have been reported to induce excystment (Darby, 1929). but naegleria cysts placed in phosphate buffers from pH 3·3 to 7 did not excyst until CO_2 was added.

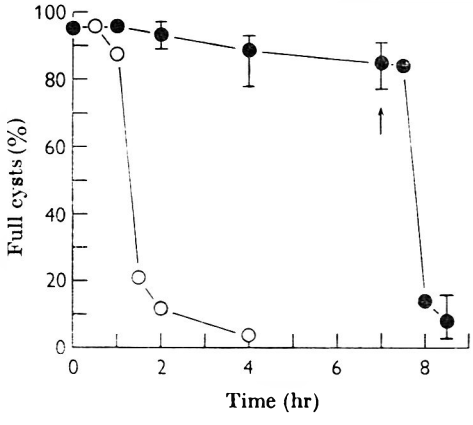

Fig. 5

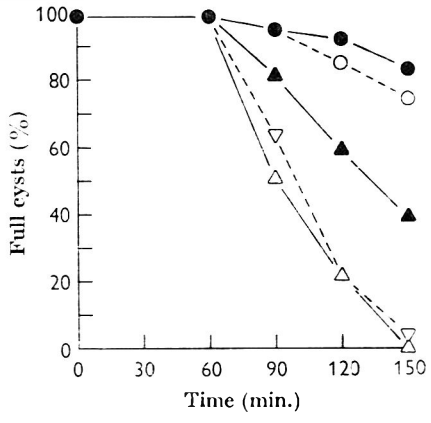

Fig. 6

Fig. 5. Effect of bicarbonate on excystment. Washed cysts were suspended at 10^5 per ml. in phosphate buffer containing various concentrations of bicarbonate and shaken at 30° with 70 strokes per minute. The closed circles give the mean values with the range, for four flasks containing 10^{-2}, 10^{-3}, 10^{-4}, and 10^{-5} M-bicarbonate. A flask was included without bicarbonate but containing 0·8 % pCO_2 (open circles). At the point indicated by the arrow the bicarbonate flasks were capped and 0·8 % pCO_2 added to each.

Fig. 6. Density-induced excystment. Washed cysts were suspended in tris (pH 7·4), and 10 ml. samples shaken at 25° in flasks capped with serum vial stoppers. The numbers of cysts $\times 10^5$ per ml. were: ●, 1·1; ○, 3·3; ▲, 11; △, 23; ▽, 55.

In the buffered solutions used the addition of CO_2 did not markedly alter the pH value (e.g. Fig. 2), so changes in pH value are not responsible for excystment. Bicarbonate ions also did not induce excystment (Fig. 5); at high concentrations the slight excystment observed was probably due to molecular CO_2 produced in the solution. The addition of CO_2 to the bicarbonate solutions induced excystment (Fig. 5), showing that bicarbonate neither stimulated nor inhibited the process. Carbonate ions, as well as carbonic acid, are produced in very small amounts at the neutral pH value used for excystment. Carbonate should be produced in similar or greater amounts in the bicarbonate solutions, which did not induce excystment. Also, changing the pH value of the excystment environment. and thus markedly altering the proportions of carbonic acid and carbonate (see Scott, 1939), did not markedly affect the rate of excystment in response to CO_2. For example, the relative rate of excystment in response to 0·6% pCO_2 in phosphate buffered solutions

at pH 5 and pH 9 was about 70% of the rate at pH 7. Thus by elimination we conclude that dissolved molecular CO_2 gives the signal for excystment.

Effect of cyst population density. In a study of the effect of cyst population density on the relative rate of excystment in response to CO_2, we found that increasing the population density in liquid eventually led to excystment at atmospheric pCO_2. The relative rate of population density-induced excystment was a function of the population density (Fig. 6). Considerable variability was encountered in the value of the population density required to obtain excystment with different batches of cysts.

Comparison of inducers of excystment

The addition of proline and an increase in the cyst population density both appeared to induce excystment by increasing the amount of CO_2 in the cyst environment. In both cases the *Aerobacter aerogenes* present in washed cyst suspensions are believed responsible for the CO_2 production. Proline can be decarboxylated or metabolized to CO_2 by *A. aerogenes*. If uninduced cysts are metabolically inactive, the aerobacter would be responsible for the metabolism of proline to CO_2. In liquid media enough CO_2 to induce excystment is produced from the stimulation of aerobacter metabolism by proline, but on agar plates the CO_2 produced diffuses away and excystment occurs only when gas exchange is prevented. Increasing the population density of cysts increases the number of aerobacters/ml., and thereby the amount of CO_2 produced/ml. by the bacteria. If these assumptions are correct, CO_2 is the active agent in all cases. Elimination of the bacteria, or removal of the CO_2 produced by them, should prevent excystment in response to proline or density.

The pCO_2 of the cyst environment can be decreased by absorbing CO_2 from the gas phase of the flask with KOH. CO_2-induced excystment did not occur in the presence of alkali absorbant. Similarly, excystment in response to increased cyst population density or added proline was inhibited by removal of CO_2 from the gas phase. KOH inhibition of excystment was dependent on sufficient shaking to ensure rapid gas exchange between liquid and gas phases. No excystment occurred in response to any inducers when KOH was present, whereas simultaneous controls without KOH did excyst.

A small amount of CO_2 (e.g. atmospheric concentration) might be essential for excystment in response to the environmental alteration produced by proline or density. This possibility, as well as the role of the aerobacter, can be tested by allowing the pCO_2 to remain at atmospheric levels but preventing the production of additional CO_2 by the bacteria. Ideally this could be achieved by studying the excystment of sterile cysts (free of metabolizing as well as colony forming bacteria), but many attempts to sterilize cysts or to obtain viable cysts by sterile cultivation of amoebae were unsuccessful. (Cysts were formed in sterile cultures on autoclaved aerobacter, but they had abnormal morphology and excysted in liquid without any stimulus.) Because of these difficulties, partial sterilization was accomplished with streptomycin, to which *Aerobacter aerogenes* is sensitive. Overnight incubation of washed cysts on NM containing 200 μg. streptomycin/ml. lowered the population of viable aerobacter from about 10^7 to less than 10^3 per 10^7 cysts plated. These cysts did not appear to be affected by streptomycin, and excysted

normally in response to CO_2. They did not, however, excyst at population densities which normally induce the excystment of untreated cysts. Streptomycin treatment also inhibited proline-induced excystment, though when streptomycin and proline were added to washed cysts simultaneously, slow excystment still occurred, suggesting that the bacteria, while unable to form colonies, were still able to metabolize proline to CO_2.

The inhibition by KOH of CO_2-, proline-, and density-induced excystment produces strong support for the hypothesis that increased pCO_2 is the stimulus for excystment in all cases. The inhibition of proline- and density- but not CO_2-induced excystment by streptomycin treatment of cysts indicates that the bacteria which contaminate the cyst suspensions are responsible for the CO_2 production.

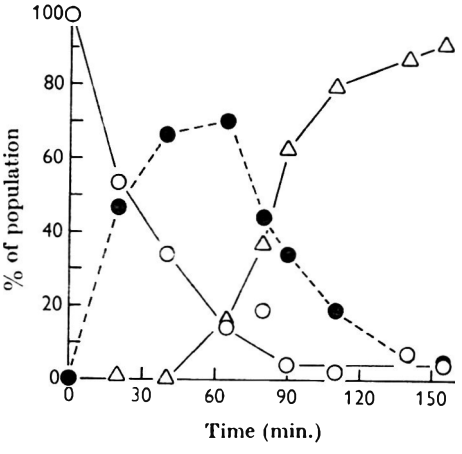

Fig. 7

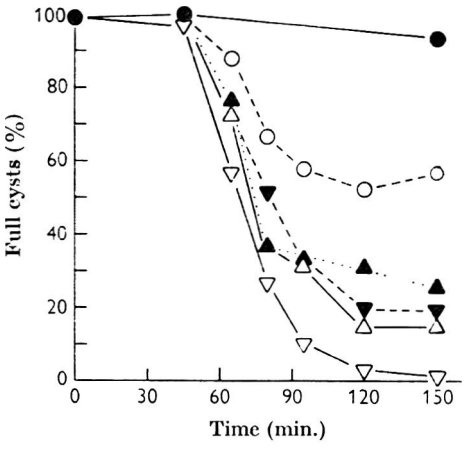

Fig. 8

Fig. 7. Temporal sequence of morphological changes during excystment. Ten ml. of washed cysts at 5×10^5 per ml. in phosphate buffer (pH 7·7) were shaken at 30° in 2·4 % pCO_2. At intervals samples of 100 cells were classified under phase contrast into the arbitrary stages described in the text: ○, stage 1; ●, stage 2; △, transitional and empty cysts.

Fig. 8. Influence of duration of exposure to CO_2 upon subsequent excystment. Washed cysts at 3×10^5 per ml. in phosphate buffer were distributed in 10 ml. samples to six flasks. One flask was left open (●), and the remaining flasks adjusted to 0·8 % pCO_2. The flasks were shaken at 30°, and at intervals a flask containing CO_2 was removed, its contents centrifuged and resuspended in 10 ml. phosphate buffer, and shaking continued at atmospheric pCO_2. In each case the transfer took about 3 min. The cysts were in elevated pCO_2 for: ○, 30; ▲, 45; △, 65; ▼, 80; and ▽, 150 min.

Response of cysts to CO_2

Phase contrast observation of living organisms revealed extensive cytoplasmic changes before excystment. In an uninduced or stage 1 cyst the cytoplasm was filled with small particles in active Brownian motion, and no contractile vacuoles were present. After exposure of cysts to CO_2, large phase-dense granules appeared in the cytoplasm (stage 2). Under standard excystment conditions, half of the cysts changed from stages 1 to 2 within about 20 min. (Fig. 7). Soon thereafter contractile vacuoles were formed, and amoeboid movement began. About an hour

after a cyst changed from stages 1 to 2, an amoeba emerged through one of the pores in the cyst, leaving behind an empty cyst wall (Fig. 7). Cysts can recognize CO_2 in their environment within 20 min. and begin the sequence of changes which lead to emergence.

In order to test whether the continual presence of increased pCO_2 is required for excystment, or whether CO_2 induced a reaction after which excystment can continue to completion independently of the presence of CO_2, cysts were exposed to an inducing concentration of CO_2 for varying times, and then shaken at atmospheric CO_2. Thirty minutes of exposure to CO_2 was sufficient to induce excystment of about 50% of a cyst population; the extent of excystment was a function of the time the cyst population was exposed to CO_2 up to about an hour (Fig. 8). Exposure to CO_2 therefore initiates a sequence of events which can continue to completion in the absence of excess CO_2.

After about 30 min. exposure to CO_2, half the cysts had reached morphological stage 2 (Fig. 7) and could complete excystment independently of increased pCO_2 (Fig. 8). This correlation suggests that the critical effect of CO_2 is to cause some early event, associated with the appearance of large granules, which results in a commitment to excystment.

DISCUSSION

Our major conclusion is that the only stimulus in the environment that naegleria cysts recognize as a signal for excystment is molecular CO_2. The fitness of this signal as a means for a phagotroph to recognize an environment in which food is available is obvious. The level of pCO_2 in an environment such as soil is a function of the rate of production of CO_2—a measure of the population density of organisms, the temperature, and other conditions influencing growth and metabolism—and the rate of loss of CO_2 by diffusion. Because of this, CO_2 would seem to be one of the few molecules whose concentration in soil would indicate when overall conditions would be favourable for naegleria amoebae to survive and grow. The water content of soil, its pH or temperature, or many of the stable organic molecules soil contains would be less sensitive signals of such an environment. This hypothesis, though it certainly does not consider the complexities of soil ecology, has a simplicity which makes it attractive. Furthermore, the levels of CO_2 found in topsoils are sufficient to induce the excystment of naegleria (see Thimann, 1963).

Ballard (1958) found that CO_2 induces germination of clover seeds. Loomis (see 1959, 1964), in a study of the role of increased pCO_2 in controlling sexual differentiation of hydra, has drawn attention to the many biological processes that are influenced by CO_2.

Other environmental variables induce the excystment of naegleria, but these appear to act by increasing the amount of CO_2 produced by bacteria in the cyst environment. Proline- and density-induced excystment in the laboratory display the effect of environmentally produced CO_2. Although cyst population density was the observed variable in density-induced excystment, it is the simultaneously altered bacterial population density that is responsible for excystment. With some cyst preparations, less responsive to density-induced excystment, excystment could be obtained by adding additional aerobacters. On the nutrient agar for naegleria, NM, it is the growth of aerobacter—and presumably the CO_2 micro-

environment created by this growth—which induces excystment of naegleria. In all cases, increased pCO_2 accounts for excystment. From this point of view, as long as bacteria are present anything which increases their rate of CO_2 production—proline, other chemicals, a temperature increase, etc.—would lead to excystment. In this connexion, it is of interest that the washed cyst suspensions used in our experiments are contaminated to the extent of about one viable aerobacter per cyst; one Aerobacter has roughly one thousandth the volume of a cyst. This relatively small quantity of aerobacter, when stimulated by proline or increased in population density, is able to produce enough CO_2 to induce excystment.

Many published studies of excystment of other organisms could be explained in the same way—i.e. that the addition of bacteria, organic acids, etc., induces excystment via CO_2. For example, Crump (1950), in a study of the ability of various bacteria to induce the excystment of a limax amoeba, stated that 'The unsuccessful attempts already recorded to induce excystment by placing cysts in culture fluid in which Aerobacter...had grown, but from which they were removed by centrifuging, suggests that the substances concerned may be so transient that they disappear as fast as they are formed, and only when there are living bacteria in the medium is enough of the stimulating material present to act successfully on the cysts'. In studying the induction of excystment of didinium by bacteria, Beers (1946) noted that 'the effective substances are highly unstable. They are probably produced at the surface of the bacteria by an enzyme system. They diffuse a short distance into the medium, where they induce excystment, but only if present in relatively high concentrations—i.e. the concentration of the bacteria must be high to induce excystment. Once in the medium the substances are rapidly altered or destroyed'. Such statements might apply to excystment induced by bacteria-produced CO_2.

Most of this study is included in a dissertation submitted to the graduate faculty of Brandeis University by M.A. in partial fulfilment of the requirements for the degree of Doctor of Philosophy. This work was done during the tenure of a Jack Cohn predoctoral fellowship, and completed while M.A. held a postdoctoral traineeship from training grant T1HD22 of the Institute of Child Health and Infant Development of the National Institutes of Health. The research was supported by a grant from the National Science Foundation.

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The Saprolegniaceae of the Environs of Blelham Tarn: Sampling Techniques and the Estimation of Propagule Numbers

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SUMMARY

Investigations of the ecology of Saprolegniaceae rely on satisfactory sampling methods. Existing procedures have been considered and new techniques, including a method for the estimation of propagule numbers, are presented. This paper shows the diversity of the communities which may exist in an area of less than 1 km.^{2}; further evidence has been provided on the constancy of these communities. From previous work distribution patterns of a mosaic type were expected; the present results suggest that several orders of such patterns exist, ranging from those within a core of soil about 5 cm. in diameter, through those of quadrats of 1 m.^{2}, to the production of a continuum several metres or even 100 m. in extent.

INTRODUCTION

Although the Freshwater Biological Association has sponsored intensive work on the biology of Blelham Tarn, a small lake in the Lake District of north-west England, it had little information on the Saprolegniaceae. This paper is the result of an invitation from Dr L. G. Willoughby to provide some data of the distribution of Saprolegniaceae in the environs of this lake. Dick (1963) recorded different species of Saprolegniaceae from different sites in south-east England. However, the regions studied were widely separated so that no information on the variety of species to be found in any one locality could be presented. Seasonal fluctuations in the occurrence of Saprolegniaceae were found by Dick & Newby (1961) and it was fortunate that the optimum periods for the isolation of Saprolegniaceae (primarily late spring, but also late summer) coincided with the weeks when it was possible to use the facilities of the Windermere Laboratory of the Freshwater Biological Association. Except when stated, results have been obtained during the 4–5 weeks between late March and early April in the years 1962 to 1965. The greater part of this paper is concerned with an evaluation of different sampling procedures, including data summarized by Dick (1964*b*). Hence the distribution data, comprising the preliminary surveys of spring and summer 1962, and a summary of information from later experiments are presented and discussed together in the next section.

Preliminary survey and summary of distribution

Topography. A map of Blelham Tarn, including the sampling sites, is given in Fig. 1. Since the topography and succession of angiosperm vegetation of Blelham Tarn and its shores have been described in detail by Macan (1949) only a brief

description will be given here. The lake is 695 m. long and up to 185 m. wide, its long axis running approximately north-east to south west. There are three principal inflow streams with drainage basins to the north-west, south-east, and south-west of the lake, respectively, together with a few minor streams mostly draining from the south. The outflow is in the north-east. The lake level may fluctuate by up to 2 m. and a large part of the shore is liable to inundation. Apart from the land lying

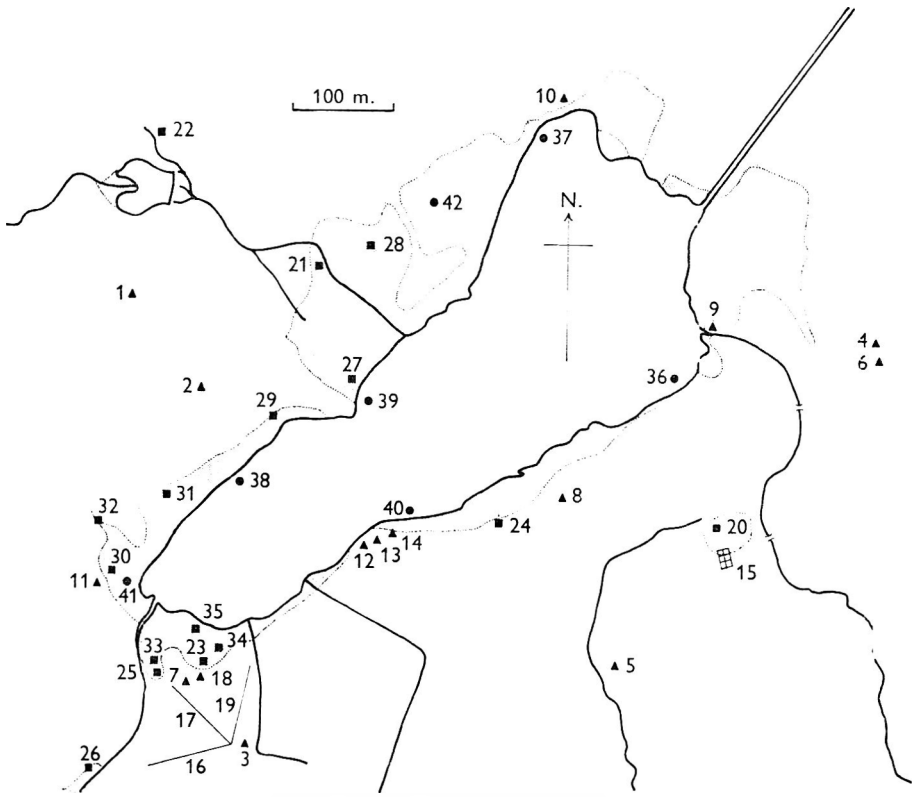

Fig. 1. Map of Blelham Tarn and its environs. Stippled regions are those liable to inundation. ▲, sites seldom or never inundated; ■, sites in areas liable to inundation; ●, sites in permanently waterlogged areas. For explanation of other symbols, see text.

between the two south-western inflows (a heavily sampled region) the land to the south rises steeply. All the land south of the lake is used for pasture and periodically has heavy dressings of cow manure and lime. There is an extensive floating vegetation platform forming the north-west shore, beyond which is some rough pasture and Pteridium-covered slopes. Blelham Bog South and Blelham Bog North occupy the two large areas liable to inundation on the northern shore, while the large area liable to inundation to the east of the lake is Juncetum and little grazed. Since intensive sampling has been carried out at site 15, special mention should be made of the isolated region liable to flooding south-east of the lake. The stream to the west is given underground drainage, presumably to the major inflow stream to the east. The surrounding land slopes steeply (approximately 30° at site 15) and after heavy rain, when the drain cannot take the flow of water, water accumulates in this

hollow to the depth of about 0·5 m. and may even spill directly into the inflow stream to the east. Periods of inundation are therefore relatively frequent, but of short duration.

Methods. Initially the sites were sampled according to the method of Dick & Newby (1961) but with two deviations from that technique. (1) Filtered sterilized lake water was used instead of glass-distilled sterile water because of the greater availability of the former. (2) In samples taken during the spring of 1962, baits of termite wings and snake skin were used in addition to hempseeds, following the recommendations of Scott (1961). This method is satisfactory for isolating the species present at a given site, as can be seen by reference to the next section of this paper. However, there are certain drawbacks to the method. First, although sixteen samples were taken from each 1 m. quadrat, they were not true replicate samples. Dick (1962) showed that 'position effects' could be found when this technique was used. Secondly, the method required tedious field sterilization of collecting equipment.

A more serious defect was apparent in the summer of 1962 when relatively deep submerged muds were sampled with coring equipment. In such sites it was not possible to use a corer more than once or twice because of the disturbance of the surface mud by the corer. Thus replicate samples had to be taken from one core. No information was then available for comparison with replicate samples from a single core in soil.

A further difficulty in sampling muds below water arose because of the differing character of some of the muds. Where there were many roots of aquatic plants even a skilled operator had to make three or four attempts before a satisfactory core was obtained. Where there was an appreciable amount of gravel it was not possible to use any coring equipment satisfactorily. Therefore, a survey of all the bottom muds had to be suspended because of improvements necessary both to the sampling technique and to the coring equipment. The remaining data given in Table 1 have been obtained from the controls of subsequent experiments on sampling procedure. No attempt has been made to obtain comprehensive data on environmental factors in this preliminary survey. From those factors which have been investigated at some of the sites studied there has been no evidence at variance with data given in Dick (1963).

Presentation of data. The sites are classified according to their liability to inundation on both the map (Fig. 1) and Table 1. The value for each species in Table 1 is the number of samples positive for that species expressed as a percentage of the total samples. Data are presented for saprophytic and hyperparasitic Saprolegniaceae with the exception of the three little-known and doubtfully placed keratinophilic species (*Aphanodictyon papillatum* Huneycutt; *Brevilegniella keratinophila* Dick; *Leptolegniella keratinophilum* Huneycutt) for which the baits were not examined.

