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THE JOURNAL OF GENERAL MICROBIOLOGY

The *Journal* will publish accounts of original research in general microbiology, i.e. the study of bacteria, microfungi, microscopic algae, protozoa, and viruses in their biological activities and, more particularly, the fundamental aspects of the study of these forms, including structure, development, physiology, genetics, cytology, systematics and ecology. Writers of papers on a specialized aspect of their subject should describe their work so that its relevance to their own science and to microbiology in general will be apparent to readers who may be unfamiliar with the particular aspect.

THE PREPARATION OF PAPERS

'Easy writing's curst hard reading.'—*Richard Brinsley Sheridan.*

'Easy reading's curst hard writing.'—*The Editors, J. gen. Microbiol.*

The Editors wish to emphasize ways in which contributors can help to avoid delays in publication.

(1) Papers must be written in English with the utmost conciseness consistent with clarity. The best English for the purpose of the *Journal* is that which gives the sense in the fewest short words.

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

(3) Authors should state the objects they had in view when the work was undertaken, the means by which they carried it out and the conclusions they draw. A section labelled 'Discussion' should be strictly limited to discussing, if this be necessary, and not to recapitulating. Many papers when first sent to the *Journal* are too long for the crucial information they contain. It is unnecessary to describe preliminary or abortive experiments.

(4) Figures and tables should be selected to illustrate the points made, to summarize, or to record important quantitative results. Well-designed tables or graphs should need little explanatory letterpress. Photographs or drawings should not be submitted unless they illustrate facts that cannot be conveniently described in the text.

(5) Authors should remember that in preparing their typescript they are giving instructions to the printer (about layout, etc.), as well as attempting to convey their meaning to their readers. The latter object will be the better attained the more carefully authors consider how their typescripts will be converted to the printed page. Ink corrections on a typescript greatly prolong the type-setter's work; the final version of a paper must if necessary be retyped to provide a clean copy for the printer. Typescripts which do not conform to the conventions of the *Journal* will be returned to authors for revision.

(6) Special attention should be given to the details below in 'Directions to Contributors'. Strict observance of these requirements will help to shorten the interval between the receipt of a paper and its publication. Where relevant the 'Suggestions to Authors, Symbols and Abbreviations and Notes on Usage and Conventions' published in the *Biochemical Journal* (1957), **66**, 1–16 should be followed. The pamphlet, *General Notes on the Preparation of Scientific Papers*, published by the Royal Society, Burlington House, Piccadilly, London, W. 1 (2*s.* 6*d.*; post free, 2*s.* 10*d.*) will be found useful.

Editors do not alter authors' typescripts except to increase clarity and conciseness, or to bring them into line with the *Journal*'s conventions. If an editorial alteration changes an author's meaning one implication is that it was expressed ambiguously. When an editor can grasp the meaning of a sentence unequivocally it may be assumed that anyone can.

DIRECTIONS TO CONTRIBUTORS

Communications. Papers submitted for publication should be sent to A. F. B. Standfast (*The Journal of General Microbiology*), Lister Institute of Preventive Medicine, Elstree, Hertfordshire, England. Communications about offprints should be addressed to The University Press, Cambridge.

General. Submission of a paper to the Editors will be held to imply that it reports unpublished work, that it is not under consideration for publication elsewhere, and that if accepted for the *Journal* it will not be published again in the same form, either in English or in any other language, without the consent of the Editors.

Form of papers Submitted for Publication. The onus of preparing a paper in a form suitable for sending to press lies in the first place with the author. Authors should consult a current issue in order to make themselves familiar with the *Journal*'s typographical and other conventions, use of cross-headings, layout of tables, etc.

Papers should be headed with the title of the paper, the names of the authors (male authors use initials, female authors use one given name in full) and the name and address of the laboratory where the work was performed.

A paper should be submitted in double-spaced typing (top copy) with a 1½ in. left-hand margin, and on paper suitable for ink corrections. The paper should in general be divided into the following parts in the order indicated: (*a*) Summary: brief and self-contained; (*b*)

Introduction; (*c*) Methods; (*d*) Results (illustrative protocols only should be included); (*e*) Discussion (if any), and general conclusions; (*f*) Acknowledgements; (*g*) References.

The position of Tables and Figures should be indicated in the typescript.

Typescripts should carry the name and address of the person to whom proofs are to be sent, and a shortened version of the paper's title, not exceeding forty-five letters and spaces in length, suitable for a running title in the published pages of the work.