It was hoped to incorporate information on saprophytic Aphanomyces species, but these species were usually seen only on the snake-skin or termite-wing baits. Oogonia of *Aphanomyces stellatus* were the only oogonia visible on these baits in rough culture conditions. Time was not available for subculturing all the Aphanomyces mycelia that were seen, but in some cases when this was done, *A. irregulare* was also identified. Subsequent experiments have shown both these species, together

Table 1. *Saprolegniaceae in the environs of Blelham Tarn*

	Sites seldom or never inundated														Sites with poor drainage at least in part				
Site no....	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15*	16*	17*	18	19*
No. of samples taken...	16	16	16	16	16	16	30	16	16	16	16	30	16	48	170	50	50	10	50
Year sampled†...	'62	'62	'62	'62†	'62	'62	'64	'62	'62	'62	'62	'63	'62	'63	'61–'65	'64	'64	'65	'64
Species‡																			
Leptolegnia eccentrica	56	44	13	—	—	6	—	—	—	—	—	—	—	—	—	—	—	—	—
Aplanopsis terrestris	6	69	—	38	69	25	7	—	13	6	—	—	—	—	15	12	6	10	—
Saprolegnia megasperma	—	—	—	—	—	6	3	—	—	13	13	—	—	—	—	6	—	—	—
Isoachlya subterranea§	—	—	—	—	—	31	20	50	—	—	—	—	31	—	22	—	—	—	—
Thraustotheca clavata	—	—	—	6	—	—	—	—	—	—	—	—	6	—	8	—	—	—	—
Geolegnia septisporangia	—	—	—	38	—	6	—	—	—	—	—	—	—	—	3	—	—	—	—
Saprolegnia terrestris	—	—	19	—	—	—	73	—	—	—	—	7	—	—	36	72	72	100	62
Brevilegnia bispora	—	—	25	—	—	—	—	—	38	—	—	—	—	—	5	18	6	—	8
Pythiopsis cymosa	—	13	—	—	75	25	93	56	13	56	94	17	25	33	39	68	50	10	18
Aplanopsis spinosa	—	31	81	100	75	75	100	100	100	100	88	100	81	83	71	80	94	90	86
Isoachlya monilifera	—	—	—	—	—	—	13	—	—	—	13	—	—	—	—	—	10	—	—
I. toruloides	—	—	—	—	—	—	—	—	6	6	6	3	—	19	—	—	—	—	—
Achlya megasperma	—	—	—	—	—	—	—	—	—	—	—	7	19	—	18	2	—	—	2
A. radiosa	—	—	—	—	—	—	—	—	—	—	—	—	6	2	1	—	—	—	1
A. apiculata	—	—	—	—	—	—	—	—	—	—	—	—	—	—	1	20	—	—	31
A. recurva	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	1	—	—	—
A. racemosa	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
A. stellata	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
A. spinosa	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
A. caroliniana	—	—	—	—	—	—	—	—	—	—	—	—	—	4	16	—	2	—	—
A. flagellata	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	4	—	6
A. (non-sexual)	—	—	—	—	—	—	—	—	—	—	—	—	—	2	15	14	—	30	6
A. americana	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Saprolegnia diclina	—	—	—	—	—	—	—	—	—	—	—	—	13	—	1	2	—	—	—
S. (non-sexual)	—	—	—	—	—	—	—	—	—	—	—	—	—	2	—	—	—	—	—
S. asterophora‖	—	—	—	—	—	—	—	—	—	—	—	—	—	—	1	8	—	—	—
S. anisospora	—	—	—	—	—	—	—	—	—	—	—	23	—	—	—	—	—	—	—
S. ferax	—	—	—	—	—	—	—	—	—	—	—	—	—	—	1	—	—	—	—
S. mixta	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
S. litoralis	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
S. turfosa	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Dictyuchus sterile	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Leptolegnia caudata	—	—	—	—	—	—	—	—	6	6	6	—	—	—	—	—	—	—	—
Aphanomyces parasiticus¶	—	—	—	—	—	—	—	—	—	—	—	—	—	—	65	—	—	—	16
Unidentified species **	—	—	—	—	—	—	—	—	—	—	—	—	—	—	2	—	—	—	—
Apodachlya minima	—	—	—	—	—	13	—	—	—	—	—	13	—	13	4	—	—	—	—
Apodachlyella completa††	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Leptomitus lacteus	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—

Table 1 (*cont.*)

	Sites in areas liable to inundation by rising lake levels or flood water from overflowing streams																Sites in permanently waterlogged areas						
Site no....	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42
No. of samples taken...	10	16	16	10	16	16	16	16	16	16	16	16	16	16	16	10	16	16	16	16	16	16	16
Year sampled†...	'65	'62	'62	'65	'62	'62†	'62	'62	'62	'62†	'62	'62†	'62†	'62	'62	'64	'62†	'62†	'62†	'62†	'62†	'62†	62
Species‡																							
Leptolegnia eccentrica	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Aplanopsis terrestris	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Saprolegnia megasperma	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Isoachlya subterranea§	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Thraustotheca clavata	—	—	—	—	—	31	—	—	—	6	—	—	—	—	—	—	—	—	—	—	6	—	—
Geolegnia septisporangia	—	—	—	—	—	—	—	—	—	—	—	—	—	31	—	—	—	—	—	—	—	—	—
Saprolegnia terrestris	20	—	—	20	—	13	6	—	—	6	—	25	—	6	6	—	—	6	—	—	—	—	—
Brevilegnia bispora	—	6	—	—	—	44	81	—	—	—	—	—	—	44	25	—	—	—	6	—	6	—	—
Pythiopsis cymosa	20	38	81	50	—	—	—	—	—	—	69	—	—	—	—	—	—	—	—	—	—	—	—
Aplanopsis spinosa	60	100	100	10	69	6	63	25	38	—	—	—	—	50	69	—	—	—	—	6	13	—	—
Isoachlya monilifera	—	—	—	—	—	—	—	38	6	—	25	—	—	—	—	—	—	—	—	—	—	—	—
I. toruloides	40	13	6	—	—	—	—	—	—	—	19	13	38	—	—	—	—	—	—	—	—	6	—
Achlya megasperma	10	—	—	10	25	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
A. radiosa	—	6	6	—	6	—	19	6	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
A. apiculata	10	13	—	50	56	19	19	6	25	81	6	—	—	—	—	—	—	—	—	—	—	—	—
A. recurva	10	—	—	—	—	—	—	75	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
A. racemosa	—	—	6	—	—	—	—	—	—	—	6	—	—	—	—	—	—	—	—	—	—	—	—
A. stellata	—	—	—	—	—	—	—	13	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
A. spinosa	—	—	—	—	—	—	—	—	—	19	63	—	6	—	—	—	—	—	—	—	—	—	—
A. caroliniana	40	—	—	—	—	—	—	—	—	6	—	—	—	—	—	—	6	—	—	—	—	—	—
A. flagellata	—	—	—	—	—	6	6	—	6	6	—	6	—	—	—	10	—	—	—	—	—	—	—
A. (non-sexual)	40	—	6	80	19	—	6	—	31	13	19	44	19	31	63	90	50	6	13	13	6	6	—
A. americana	—	—	—	—	—	—	—	—	6	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Saprolegnia diclina	—	19	—	—	—	—	—	6	44	—	13	—	—	—	—	—	—	—	13	19	—	—	—
S. (non-sexual)	20	13	—	—	—	6	—	—	—	—	38	—	—	—	—	—	13	—	—	13	19	—	—
S. asterophora‖	—	—	—	—	—	6	—	19	—	—	—	—	38	19	—	—	—	—	—	—	—	69	69
S. anisospora	40	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
S. ferax	—	6	—	—	—	—	—	—	—	6	—	—	—	—	—	—	—	—	6	—	—	—	—
S. mixta	—	—	—	—	—	—	—	—	—	6	—	—	—	—	—	—	—	—	—	—	—	—	—
S. litoralis	—	—	—	—	—	—	—	—	—	25	—	—	—	—	—	—	—	—	—	—	6	—	—
S. turfosa			—	—	—	—	—	—	—	—	19	—	—	—	—	—	—	—	—	—	—	—	25
Dictyuchus sterile	—	6	—	—	—	—	25	6	—	—	—	—	—	—	6	—	—	19	13	25	—	—	—
Leptolegnia caudata	—	—	—	—	—	—	—	—	—	—	—	13	19	—	—	—	13	—	25	25	—	—	—
Aphanomyces parasiticus¶	60	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Unidentified species**	—	—	—	—	—	—	—	—	—	—	—	—	—	6	—	—	—	—	—	—	—	—	—
Apodachlya minima	—	6	—	—	—	—	—	—	—	—	—	—	—	13	—	—	—	—	—	—	—	—	—
Apodachlyella completa††	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	6
Leptomitus lacteus	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	6	—	—	—	—	—

* Reference should be made to Fig. 1 for the extent of these sites.
† Sites were sampled in late March or early April except when daggered. These sites were sampled in late August or early September.
‡ In addition to the species in this list, *Geolegnia inflata* has been isolated from site 25. See Table 4.
§ This species is identical with *Isoachlya itoana* of earlier papers by the author.
‖ This species is not identical with that described in Dick (1960*b*).
¶ Saprophytic species of *Aphanomyces* (*A. irregulare*, *A. stellatus*) are not included in this table because of the special baiting procedures required. Species of Aphanomyces were looked for, but not found in sites 1, 2, 4, 5 and 32. Baits for Aphanomyces were not used in sites 15–20, 23, 29, 32 or 35–41.
** This species is identical with isolate 3C1 in Dick (1963).
†† A full description of this species is given in Dick (1964*a*).

with apparently sterile forms, to occur at a number of sites. At most sites where Aphanomyces species were recorded, they occurred in the majority of replicates. The uncertain value which can be placed on the records for these species has necessitated their omission from Table 1.

Leptomitaceae were recorded occasionally on hempseed baits and have been included in Table 1.

Although Pythiaceae were not included in this survey, data were obtained on Pythiogeton species in later experiments and considered worthy of inclusion, since the genus is infrequently isolated.

Discussion. It is not possible to give a detailed commentary on every species or every site. Nevertheless, an examination of the grouping of the sites on the basis of their liability to inundation is useful. From this, it can be seen that there is considerable agreement with Dick (1963) on two major points. In the first place, it was the wetter sites which yielded the more varied species lists. Secondly, the distribution of species was largely independent of the genera to which the species belong. This was especially true of Saprolegnia species; *Saprolegnia asterophora* and *S. megasperma* with two species which were again shown to have conspicuously different distributions. The same phenomenon is also seen with *Leptolegnia caudata* and *L. eccentrica*. On the other hand, Achlya species, with the notable exception of the non-sexual Achlya, predominated in the sites liable to inundation.

In the present survey, the waterlogged sites were frequently dominated by either non-sexual Achlya or, in bog conditions, *Saprolegnia asterophora*. Other species which occurred in these sites were those isolated more frequently from the periodically flooded sites. In contrast to this difference of frequency, there were distinctive species, e.g. *Aplanopsis terrestris*, *Isoachlya subterranea*, *Leptolegnia eccentrica* and *Saprolegnia megasperma*, in well-drained soils. These species were found in association with the more widespread species *Aplanopsis spinosa* and *Pythiopsis cymosa*. It may be significant that some of these species showed little overlap of distribution.

Some local differences of distribution appear to be superimposed on the primary pattern discussed above. For example, *Aplanopsis spinosa* was almost completely absent from the land to the north-west of the lake, while the same region appeared to be most favourable for both Leptolegnia species, *Leptolegnia eccentrica* occurring in the drier sites. Similarly, *Brevilegnia bispora* and *Saprolegnia terrestris* were most frequently isolated from sites in the field at the western end of the southern shore and in the region of sites 15 and 20 in the south-east.

With the exception of *Isoachlya monilifera*, *Leptolegnia caudata* and *Saprolegnia terrestris*, the species found in the environs of Blelham Tarn were also found in south-east England.

Sampling methods

The sampling procedure of Dick & Newby (1961) seems the best general method for isolating a wide range of Saprolegniaceae from soil, but since certain disadvantages became manifest during the preliminary survey (previous section), an investigation of possible modifications to this technique was made. The principal objections to any baiting technique rest on the possibility of competition amongst fungal propagules for the nutrient supplied, and the possible selectivity of the nutrient for

particular species. The first essential is to provide a method which gives the consistent and reproducible results which are necessary before any fluctuating errors associated with these possibilities can be dismissed. The method of Dick & Newby satisfies this requirement for a defined area, but does not do this for the single sample, which is the only feasible means of sampling bottom muds. Therefore, the first requirement was to test the effect of homogenizing samples. This would be unlikely to present problems for bottom muds, but the effect on soils was not predictable. The second requirement, which follows from the first, was that data must be provided from true replicates. Once these requirements have been fulfilled it is possible to assess the relative merits of different baits, the effect of dilution of the sample and the optimum duration of contact between the bait and the sample.

Fractionation of the sample. The first experiments were concerned with forming a homogenate from soil. Soil from the top 5 cm. includes stones of various sizes and large pieces of plant material. No apparatus which could readily be sterilized was available for blending such a complex mixture. Thus it was decided to wet the soil and use an electrically driven stirrer. This produced three fractions of soil: a supernatant suspension of minute particles in water, a slurry of sedimenting fine particulate matter and a deposit of stones and coarse plant debris. The value of the three fractions as sources of propagules of Saprolegniaceae was first assessed.

Soils from three separate sites (4, 25, 32) were treated by the procedure outlined below and soil from site 14 was similarly sampled three times at weekly intervals. These experiments were designed to investigate both the fractionation of soil and the possible methods of collecting soil samples. There are two sets of data for each collection; the method of Dick & Newby was used as a control.

The fractionation of the soil was made by the following procedure. Soil with a known fresh weight of 50–150 g. was placed in a 1000 ml. conical beaker with 250 ml. sterile lake water. This was stirred with a brass paddle attached to an Anderman multispeed stirrer, set at speed mark 35, for 5 min. The soil-water mixture was passed through a sterile sieve with a mesh of 1 mm. and the debris held back was washed with a further 250 ml. sterile lake water. Plant material still remaining on the sieve was designated *coarse plant debris*. The sieved matter was returned to the conical beaker and allowed to stand for 15 min. and the *supernatant fluid* was drawn off, leaving the sedimented fine particulate *slurry*. It is not claimed that the method gives complete separation of the fractions; for example, further washings would be required to free the coarse plant debris from attached small particles.

The plant debris was divided into 16 approximately equal portions; each portion was placed in a sterile Petri dish, 25 ml. sterile lake water added and each dish baited with three hempseeds. Sixteen 25 ml. samples of the supernatant fluid were drawn off; each sample was placed in a sterile Petri dish and baited with three hempseeds. Sixteen 10 ml. samples of slurry were similarly plated out, each with the addition of 15 ml. sterile lake water and three hempseeds as bait. Results of these experiments are given in Tables 2–5.

A comparison of the Saprolegniaceae isolated from the coarse plant debris and from the slurry from each blended soil sample shows that there is no evidence that different species were isolated by the two treatments. On the other hand, the supernatant fluid gave a markedly different result. Apart from a few isolated

Table 2. *Isolation of Saprolegniaceae by using different sampling procedures*

Three collections (*a*, *b* and *c*) taken at 7-day intervals from the same 1 m. quadrat at site 14. A, 16 cores treated individually; B, 16 cores blended to give 16 replicates; C, a large central core blended to give 16 replicates.

Species	Collection *a*						Collection *b*						Collection *c*					
	A	B		C			A	B		C			A	B		C		
Treatment	Control	Coarse plant debris	Slurry	Coarse plant debris	Slurry	Supernatant fluid	Control	Coarse plant debris	Slurry	Coarse plant debris	Slurry	Supernatant fluid	Control	Coarse plant debris	Slurry	Coarse plant debris	Slurry	Supernatant fluid
Aplanopsis spinosa	13	14	16	8	16	—	13	13	16	8	16	—	14	7	16	2	16	— 2
Pythiopsis cymosa	4	8	8	14	7	—	3	2	3	12	4	—	9	13	9	12	14	
Isoachlya torulosides	3	4	16	5	4	—	2	1	3	6	10	—	4	3	6	2	7	— —
I. subterranea	—	3	1	1	—	—	—	—	—	6	7	—	—	—	1	2	4	— —
Apodachlya minima	4	1	1	—	—	—	2	1	—	—	—	—	—	—	—	—	—	— —
Achlya caroliniana	—	—	1	—	—	—	1	—	—	—	—	—	1	—	—	—	—	— —
A. radiosa	—	—	—	—	—	—	1	—	—	—	—	—	—	—	—	—	—	— —
A. (non-sexual)	1	—	1	—	—	—	—	—	—	—	1	—	—	—	—	—	—	— —
Brevilegnia bispora	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	— —
Saprolegnia diclina	—	—	—	—	—	1	—	—	—	—	—	—	—	—	—	—	—	— —
S. (non-sexual)	—	—	—	—	—	—	1	—	—	—	—	—	—	—	—	—	—	— —
S. megasperma	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	1	—	— —
Aphanomyces spicules	1	—	—	—	—	5	—	1	—	—	—	1	1	1	—	1	—	10 11

records for *Pythiopsis cymosa*, *Saprolegnia terrestris* and *S. diclina*, the baits were not colonized by species isolated from the control, the slurry or the coarse plant debris. However, Aphanomyces species were isolated; these species were never isolated from the slurry and were hardly represented in either the coarse plant debris or the control plates. It should be noted that when Aphanomyces species were isolated from the supernatant fluid, the counts were relatively high. The second collection from site 14 gave very poor counts for Aphanomyces as compared with

Table 3. *Isolation of Saprolegniaceae from a 1 m. quadrat by using different sampling procedures at site 4*

A, 16 cores treated individually; B, 16 cores blended to give 16 replicates; C, a large central core blended to give 16 replicates.

	Treatment						
	A	B			C		
Species	Control	Coarse plant debris	Slurry	Supernatant fluid	Coarse plant debris	Slurry	Supernatant fluid
Aplanopsis spinosa	16	11	15	—	7	16	—
A. terrestris	6	—	7	—	2	11	—
Geolegnia septisporangia	6	—	9	—	—	4	—
Thraustotheca clavata	1	1	1	—	4	15	—

Table 4. *Isolation of Saprolegniaceae from a 1 m. quadrat by using different sampling procedures at site 25*

A, 16 cores treated individually; B, 16 cores blended to give 16 replicates; C, a large central core blended to give 16 replicates.

	Treatment						
	A	B			C		
Species	Control	Coarse plant debris	Slurry	Supernatant fluid	Coarse plant debris	Slurry	Supernatant fluid
Thraustotheca clavata	5	7	16	—	12	15	—
Saprolegnia terrestris	2	15	13	5	6	5	—
Brevilegnia bispora	7	—	11	—	3	15	—
Achlya apiculata	3	—	1	—	2	10	—
Aplanopsis spinosa	1	—	—	—	4	1	—
Saprolegnia ferax	—	2	2	—	—	—	—
S. asterophora	1	1	—	—	1	—	—
Geolegnia septisporangia	—	—	1	—	—	1	—
Saprolegnia (non-sexual)	1	1	—	—	—	—	—
Achlya (non-sexual)	—	—	2	—	—	—	—
A. flagellata	1	1	—	—	—	—	—
A. caroliniana	—	1	—	—	—	—	—
Dictyuchus sterile	—	—	—	—	1	—	—
Unidentified species	—	—	1	—	—	—	—
Geolegnia inflata	—	—	1	—	—	—	—
Aphanomyces stellatus	—	—	—	8	—	—	11

the first and third collections, but isolations of the other species did not show such fluctuations.

Several samples of supernatant fluid were centrifuged and the deposits baited. No species were isolated other than those mentioned above. The hempseeds supported large numbers of small and large ciliates and bacteria and it was assumed that any saprolegniaceous propagules would have been brought down. These other organisms were not a barrier to saprolegniaceous colonization of the hempseed, since subsequent inoculation of the dishes with Achlya zoospores resulted in vigorous colonization within 24 hr. The total absence of the abundant species *Aplanopsis spinosa* from the supernatant fluid fraction should be noted, since this species is not known to form zoospores (Dick, 1960*a*).

Table 5. *Isolation of Saprolegniaceae from a 1 m. quadrat by using different sampling procedures at site 32*

A, 16 cores treated individually; B, 16 cores blended to give 16 replicates; C, a large central core blended to give 16 replicates.

	Treatment						
	A	B			C		
Species	Control	Coarse plant debris	Slurry	Super-natant fluid	Coarse plant debris	Slurry	Super-natant fluid
Leptolegnia caudata	3	11	9	—	5	7	—
Isoachlya toruloides	6	3	4	—	1	6	—
Saprolegnia asterophora	6	2	1	—	5	1	—
Achlya (non-sexual)	3	1	6	—	2	11	—
Saprolegnia litoralis	—	—	2	—	1	3	—
S. diclina	—	—	2	—	—	—	—
S. (non-sexual)	—	—	3	—	—	—	—
Achlya spinosa	1	—	—	—	—	—	—

These results suggest that the supernatant fluid fractions contained zoospores but no other saprolegniaceous propagules. Whether these zoospores existed as such in the soil before the collection was made, or whether they were released from mature zoosporangia by the subsequent treatment cannot be stated. Nevertheless, it is certain that they could not have been produced from mature resting propagules which had not started to germinate before the collection was made, since the time between collection and plating-out was always less than 4 hr.

Although the supernatant fluid fraction appeared to provide a selective method for isolating Aphanomyces species, it was not satisfactory for assessing the range of species present in a given soil. There is no evidence that coarse plant debris provides a source of propagules better than the control or the slurry. Therefore, it was decided to reject these two fractions when the sampling procedure was to be used for preparing a list of species from a given soil. Sampling for Aphanomyces species must be performed separately when information on these is required.

The slurry used in all subsequent experiments was obtained by using the procedure outlined above.

Characterization of sites by a single core. The above experiments provided data on the effect of varying the procedure for collecting the sample in the field. These data

Table 6. *A comparison of the reliability of species counts from replicate plates by using the slurry from a blended soil, and the method of Dick & Newby (1961)*

Data from Tables 2–5. A, 16 cores treated individually; B, 16 cores blended to give 16 replicates; C, a large central core blended to give 16 replicates.

	A						B						C					
Sites...	13*a*	13*b*	13*c*	4	25	32	13*a*	13*b*	13*c*	4	25	32	13*a*	13*b*	13*c*	4	25	32
Species																		
Achlya radiosa	—	1	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Aphanomyces stellatus	1	—	1	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Achlya caroliniana	—	1	1	—	—	—	1	—	—	—	—	—	—	—	—	—	—	—
Apodachlya minima	4	2	—	—	—	—	1	—	1	—	—	—	—	—	—	—	—	—
Isoachlya subterranea	—	—	—	—	—	—	1	—	1	—	—	—	—	7	4	—	—	—
Pythiopsis cymosa	4	3	9	—	—	—	8	3	9	—	—	—	7	4	14	—	—	—
Aplanopsis spinosa	13	13	14	16	1	—	16	16	16	15	—	—	16	16	16	16	1	—
A. terrestris	—	—	—	6	—	—	—	—	—	7	—	—	—	—	—	11	—	—
Geolegnia septisporangia	—	—	—	6	—	—	—	—	—	9	1	—	—	—	—	4	1	—
Thraustotheca clavata	—	—	—	1	5	—	—	—	—	1	16	—	—	—	—	15	15	—
Saprolegnia terrestris	—	—	—	—	2	—	—	—	—	—	13	—	—	—	—	—	5	—
Brevilegnia bispora	—	—	—	—	7	—	—	—	—	—	11	—	—	1	—	—	15	—
Achlya apiculata	—	—	—	—	3	—	—	—	—	—	1	—	—	—	—	—	10	—
Saprolegnia ferax	—	—	—	—	—	—	—	—	—	—	2	—	—	—	—	—	—	—
Geolegnia inflata	—	—	—	—	—	—	—	—	—	—	1	—	—	—	—	—	—	—
Unidentified species	—	—	—	—	—	—	—	—	—	—	1	—	—	—	—	—	—	—
Achlya flagellata	—	—	—	—	1	—	—	—	—	—	—	—	—	—	—	—	—	—
Saprolegnia (non-sexual)	—	1	—	—	1	—	—	—	—	—	—	3	—	—	—	—	—	—
Achlya (non-sexual)	1	—	—	—	—	3	1	—	—	—	2	6	—	—	—	—	—	11
Saprolegnia asterophora	—	—	—	—	1	6	—	—	—	—	—	1	—	—	—	—	—	1
Leptolegnia caudata	—	—	—	—	—	3	—	—	—	—	—	9	—	—	—	—	—	7
Isoachlya toruloides	3	2	4	—	—	6	16	3	6	—	—	4	4	10	7	—	—	6
Saprolegnia litoralis	—	—	—	—	—	—	—	—	—	—	—	2	—	—	—	—	—	3
S. diclina	—	—	—	—	—	—	—	—	—	—	—	2	—	—	—	—	—	—
Achlya spinosa	—	—	—	—	—	1	—	—	—	—	—	—	—	—	—	—	—	—

are summarized in Table 6. Treatment A was the method of Dick & Newby in which 16 cores were collected from evenly spaced points on a grid over a 1 m. quadrat, each core being plated and baited individually. Treatment B was a similar collection of 16 cores, each of about 8 g. fresh weight from adjacent points on the grid; these were blended to give about 200 ml. slurry. Treatment C was the collection of a single large core central to the quadrat and of the same fresh weight as the 16 cores of treatment B (i.e. 150 g.) and blended as above. Sixteen replicates were taken of slurries from treatments B and C.

Examination of Table 6 shows that at every site the three treatments gave comparable species lists. A detailed analysis is only possible for the three collections at site 14. Using the table of confidence limits (at the 0·95 level) for the expectation of a Poisson variable, it can be seen that there were no significant differences between the counts of the three collections for any of the three treatments. The counts for the two most commonly isolated species show no significant differences between the treatments, but the counts for the two Isoachlya species are significantly lower in treatment A.

At all sites, there were significant differences in the counts for one or two species, providing evidence of 'position effect' in the siting of the single central core: *Thraustotheca clavata* at site 4; *Isoachlya subterranea* at site 14; *Achlya apiculata* at site 25; non-sexual Achlya at site 32. This observation, together with the general similarity of the central core results with the quadrat samples, and also in conjunction with the findings of Dick (1962) suggests that there is a repeating mosaic pattern of small order for saprolegniaceous propagules over areas of at least 1 m^2. Any fluctuations were of a quantitative rather than a qualitative nature. The method gives an arbitrary guide to abundance, but because of the possible selectivity of a particular nutrient source for a given species or type of propagule, it does not seem advisable to present data on abundance on a statistical basis from a single sampling.

Replicates from the slurry of a single core form an acceptable alternative to the method of Dick & Newby in providing an assessment of the species of Saprolegniaceae present in the soil from which the core was taken. Furthermore, the number of replicates can be increased or decreased according to the emphasis to be placed on relative abundance amongst common species or on the isolation of additional, rarer, species. In subsequent experiments the number of replicates was decreased from 16 to 10.

Baiting the sample. Hempseeds have been used extensively for isolating Saprolegniaceae; Saprolegniaceae can be found growing on dead insects in water and Aphanomyces is said to be most readily isolated with snake-skin bait. However, no comparisons of the relative efficiency of these baits have been published. Two sites (7, 12) were therefore examined to provide this information. At both sites three large cores were collected 4 m. apart. The cores were blended separately and for each substrate 10 replicates of slurry were prepared. At site 12 hempseed and ant pupae were the substrates used; at site 7 hempseed, ant pupae and snake-skin baits were added. The results from site 7 are given in Table 7.

In both experiments the similarity between the species counts from hempseed and from ant pupae was marked. With either bait it was apparent that *Pythiopsis cymosa* was less common at site 12 than at site 7. It was also obvious that snake skin

Table 7. *A comparison of the efficiency of plant baits and animal baits of different nutrient content in the isolation of Saprolegniaceae from the slurry of a blended soil*

Data from 10 replicates of each treatment from each of 3 cores (*a*, *b*, *c*) 4 m. apart at site 7.

	Cores											
	a			*b*			*c*					
	Bait											
Species	Hemp-seed	Ant pupae	Snake skin	Hemp-seed	Ant pupae	Snake skin	Hemp-seed	Ant pupae	Snake skin	Total from hemp-seed	Total from ant pupae	Total from snake skin
Pythiopsis cymosa	10	10	6	10	10	4	8	10	1	28	30	11
Aplanopsis spinosa	10	9	—	10	10	1	10	10	—	30	29	1
Saprolegnia terrestris	6	7	5	6	4	6	10	10	10	22	21	21
Isoachlya subterranea	3	4	—	1	3	—	2	—	—	6	7	—
I. monilifera	4	2	—	—	—	—	—	—	—	4	2	—
Saprolegnia megasperma	—	—	—	1	2	2	—	1	—	1	3	2
Aplanopsis terrestris	—	—	—	2	—	5	—	—	1	2	—	6
Aphanomyces species	—	—	6	—	—	8	—	—	9	—	—	23

gave a quite different impression of the species present; Aphanomyces species were prominent on this, and were the only species isolated on snake skin but not on the other baits. Other Saprolegniaceae can also colonize snake skin, but their growth is depauperate and they are difficult to identify. The absence of *Aplanopsis spinosa* from snake-skin baits was as notable as the presence of the Aphanomyces species. Since these same Aphanomyces species were isolated by using hempseeds in the fractionation experiments, this difference cannot be ascribed to a basic nutrient requirement provided only by snake skin. Thus, it would appear that species of the genus *Aphanomyces* were the only species for which competition was a decisive factor. It is clear that information on this genus cannot be obtained in the same way as information on the Saprolegniaceae as a whole.

Table 8. *Isolation of Saprolegniaceae from different dilutions of the slurry of a blended soil*

Data from the region of site 15 (1964). Each Petri dish contained 25 ml. diluted slurry. Ten replicates of each dilution.

Slurry ml./dish...	10	5	2·5	1
Species				
Aphanomyces parasiticus	10	10	10	9
Achyla megasperma	7	9	9	4
Aplanopsis spinosa	3	6	6	9
Achlya (non-sexual)	4	3	4	3
Saprolegnia terrestris	4	3	3	4
Achlya caroliniana	2	4	2	5
Saprolegnia ferax	2	2	3	1
Unidentified species	2	—	—	—
Saprolegnia asterophora	1	—	—	—
Apodachlya minima	2	2	—	—
Pythiogeton sp.	2	3	1	5

Table 9. *Isolation of Saprolegniaceae from different dilutions of the slurry of a blended soil*

Data from the region of site 15 (1965). Each Petri dish contained 25 ml. diluted slurry. Ten replicates of each dilution.

Slurry ml./dish...	10	2	1	0·4	0·1
Species					
Aplanopsis spinosa	10	10	9	10	9
A. terrestris	8	7	7	8	5
Thraustotheca clavata	4	3	5	6	7
Aphanomyces parasiticus	7	7	6	1	1
Pythiopsis cymosa	—	—	1	2	1
Isoachlya subterranea	—	1	1	—	1
Geolegnia septisporangia	1	—	—	1	1
Achlya caroliniana	—	—	—	1	—

Dilution of the sample. Competition for nutrients is likely to be affected by the total number of propagules in the vicinity of a bait. Four experiments at sites 32 35 and 15 (2) were designed to investigate the effect of dilution of slurry on the relative counts of Saprolegniaceae isolated. The results from site 15 are given in Tables 8 and 9.

The preliminary experiment at site 32 indicated that a decrease of the volume of slurry from 10 ml./dish to 2·5 ml./dish had little effect on the species list; the most abundant species were present throughout and it would not be expected that the rarer species would show consistent trends. At greater dilutions the isolations of all species were unpredictable, and no isolations were made when each dish contained only 0·3 ml. of the original slurry. Two subsequent experiments were made at sites 35 and 15 (Table 8) in conjunction with investigations on the optimum duration of contact between bait and slurry. There were no significant differences between species counts from dishes containing either 10 ml. slurry/dish or 1 ml./dish. In both these experiments a Pythiogeton species was prominent; with this species also there was no significant difference in the incidence of isolations with dilution.

The second of these experiments at site 15 (Table 8) was of interest because of the presence of the hyperparasite, *Aphanomyces parasiticus*. In the plates where the slurry was less diluted, there were many parasitized hyphae. In dishes containing heavily parasitized material the bacterial contamination was so high that the saprophytic fungi were moribund within 10 days. Since *Aplanopsis spinosa* needs at least 10 days before oogonia are formed, the parasitism depressed the counts for this species in those plates which contained most slurry. The identification of other hosts was also difficult. (A list of hosts was published by Dick, 1964*c*. In addition to those hosts mentioned, *Aphanomyces parasiticus* has now been found in hyphae of *Thraustotheca clavata*.) With greater dilutions of slurry most host hyphae reached maturity and produced oogonia. Site 15 was visited a year later when the test of dilution also served as a control for preliminary estimations of propagule numbers; the data are given in Table 9. *A. parasiticus* was still present, but in no case was the attack so heavy as to affect identification. This experiment also showed that the majority of the species could be isolated when only 0·1 ml. of slurry was used in each dish. A comparison of Tables 8 and 9 shows the unpredictability of isolations of the rarer species, irrespective of dilution.

The principal conclusion from these experiments is that the amount of slurry added to each plate for baiting is not a critical factor which affects the species or species counts. Thus, it appears unlikely that competition for baits is ever a factor in the non-isolation of a given species. If an assessment is required of the species present in a given soil, it is suggested that an excess of slurry should be used since this is detrimental to species counts only when parasites are present, while misleading species lists may result when too great a dilution of slurry is used.

Duration of contact between bait and slurry. To complete the survey of possible modifications to the sampling procedure, it was necessary to know whether there was an optimum period for contact between bait and slurry. Two experiments with slurry from sites 35 and 15 were made in which the baits were removed, washed and incubated in fresh sterile lake water after contact periods of 1, 3 and 6 days. Growth on the baits was so dense after 6 days that washing was difficult. It would be impossible to remove the slurry completely after longer periods of contact, with the result that identification would be made difficult by adhering particles of slurry. Results from site 15 are given in Table 10. It can be seen that the relatively short period of contact of 1 day was sufficient to obtain an adequate representation of the species present. However, it is significant that *Aphanomyces parasiticus* was absent from the 1-day-contact replicates, although it was present in most replicates

after the 3-day-contact. It was noted that the infection was not so heavy as in the 6-day-contact replicates, and this did not result in such a rapid deterioration in the condition of the cultures; a result reflected in the higher counts for susceptible species in the 3-day-contact replicates. It is apparent that a decrease in the time of contact within the limits of the experiment had as little effect on the species isolated as did the dilution of slurry. To be certain that parasitic species will be isolated when present in the slurry, a contact period of 3–6 days is recommended.

Table 10. *Isolation of Saprolegniaceae after different periods of contact between slurry of a blended soil and hempseed baits*

Data from 10 replicates of each treatment. Slurry from a core in the region of site 15.

	Time of contact (days)		
	1	3	6
Species			
Aphanomyces parasiticus	—	7	10
Achlya megasperma	7	9	7
Aplanopsis spinosa	8	9	3
Achlya (non-sexual)	5	4	4
Saprolegnia terrestris	1	1	4
Achlya caroliniana	3	—	2
Saprolegnia ferax	—	—	2
Unidentified species	—	—	2
Saprolegnia asterophora	—	—	1
Aplanopsis terrestris	1	1	—
Geolegnia septisporangia	—	1	—
Apodachlya minima	—	—	2
Pythiogeton sp.	4	2	2

The estimation of propagule numbers

The successful isolation of all principal saprolegniaceous species from dilute slurry indicated that propagules were sufficiently numerous for estimates of their number to be made. A saprolegniaceous isolate has never been made from soil by using accepted fungal plating techniques, although Willoughby (1962) successfully assayed zoospores of Saprolegniaceae in natural waters. The hyphal isolation technique of Warcup (1955) has shown that saprolegniaceous hyphae can be isolated from soil, but this technique does not provide a method for estimating propagule numbers. The fractionation experiments reviewed in the section on *Sampling methods* above indicated that zoospores were not the principal propagules present in soils. However, spherical oospores or oogonia are unlikely to be obvious by direct observation amongst other particles. Hence the first requirement was to discover whether saprolegniaceous propagules could be induced to germinate when embedded in nutrient agar. Two properties would be required of such an agar: its nutrient content must be known to support a wide range of fungi, and it must be transparent. Willoughby (1962) recommended the use of oatmeal agar, which is opaque, for zoospore assay. Emerson (1958) recommended the Corn Meal Agar of Baltimore Biological Laboratories as being of exceptional transparency and suitable for many aquatic fungi, and this was the agar chosen for the present work. The method used in the following experiments was to incorporate 2·5 ml. diluted slurry

in 10 ml. corn meal agar made up with 80% of the recommended volume of glass-distilled water.

In a preliminary experiment two agar plates were prepared with slurry from the dilution experiment reported in Table 9. The diluted slurry was obtained by shaking 4 ml. of slurry with 46 ml. sterile lake water. Observations were made 24 hr after the plates had been poured.

It was found that saprolegniaceous hyphae could be distinguished from those of Pythiaceae by their greater diameter, lesser tendency to branching and the greater angle between any branch and its subtending hypha; they could be distinguished from those of Mucorales by the absence of rhizoid-like branch systems of tapering hyphae. Further, it was apparent that considerable growth had taken place during the 24 hr, the length of a hypha commonly exceeding 5 mm. Suspected saprolegniaceous mycelia were cut out, placed in a drop of water and baited with a hempseed. Mycelia identified from the two plates were: *Aplanopsis spinosa* 36, *A. terrestris* 20, *Isoachlya subterranea* 1. In one of the plates a growing leptomitaceous hypha was seen; no special attempt was made to cultivate it, and it did not become established on the hempseed.

Thus, alternative ways of estimating propagule numbers were possible: either the mycelia could be counted by direct observation after 24 hr, or by using a suitable dilution of slurry the agar could be cut up into standard sized blocks, each of which would be baited with a hempseed in water. The following experiment was designed to compare these alternatives, to confirm that random variation could be assumed between replicate agar plates, to ascertain propagule numbers on the basis of the dry weight of the soil, and to examine the origins of the mycelia observed.

A core of soil from the drier part of site 15 was collected in a plastic bag, and mixed by manipulation through the bag. Two equal parts by weight, of about 50 g. fresh weight, were taken; one was used to calculate the dry weight, the other was treated to produce slurry. After separation, the volume of slurry was measured and the slurry then re-stirred and two dilutions of slurry prepared by pipetting 5 ml. slurry into 95 ml. sterile lake water. To provide the control, one of these samples was plated out at 10 ml./Petri dish, with the addition of a further 20 ml. of sterile lake water/dish. Each dish was baited with three hempseeds. Twelve 2·5 ml. samples were transferred from the other dilute slurry to Petri dishes and incorporated in agar.

A ruled grid, giving fifty $0{\cdot}5 \times 1{\cdot}0$ cm. rectangles was drawn on the bottom of each Petri dish. The 25 small blocks of agar on one half of the grid were cut out and each placed in a separate drop of water with a hempseed balanced on top of the block. These cultures were examined after 48 hr and those showing no Saprolegniaceae rejected. The remaining cultures were kept until the saprolegniaceous fungi could be identified. The other 25 blocks were not cut out, but examined *in situ* after 24 hr, and the number and origins of saprolegniaceous mycelia were recorded. (In subsequent experiments standard sized blocks $0{\cdot}5 \times 0{\cdot}5$ cm. were cut out.) The results are given in Table 11. The application of the χ^2 test to these results shows that the hypothesis of random distribution according to the Poisson law is not disproved.

Since certain fractions of soil, which were known to contain some propagules, had been rejected, it is only possible to estimate the *minimum propagule number* from the tables of fiducial limits. Hence it was not necessary to apply a correction for

Table 11. *A comparison of the analysis of saprolegniaceous propagule numbers by direct observation and by baiting*

Data from 12 replicate agar plates incorporating slurry from the region of site 15. For further explanation, see text.

Direct observation of agar blocks			Agar blocks baited with hempseeds			
No. of blocks examined	No. of blocks with Sapro-legniaceae	No. of mycelia	No. of blocks examined	No. of blocks with Sapro-legniaceae	*Aplanopsis spinosa*	*Apianopsis terrestris*
25	9	11	25	12	9	3
25	11	11	25	10	7	4
25	4	4	25	10	8	3
25	9	9	25	5	4	1
25	5	5	25	9	8	2
25	7	7	25	12	9	5
25	7	8	25	7	6	1
25	6	7	25	15	13	5
25	9	12	25	6	7	2
25	8	8	25	9	5	1
25	11	12	25	9	8	4
25	13	14	25	14	12	3
300	99	108	300	118	96	34

multiples counted as one when reading fiducial limits, provided that the dilution was suitable. The volume of slurry given by a soil varies with the particle size range and the organic content of the sample, therefore, it is preferable to express the results in terms of the *dry soil equivalent*. The minimum propagule number/g. dry soil equivalent is given by:

$$\frac{\begin{pmatrix}\text{minimum}\\ \text{no. of}\\ \text{propagules}\end{pmatrix} \times \begin{pmatrix}\text{area of}\\ \text{Petri}\\ \text{dish}\end{pmatrix} \times \begin{pmatrix}\text{total volume of}\\ \text{slurry from } x \text{ g.}\\ \text{fresh weight soil}\end{pmatrix}}{\begin{pmatrix}\text{ml. slurry multiplied}\\ \text{by dilution factor per}\\ \text{Petri dish}\end{pmatrix} \times \begin{pmatrix}\text{area}\\ \text{of}\\ \text{block}\end{pmatrix} \times \begin{pmatrix}\text{no.}\\ \text{of}\\ \text{blocks}\end{pmatrix} \times \begin{pmatrix}\text{dry weight}\\ \text{of } x \text{ g. fresh}\\ \text{wt. soil}\end{pmatrix}}.$$

From this formula, the minimum propagule number/g. dry soil equivalent is 616 by direct observation, or 745 by baiting. Although the number of propagules observed directly was lower than the number arrived at by the baiting method, the difference is not significant. The baiting technique enables specific identifications to be made and is more suitable for routine sampling. From the baiting technique results it can be seen that there was a significant difference between the number of propagules of *Aplanopsis spinosa* and that of *A. terrestris*. In this connexion it is interesting to record that the control gave counts of 10 for *Aplanopsis spinosa*, 5 for *A. terrestris* and 2 for *Aphanomyces parasiticus*. It would not be expected that the last species would appear on assay plates unless it had an extremely high potential inoculum.

The result of the examination of propagule origins is given in Fig. 2. From the

above discussion it can be assumed that these mycelia are of Aplanopsis. A large percentage of the particles from which these mycelia originated were of the same order of size as the oogonia of Aplanopsis. Further, on a number of occasions the hypha could be seen to be dilated at the point of conjunction with the particle in a manner indicating an origin other than from a segment of vegetative mycelium. Minute organic particles adhering to the propagules precluded a positive identification, but the evidence strongly suggests that the propagule is the resting spore. The oogonium of *Aplanopsis spinosa* is spiny, while that of *A. terrestris* is more or less spherical with a variable number of blunt papillae. Both normally have only one oospore within the oogonium, so it was not possible to distinguish between the oospore and the oospore within the oogonium in this experiment.

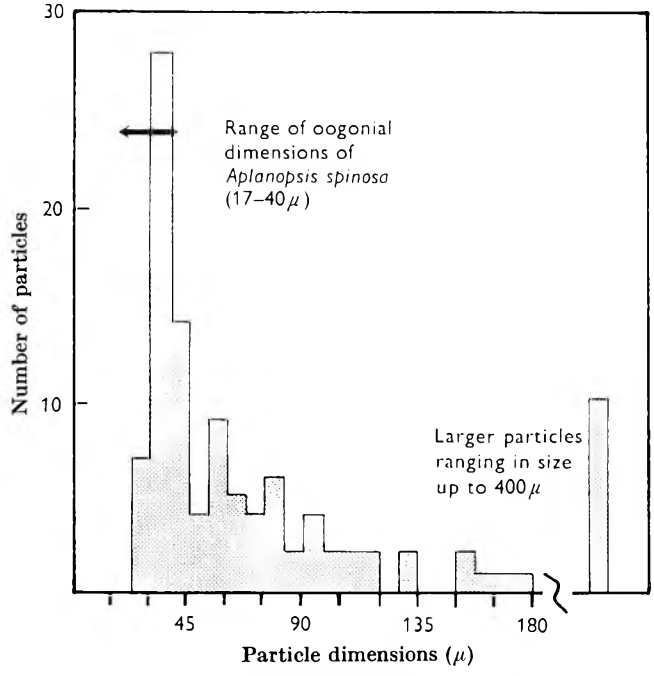

Fig. 2. Histogram of mean dimensions of particles giving rise to saprolegniaceous hyphae. Data from direct observation of the mycelia of column 3 in Table 15. The origins of two mycelia were uncertain.

In one instance, a mycelium with many principal hyphae giving a colony of 8 mm. diameter became established within 24 hr. The origins of these hyphae were from a large particle 375 $\mu \times 150$ μ; in this case it is possible that the propagule was actively growing mycelium. In another instance, the origin could not be determined although all hyphal tips were traced back. In this case the propagule may have been a hyphal segment. (Had zoospore-forming Saprolegniaceae been recorded from the control, a zoospore as origin might also be suggested.)

Experiments with the agar block + hempseed technique on other soils indicated that minimum propagule numbers varied from site to site, and also that propagules of zoospore-forming Saprolegniaceae were less numerous than would be inferred from slurry samples baited directly with hempseeds. A comparison of

Table 12. *A comparison of hempseed-baited slurry, with agar incorporating different dilutions of slurry, as methods for isolating and estimating the propagule numbers of Saprolegniaceae*

Data from the region of site 15. For further explanation, see text.

Species	Hempseed baited slurry (10 replicates)	5 replicate agar plates, each incorporating 0·625 ml. slurry. 20 agar blocks examined from each plate					5 replicate agar plates, each incorporating 0·125 ml. slurry. 20 agar blocks examined from each plate				
Aplanopsis spinosa	10	15	17	15	11	16	3	2	5	4	4
Saprolegnia terrestris	10	2	—	1	2	1	—	—	2	—	—
Aplanopsis terrestris	9	3	8	4	11	6	1	1	1	2	1
Thraustotheca clavata	4	—	—	—	—	—	—	1	—	—	—
Achlya caroliniana	2	—	—	—	—	—	—	—	—	—	—
Isoachlya subterranea	1	—	—	—	—	—	—	—	—	—	—

Table 13. *A comparison of hempseed-baited slurry, with agar incorporating different dilutions of slurry, as methods for isolating and estimating the propagule numbers of Saprolegniaceae*

Data from site 18. For further explanation, see text.

Species	Hempseed baited slurry (10 replicates)	5 replicate agar plates, each incorporating 0·625 ml. slurry. 20 agar blocks examined from each plate					5 replicate agar plates, each incorporating 0·125 ml. slurry. 20 agar blocks examined from each plate				
Aplanopsis spinosa	9	1	5	5	2	6	—	—	2	—	1
Saprolegnia terrestris	10	2	—	—	1	1	—	—	—	1	—
Achlya (non-sexual)	3	—	—	—	—	—	—	—	—	—	—
Aplanopsis terrestris	1	1	2	1	2	1	—	—	—	—	—
Pythiopsis cymosa	1	—	—	—	—	—	—	—	—	—	—

Tables 12 and 13 shows that propagule numbers should be calculated from the greater dilution in Table 12, and from the lesser dilution in Table 13. Minimum propagule numbers/g. dry soil equivalent were 1006 and 305, respectively. To obtain more than sporadic isolations of even the most abundant zoospore-forming Saprolegniaceae, the lesser dilutions must be used. These dilutions are not always suitable for assay purposes, and the amount of undesirable organisms is excessive. One isolate was made of Pythiogeton by this technique.

This method for assaying propagule numbers reveals a major weakness in the direct hempseed-baiting technique. The bait must remain in contact with the slurry for several hours, in order that hyphae of Saprolegniaceae may be firmly attached before the bait is washed to free it from slurry particles. It is evident that this time is sufficient for resting propagules of zoospore-forming Saprolegniaceae to germinate and release zoospores, thereby giving an undeterminable increase to the potential inoculum. Nevertheless, an enormous number of agar blocks would have to be examined to provide both an estimate of propagule numbers and a species list. Thus, the direct hempseed-baiting technique is not superseded by this assay method, but is complemented by it.

Table 14. *Data from 3 transects (sites 16, 17 19) originating from a common point (see Fig. 1)*

Cores were taken at 21 m. intervals. Ten replicates were prepared from each core.

	Sites														
	16					17					19				
	a	*b*	*c*	*d*	*e*	*a*	*f*	*g*	*h*	*i*	*a*	*j*	*k*	*l*	*m*
Species															
Aplanopsis spinosa	7	10	6	7	10	7	10	10	10	10	7	10	10	10	6
Saprolegnia terrestris	10	8	10	8	—	10	10	4	8	4	10	9	—	2	10
Pythiopsis cymosa	8	10	2	4	10	8	3	—	4	10	8	—	—	1	—
Achlya apiculata	—	—	3	7	—	—	—	—	—	—	—	—	7	2	8
Achlya (non-sexual)	—	—	2	5	—	—	—	—	—	—	—	—	3	—	—
Brevilegnia bispora	1	—	—	4	4	1	—	1	1	—	1	—	3	—	—
Aplanopsis terrestris	—	—	5	1	—	—	1	—	—	2	—	—	—	—	—
Aphanomyces parasiticus	—	—	—	—	—	—	—	—	—	—	—	—	1	—	7
Isoachlya monilifera	—	—	—	—	—	—	—	5	—	—	—	—	—	—	—
Achlya flagellata	—	—	—	—	—	—	—	2	—	—	—	—	1	—	2
Saprolegnia asterophora	—	—	—	4	—	—	—	—	—	—	—	—	—	—	—
S. megasperma	1	—	—	—	2	—	—	—	—	—	—	—	—	—	—
Achlya megasperma	—	—	—	1	—	—	—	—	—	—	—	—	1	—	—
Saprolegnia diclina	—	—	—	1	—	—	—	—	—	—	—	—	—	—	—
Achlya caroliniana	—	—	—	—	—	—	1	—	—	—	—	—	—	—	—

Transect sampling

Surveys of submerged soils or the bottom muds of lakes cannot be made by the quadrat technique of Dick & Newby (1961). Sampling must be done by taking single cores at intervals along a predetermined line or lines. A similar sampling programme would be necessary for surveys designed to investigate large-scale distribution patterns on land. Two examples of the application of transect sampling are given blow. The results given in Tables 14 and 15 were obtained by the direct hempseed–baiting technique on slurries from single cores.

Table 15. *Data from site 15. Cores taken 6 m. apart on a grid 18 m. × 12 m. sited on a slope (see Fig. 1).*

Ten replicates were prepared from each core. The table reads south to north and each column reads east to west.

	Site											Total counts east to west				Total counts south to north		
Species	*a*	*b*	*c*	*d*	*e*	*f*	*g*	*h*	*i*	*j*	*k*	*l*							
Aphanomyces parasiticus	10	2	—	9	10	10	9	9	10	10	9	3	12	29	28	22	38	30	23
Aplanopsis spinosa	10	10	10	10	8	8	—	2	8	1	4	8	30	26	10	13	21	24	34
Pythiopsis cymosa	8	6	2	5	8	7	6	2	5	5	8	5	16	20	13	18	24	24	19
Saprolegnia terrestris	—	—	—	—	1	2	10	—	9	10	6	3	—	3	19	19	20	7	14
Isoachlya subterranea	4	7	2	5	1	9	1	6	2	—	—	—	13	15	9	—	10	14	13
Achlya megasperma	—	—	—	—	—	—	2	10	1	9	1	—	—	—	13	10	11	11	1
A. caroliniana	—	2	—	—	5	4	5	—	—	1	1	1	2	9	5	3	6	8	5
Achlya (non-sexual)	—	—	—	1	3	1	1	—	1	—	4	2	—	5	2	6	2	7	4
Brevilegnia bispora	—	—	1	—	—	—	2	—	1	5	—	—	1	—	3	5	7	—	2
Achlya apiculata	—	—	—	—	—	—	1	—	—	2	4	—	—	—	1	6	3	4	—
Saprolegnia ferax	—	—	—	—	—	—	—	4	—	—	1	—	—	—	4	1	—	5	—
Thraustotheca clavata	—	—	5	—	—	—	—	—	—	—	—	—	5	—	—	—	—	—	5
Geolegnia septisporangia	—	—	2	—	—	—	2	—	—	—	—	—	2	—	2	—	2	—	2
Apodachlya minima	—	1	—	2	—	—	—	—	—	—	—	1	1	2	—	1	2	1	1
Saprolegnia diclina	—	—	—	—	—	—	—	—	—	—	1	—	—	—	—	1	—	1	—
Achlya radiosa	—	—	—	—	—	—	—	—	—	—	1	—	—	—	—	1	—	1	—
Aplanopsis terrestris	—	—	—	1	—	—	—	—	—	—	—	—	—	1	—	—	1	—	—
Unidentified species	—	—	—	—	—	—	—	—	1	—	—	—	—	—	1	—	—	—	1

The line transects comprising sites 16, 17 and 19 were set out in the relatively flat field at the western end of the tarn. Reference to the species lists from sites surrounding the transect area (see section, *Preliminary survey and summary of distribution*) reveals that three species were common to all three sites. These species were *Aplanopsis spinosa*, *Saprolegnia terrestris* and *Brevilegnia bispora*. These three species were those most consistently isolated from the transect samples, in spite of a time lapse of two years between samplings and the use of a different sampling method at different sites. Also, the transect samples showed *Pythiopsis cymosa* to be widely distributed in the field, although the frequency with which it was recorded varied from transect to transect.

In connexion with the suggestion in the section *The estimation of propagule numbers* that species represented by propagules which had a capacity for rapid production of zoospores would be favoured by the direct baiting technique, it is of interest to note that certain species (e.g. *Saprolegnia terrestris*, *Achlya apiculata*) were seldom recorded with low counts, although they were not recorded from all sites. The absence of such species from a given site may probably be of greater ecological significance than the variation of counts between other sites.

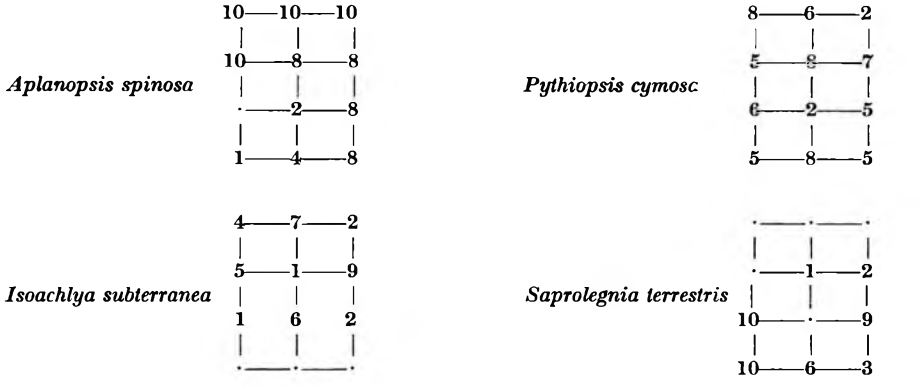

Fig. 3. Maps of the distribution of the four most frequently occurring saprophytic species of Saprolegniaceae from cores 6 m. apart on a grid 18 m. × 12 m. sited on a slope. Data from Table 19.

There are differences between the species lists from the sites along these transects, but no obvious pattern of distribution emerges. The complex topography of a relatively flat field with shallow depressions, probably with poor drainage, is reflected by the differences between these species lists.

The second series of three transects formed a grid on a sloping site (see Fig. 1). Summation of the counts from the three cores at each level going down the slope (total counts east to west) and of the counts from the four cores forming each transect down the slope (total counts south to north) enabled the effect of the slope on the distribution pattern to be assessed. There were very few significant differences between the counts of species from the three transects, but significant differences occurred between the counts for the majority of the species at one or other of the four levels down the slope. Different species had different distributions. This can be most clearly seen in the diagrams of Fig. 3. *Aplanopsis spinosa* was most

abundant at the top of the slope, and possibly least abundant at the bottom, to the north-east. *Isoachlya subterranea* was similarly confined to the upper parts of the slope. On the other hand, *Saprolegnia terrestris* was isolated only at the bottom of the slope, while *Pythiopsis cymosa* was uniformly distributed. Thus, a correlation can be seen between the pattern of distribution and the topography of the site. However, it is not possible to state what environmental factors, controlled by the slope of the terrain, exerted the primary influence on this distribution pattern. It is apparent that transect sampling can provide a useful method for investigating large-scale distribution patterns.

Discussion of sampling techniques

Saprolegniaceae have hardly ever been recorded by the routine soil plating techniques used for the preparation of species lists and the estimation of propagule numbers of fungi in soil. Dick & Newby (1961) showed that a baiting technique can reveal differences between species lists of Saprolegniaceae and that relative abundances can be assessed. However, Willoughby (1962) suggested that there was no certainty that saprolegniaceous propagules would locate a discrete substratum, or that they would become established in the face of competition. The results above have shown that hempseed baiting is a highly efficient method of isolating species of Saprolegniaceae present, and that neither the discrete nature of the substrate nor the factor of competition introduce appreciable errors. Furthermore, the advantages and disadvantages of the baiting technique in assessing relative abundances of species has been clarified: zoospore-forming Saprolegniaceae are favoured by this technique, but not to the exclusion of other species.

The development of a soil fractionation technique has made possible an investigation into the source, nature and number of propagules of Saprolegniaceae in soils. The results from soil fractionation agree with those of Williams (1963) in that there was no species difference observed between the different fractions, but exceptions must be made in respect of Aphanomyces and Aplanopsis. Williams recorded longer species lists from washed soil particles than from the wash water, and noted that this increase included sterile forms. May these have included Saprolegniaceae, which are frequently sterile on agar media? An examination of slurry incorporated in agar has shown that saprolegniaceous mycelia originate predominantly from small soil particles of size comparable to oospores or oogonia. Thus it appears that the majority of propagules are neither zoospores nor mycelia. Zoospores may be present in the original soil and it is possible that their numbers could be estimated by assay of the supernatant fluid fraction. However, the dilution factor would be so small that contamination by other micro-organisms might well be too great for reliable assay.

The factors which affect the isolation of Aphanomyces are not yet understood. The isolation of the same Aphanomyces species from supernatant fluid fractions by hempseed bait and from slurries by snake-skin bait suggests that both zoospores and resting spores were present in these soils. It is also unlikely that the nutrient source is critical, except in respect of the competitive advantage apparently conferred by a nutrient source less favourable for other Saprolegniaceae. While Saprolegniaceae in general will germinate on agar or colonize discrete baits within 24–48 hr, it is possible that a longer incubation period is essential for Aphanomyces. Scharen (1960) found that an incubation period of 6 to 24 days was necessary for

oospores of the pea-root parasite *Aphanomyces euteiches* when embedded in organic particles. A "horse-dung + pea-sand leachate" medium was used in this experiment, although cultures were grown in corn-kernel water media. Incubation periods of more than 48 hr are not feasible with the assay method described above because of contamination by other micro-organisms.

For the first time it has been possible to estimate propagule numbers of Saprolegniaceae in soil. Minimum propagule numbers of 745/g. dry soil and 1056/g. dry soil were recorded from the upper and lower parts of the slope at site 15 respectively, and 305/g. dry soil from site 18. The percentage water content of these soils (dry-wt. basis) was 84, 85 and 179%, respectively. One species, *Aplanopsis spinosa*, was responsible for well over half of these totals in each case. No figures are available for other fungal propagules from these soils, but a commonly quoted number for all fungal units in fertile agricultural soil is 400,000/g. On this basis Saprolegniaceae must be regarded as forming a relatively insignificant part of the total fungus flora. However, in terms of potential biological activity, as a Phycomycete primary colonizer of substrates, *Aplanopsis spinosa* may not be so regarded. For example, Boosalis & Scharen (1959) showed by an elaborate screening technique, that 100 g. of soil from a field with a high index of pea-root-rot contained on average 55 fragments of plant tissue with *Aphanomyces euteiches*; most of these fragments contained 10–30 oospores, that is roughly equivalent to 15 oospores/g.

Dr L. G. Willoughby has recorded (unpublished data obtained by his sector assay technique) that a very high percentage of the propagules from lake margin muds are of *Aplanopsis spinosa*. He has estimated total numbers of saprolegniaceous propagules at 1300/l. surface mud and has postulated up to a 100-fold increase on this value for certain marginal soils. For comparison, minimum propagule numbers/l. slurry found in the present work were 332,500 for the top of site 15, 411,200 for the lower part of site 15 and 72,470 for site 18.

It is, therefore, proposed that the direct hempseed-bait technique should be used for isolating species of Saprolegniaceae. When estimations of propagule numbers are required, the hempseed baited agar block assay is recommended. These methods do not give information on the activity of these fungi, since they sample total propagules, the majority of which are resting propagules. It may be possible to estimate zoospore numbers by using the supernatant liquid fraction, but to do this would be to ignore the commonest species, *Aplanopsis spinosa*.

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Further Studies on Germination of Sporangiospores of *Rhizopus arrhizus*

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SUMMARY

Although sporangiospores of *Rhizopus arrhizus* do not swell or produce germ-tubes in distilled water, when they are suspended in heavy water the water in the spore is exchanged with heavy water in the medium. Spores swell and some produce germ-tubes in a glucose solution. Maximum germination occurred in the presence of utilizable carbon and nitrogen sources and suitable compounds containing phosphate, sulphate, potassium and magnesium ions. Germination was accompanied by a considerable increase in oxygen uptake and by the time the germ-tube emerged, the dry weight had increased by about 500%. Respiratory inhibitors (2,4-dinitrophenol, sodium azide, potassium cyanide) inhibited germination; sodium azide inhibited both the oxygen uptake and the dry weight increase.

Electron microscope studies showed structural changes in germinating *Rhizopus arrhizus* spores similar to those reported in other Rhizopus species: a new inner wall layer was formed, and changes in form and probably number of mitochondria occur. The effects of either anaerobic conditions or media containing sodium azide, on fine structure of germinating *R. arrhizus* spores were similar; mitochondrial multiplication ceases, mitochondrial cristae became a disorganized collection of undulating 'plates', and the nuclear membrane became split in places, thus giving rise to small vacuoles between the two electron-dense layers of the membrane.

INTRODUCTION

Some aspects of the physiology of germination of sporangiospores of *Rhizopus arrhizus* Fischer have already been reported by Ekundayo & Carlile (1964), who found that initiation of germination required the presence of glucose or fructose; maximum germination required in addition a nitrogen source, PO_4^{3-} and K^+ or Na^+. On media containing these nutrients, the spore increased in length from 5·5 to 13·5 μ in 8 hr. Anaerobic conditions inhibited germination. Weber & Ogawa (1965) observed that only proline, in the absence of phosphate, stimulated germination of *R. arrhizus* spores among the individual amino acids, sugars, organic acids, vitamins and inorganic nitrogen sources tested in their studies. Hawker & Abbott (1963*b*) described changes in fine structure during germination of sporangiospores of *R. nigricans* (= *R. stolonifer*) and *R. sexualis*. Washed spores of these species also failed to germinate in distilled water (Abbott, personal communication). The present investigations extended these various lines of study.

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METHODS

General. The preparation of media and inocula and the assessment of spore germination were as previously described (Ekundayo & Carlile, 1964).

Permeability studies with heavy water (deuterium oxide) as tracer. Spores, filtered, centrifuged and dried over P_2O_5 at 0·05 torr in an Edwards High Vacuum Unit, were suspended in 1 ml. D_2O in a centrifuge tube covered with aluminium foil and incubated at 30° for 18 hr. The D_2O was decanted and distilled under reduced pressure in a micro-distillation apparatus. The density of the distillate was determined by the gradient tube method (Shaw, 1955). 50 ml. CCl_4 were put in a 100 ml. graduated cylinder, 50 ml. Kerosene were added to the top of the carbon tetrachloride, and the interface between them was stirred and allowed to stand for 24 hr in a water-bath at 20° to allow the density gradient to become established. The density of D_2O was calibrated by introducing drops of known concentrations of D_2O into the CCl_4+Kerosene mixture and measuring the positions of the drops with a travelling microscope and vernier. Drops of the distillate were then added, their equilibrium position determined and the equivalent D_2O concentration was read from the calibration graph. The percentage exchange was taken as the difference between the percentage of D_2O in the distillate from spore suspension and from 100% D_2O control. The theoretical full exchange value was derived from the equation:

$$\% \text{ full exchange} = \frac{x}{x+y} \times 100,$$

where x = wt. (mg.) of water from spore sample at 100° for 24 hr;
y = wt. (mg.) of 1 ml. D_2O at room temperature.

Electron microscopy. Ungerminated Rhizopus spores were not always well fixed after 24 hr in 2% $KMnO_4$, glutaraldehyde or osmic tetroxide. Satisfactory fixation was achieved by shaking the spore suspension in 4% $KMnO_4$ in veronal buffer pH 7·4 with Ballotini glass balls in a Mickle disintegrator for 30 min. and leaving the spores in the fixative at room temperature for a further 3½ hr. The spore suspension was then centrifuged at 2000 rev./min. for 5 min. to separate the glass balls. The supernatant was decanted and centrifuged at 4000 rev./min. for 15 min. and the spore deposit washed with distilled water. Only broken or damaged spores subsequently showed internal structure; it was, therefore, assumed that the fixative penetrated through the cracks in the walls.

Germinated spores were fixed with 2% $KMnO_4$ in veronal buffer pH 7·4 (Luft, 1956) for 4 hr at room temperature.

After fixation the spores were dehydrated with acetone, embedded in vestopal W, sectioned, and observed with either an Akashi TRS-50 or a Siemens Elmiskop I electron microscope.

RESULTS

Permeability of Rhizopus arrhizus spores

Ekundayo & Carlile (1964) showed that *R. arrhizus* spores did not swell or produce germ-tubes in sterile distilled water; maximum swelling and germ-tube production by all spores required the presence of utilizable carbon and nitrogen sources and compounds containing phosphate, potassium or sodium ions.

In the present studies, wetting the spores with Gemex Z-11, Manoxol OT, Nonidet P42 or Teepol either before or during their incubation in water did not make them swell or produce germ-tubes in water.

Experiments in which spores were suspended in solutions of the standard medium containing high concentrations of $(NH_4)_2SO_4$ showed that saturated solutions of $(NH_4)_2SO_4$ did not plasmolyse ungerminated *Rhizopus arrhizus* spores. Spores which had been incubated for about 3 hr on the standard medium were, however, plasmolysed by standard medium containing 3·0 M-$(NH_4)_2SO_4$.

Heavy water was used as a tracer to find whether ungerminated *Rhizopus arrhizus* spores could exchange the water in the spore with heavy water in the medium. The results are given in Table 1. Experimental values were higher than theoretical figures obtained by the use of the equation given under Methods; full exchange values were always obtained. This suggests that *R. arrhizus* spores are permeable to water even though they do not swell in it in the absence of suitable nutrients.

Vacuum-dried spores did not swell or produce germ-tubes in distilled water; but all the spores germinated in the standard medium. The ability of the spores to exchange their water with the heavy water in the medium, demonstrated above, was therefore not due to an effect of drying on the permeability of the spore wall.

Table 1. *The estimation of heavy water (deuterium oxide) in permeability studies with Rhizopus arrhizus spores*

Wt. (mg.) of vacuum-dried spores	Wt. (mg.) of oven-dried spores	Wt. (mg.) of water in spores = $\frac{\%\ \text{water} \times M}{100}$	Theoretical full-exchange value (%)	Experimental exchange value (%)
389·3	282·0	107·4	9·7	15·4
296·9	167·1	129·8	11·5	17·8

M = wt. (mg.) of oven-dried spores

Table 2. *The effect of respiratory inhibitors on germination of Rhizopus arrhizus on standard medium at 30°*

Chemical inhibitor in medium	Least molar concentration inhibiting germination	Average spore diameter (μ) time 0	Average spore diameter (μ) at 8 hr	Spores (%) with germ-tubes at 8 hr
2,4-Dinitrophenol	5×10^{-3}	5·5	6·0	15
Sodium azide	10^{-3}	5·5	5·5	5
Potassium cyanide	5×10^{-3}	5·5	8·0	30

Effect of respiratory inhibitors on spore germination

Mandels & Darby (1953) reported the inhibition by sodium azide of spore swelling in *Myrothecium verrucaria* in the basal nutrient medium.

The effect of 2,4-dinitrophenol, sodium azide and potassium cyanide on the germination of *Rhizopus arrhizus* spores was, therefore, investigated. A decimolar solution of each inhibitor in liquid standard medium was made and samples of it were withdrawn into different Petri dishes. Melted standard medium agar was added to each Petri dish to a total volume of 20 ml. and allowed to solidify. Four sterile cellophan strips were placed on the solidified medium in each Petri dish

and two drops of spore suspension were spread on each strip. After 8 hr the least concentration of each inhibitor causing incomplete swelling and/or less than 50% germ-tube production was determined. 2,4-Dinitrophenol, sodium azide and potassium cyanide concentrations up to about 10^{-3}M inhibited both spore swelling and germ-tube emergence in *R. arrhizus* (Table 2).

Effect of 10^{-3} M-NaN_3 on germinating Rhizopus arrhizus spores

Experiments in which ungerminated spores were placed in contact with the inhibitor for different periods, after which they were transferred to fresh media not containing the inhibitor, showed that *R. arrhizus* spores on the standard medium containing 5×10^{-3} M-NaN_3 remained unswollen and without germ-tubes for at least 24 hr, but if then transferred to the standard medium not containing the inhibitor, all spores swelled and produced germ-tubes after a further 8 hr. The effect of the inhibitor on the ungerminated spore was, therefore, not permanent.

The following experiment was accordingly made to investigate the effect of 10^{-3} M-NaN_3 on spores which had been incubated for 3 hr on the standard medium. Such partially swollen spores were transferred either to the standard medium containing 10^{-3} M-NaN_3 or to water agar (with or without 10^{-3} M-NaN_3) for 5 hr. The results are given in Table 3. Spores transferred to the standard medium containing 10^{-3} M-NaN_3 did not swell further or produce germ-tubes, but spores transferred to water agar, with or without the inhibitor, produced germ-tubes but stopped swelling. This suggested that NaN_3 was taken into the spores only when utilisable exogenous nutrients were present.

Table 3. *Effect of 10^{-3} M-NaN_3 on germinating Rhizopus arrhizus spores at 30°*

	Condition at time of transfer (3 hr)		Condition at 8 hr	
Treatment	Average spore diam. (μ)	Spores (%) with germ-tubes	Average spore diam. (μ)	Spores (%) with germ-tubes
3 hr on standard medium, then:				
5 hr on medium + 10^{-3}M-NaN_3	7·5	0	8·0	0
5 hr on water agar + 10^{-3}M-NaN_3	7·5	0	7·5	90
5 hr on water agar	7·5	0	8·0	100
5 hr on fresh standard medium	7·5	0	13·5	100
All 8 hr on standard medium	—	—	13·5	100
All 8 hr on plain agar	—	—	5·5	0

Dry weight changes during germination

Dry weight was determined at intervals during the germination of *Rhizopus arrhizus* spores in the standard medium with or without sodium azide. 25 ml. samples of spore suspension in water were measured into 150 ml. Erlenmeyer flasks containing either 25 ml. of double-strength standard medium with or without 10^{-3} M-NaN_3 (to maintain the usual standard medium nutrient level on dilution with spore suspension), or 25 ml. of water. Four replicates of each treatment were set up. Air, filtered by passing through cotton wool plugs, was bubbled through the suspensions which were maintained at $30 \pm 1°$. After 8 hr, the contents of

similarly treated flasks were pooled and filtered through a sterile sintered crucible of fine porosity lined with sterile glass paper of known weight. Spores were washed with distilled water and then acetone, and dried at 100° for 6 hr. A considerable increase (about 500%) in dry weight accompanied the germination of *R. arrhizus* spores in the standard medium, but in the presence of 10^{-3} M-NaN_3, no increase in dry weight occurred (Table 4).

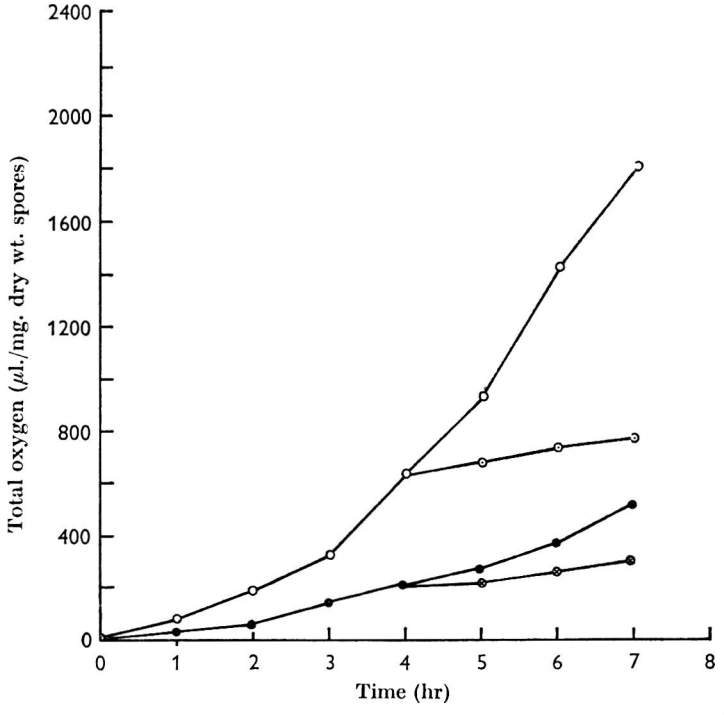

Fig. 1. Effect of sodium azide on oxygen uptake by *Rhizopui arrhizus* spores at 30°. ○—○, standard medium; ●—●, standard medium + sodium azide at time zero; ⊙—⊙, standard medium + sodium azide at 4 hr; ⊗—⊗, distilled water.

Table 4. *Dry weight changes during germination of R. arrhizus spores*

Treatment	Weight (mg.) of spores
Spores at time 0	1·6
Spores in sterile distilled water for 8 hr	1·5
Spores in standard medium for 8 hr	8·1
Spores in standard medium + 10^{-3} M-NaN_3 for 8 hr	1·8
Spores on standard medium for 3 hr	3·7
Spores in standard medium far 3 hr, then 5 hr in medium containing 10^{-3} M-NaN_3	3·5

Respiration of Rhizopus arrhizus spores

As it had been found that respiratory inhibitors (2,4-dinitrophenol, sodium azide, and potassium cyanide) inhibited germination of *R. arrhizus* spores, the following experiments were made to investigate the dependence of spore germination on respiration. Measurements of oxygen uptake were made with Warburg

respirometers (Umbreit, Burris & Stauffer, 1957). The results are summarized in Fig. 1. Oxygen uptake by *R. arrhizus* spores in the standard medium was very high; in water, oxygen uptake was low and could be estimated only by using thick spore suspensions. 10^{-3} M-NaN_3 inhibited oxygen uptake. Weber & Ogawa (1965) observed that 10^{-3} M-NaN_3 and 10^{-3} M-NaF completely inhibited oxygen uptake, whereas 2,4-dinitrophenol (10^{-3}M) stimulated respiration of *R. arrhizus* spores in proline-phosphate mixtures.

Electron microscope studies

Hawker & Abbott (1963*b*) studied the maturation and germination of sporangiospores of *Rhizopus nigricans* (*R. stolonifer*) and *R. sexualis*. *R. arrhizus* spores have been similarly studied; in Plate 1, fig. 1. can be seen changes in fine structure of germinating sporangiospores (on standard medium) similar to those observed by Hawker & Abbott (1963*b*) in *R. stolonifer*. A new inner wall (IW) has been formed, and the large mitochondria (M) have occasional constrictions along the longer axis. Spores germinating on glucose agar contained similar structures, but no changes in spore fine structure were observed in spores on water agar.

Effect of sodium azide and anaerobic conditions on germinating spores. Rhizopus arrhizus spores which had been incubated on the standard medium for 3 hr and then transferred for 5 hr either to standard medium in a sealed system filled with nitrogen or to standard medium containing 10^{-3} M-NaN_3 usually stopped swelling and producing germ-tubes. Fine structure of the spores so treated, was, therefore, studied and it was found that mitochondrial division ceased, as shown by the large size of these organelles (Pls. 1, 2; figs. 2–4). Cristae which appeared as nearly parallel 'plates' in control spores became a disorderly collection of undulating plates (Pl. 2, figs. 3, 4). The nuclear membrane became split in places, thus giving rise to small vacuoles between the two electron-dense layers of the membrane. Gale & McLain (1964) observed similar effects on spores of *Candida albicans* exposed to thiobenzoate; and Hirano & Lindegren (1961, 1963) observed that mitochondria of *Saccharomyces cerevisiae* developed some abnormalities in partially anaerobic conditions, the mitochondrial cristae becoming replaced by a complex system of inner membranes. The finding that sodium azide and anaerobic conditions have similar effects on fine structure of *R. arrhizus* spores is interesting. If NaN_3 inhibits spore germination by its inhibitory action on the cytochrome oxidase-cytochrome c oxygen transfer system thus causing anaerobic conditions to occur (Stannard & Horecker, 1948) it might be expected that anaerobic conditions would have the same effect as that of NaN_3 on spore structure.

The microfibrillar structure of R. arrhizus cell-walls. Hawker & Abbott (1963*b*) had observed that a thin inner wall consisting of tangentially arranged elements, resembling the wall of the vegetative hypha (Hawker & Abbott, 1963*a*), but differing from the original spore wall, was laid down during germination of *Rhizopus stolonifer* and *R. sexualis* spores.

A similar wall-layer was seen in germinating *Rhizopus arrhizus* spores. A comparative study of shadowed preparations of isolated cell walls of ungerminated and germinating spores and of young hyphae (about 16 hr old) by Dawson's (1949) method showed that the microfibrils of *R. arrhizus* cell walls were oriented in a

rather irregular and random fashion and no clear differences in microfibrillar structure of original spore walls, new inner spore walls and hyphal walls was detected.

DISCUSSION

The initiation of germination in *Rhizopus arrhizus* spores has been shown to require a utilizable carbon compound (Ekundayo & Carlile, 1964). Earlier, it was thought that the metabolism of the carbon compound, probably at the spore surface, brought about induced permeability of the spore walls to mineral ions and water. Heavy water exchange experiments have since shown that *R. arrhizus* spores can exchange the water in the spore with heavy water in the medium in the absence of carbon compounds. This suggests that the spore is already permeable to water, and, probably, to mineral ions. Spore swelling, however, involves an increase in spore size and requires the spore wall to be extensible. Since swelling occurs on media containing utilisable carbon compounds (e.g. glucose) without any mineral salts, glucose is sufficient to induce the walls of *R. arrhizus* spores to become more extensible.

Nickerson's (1963) work on yeast suggested an explanation of how glucose might bring about changes in the Rhizopus spore wall and thereby make it become extensible. Plasticity of the yeast wall was thought to result from a reduction of disulphide linkages in the protein component of the wall to sulphydryl groups, the ultimate reducing agent being $NADH_2$ generated from glucose. If this theory applies to Rhizopus, a disulphide-containing protein complex must be present in the spore wall and the spore must contain enzyme systems capable of reducing disulphide linkages. Results of investigations by other workers support such a possibility: Crook & Johnston (1962) demonstrated the presence of protein-carbohydrate complexes in a wide range of fungal cell-walls. Bartnicki-Garcia & Reyes (1964) demonstrated the presence of such complexes in *Mucor rouxii* which, like Rhizopus, is a member of the Mucoraceae. Hatch & Turner (1960) showed that in the presence of systems to provide $NADH_2$ or $NAPH_2$, pea-seed protein disulphide reductases reduced protein disulphide linkages. Robson & Stockley (1962), by using autoradiographic methods detected the presence of sulphydryl groups in the cell walls of many fungi.

If the above hypothesis is correct, swelling in Rhizopus spores would result from glucose-induced increased plasticity of the spore wall. Once wall plasticity is achieved, the wall stretches in response to water intake caused by the high osmotic pressure in the spores. The observations (1) that Rhizopus spores can exchange the water in the spore with heavy water in the medium and (2) that Rhizopus spores which have been incubated on the standard medium for about 3 hr, with probable dilution of their contents, become plasmolysed when placed in hypertonic salt solutions, support the view that these spores take in water by osmosis. It must be emphasised, however, that these observations prove only that water can be extruded from *Rhizopus* spores by osmotic methods. Systems are known in which water intake and extrusion take place by different processes, e.g. in amoeba in which water enters the organism passively but its excretion through the contractile vacuole requires respiratory energy.

Spore swelling in Rhizopus is accompanied by a considerable increase in oxygen

uptake and an increase of about 500% in dry weight of the spores. It is inhibited by respiratory inhibitors (Table 2, and Weber & Ogawa, 1965) and anaerobic conditions. It is thus likely that synthetic processes occur during spore swelling. Indeed, the formation of new internal structures can be demonstrated by electron microscopy during swelling. If the spores are low in nutrient content, such synthesis may account for the dependence of Rhizopus spores on exogenous nutrients for spore swelling. Anaerobic conditions and respiratory inhibitors may prevent swelling by blocking the metabolic processes necessary for the initiation of germination.

The above discussion suggests that glucose is needed for the germination of Rhizopus spores for two reasons:

(i) To generate $NADH_2$, to reduce disulphide linkages in the spore wall and permit wall extensibility and hence osmotic swelling.

(ii) As a source of carbon and energy for the synthesis of the new inner wall of the spore which subsequently becomes the germ-tube wall.

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Fig. 1

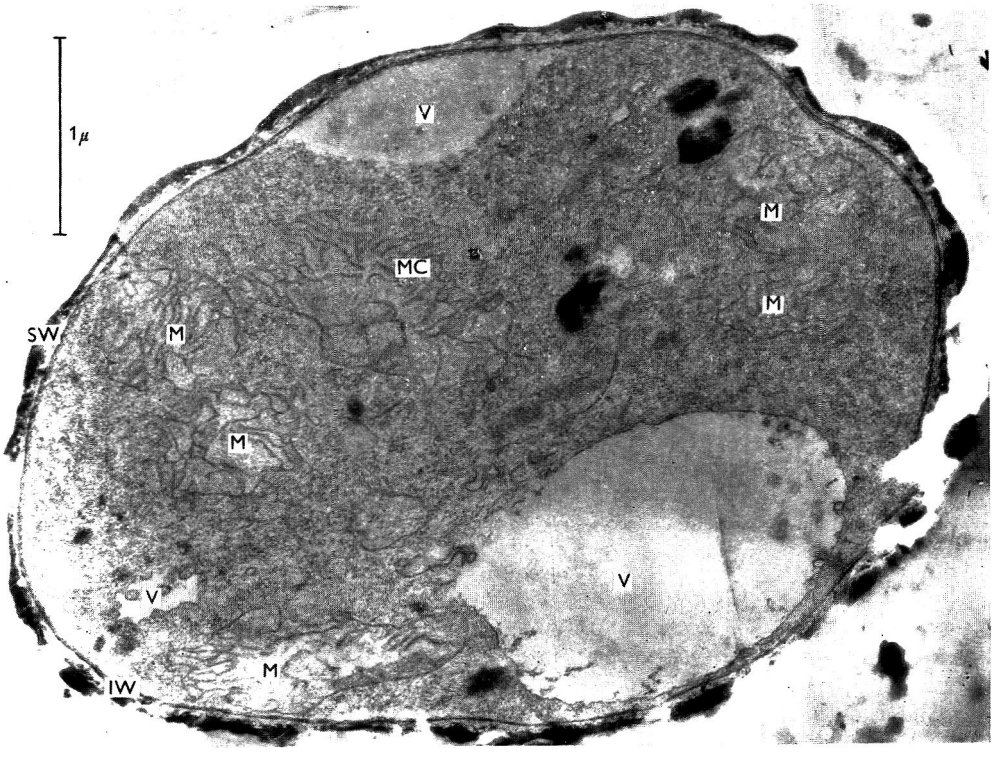

Fig. 2

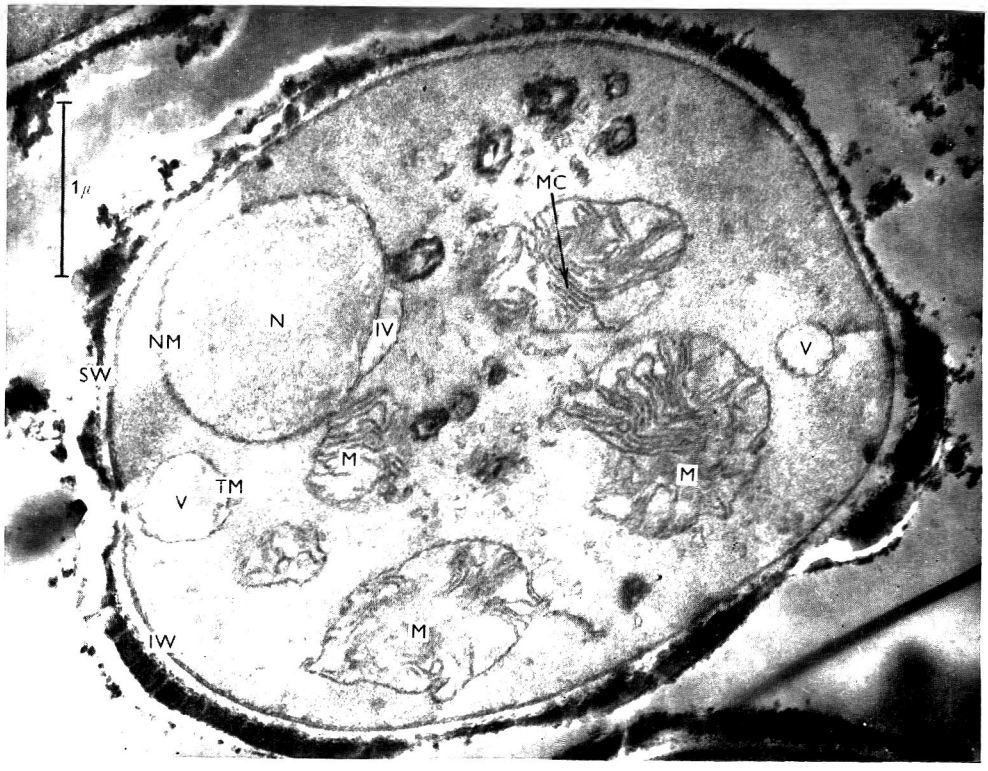

Fig. 3

Fig. 4

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EXPLANATION OF PLATES

Electron micrographs of *Rhizopus arrhizus*.

SW = spore wall, IW = Inner wall, N = nucleus, NM = nuclear membrane, M = mitochondria, MC = mitochondrial cristae, P = plasmalemma, TM = tonoplast, V = vacuole, IV = intramembranous vacuole.

PLATE 1

Fig. 1. Approximately transverse section through sporangiospore incubated on standard medium for 3 hr; showing typical nuclei, development of inner wall and mitochondria (e.g. M_1) with constrictions along the longer axis.

Fig. 2. Section through a spore incubated on standard medium for 3 hr followed by 5 hr on standard medium containing 10^{-3} M-NaN_3. Note presence of disorganized mitochondrial cristae, vacuoles have appeared in cytoplasm.

PLATE 2

Fig. 3. Section through a spore incubated on standard medium for 3 hr followed by 5 hr in N_2-saturated standard medium. Note mitochondrial cristae have become disorganized and nuclear membrane split in places thus giving rise to small vacuoles between the two electron-dense layers of the membrane. Features are essentially similar to those observed in spores placed under anaerobic conditions (Fig. 2).

Fig. 4. Two mitochondria from Fig. 3, enlarged. Note outer electron-dense layer of mitochondrial menbrane has disappeared in some places and mitochondrial cristae have become a collection of undulating 'plates'.

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The Effect of Substituted Phenoxyacetic and Phenoxybutyric Acids on the Growth and Respiration of *Aspergillus niger*

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SUMMARY

Substituted phenoxyacetic and phenoxybutyric acids stimulated growth of *Aspergillus niger* at low concentrations (10^{-6} M) and inhibited growth at higher concentrations (10^{-4} M). The inhibition of growth was paralleled by an inhibition of endogenous and mitochondrial respiration. The main site of inhibition appears to be at that part of the respiratory chain which involves ubiquinone.

INTRODUCTION

Substituted γ-phenoxybutyric acids are used in agriculture for the control of dicotyledonous weeds in growing crops. Such selectivity apparently depends upon the conversion by β-oxidation of 4-chloro-2-methylphenoxybutyric acid (MCPB) to the active 4-chloro-2-methylphenoxyacetic acid (MCPA) within the tissues of susceptible weeds but not in the crop plants (Wain, 1955, 1957; Fawcett, Wain & Wightman, 1960). It is inherent in this butyrate→acetate degradation concept that MCPB cannot be more toxic to plant life than MCPA.

Recently Shennan & Fletcher (1965) have tested *in vitro* the effect of substituted phenoxy acids on the growth of selected species of bacteria, actinomycetes, fungi and micro-algae, and have shown that at low (herbicidal) concentrations there was no harmful effect on the micro-organisms. However, at concentrations greater than 10^{-4} M there was a progressive increase in the toxicity of MCPB to most of the micro-organisms, whereas at similar concentrations MCPA had considerably less effect. These authors suggest that MCPB has in its own right an inherent protoplasmic toxicity at relatively high concentrations in addition to its hormonal effect at low concentrations when converted to MCPA. The present paper reports work done on the inhibitory action of high doses of MCPB and MCPA on the respiratory system of the filamentous fungus *Aspergillus niger* in an attempt to elucidate the site of the inhibition.

METHODS

Fungal material. The strain of *Aspergillus niger* van Tieghem used in this experimental work was obtained from the departmental stock cultures. Stock cultures of this fungus were maintained on potato glucose (PG) agar slopes in screw-cap bottles at 26°. Subcultures were made periodically by transferring large numbers of conidia to agar slopes. Liquid cultures were inoculated with 2% of their volume of a heavy conidial suspension (about 10^6 conidia/ml.), using a conidial suspension from 7-day cultures.

For experimental work the culture medium used was: Oxoid malt extract, 3 g.; yeast extract 3 g.; proteose peptone 5 g.; glucose 10 g.; distilled water to 1 l. Samples (25 ml.) of this liquid were added to 100 ml. conical flasks, plugged with non-absorbent cottonwool and sterilized (15 min., 15 lbs.). Flasks were incubated at 26° for 4 days on a shaking machine operating at 200 rev./min. Test compounds were added to the flasks before sterilization.

Mycelial dry weights were determined by filtration by suction through previously weighed Whatman no. 1 filter paper, drying the mycelial mat for 4 hr at 125°, cooling in a desiccator, and reweighing. Corrections were made for filter-paper weight lost on drying (about 5%). The results expressed as mg. dry-wt. mycelium.

Enzyme extraction. Mycelium for enzyme studies was grown in static liquid culture for 48 hr at 26°. The culture medium was removed by suction filtration, and the mycelium rinsed in tap water and blotted dry.

The mitochondrial preparation was isolated by grinding mycelium with twice its weight of sand in a chilled mortar with a solution containing 0·6 M-sucrose + 0·2 M-tris buffer + 0·005 M-ethylenediaminetetracetic acid (EDTA) + 0·01 M K_2HPO_4 previously adjusted to pH 7·8 with NaOH. The homogenate was passed through several layers of moist butter muslin and clarified by centrifugation at 1500*g* for 10 min. in an M.S.E. 'High Speed 17' refrigerated centrifuge, to remove coarse cell debris and nuclei. The mitochondrial preparation was then sedimented from the supernatant fluid by centrifugation at 20,000*g* for 30 min., and the deposit was re-suspended in a small volume of 0·4 M-sucrose in 0·05 M-phosphate buffer (pH 7·8).

Manometric assay of enzyme activities. Gaseous exchange was measured by the direct method of Warburg. Oxygen uptake was done in flasks containing 0·2 ml. of 6 N-KOH in the centre wells to absorb the CO_2 evolved. The mycelial discs used for respiratory studies were obtained from mycelium grown at 26° for 48 hr. The discs (1 cm. diameter) were washed in tap water, blotted free from excess water and added to the main compartment of the flask containing 2·0 ml. 0·1 M-phosphate buffer (pH 6·0). Test compounds in aqueous solution were added in samples of 0·2 ml. After all additions had been made, the total volume of the reaction mixture was brought to 3·0 ml. by adding distilled water. The gas phase was air; all measurements were made at 30°.

The reaction mixtures for the manometric determination of succinate oxidase activity and component electron transfer reactions are given below. The reactions were done at 30° after an equilibration period of 5 min. and the oxygen uptake measured during 20 min. Where the oxygen uptake was due to autoxidation of the reduced methylene blue or phenazine methosulphate to H_2O_2, the rates were halved to make them directly comparable with those of succinate oxidase. Cytochrome oxidase was measured according to Mackler & Green (1956).

Succinate oxidase. 0·06 M-KH_2PO_4 + Na_2HPO_4 buffer (pH 7·5), 0·03 M-sodium succinate, $9{\cdot}5 \times 10^{-5}$ M-cytochrome *c* and 2–3 mg. mitochondrial protein.

Succinate-methylene blue reductase. As for succinate oxidase except substituting cytochrome *c* with 1·6 mM-methylene blue and 1·7 mM-KCN to inhibit cytochrome oxidase.

Succinate-methylene blue reductase mediated by ubiquinone. As for succinate-methylene blue reductase + 3×10^{-5} M-ubiquinone in 0·1 ml. ethanolic solution. The addition of the ubiquinone resulted in a 3- to 5-fold increase in oxygen uptake.

Succinate-methylene blue reductase mediated by phenazine methosulphate. As for succinate-M.B. reductase + 4 mM-phenazine methosulphate.

Succinate-phenazine methosulphate reductase. As for succinate-methylene blue reductase except replacing methylene blue with 4 mM-phenazine methosulphate.

Spectrophotometric assays of enzyme activities. All assays were run at 30° for 2 min. Rates were corrected from blank determinations done in the absence of substrate.

Succinate-cytochrome c reductase. 20 μmoles sodium succinate were added to a reaction mixture containing: 0·1–0·3 mg. mitochondrial protein; 100 μmoles $KH_2PO_4 + K_2HPO_4$ buffer (pH 7·4); 0·1 ml. 1% (w/v) cytochrome *c*; 10 μmoles KCN in a total volume of 3 ml. The rate of oxidation of succinate by cytochrome *c* was determined by following the reduction of the cytochrome *c* by the increase in extinction at 550 mμ.

Nicotinamide adenine dinucleotide ($NADH_2$) oxidase. 0·2 μmoles $NADH_2$ was added to a reaction mixture containing 0·5–1·0 mg. mitochondrial protein, in 100 μmoles KH_2PO_4-K_2HPO_4 buffer, pH 7·4. The reaction was followed by measuring the decrease in extinction at 340 mμ.

$NADH_2$-cytochrome c reductase. As for succinate-cytochrome *c* reductase with the exception that 0·2 μmoles $NADH_2$ replaced the sodium succinate.

In all assays enzyme activity was proportional to the amount of enzyme added. Protein concentrations of mitochondrial preparations were determined by the method of Ma & Zuazaga (1942).

RESULTS

Effect of 4-chloro-2-methylphenoxy butyric acid (MCPB) and 4-chloro-2-methylphenoxyacetic acid (MCPA) on mycelial growth

The results in Fig. 1 show the effect of MCPB and MCPA on the growth of *Aspergillus niger*. In each case there was a stimulation of growth at low concentrations and an inhibition of growth at higher concentrations. A similar growth pattern was obtained when conidiospores were allowed to germinate for 24 hr before the addition of the phenoxy acids. This would suggest that the stimulatory/inhibitory effect of the compounds was being exerted against mycelial growth rather than on spore germination.

Effect of MCPB and MCPA on endogenous and mitochondrial respiration

When mycelial discs were incubated with different concentrations of the substituted phenoxy acids there was a progressive decrease with time in endogenous respiration (Table 1). At the highest concentration of MCPB (5×10^{-3} M) there was marked inhibition of endogenous respiration after only 30 min. of incubation. At this concentration prolonged periods of incubation resulted in complete inhibition of respiration. At the highest concentration of MCPA tested (5×10^{-3} M) and an incubation period of 20 hr the maximum inhibition was 52%.

Mitochondrial preparations had considerable succinate oxidase and $NADH_2$ oxidase activities. Both oxidases were sensitive to potassium cyanide, and succinate oxidase to malonate. For inhibitor studies mitochondrial preparations and the substituted phenoxy acids were incubated at 2° for 30 min. before measurement

of enzyme activity. The oxidations of succinate and $NADH_2$ were inhibited by both compounds, though in each case the inhibition by MCPB was much greater than by MCPA (Table 2). The inhibitory effect of low concentrations of MCPB (5×10^{-4} M) could be enhanced by prolonged incubation with enzyme preparations.

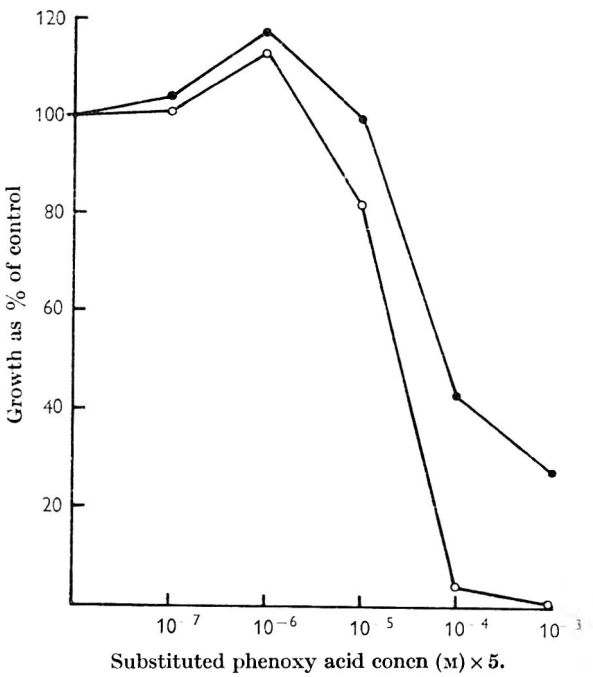

Fig. 1. The effect of 4-chloro-2-methylphenoxybutyric acid (MPCB) and 4-chloro-2-methylphenoxyacetic acid (MCPA) on the growth of *Aspergillus niger*. Cultures were grown under shaking conditions for 4 days at 26°. Growth was measured as mycelial dry weight and results expressed as percentage of controls which did not contain MCPB or MCPA. (●), MCPA; (○), MCPB.

Table 1. *The effect of 4-chloro-2-methylphenoxyacetic acid (MCPA), and 4-chloro-2-methylphenoxybutyric acid (MCPB), on endogenous respiration of mycelial discs of Aspergillus niger*

Incubation period (hr)	MCPB (M)			MCPA (M)		
	5×10^{-5}	5×10^{-4}	5×10^{-3}	5×10^{-5}	5×10^{-4}	5×10^{-3}
0·5	0	0	35	0	0	0
2*	0	15	85	0	0	10
20*	27	52	100	0	0	50

* Respiration was measured for the last hour of each incubation period.

Effect of MCPB and MCPA on the component electron transport enzymes

The component enzymes of the electron transport chain are known to be an integral part of the inner mitochondrial membrane and for this reason it is difficult to test the inhibitors against individual enzymes. This difficulty can be partially overcome by the use of artificial electron acceptors such as methylene blue, phena-

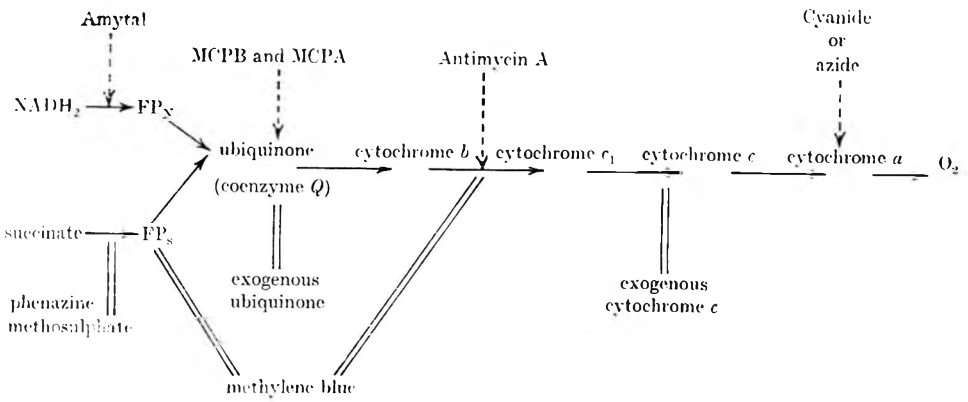

Fig. 2. A generalized scheme of electron transport. The full arrows show direction taken by the two reducing equivalents from substrate to oxygen. The broken arrows show the point of action of inhibitors. Double lines indicate regions at which artificial electron acceptors can participate in the electron flow. Abreviations: $NADH_2$, reduced nicotinamide adenine dinucleotide; FP_S succinic dehydrogenase flavoprotein; FP_N, nucleotide dehydrogenase flavoprotein.

Table 2. *Effect of 4-chloro-2-methylphenoxybutyric acid (MCPB), and 4-chloro-2-methylphenoxyacetic acid (MCPA) on the respiratory oxidases of mitochondria from Aspergillus niger*

Mitochondria and the substituted phenoxy acids were incubated at 2° for 30 min. before assaying activity.

	Succinate oxidase		NADH oxidase	
Inhibitor conen (M)	MCPB	MCPA	MCPB	MCPA
	inhibition (%)			
5×10^{-5}	0	0	0	0
5×10^{-4}	10	0	10	0
5×10^{-3}	95	10	90	8

zinc methosulphate, ubiquinone (Co. Q) and cytochrome *c* to tap into the respiratory chain, and in this way to measure electron flow over known sections of it. In this way the approximate site of action of an inhibitor can be located. The use of methylene blue as a hydrogen acceptor in the manometric assay of the succinic dehydrogenase complex of mitochondrial preparations was described by Slater (1949). The dye appears to be a relatively inefficient acceptor since its rate of reduction is only a small fraction of the succinate oxidase rate. Methylene-blue reduction is greatly increased when other electron acceptors such as phenazine methosulphate or ubiquinone are also present in the reaction system. It is considered that the main site of action of methylene blue is between the succinate flavoprotein and the antimycin A-sensitive region. In this way it overlaps on to the common pathway of succinate oxidase and $NADH_2$ oxidase (Fig. 2).

The results of the experiments on the effect of the substituted phenoxy acids on the component enzymes of succinate oxidase and $NADH_2$ oxidase are shown in Table 3. These results suggest that there are possibly two sites of action of the compounds:

one is before the site of action of phenazine methosulphate (about 25% inhibition); the second between the site of action of phenazine methosulphate and methylene blue (about 85% of residual activity).

Table 3. *Effect of 4-chloro-2-methylphenoxybutyric acid (MCPB) and 4-chloro-2-methylphenoxyacetic acid (MCPA), on the component enzymes of the succinate oxidase and $NADH_2$ oxidase systems of Aspergillus niger*

Inhibitor concentration in each case was 5×10^{-3} M.

Enzyme system	MCPB Inhibition (%)	MCPA Inhibition (%)
Succinate oxidase	90	10
Succinate-methylene blue reductase	85	10
Succinate-methylene blue reductase (mediated by ubiquinone)	70	8
Succinate-methylene blue reductase (mediated by phenazine methosulphate)	25	5
Succinate-phenazine methosulphate reductase	20	0
Succinate-cytochrome *c* reductase	80	10
Cytochrome oxidase	0	0
$NADH_2$ oxidase	85	10
$NADH_2$-cytochrome *c* reductase	65	8

DISCUSSION

The effects of the two substituted phenoxy acids on the growth of *Aspergillus niger* show that each compound stimulated growth at low concentrations and inhibited at higher concentrations. The stimulation of growth by MCPB may be due to the presence of a β-oxidizing system within the fungus which converts the butyric to the active acetic homologue. Abundant evidence has accumulated which substantiates the β-oxidation pathway for fatty acid degradation by many micro-organisms. The actinomycete *Nocardia opaca* can bring about the β-oxidation of ω-phenoxyalkanecarboxylic acids (Webly, Duff & Farmer, 1955), while *A. niger* and *Sclerotinia laxa* can degrade ω-(2-naphthoxy)-*n*-alkylcarboxylic acid by β-oxidation (Byrde, Harris & Woodcock, 1956; Byrde & Woodcock, 1958).

The results in Tables 1–3 indicate that the inhibitory effect of the higher concentrations of the substituted phenoxy acids on the growth of *Aspergillus niger* were paralleled by a corresponding inhibition of the normal respiratory processes. Endogenous respiration was progressively inhibited with prolonged incubation with the compounds, and in a similar manner the essential components of the respiratory electron transport chain (succinate oxidase, $NADH_2$ oxidase) were also inhibited. In all cases the degree of inhibition was always greater with MCPB than with an equimolar concentration of MCPA.

Since the relative inhibition of succinate oxidase and $NADH_2$ oxidase was approximately the same, it is logical to consider that the main site of inhibition of the electron transport occurs at some point common to both electron pathways. Furthermore, the high degree of inhibition of succinate-methylene blue reductase implies that this inhibition occurs between the flavoprotein and antimycin A-sensitive area of the respiratory chain.

There is now much evidence which suggests that ubiquinone is a functional component of the mitochondrial respiratory chain, occupying a position on the oxygen side of the $NADH_2$ and succinate flavoproteins and on the substrate side of cyto-

chrome *c* (Redfearn, 1961*a*; Hatefi, 1963). Ubiquinone is widely distributed in micro-organisms and readily undergoes oxidation and reduction (Lester & Crane, 1959; Morton, 1961; Crane, 1961; Brodie, 1961; Lavate, Dyer, Springer & Bentley, 1962; Osnitskaya, Threlfall & Goodwin, 1964). In the present study it has been found that ubiquinone can be used to mediate the reaction between the respiratory chain and methylene blue. This stimulation of succinate-methylene blue reduction by exogenous ubiquinone is generally considered to be due to a direct reaction with the flavoproteins or the complex which may be designated ubiquinone reductase (Redfearn, 1961*b*). In the presence of MCPB and MCPA this ubiquinone mediated electron transport in *Aspergillus niger* was inhibited 70 and 8%, respectively. Thus it would appear that the main site of inhibition of the substituted phenoxy acids is at that part of the respiratory chain which involves ubiquinone, perhaps the enzyme complex ubiquinone reductase. There may also be other minor sites of action before and beyond this point since both succinate oxidase and $NADH_2$ oxidase were inhibited to a greater extent than was the ubiquinone mediated succinate-methylene blue reduction.

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The Internal Membranes of *Caulobacter crescentus*

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SUMMARY

The intracytoplasmic membranous structures previously observed in various caulobacter species by electron microscopy of thin sections were studied further by electron microscopy of whole organisms of *Caulobacter crescentus* negatively stained with phosphotungstate. The large, complex mesosomes are intrusions of the cell membrane. They appeared, from their central location in dividing cells, to play a role in division. Smaller and less involuted intrusions of the membrane became abundant in oxygen-limited cells, which also had an abnormally high content of haem pigments. These smaller intrusions may therefore be a structural reflexion of an increased content in the membrane of components of the respiratory electron transport system. The membranous organelle which occurred at the site of stalk formation and appeared to be connected with the membranous core of the stalk was not penetrated by phosphotungstate, and therefore had an organization different from that of the mesosome.

INTRODUCTION

A study of the cellular organization of caulobacters belonging to the genera *Caulobacter* and *Asticcacaulis* by electron microscopy of thin sections has shown that these bacteria characteristically contain membranous organelles (Poindexter & Cohen-Bazire, 1964). Large mesosomes were frequent, particularly near the site of cell division, and a membranous structure of variable size occurred at the base of the stalk and was seemingly continuous with the membranes that composed the core of the stalk itself. It was not easy to ascertain the origin and arrangement of these membranous elements from the examination of their profiles in thin sections, and we have accordingly explored another technique for their study.

Zwillenberg (1964) and Bladen, Nylen & Fitzgerald (1964) examined by electron microscopy whole bacteria treated with a solution of potassium phosphotungstate. By virtue of its electron density, phosphotungstate serves as a negative stain. It cannot enter the protoplast; hence, if it is able to penetrate the cell wall, it reveals by its opacity the contours of the cell membrane and its intrusions. Zwillenberg examined a considerable variety of true bacteria, both Gram positive and Gram negative, by this method and found that only four organisms (all Gram positive) possessed walls permeable to phosphotungstate. In the other 8 bacteria he examined, phosphotungstate acted as a negative stain for the whole organism, revealing only the surface structure of the wall. Preliminary trials of this method were conducted with *Caulobacter bacteroides* strain CB 11a, *C. fusiformis* strain CB 27, and *C. crescentus*

strain CB 15, representative of the main structural subgroups in the genus *Caulobacter* (Poindexter, 1964). Only *C. crescentus* proved to possess a wall permeable to phosphotungstate, and it was accordingly used for the experiments reported here.

METHODS

Bacteriological methods. Caulobacter crescentus strain CB 15 (Poindexter, 1964) was grown in a liquid medium containing, g./l. of tap water: Bactopeptone 2, Difco yeast extract 1, and $MgSO_4.7H_2O$, 0·2. All cultures were incubated at 30°. Growth was estimated turbidimetrically, by using a Klett–Summerson colorimeter with a red filter (*c.* 660 mμ).

Bacteria were harvested in three different physiological states for electron-microscopic examination. *Normal bacteria* were derived from fully aerated flask cultures, incubated on a New Brunswick rotary shaker, and harvested during the course of exponential growth. *Oxygen-limited bacteria* were grown in weakly aerated flask cultures, incubated on a slow shaker (80 strokes/min., 2·5 cm. amplitude), and harvested after growth had attained a density sufficient for oxygen to become the limiting nutrient. *Carbon-limited bacteria* were grown in flasks of the standard medium which had been diluted fivefold with 0·002 M-phosphate buffer (pH 6·8) containing, g./l.: NH_4Cl, 0·25 and $MgSO_4.7H_2O$, 0·1. The cultures were fully aerated (incubation on a New Brunswick rotary shaker) and harvested shortly after entry into the stationary phase. Controls showed that the cessation of growth reflected exhaustion of the available carbon and energy sources, and that oxygen was at no point a limiting nutrient.

Electron-microscope examination. The bacteria were harvested by centrifugation and resuspended in distilled water containing 5 g. ammonium acetate and 2 g. sucrose/l. A drop of this suspension was placed on a carbon-stabilized formvar-coated 300-mesh copper grid, and most of the liquid was withdrawn by touching the edge of the drop with a piece of filter paper. A drop of 1% (w/v) potassium phosphotungstate containing 0·2% (w/v) sucrose and adjusted to pH 7·0 was immediately added, and most of the liquid again withdrawn. The air-dried specimens were examined within 24 hr in a Siemens Elmiskop I operating at 80 kV.

Measurements of protein and haem. Suspensions of bacteria in distilled water were treated for 10 min. in the MSE ultrasonic disintegrator. The protein contents of suitably diluted samples were determined by the method of Lowry, Rosebrough, Lewis Farr & Randall (1951). Other samples were used to prepare pyridine haemochromogen by the method of Elliot & Keilin (1963), as modified by Omura & Sato (1964). The spectral difference between the oxidized form and the dithionite-reduced form of the haemochromogen was measured with a Cary 14 recording spectrophotometer, and the total haem content was calculated from the absorption difference between the peak of the α band (557 mμ) and the minimum between the α and β bands, assuming an extinction coefficient of 22 cm.$^{-1}$ mM^{-1} (Jones, 1963).

RESULTS

The structure of normal Caulobacter crescentus

Plates 1, 2, 3, figs. 1–5 show typical appearances in phosphotungstate-stained preparations of bacteria growing exponentially in standard medium under conditions

where oxygen is not a limiting nutrient. The cell wall is typically somewhat distorted: irregular, bleb-like extensions (probably derived from the plastic outer layer characteristic of the walls of Gram-negative bacteria) are visible at many points. The contour of the protoplast, sharply revealed by the penetration of phosphotungstate to its outer surface, is smooth and regular. The only visible intracytoplasmic structures are irregularly distributed spherical inclusions that differ intrinsically from the surrounding cytoplasm in their electron opacity. Since the caulobacters characteristically contain deposits of both volutin and poly-β-hydroxybutyric acid (Poindexter, 1964), we tentatively interpret the small, electron-dense inclusions (v) as volutin, and the larger, electron-transparent inclusions (p) as poly-β-hydroxybutyric acid.

The most conspicuous structural elements revealed in these preparations are the large mesosomes (LM), which were deeply penetrated by phosphotungstate so that their number, location, and complex internal structure could be readily observed. Evidently, they represent infoldings of the cytoplasmic membrane. In dividing bacteria, examples of which occur in Pls. 1, 2, 3, figs. 1–4, a single large mesosome occupies the equatorial constriction and extends for some distance into the adjoining cytoplasm on each side of it. The appearance of the mesosome in late division stages (Pl. 2, fig. 3, and Pl. 3, fig. 4) indicates that it was bisected on completion of division, each daughter cell initially containing a single mesosome, derived from this bisection, at the undifferentiated pole (i.e. the pole that bears neither a stalk nor a flagellum).

After the daughter cells have separated, the mesosome apparently undergoes rapid changes in location and structure. This can be inferred from the mesosomal arrangements in Pl. 3, fig. 5, which shows a rosette containing four recently liberated, motile, apical organisms (swarmers). The formation of rosettes in caulobacter results from the adhesion of swarmers at their flagellated poles, which carry holdfast material; as growth proceeds, each cell in the rosette loses its flagellum and develops a stalk at the previously flagellated pole (Stove & Stanier, 1962). Hence a rosette composed of recently liberated swarmers can be unambiguously recognized, not merely by the shortness of the constituent bacteria, but by the presence of flagella and the absence of stalks. Furthermore, because adhesion always involves the flagellated poles of the participating swarmers, the outer poles of the bacteria in such a rosette can be identified as 'new' poles, formed at the immediately preceding division. Of the four cells in Pl. 3, fig. 5, only the upper one contains a single mesosome at the outer pole, the position that we infer to be characteristic of the immediate post-divisional state. In the cell at the lower right, there is one large, bi-lobed mesosome close to the outer pole, and a second, smaller mesosome not far from the inner pole. In the two other cells, there is a single mesosome close to the flagellated pole. These positional variations might have been brought about either by migration of the post-divisional mesosome, or by its reabsorption and the formation of a new mesosome at another point on the surface of the membrane.

The membranous organelle which lies at the base of the stalk and is seemingly continuous with the membranous core of the stalk (see Pl. 6, figs. 10, 11) frequently has a structure indistinguishable from that of the mesosome in thin sections; occasionally, its component membranes are more regularly arranged, more or less parallel to the long axis of the bacteria (Poindexter & Cohen-Bazire, 1964). In phosphotungstate-treated bacteria, neither the organelle itself nor the membranous core

of the stalk is appreciably penetrated by phosphotungstate. This can be seen with particular clarity in Pl. 1, fig. 2, where the protoplast has retracted slightly from the wall at the stalked pole, so that its surface is sharply outlined by negative contrast. As is also particularly evident in this figure, the core of the stalk shows up by negative contrast with the enclosing, phosphotungstate-permeated wall of the stalk. These observations indicate that the system of membranes occupying the stalked pole of the bacteria and extending into the core of the stalk do not (in contrast to the mesosome) represent a simple invagination of the cell membrane.

Effects of oxygen starvation on membrane structure

Earlier unpublished electron-microscopic studies of thin sections suggested that membranous intrusions are particularly abundant in caulobacter which have been grown in low concentrations of oxygen. Since these bacteria are strict aerobes, oxygen is an essential nutrient, so that the observed abundance of membranes in such cells could be a non-specific consequence of the slowing down or cessation of division. However, no such proliferation of membranes could be detected in sections of cells that had entered the stationary phase as a result of the depletion of the carbon and energy source. Negative staining with phosphotungstate completely confirmed the earlier observations on thin sections. Bacteria which have entered the stationary phase as a result of carbon depletion are essentially indistinguishable in both size and mesosomal structure from normal bacteria harvested during the exponential phase of growth; but bacteria from oxygen-limited cultures have a completely different aspect, illustrated in Pls. 4, 5, figs. 6–9.

Oxygen limitation evidently deranges cell growth and division. Most of the bacteria are greatly elongated (up to eight times as long as normal), and bear unusually short stalks; swarmers are very rarely observed. These elongated bacteria contain large numbers of membranous intrusions which are freely penetrated by phosphotungstate, and which vary greatly in size, shape and internal complexity. Some show the characteristic internal complexity of mesosomes; others seem to be simple sac-like structures. As many as 30 may occur in a single bacterium. Division figures are relatively rare in oxygen-limited bacteria; but when they do occur (see Pl. 5, fig. 8) the plane of constriction is characteristically occupied by a single large mesosome, entirely analogous in position and structure to the mesosome of a dividing normal bacterium. It should be specially noted that the development of the large mesosome in a dividing oxygen-limited bacterium is not accompanied by the disappearance of the smaller membranous intrusions, which are clearly evident at the other points on the surface of the protoplasts of dividing bacteria. Division in oxygen-limited bacteria is often highly asymmetric, as seen in the upper organism of Pl. 5, fig. 8; and in such cases the large mesosome still occupies the plane of constriction.

There is much evidence that in aerobic bacteria the cell membrane is the site of the respiratory electron transport system (see Hughes, 1962, for a review). Lenhoff, Nicholas & Kaplan (1956) have demonstrated that in *Pseudomonas fluorescens* oxygen limitation leads to a substantial increase in the cellular content of cytochrome *c*. These facts suggested a possible explanation for the abundance of membranous intrusions characteristic of oxygen-limited caulobacters. If, as in *P. fluorescens*, oxygen limitation increases the differential rate of synthesis of com-

ponents of the respiratory electron transport system, intrusion of the cytoplasmic membrane may be necessary to accommodate the increased cellular content of these membrane-bound enzymes. An analogous situation has been shown in *Rhodospirillum rubrum* and other non-sulphur purple bacteria, where the content of membrane-bound photosynthetic pigments, which can be greatly varied in response to environmental factors, is correlated with the extent of the internal membrane system (Cohen-Bazire & Kunisawa, 1963; Cohen-Bazire, 1963).

Table 1. *Variation of the specific haem content of Caulobacter crescentus in response to changes in the conditions of growth*

	Haem content, μmoles/mg. cellular protein		
Conditions of growth...	Aerobic exponential	Oxygen-limited	Carbon-limited
Expt. 1	0·182	0·415	0·25
Expt. 2	0·187	0·5	—
Expt. 3	0·20	0·53	—

In order to obtain some indication of the cellular content of respiratory enzymes in *Caulobacter crescentus*, we determined the haem content, measured as pyridine haemochromogen. As shown in Table 1, oxygen-limited bacteria contained more than twice as much haem on a protein basis as normal. Carbon-limited bacteria had a slightly greater haem content than normal, but the value was far below that characteristic of oxygen-limited organisms. These findings are accordingly consistent with the functional interpretation of the abundance of membranous intrusions in oxygen-limited caulobacters that has been outlined above.

DISCUSSION

Caulobacter crescentus contains at least two kinds of membranous organelles, distinguishable both by their location in the organism and by their structure. One type of organelle is situated at the site of stalk formation, and is probably connected with the membranous core of the stalk. This membrane system is not penetrable by phosphotungstate, and cannot therefore be interpreted as a direct intrusion of the cytoplasmic membrane. We propose to term it the *stalk organelle*.

The other type of organelle, for which we adopt the term 'mesosome', is a complex intrusion of the cytoplasmic membrane, readily penetrable by phosphotungstate. In exponentially growing populations, there are typically only one or two mesosomes in the interdivisional bacteria, and only one in dividing bacteria, invariably associated with the site of division. In oxygen-limited populations there are many more mesosomes in each bacterium, and they are more varied in size and structure. Since oxygen depletion leads to a substantial increase in the cellular haem content, we suggest that this proliferation of membranous intrusions reflects an increased content of membrane-bound respiratory enzymes, induced by low oxygen concentration.

In Gram-positive bacteria there is much evidence that mesosomes may play a role in the processes of nuclear and cell division (FitzJames, 1965), and their frequent association with transverse septa has led several workers to suggest that they may function in septum formation (van Iterson, 1961; Glauert, Brieger & Allen,

1961; Imaeda & Ogura, 1963). In 1963 Jacob, Brenner & Cuzin proposed that the bacterial chromosome may be replicated at an attachment site on the cell membrane, and that physical separation of the daughter chromosomes may result from interstitial growth of the membrane between their attachment points. The first direct evidence in support of this hypothesis was produced by Ryter & Jacob (1964), who demonstrated a constant topological association and probable physical attachment between the nucleoplasm and the mesosome in *Bacillus subtilis*. The invariable location of the mesosome at the site of cell division in *Caulobacter crescentus* is certainly compatible with the notion that it has important functions in division, and provides the first indication of a mesosomal role in the division of Gram-negative bacteria.

When oxygen-limited organisms of *Caulobacter crescentus* divide, the small intrusions characteristically formed as a result of oxygen limitation remain dispersed on the surface of the protoplast, even though a single large mesosome occupies the site of division. The increase in haem content which accompanies this cytological modification provides evidence that mesosomes can function as areas of concentrated respiratory activity; this is in accord with the cytochemical observations of van Iterson & Leene (1964) in *Bacillus subtilis*. Further, one of us (J.S.P.) has found that phosphate limitation similarly results in the proliferation of small, peripheral mesosomes. In light of the studies on oxygen limitation in these strictly aerobic bacteria, this suggests that restrictions of energy metabolism are responsible for formation of the small mesosomes, and that their formation may be controlled by a high-energy phosphate compound within the cell.

The accumulating observations on bacterial membrane systems suggest that the term 'mesosome' may in fact encompass organelles that have different functions. Some mesosomes, derived by intrusion of the cell membrane, may have specific roles in cell division, while others of the same derivation serve to extend the surface of the membrane in order to accommodate a large complement of respiratory enzymes. The stalk organelle, which is superficially similar to mesosomes in structure, provides evidence that membrane organelles may have a third function, i.e. participation in differentiation of subcellular structures. The significance of the apparent independence of this organelle from the cell membrane is not clear.

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EXPLANATION OF PLATES

All figures are electron micrographs of *Caulobacter crescentus* strain CB 15. Plates 1–5, negative-stained with phosphotungstate. Plate 6, thin sections of osmium-fixed caulobacters, embedded in Vestopal and post-stained with lead hydroxide.

Abbreviations used in figures: S, stalk; e, core of stalk; cb, cross-band in wall of stalk; f, flagellum; H, holdfast; v, volutin granule; p, poly-β-hydroxybutyric acid granule; M, mesosome; LM, large mesosome; W, cell wall; mo, membranous organelle.

PLATE 1

Dividing organisms from a culture in exponential growth.

Fig. 1. Note the large mesosome at the equator of the organism, symmetrically distributed between the two future daughter cells. The holdfast material at the tip of the stalk is clearly shown. Magnification, × 36,000.

Fig. 2. The large mesosome is somewhat unequally distributed between the two future daughter cells. At the stalked pole the continuity between the protoplast (containing the membranous organelle at the base of the stalk) and the core of the stalk itself is particularly clear. Magnification, × 40,000.

PLATE 2

Fig. 3. Caulobacters from a culture in the course of exponential growth. The two stalked organisms are adherent to a common holdfast. The upper organism, in a late stage of division, has a single large mesosome at the site of constriction. The lower organism, in the interdivisional state, contains a large mesosome near the apical pole, and a much smaller one near the basal, stalked pole. Cross-bands are visible in the stalks of both organisms. Magnification, × 36,000.

PLATE 3

Rosettes of caulobacters from a culture in the course of exponential growth.

Fig. 4. A mature rosette, composed of three stalked bacteria at different stages of division. In each organism there is one large mesosome, located in the area of division. Magnification, × 39,000.

Fig. 5. A newly formed rosette, composed of four swarmers, adherent by the flagellated poles. Note the differences in the number and position of the mesosomes. Magnification, × 30,000.

PLATE 4

Caulobacters from oxygen-limited cultures. Note the cellular elongation, the short stalks, and the numerous small mesosomes.

Fig. 6. Electron-transparent material appears to have exuded from the mesosomes and to have accumulated between the surface of the protoplast and the cell wall. Magnification, × 24,000.

Fig. 7. The diversity of mesosomal size and shape is particularly striking in this preparation. Two cross-bands in the wall of the stalk of the right-hand organism are shown very clearly. Magnification, × 20,000.

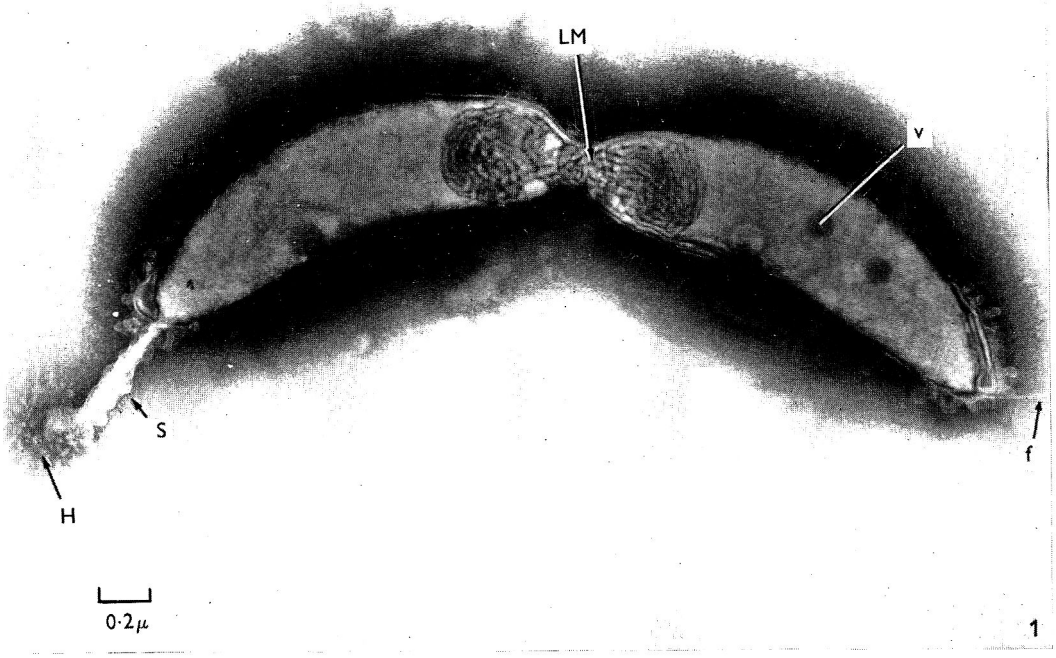
PLATE 5

Fig. 8. Rosette composed of three caulobacters from an oxygen-limited culture. Two organisms are dividing, and contain characteristic large mesosomes at the sites of division, as well as smaller mesosomes elsewhere. In the bacterium at left the division is highly asymmetric. Magnification, × 24,000.

Fig. 9. A group of stalked bacteria from an oxygen-limited culture, showing the diversity in size and form of the mesosomes, and the shortness of the stalks. Magnification, × 27,000.

PLATE 6

Figs. 10 and 11. Thin sections of caulobacters from an oxygen-limited culture. The membranous organelle at the base of the stalk, which cannot be seen in whole organisms stained with phosphotungstate, is evident in two bacteria. Profiles of mesosomes are also abundant. Magnification, × 84,000.



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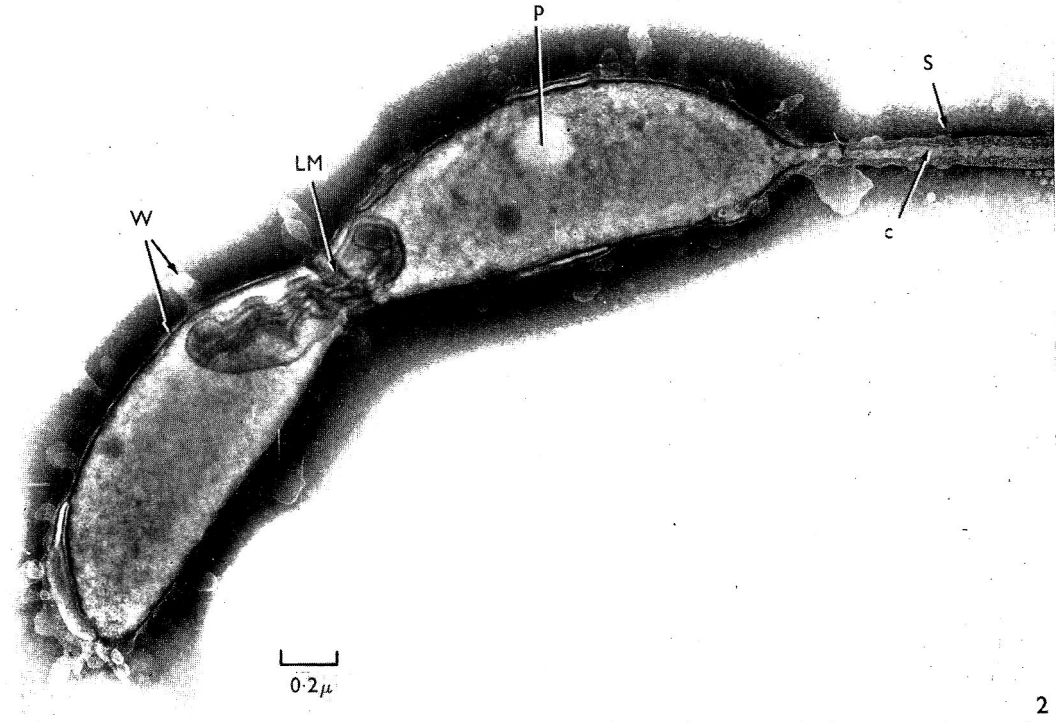

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G. COHEN-BAZIRE, R. KUNISAWA AND J. S. POINDEXTER (*Facing p.* 308)


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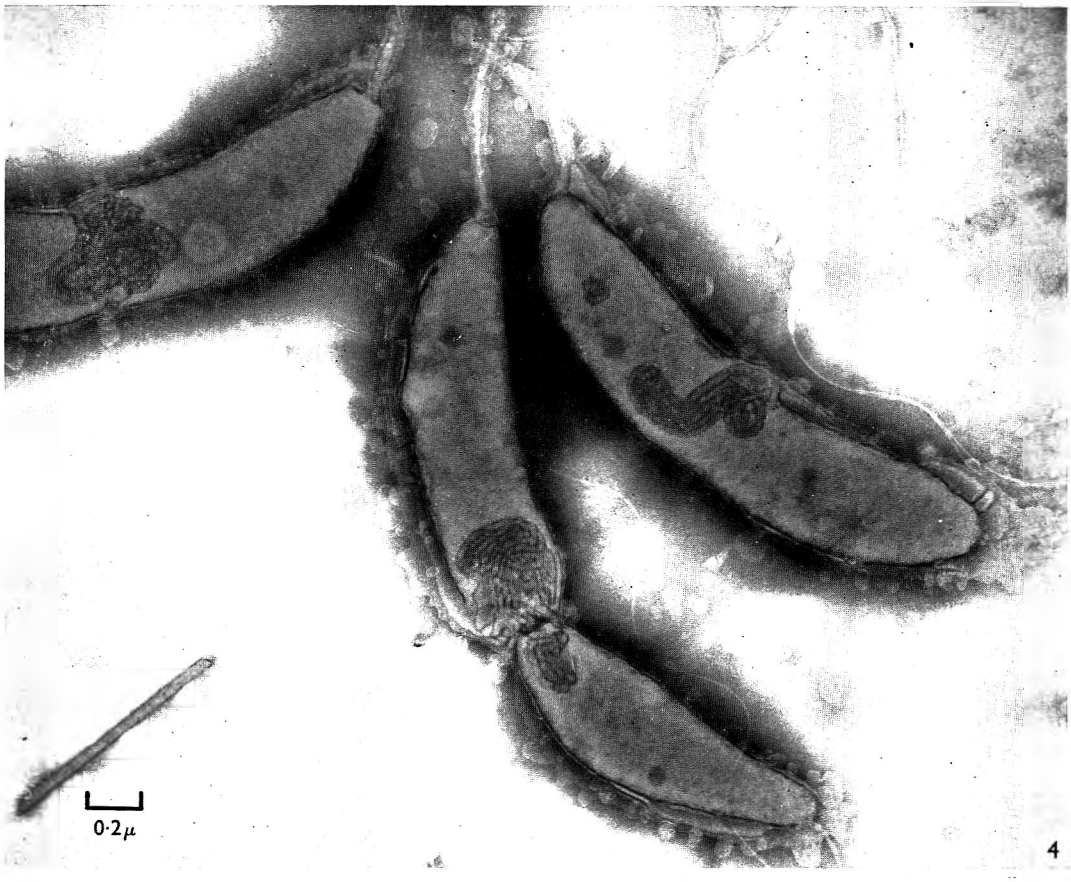

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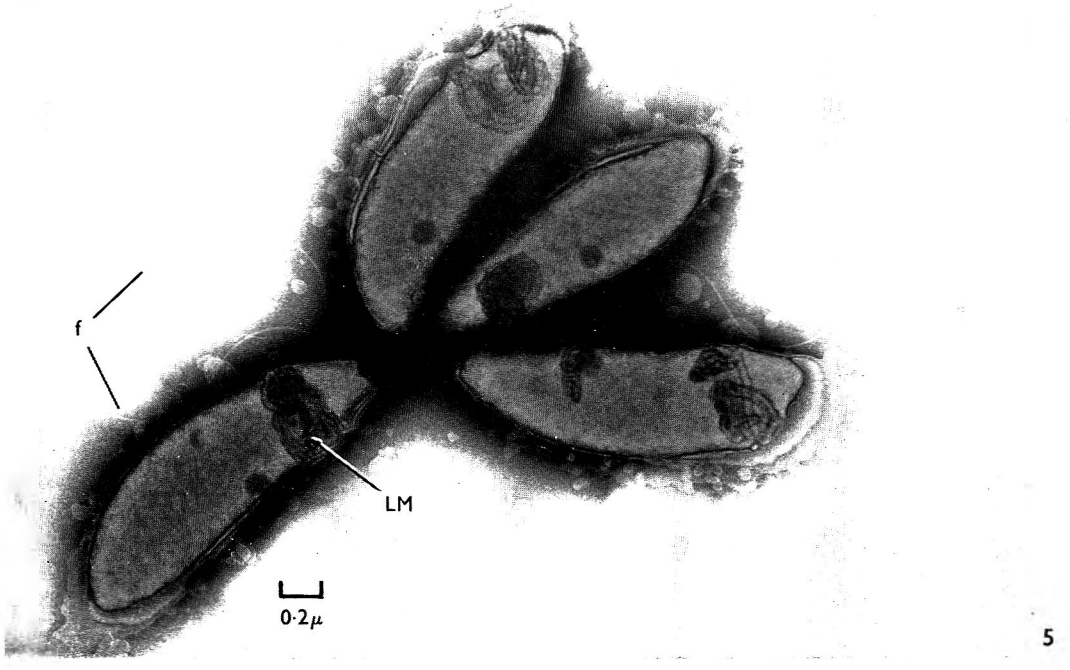

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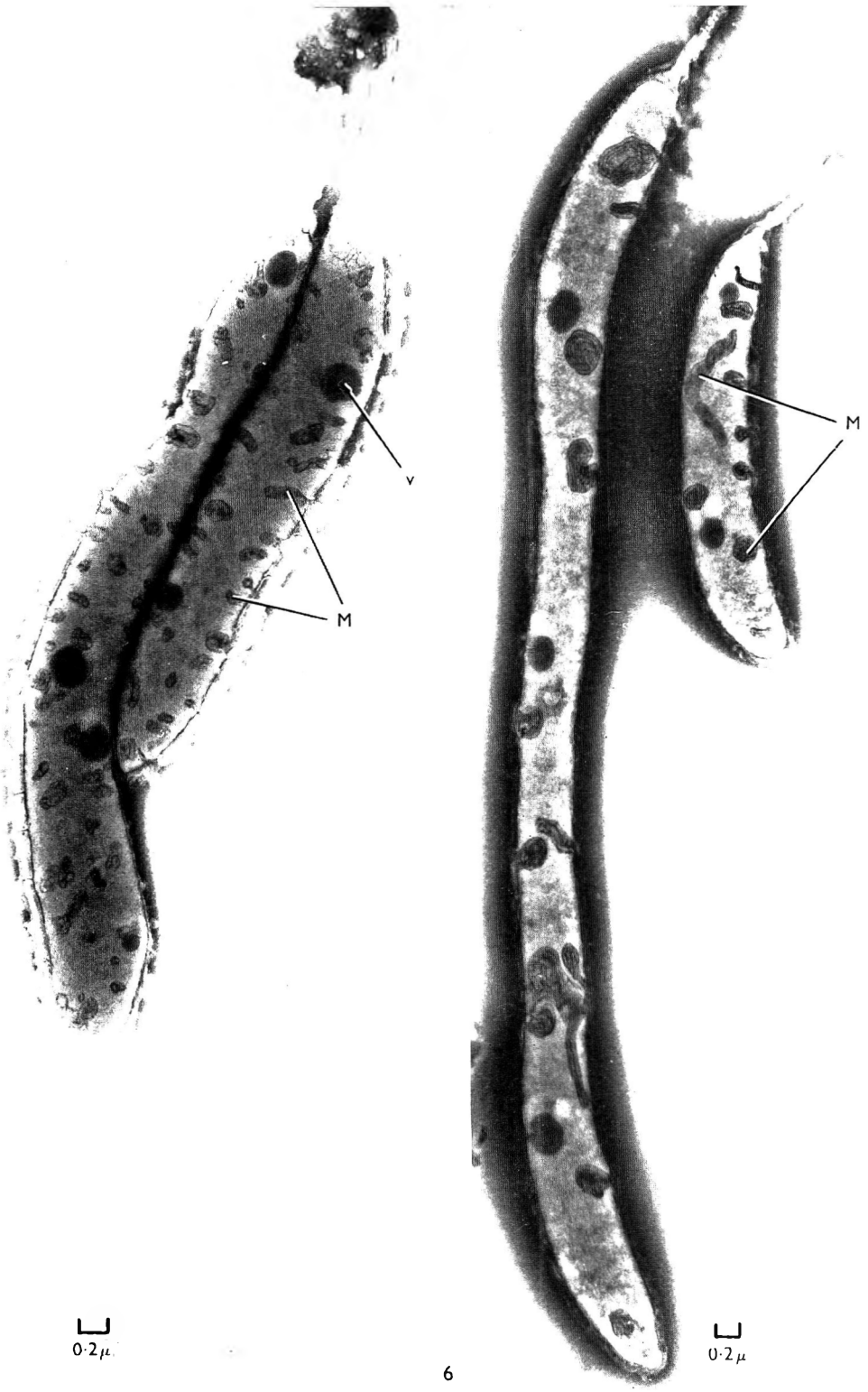

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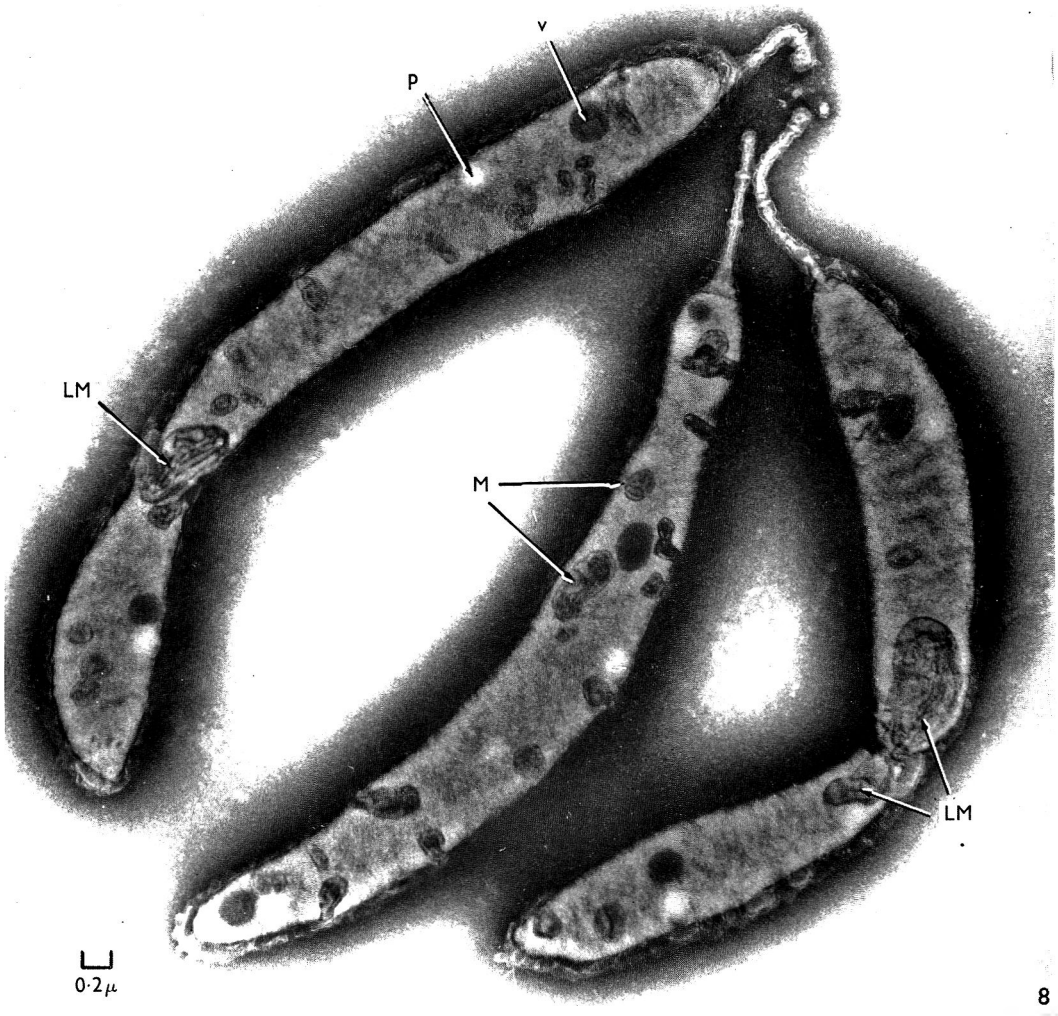

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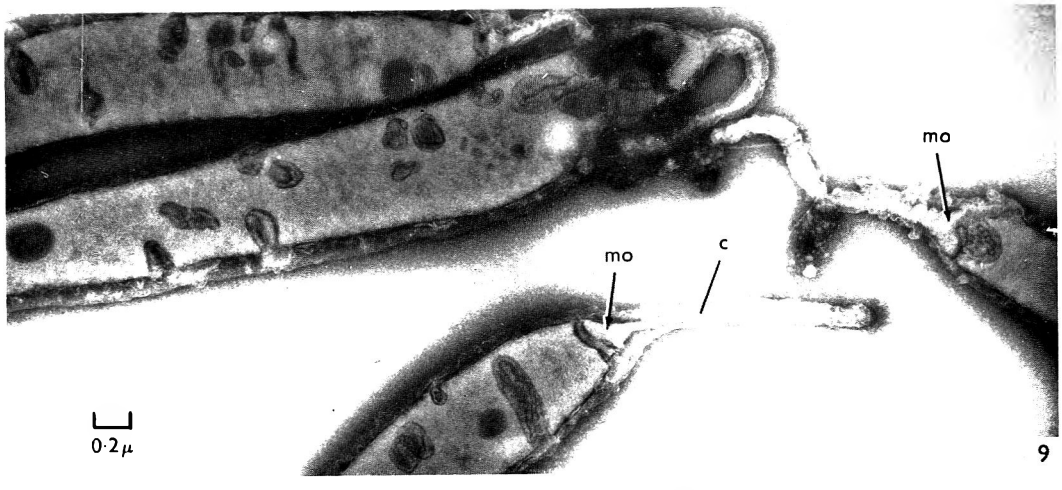

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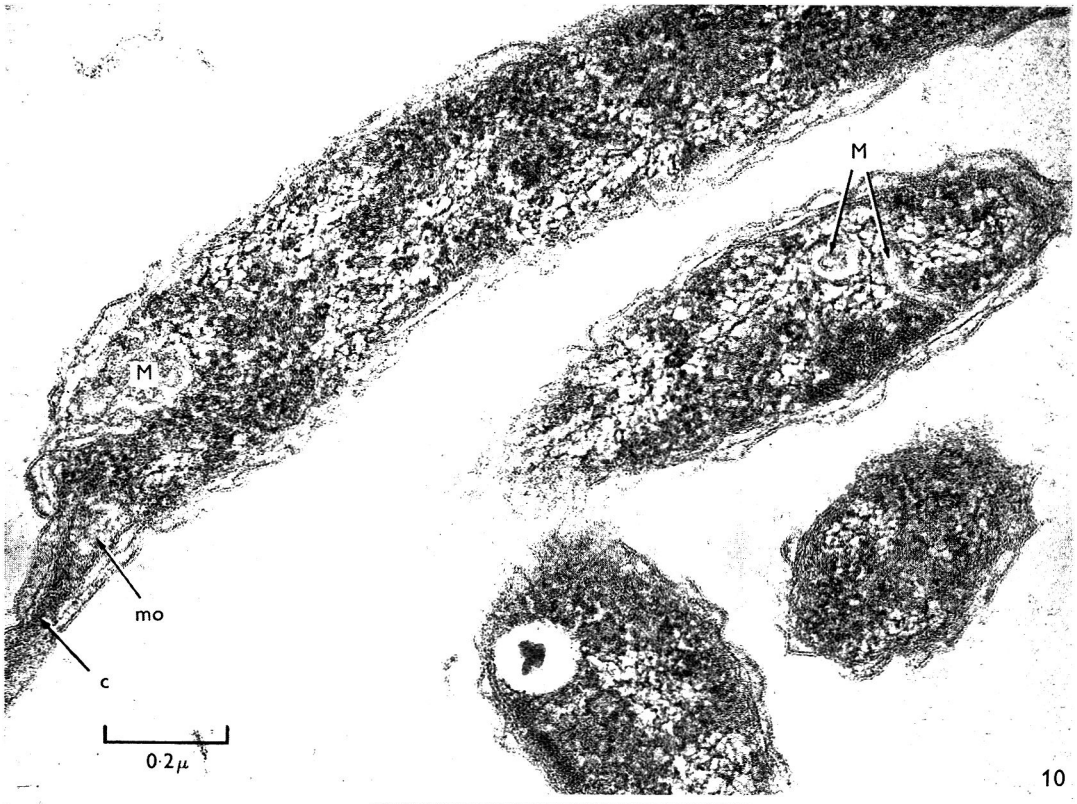

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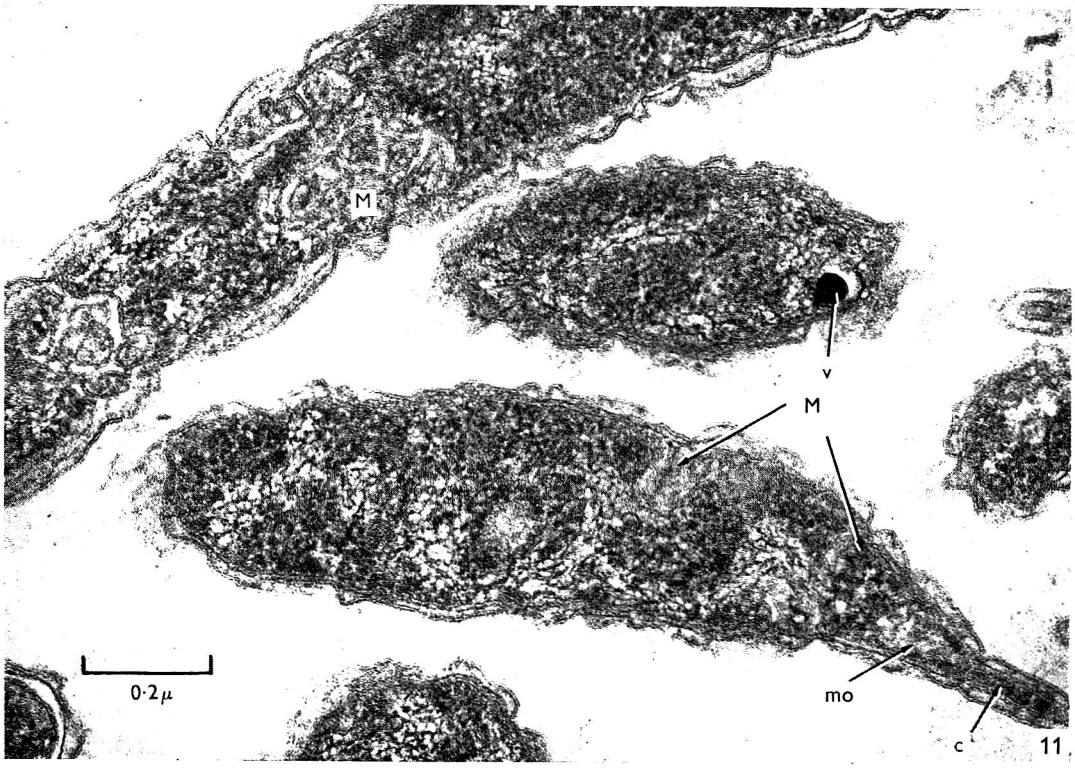

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The Action of Chloroform-killed Suspensions of Enteropathogenic *Escherichia coli* on Ligated Rabbit-gut Segments

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SUMMARY

Suspensions of enteropathogenic *Escherichia coli* and organisms of the same O-antigen group were tested for ability to cause dilatation of the ligated gut segment of rabbit small intestine. Suspensions treated with penicillin or by disintegration in a Mickle shaker were unsuccessful, since it was impossible to obtain sterile material. Suspensions killed with toluene did not cause dilatation, but chloroform-killed cultures did so when the living culture also gave a positive reaction, whereas chloroform-killed cultures of negative living strains were also negative. Chloroform-killed positive suspensions lost their gut-dilatation effect on keeping; this loss ran parallel to loss of esterase activity, though the esterase was not responsible for the dilatation effect.

INTRODUCTION

Previous work on the enteropathogenicity of *Escherichia coli* showed that certain serotypes isolated from babies with diarrhoea caused dilatation when living cultures were injected into ligated loops of rabbit small intestine (De, Bhattacharya & Sarkar, 1956). On the other hand, the same serotypes which had been isolated from healthy children, animals or other sources, had no action on rabbit gut segments (Taylor, Wilkins & Payne, 1961). Cell-free filtrates and sterile material obtained by repeated freezing and thawing of positive cultures did not produce any effect on ligated rabbit gut (Taylor, Maltby & Payne, 1958). Nevertheless, we believed that it should be possible to devise a method of killing cultures so that preparations of enteropathogenic *E. coli* would retain their ability to cause a positive rabbit gut reaction. If such material could be prepared it should be possible to investigate some of the pathological processes in diarrhoea; in addition it might be possible to identify enteropathogenic *E. coli* by methods other than classical antigenic analysis. Previous work had shown that cultivation in sterile milk under micro-aerophilic conditions gave the best results. These cultural methods were used in the present investigation.

Various methods of preparing killed suspensions were tried; neither disintegration in a Mickle shaker nor treatment with penicillin were successful, for while cultural tests did not show any living bacteria, positive cultures were obtained from the rabbit gut.

Sterile suspensions of enteropathogenic *Escherichia coli* were obtained by treatment with toluene or chloroform. However, experiments showed that toluene-

killed suspensions did not have any effect on the rabbit gut segment, whereas chloroform-killed suspensions caused dilatation. Therefore the work described in this paper was done with chloroform-killed suspensions.

METHODS

Strains of Escherichia coli. Table 1 gives the source, whether the patients suffered from diarrhoea, and the serotypes of *Escherichia coli* used in this work. Four positive strains known to cause dilatation of ligated rabbit gut segments and four negative strains known to be inactive were used.

Table 1. *Source and serotype of strains of Escherichia coli*

Strain	Serotype	Source
'Positive'* strains		
E65	026.B6.H−	Baby, gastroenteritis
Aberdeen	055.B5.H6	Baby, gastroenteritis
E122/63	0111.B4.H2	Baby, gastroenteritis
D5301	0128.B12.H2	Baby, gastroenteritis
'Negative'* strains		
E28/65	026.B6.H32	Chicken
E686/64	055.B5.H−	Chicken
D2101	0111.B4.H12	Healthy baby
E21/63	0128.B12.H2	Healthy woman

* Positive strains are those known to produce dilatation in rabbit gut when used as live suspensions; negative strains do not.

Strains were maintained at room temperature (about 18°) on Dorset's egg medium in screw-capped bottles. Before use a strain was subcultured twice serially into sterile milk, in screw-capped bottles which were almost filled with fluid; during incubation the cap was screwed down tightly to maintain micro-aerophilic conditions. Cultures were incubated at 37° for about 16 hr and 1 ml. of such a culture of each strain shown in Table 1 was injected into a rabbit gut segment.

Treatment with chloroform. Cultures were killed by the addition of 1/5 volume chloroform; the mixture was shaken mechanically for 1 hr at 37°. The sterility of the mixture was tested on blood agar and MacConkey agar plates, by the inoculation of 1 ml. to 100 ml. nutrient broth, and in addition was demonstrated by the fact that bacteria could not be isolated from the contents of rabbit gut segments 24 hr after injection.

In an early experiment chloroform was removed by blowing sterile air or nitrogen through the mixtures before injecting the material into the ligated gut loops but, probably due to the lability of the enterotoxic material, no dilatation was observed in these cases.

All chloroform-treated preparations were either injected into the ligated gut loops within 30 min. of being taken from the shaker or were kept at 4° for various lengths of time and brought to room temperature just before injection (Table 2).

Estimation of esterase. Immediately before injection into rabbit gut segments, all material was tested for esterase activity as measured by a method adapted from that described by Baillie & Norris (1963). To 2 ml. maleic acid + tris buffer (pH 5·8) was added 0·2 ml. of 1 % α-naphthylacetate in 50 % (v/v) aqueous acetone followed by

one drop of culture material under test. This mixture was incubated for 1 hr at 37° after which 0·2 ml. of a freshly prepared 1 % solution of Brentamine fast Blue B in distilled water was added. The immediate production of a red colour which slowly faded indicated the presence of esterase activity.

Preparation of rabbit ligated gut loops. Copenhagen white male rabbits (1·3–1·5 kg.) from a closed colony were used throughout these experiments. The test materials were injected into separate ligated gut loops as described by Taylor *et al.* (1958). In each rabbit four segments were used, each being separated from other test segments by unused portions of gut. In each rabbit one control segment was inoculated with 1 ml. of living 16-hr milk culture of strain E 65 and a second control segment with strain D 2101; these are known positive and negative strains respectively; the reaction to these cultures determined the suitability of each rabbit used. The additional segments were used for testing the chloroform-treated milk cultures of four positive strains and four negative strains; chloroform-treated sterile milk was used as a control. This control material consisted of sterile milk treated with chloroform by the same method as used for killing cultures, 1 ml. being injected into the ligated gut segment.

Recording of results. Different rabbits produce a different amount of exudate to a given dose of the positive control material tested under identical conditions. To make a comparison between tests done on different rabbits the volume of exudate obtained in loops injected with test material was divided by the volume of exudate in the control positive loop.

At post-mortem examination each segment of ligated gut, both inoculated and uninoculated, was cultured on blood agar and MacConkey agar plates. All uninoculated loops and those inoculated with chloroform-treated material were found to be sterile. Loops injected with living cultures were shown to contain a pure culture of the organisms injected. Occasionally a rabbit was found to have an adventitious small-gut infection with a strain of *Escherichia coli*; the results from such rabbits were discarded.

RESULTS

Injection of chloroform-treated sterile milk into ligated rabbit gut segments was done five times in five different rabbits; a positive result was never obtained, although the expected result was obtained with the control positive strain E 65. Table 2 gives the results of the tests, which show that dilatation only occurred in those loops injected with chloroform-killed suspensions of the four positive strains, those which when living, caused dilatation. Dilatation was never observed with any chloroform-treated suspensions from the four negative strains, i.e. those which did not cause dilatation when alive.

Dilatation was not always observed from chloroform-treated suspensions of the four positive strains after they had been kept at 4° for various periods. Such material from three of the four strains tested when kept for 1 day did not give a reaction. The fourth strain, E 65, continued to give a positive reaction after 2 days, but was negative after 3 and 4 days. The gradual loss of effect of stored chloroform-treated material shown by strain E 65, namely, a change from exudate ratio 1 when tested immediately to 0·5 when tested at 2 days, suggests that the activity is easily lost.

All cultures when tested within 30 min. of chloroform treatment were found to

be esterase-positive, irrespective of whether or not they were capable of causing gut dilatation, so we do not consider that esterase causes gut dilatation. Nevertheless, esterase may be useful as a marker in material known to cause a positive reaction, because the loss of esterase activity appears to run parallel with loss of gut-dilating activity.

Table 2. *Effect of chloroform-killed cultures of Escherichia coli on rabbit gut segments*

Strain	Antigenic structure	Time of chloroform treatment	Volume (ml.) of exudate in test loop (*A*)	Volume (ml.) of exudate in positive control loop (*B*)	Ratio *A*:*B*	Esterase activity
Positive strains						
E65	0·26.B6.H–	< ½ hr	1	1	1	+
		1 day	2	3	0·7	+
		2 day	1	2	0·5	+
		3 day	0	2	.	–
		4 day	0	2	.	–
Aberdeen β	0·55.B5.H6	< ½ hr	8	1	8	+
		1 day	0	1	.	–
		3 day	0	1	.	–
E122/63	0111.B4.H2	< ½ hr	10	1	10	+
		1 day	0	1	.	–
D5301	0128.B12.H2	< ½ hr	20	1	20	+
		1 day	0	1	.	–
Negative strains						
E28/65	026.B6.H32	< ½ hr	0	2	.	+
E686/64	055.B5.H–	< ½ hr	0	2	.	+
D2101	0111.B4.H12	< ½ hr	0	2	.	+
		1 day	0	3	.	–
		3 day	0	3	.	–
		4 day	0	3	.	–
E21/63	0128.B12.H2	< ½ hr	0	1	.	+
		1 day	0	1	.	–

+ = esterase present; – = esterase absent.

The lability of the gut-dilating factors was confirmed by the fact that positive material treated by bubbling air or nitrogen through it for 10 min. was then inactive.

Positive material, frozen rapidly by surrounding the container and keeping in crushed solid CO_2, when brought to room temperature after 1 week and injected into a rabbit gut segment, gave a positive result. However, when similar material was freeze-dried and reconstituted a week later it was inactive.

DISCUSSION

The results show that from the four strains of *Escherichia coli* isolated from cases of infantile diarrhoea a material could be obtained which produced an effect previously associated with living cultures of these organisms. Also, this active material was labile and its effect lost on keeping and by freeze-drying or bubbling with oxygen or nitrogen. This active material was absent from the four strains with

similar somatic structures which were isolated from sources other than cases of infantile diarrhoea. Also these non-active strains when treated with chloroform did not in any instance cause gut dilatation. Thus it is considered that the effects observed with the four strains from infantile diarrhoea were due to this active material and not due to the chloroform or due to the endotoxin which is related to the known O antigen. Each enterotoxic strain was controlled by a non-enteropathogenic strain belonging to the same O-antigen group.

This gut-dilating activity of some strains was either more easily lost on keeping or produced in different amounts. This latter view is questionable since, if the amount of active material present is proportional to the volume of exudate produced, then strain D 5301 would appear to be the most active. On the other hand, the keeping qualities of material from strain D 5301 do not compare with those of strain E 65, for the activity of the former was lost in 24 hr, whereas the latter remained active for periods up to 2 days (Table 2). In those suspensions where this active material was present it was also shown that, simultaneously with the loss of this gut-dilating activity, there was loss of esterase activity. It is considered that whatever is responsible for the destruction of the one activity is also responsible for the destruction of the other.

The finding that *Escherichia coli* strains of similar serotype will either possess this gut-dilating activity or not, depending on whether they originated from cases of infantile diarrhoea or not, seems to clarify the frequent observation that serotypes known to be associated with infantile diarrhoea are also found in healthy babies. It appears therefore that the enteropathogenicity is dependent on the possession by the strain of *E. coli* of this active material. Having found a method for obtaining rabbit gut-dilating active material it is considered that the way is open to investigate the nature of this material and its relation to the ability of the strain to cause infantile diarrhoea, and thus further to devise simpler methods for the identification of enteropathogenic *E. coli*.

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With 1 *plate*
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Methicillin-Resistant Staphylococci: Genetics of the Minority Population

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SUMMARY

A methicillin-resistant strain of *Staphylococcus aureus* (no. 5982) contained cocci with differing intrinsic resistances to methicillin. Most of the population was slightly more resistant than a typical methicillin-sensitive staphylococcal isolate, but a few were mutants with ability to grow at markedly increased concentrations of methicillin (500 μg./ml. or more). Penicillinase production was not essential for methicillin resistance since a penicillinase-negative variant of strain 5982 was able to produce penicillinase-negative strains with high degrees of methicillin resistance. Methicillin-sensitive strains also gave rise to mutants with increased intrinsic resistance to methicillin, but, in the mutants from methicillin-resistant strains, the increase in resistance was much greater. The mutants were characteristically slower growing than the wild type. Many were small colony variants which reverted to the parent type when passed in liquid media. With 5% NaCl-methicillin plates strain 5982 exhibited a phenotypic increase in resistance. On such plates a few L-type colonies were produced at high concentrations of methicillin.

INTRODUCTION

Methicillin is effective against penicillinase-producing staphylococci because of its relative resistance to penicillin-β-lactamase, the staphylococcal enzyme which inactivates benzylpenicillin. Although at high concentrations methicillin is inactivated at a rate about one-thirtieth that of benzylpenicillin, the extremely poor affinity of the enzyme for methicillin (Novick, 1962; Richmond, 1963) probably results in negligible inactivation under clinical conditions. A few naturally occurring methicillin-resistant staphylococci have been isolated, as first reported by Jevons (1961) and on occasion have given rise to severe illness (Stewart & Holt, 1963). Such isolates have been reported to be penicillinase producers; hence they were capable of some degree of methicillin inactivation. However, the findings of Sutherland & Rolinson (1964) indicated that methicillin-resistant staphylococci, which had been isolated by several laboratories in England and in Europe, consisted of a majority population with a comparatively slight increase in methicillin resistance, and a minority population with a much greater increase in resistance. In the work described here the characteristics of the minority population were further investigated and compared with methicillin-sensitive strains.

METHODS

Media. Trypticase soy agar plates (TSA, Baltimore Biological Laboratories) containing methicillin were prepared by adding various amounts of methicillin to liquid TS agar cooled to 45°. Liquid cultures were made in trypticase soy (TS) broth.

Organisms. The methicillin-resistant *Staphylococcus aureus* (no. 5982), was originally isolated by Eriksen & Erichsen (1963) and was kindly supplied by Drs Kagan and Martin. The methicillin-sensitive strains were the penicillin-sensitive Oxford staphylococcus (209 P) and a penicillin-resistant clinical isolate, no. 97990. Stock cultures were kept by monthly passage of an isolated colony on TS agar. Suspected mutants were streaked on TS agar from isolated colonies for at least three successive transfers before testing.

Inoculation of plates. Overnight broth cultures were diluted in TS broth and streaked linearly in 0·05 ml. amounts on methicillin plates, 4 dilutions/plate. The plates were read daily for 2 days. When hypertonic media were used or when small colony variants were being studied, incubation was continued for 6 days. The methicillin-resistance of the small colony variants was studied by fishing an isolated colony into 2 ml. of TS broth which was considered as the undiluted inoculum.

RESULTS

The minority population consisted of organisms which grew at concentrations of methicillin which inhibited the majority (Table 1). After incubation for 1 day only a few of the minority population were visible on methicillin plates, but by 2 days the number of colonies was of the same order as that obtained after more prolonged

Table 1. *Colony counts of a methicillin-sensitive strain of Staphylococcus aureus, no. 5982, on methicillin plates*

Dilutions of an overnight culture were inoculated in 0·05 ml. amounts on methicillin TS agar plates and incubated for 2 days. + = > 100 but discrete, + + = too numerous to count. + + + = confluent growth.

Methicillin (μg./ml.)	Dilution of culture 10^{-6}	10^{-4}	10^{-2}	Undiluted
	colony count			
0	62	+ +	+ + +	+ + +
5	60	+ +	+ + +	+ + +
12·5	0	3	+	+ + +
25	0	1	+	+ + +
50	0	1	39	+ + +
125	0	0	15	+ +
250	0	0	9	+
500	0	0	2	+

incubation. Except for an occasional small colony isolated from a barely inhibitory concentration, colonies isolated from the minority population proved to be mutants with increased intrinsic resistance to methicillin. In colonial appearance the parent strain was a typical *Staphylococcus aureus*. The mutants, however, varied in colour from off-white to a deeply pigmented yellow-orange when plated either on methicillin agar or on TS agar.

Considerable variation in the number of colonies isolated on the lower concentrations of methicillin was found in successive experiments. At methicillin 5 μg./ml. the results were especially variable. In some experiments the number of colonies was the same as on TS agar while in others there were 10^4-fold decreases in count. Hence, it is not possible to give a precise estimate of the number of mutants from the data in Table 1. Experiments done with ten-fold bacterial dilutions indicated that the colony count was not always proportional to the dilution factor. Inoculation of several plates at the same dilution indicated that the number of plates incubated in a stack, the place of the stack in the incubator, and the number of plates in the incubator influenced the colony count. Similar variability in quantitative results was found with *Escherichia coli* and ampicillin by Seligman & Hewitt (1965).

Isolates from barely inhibitory concentrations of methicillin exhibited degrees of increased resistance, varying from 12·5 μg./ml. to greater than 500 μg./ml. In Table 2 the resistance pattern of a mutant (no. 5982/M 10) isolated from a 500 μg./ml., plate illustrates that the number of colony-forming particles able to grow on 500 μg. plates was substantially greater than with the parent strain. A spontaneously occurring back mutant (no. 5982/M 10/399) was of particular interest, since it grew as a confluent streak on a 500 μg. plate when an undiluted overnight culture was inoculated, but the majority of its population was inhibited by 12·5 to 25 μg.; and at 50 to 500 μg. only a few colonies were produced at a 10^{-2} dilution (Table 2).

Table 2. *Minority populations mutants plated on methicillin plates*

Strain 5982/M 10 was isolated from a methicillin 500 μg./ml. plate. Strain 5982/M 10/399 was isolated from 5982/M 10 on TS agar. Method and terminology as in Table 1.

	5982/M 10				5982/M 10/399			
	Dilution				Dilution			
Methicillin (μg./ml.)	10^{-6}	10^{-4}	10^{-2}	Undiluted	10^{-6}	10^{-4}	10^{-2}	Undiluted
	Colonies							
0	39	++	+++	+++	34	++	+++	−++
5	38	++	+++	+++	24	++	+++	+++
12·5	37	++	+++	+++	0	19	+++	+++
25	37	++	+++	+++	0	1	+++	+++
50	12	++	+++	+++	0	0	18	+++
125	12	++	+++	+++	0	0	7	+++
250	17	++	+++	+++	0	0	5	+++
500	10	++	+++	+++	0	0	0	+++

Such a marked inoculum effect with confluent growth of a concentrated inoculum is characteristic of penicillinase activity; however, significant inactivation of methicillin could not be demonstrated by iodometric titration (Perret, 1954) or by a modified Gots test (Gots, 1945).

After numerous serial transfers of isolated colonies on TS agar strain 5982 lost its ability to form penicillinase as judged by a negative Gots test, a negative Novick test (Novick, 1963; Novick & Richmond, 1965), and lack of ability to grow as a confluent streak of concentrated inocula on concentrations of penicillin

which were inhibitory to the majority. Comparison with a penicillinase-positive variant of an earlier passage revealed that both strains had a similar pattern of methicillin resistance, except that growth of the penicillinase-negative variant was frequently less on the 5 μg. plates. Isolates of the penicillinase-negative variant from 500 μg. plates proved to be mutants with large increases in methicillin resistance, comparable to their penicillinase-producing counterparts. Both the penicillinase-positive and -negative variants were resistant to streptomycin and tetracycline but were sensitive to erythromycin, kanamycin or chloramphenicol. Additional studies on a series of such penicillinase-negative, methicillin-resistant mutants will be reported elsewhere.

Four methicillin-resistant penicillinase-positive mutants were tested after serial passage in antibiotic-free media. The three highly resistant strains reverted to a population with a resistance pattern similar to that of the parent strain. The fourth, in which the increased resistance was of relatively low degree, maintained its resistance after fourteen serial passages. (Seligman, 1966)

Table 3. *Methicillin-sensitive staphylococci on methicillin plates*

Strain 209 P is penicillinase-negative. Strain 97990 is penicillinase-positive. Method and terminology as in Table 1.

	209 P				97990			
	Dilution				Dilution			
Methicillin	10^{-6}	10^{-4}	10^{-2}	Undiluted	10^{-6}	10^{-4}	10^{-2}	Undiluted
(μg./ml.)	Colonies							
0	84	+ +	+ + +	+ + +	61	+ +	+ + +	+ + +
1	1	2	+ +	+ + +	49	+ +	+ + +	+ + +
2	0	0	50	+ +	17	+ +	+ + +	+ + +
3	0	0	13	+ +	0	0	9	+ +
4	0	0	5	+ +	0	0	0	14
5	0	0	0	0	0	0	0	1

Step-wise resistance

Mutations to increased resistance to the penicillins usually consist of relatively small increments in step-wise resistance in both Gram-positive (Demerec, 1945; Hotchkiss, 1951) and Gram-negative organisms (Roantree & Steward, 1965; Seligman & Hewitt, 1965; Banič, 1959). However, the relative increment in resistance from 5 μg./ml. to greater than 500 μg./ml. in many of the methicillin-resistant mutants was much greater than that found with mutations to the other penicillins. Consequently, such mutations to methicillin resistance resembled the one-step mutations to high degrees of resistance which are characteristic of many streptomycin-resistant mutants. In contrast, the penicillinase-negative Oxford staphylococcus (209 P) and a typical penicillinase-producing clinical isolate contained majority populations which were inhibited by methicillin 2 and 3 μg./ml., respectively, and minority populations almost completely inhibited by methicillin 5 μg./ml. (Table 3). These strains also produced mutants which, after at least 3 and 5 steps respectively, consisted predominantly of populations resistant to methicillin 25 and 10 μg./ml.

Small colony variants

Small colonies appeared on methicillin plates after incubation for 1 to 4 days but subculture and further observation revealed that they had diverse genetic backgrounds. Some, particularly in crowded areas at borderline inhibitory concentrations, proved to be phenotypic variants of the parent strain. Others grew to larger size on more prolonged incubation and were mutants with increased methicillin resistance. On TS agar they had a slower growth rate than the parent strain. In the third group, especially those which first became visible after incubation for 3–4 days on methicillin plates, the final size of a colony on TS agar was distinctly smaller than the parent strain. These small colony mutants are comparable to the small colony variants described after exposure to benzylpenicillin (Youmans, Williston & Simon, 1945). On TS agar their colonies were regular and the morphology by Gram stain was normal. Subinhibitory concentrations of methicillin were sometimes associated with abnormally swollen, variably sized, cocci (Barber, 1964).

The small colony variants described here were almost always more resistant to methicillin than was the parent strain. Some grew in undiminished numbers in methicillin up to 500 μg./ml., the highest concentrations at which most strains were tested. In the more heavily streaked areas of TS agar plates, larger colonies were seen occasionally and when isolated usually proved to be back mutants giving colonies larger than their small-colony variant (Fig. 1). Continued incubation resulted in an increase in the number of larger colonies, particularly in the less heavily inoculated areas; this finding is attributable in part to intracolonial mutation (Fig. 2). Frequently, the back mutants had also regained the pigmentation and methicillin sensitivity of the parent strain, but some retained the altered pigmentation and sometimes the approximate methicillin resistance of their small colony variant. Small colony variants have been maintained by passage of isolated colonies on TS agar for more than 25 consecutive streakings. When grown in broth, however, the faster-growing back mutants became the predominant organisms. Such overgrowth was sometimes significant after one transfer and, together with phenotypic variation and intracolonial mutation, accounts for previous difficulties in estimating antibiotic resistance in the small colony variants (Youmans *et al.* 1945).

Resistance in hypertonic media and L*-type growth*

Growth in media containing 5% (w/v) NaCl has been reported to increase the resistance of methicillin-resistant staphylococci (Barber, 1964). In the present work experiments with methicillin plates containing 5% (w/v) NaCl usually revealed growth of a majority of the parent population on methicillin 50 μg./ml. plates, roughly a tenfold increase. Isolates from such plates did not exhibit increased methicillin resistance, thereby confirming the phenotypic nature of increased resistance of the majority population on hypertonic media. By contrast, growth of the minority population was inhibited on hypertonic methicillin plates. Small numbers of L-type colonies were isolated on methicillin + NaCl + TS agar plates, especially at methicillin 500–1000 μg./ml. Such colonies were similar to the ones previously described from methicillin-resistant staphylococci (Kagan, Martin & Stewart, 1964) and could be propagated on methicillin + NaCl + TS agar plates. Added animal serum was not essential for the isolation or propagation of L-type growth.

DISCUSSION

The mechanism of methicillin resistance has been a controversial subject. Several workers have shown that methicillin-resistant strains can inactivate methicillin to a greater degree than can methicillin-sensitive strains (Eriksen & Erichsen, 1964*b*; Gravenkemper, Brodie & Kirby, 1965). However, in these experiments the concentrations of methicillin were usually such that greater growth of the methicillin-resistant strains was permitted. Disproportionate increase in penicillinase production might account for increased inactivation of methicillin. Under appropriate conditions (Ayliffe & Barber, 1963; Knox & Smith, 1963), comparable amounts of methicillin were destroyed by both methicillin-sensitive and methicillin-resistant strains. Furthermore, it is clear that the degree of inactivation of methicillin could not account for the increase in methicillin resistance observed in the highly resistant mutants. The penicillinase-negative variant which maintained the ability to produce highly resistant mutants indicates that penicillinase production did not contribute significantly to such methicillin resistance. Eriksen & Erichsen (1964*a*), however, encountered a penicillinase-negative variant of a methicillin-resistant strain which, in their studies, showed a decrease in methicillin resistance. Penicillinase-negative variants in methicillin-sensitive strains, and presumably in the current methicillin-resistant strain, result from the loss of an extra-chromosomal element or plasmid (Novick, 1963).

A characteristic of significant penicillinase production is the ability of surface colonies from heavy inocula to grow as confluent streaks at concentrations of penicillin considerably greater than that which inhibited the production of isolated colonies. As already mentioned, a mutant was isolated which showed such an inoculum effect on methicillin plates but in which a significant degree of methicillin inactivation was not found. The explanation of such an inoculum effect remains conjectural; it might result from intercellular utilization of enzymes involved with cell wall synthesis. Barber (1964) observed results consistent with an inoculum effect in methicillin-resistant staphylococci. However, our studies, in confirmation of those of Sutherland & Rolinson (1964), indicate that most resistant mutants are able to grow as isolated colonies on methicillin plates. Although it is difficult to give a precise estimate of mutant frequency (see above), in both methicillin-sensitive and methicillin-resistant strains, approximately one organism in 10^4 is a mutant. With similar techniques the order of magnitude of the frequency of resistant mutants of *Escherichia coli* to ampicillin (Seligman & Hewitt, 1965) was found to be similar to that found in the present work for methicillin resistance in staphylococci. Therefore, the methicillin-resistant staphylococci do not appear to be characterized by an excessive rate of mutation for this marker. The majority of organisms in a population of the methicillin-resistant strain is more resistant than the majority in the sensitive strain, but this difference is relatively slight. Accordingly, the distinguishing property of the resistant strain is its capacity to mutate to greatly increased methicillin resistance.

Step-wise increase in resistance to penicillin was found by Banič (1959) to be polygenic. It has not been determined whether more than one locus is capable of mutating to the currently described large-step increase in methicillin resistance. The alterations in biosynthetic processes present in the naturally-occurring resistant

strains which are associated with the modest increase in resistance may be those which permit mutation to greatly increased methicillin resistance. Study of such altered metabolic pathways may reveal additional evidence of the biochemical defect resulting from the action of penicillin.

The plethora of variants with diminished colonial size indicates that the metabolic pathways associated with methicillin resistance usually result in a slower growth rate in antibiotic-free media. The term G colony (Hadley, Delves & Klimek, 1931), sometimes used in association with small colony variants, does not seem appropriate since it was originally used to describe small colony variants thought to reproduce by gonidia.

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EXPLANATION OF PLATE

Fig. 1. Staphylococcal small-colony variants streaked on TS agar and incubated for 4 days. Larger colonies in crowded areas are back mutants with increased colonial size. In the sparser areas the disproportionately large colonies frequently contained intracolonial mutants.

Fig. 2. Staphylococcal intracolonial mutant developing in an isolated small-colony variant. The drawing illustrates the change which had occurred since the previous day's incubation. The small-colony variant was distinctly more pigmented than the faster-growing back mutant.

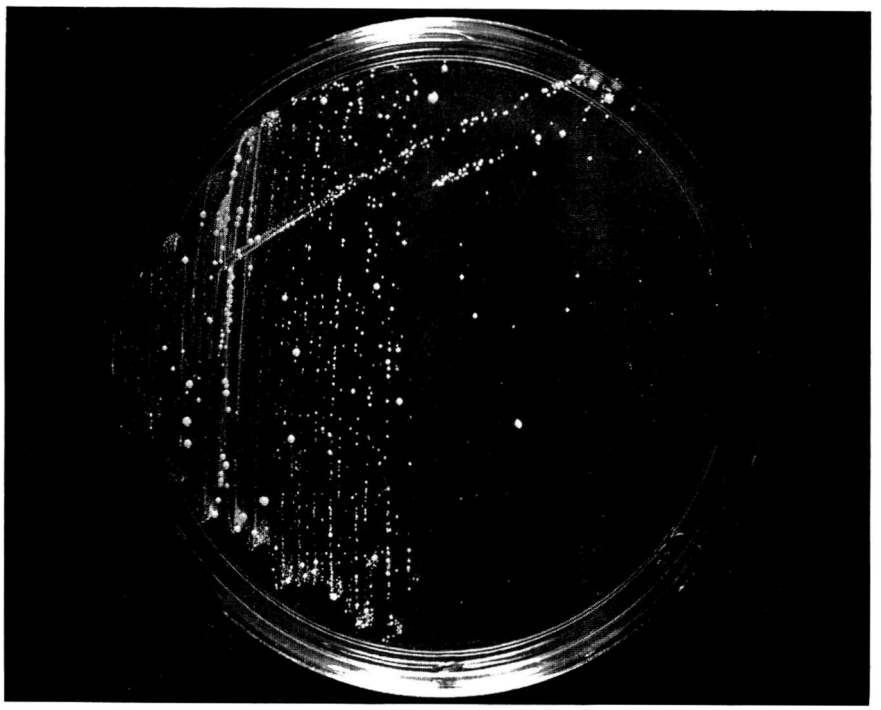

Fig. 1

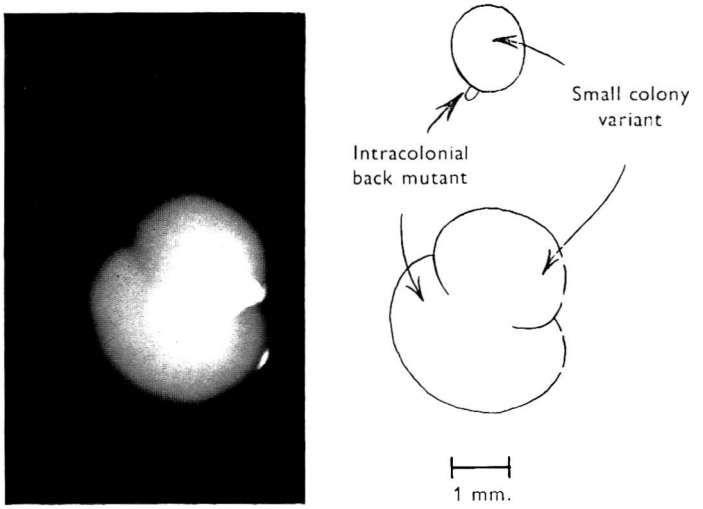

Fig. 2