References. References in the text are cited thus: Brewer & Stewer (1942), (Brewer & Stewer, 1942). Where a paper to be cited has more than two authors, the names of all the authors should be given when reference is first made in the text, e.g. (Brewer, Stewer & Gurney, 1944), and subsequently as (Brewer *et al.* 1944); but papers with more than four authors may be cited, e.g. (Cobley *et al.* 1940) in the first instance. Where more than one paper by the same author(s) has appeared in one year the references should be distinguished in the text and the bibliography by the letters *a*, *b*, etc. following the citation of the year (e.g. 1914*a*, 1914*b*, or 1914*a*, *b*).

References at the end of the paper should be given in alphabetical order according to the name of the first author of each publication, and should include the title of the paper. Titles of journals should be abbreviated in accordance with the *World List of Scientific Periodicals*, 4th edn. (1965). References to books and monographs should include year of publication, title, edition, town of publication and publisher, in that order. It is the *duty of the author to check his references* and see that the correct abbreviations are used.

Illustrations. Illustrations and diagrams should be approximately *twice the size of the finished block*, each on a separate sheet, bearing the author's names, short title of the paper and Plate or Figure numbers on the back. Diagrams should be drawn in indian ink on plain white paper, Bristol board, faintly *blue*-lined paper, or tracing linen (but not plastic tracing linen) with letters, numbers, etc. written lightly in pencil. Lettering should be clear of the diagram and indicate by blue pencilled lines the desired position. Caption and legend should be typed on a sheet separate from the illustration and numbered to correspond. Drawings and photographs should include a statement of magnification. Photographs should be well-contrasted prints on glossy paper, and should be chosen for size and number, bearing in mind layout on the finished Plate; layout should be indicated. Coloured plates must be paid for by the author.

Tables. Tables should carry headings describing their content and be comprehensible without reference to the text. Each table should be typed on a separate sheet and its approximate position in the text indicated on the typescript.

Symbols and Abbreviations. Authors should refer to current issues of *The Journal of General Microbiology* for information in this connection. Attention is particularly drawn to the following points: degrees Centigrade are written, e.g. 100°, not 100°C.; hr, min., sec. (singular and plural); M = molar; m (milli-) = 10^{-3} and μ (micro-) = 10^{-6}; ml. (millilitre) should be used instead of c.c., and μg. (microgram) instead of γ; N = normal (of solutions); No. or no. = number. Ratios should be written 1:10; dilutions, 1/10.

Chemical Formulae. These should be written as far as possible on one line. The chemical nomenclature adopted is that followed by the Chemical Society (*J. chem. Soc.* 1936, p. 1067). With a few exceptions the symbols and abbreviations are those adopted by a committee of the Chemical, Faraday, and Physical Societies in 1937 (see *J. chem. Soc.* 1944, p. 717). Care should be taken to specify exactly whether anhydrous or hydrated compounds were used, i.e. the correct molecular formation should be used, e.g. $CuSO_4$, $CuSO_4.H_2O$ or $CuSO_4.5H_2O$.

Descriptions of Solutions. The concentrations of solutions are preferably defined in terms of normality (N) or molarity (M). The term '%' must be used in correct sense, i.e. g./100 g. of solution. For 'per cent of volume', i.e. ml./100 ml., the term '% (v/v)' should be used, and for weight of a substance in 100 ml. of solution, the term '% (w/v)'.

Proprietary Substances and Materials. At first mention, the correct designation of a manufacturer should be given in the text, with address to enable other workers to obtain the product mentioned.

Nomenclature of Amino Acids and Vitamins. The rules published in the *Biochemical Journal* (1952), **52**, 1–2, should be followed.

Nomenclature and Descriptions of Micro-organisms. Binomial Latin names of micro-organisms, the generic name only with a capital, must be used in accordance with International Rules of Nomenclature; in full at the first mention in each paragraph and in the Summary but in subsequent mention with the generic name abbreviated. Single initial letter abbreviations are used where they are not ambiguous. Binomials should be underlined in the typescript. Scientific epithets or trivial names are not underlined and should be without capitals.

Descriptions of new species of cultivable microbes should not be submitted unless an authentic specimen of a living culture has been deposited in a recognized culture collection

The word 'generation' should not be used synonymously with 'subculture'. For an agreed use of terms like strain, type, variant, phase, etc., see the International Bacteriological Code of Nomenclature, Section 1, Rules 7 and 8.

Except for good reasons, micro-organisms should be designated by the names used in the works listed below. When other authorities are followed, they should be cited whenever obscurity might result from their use.

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

PLANT PATHOGENIC FUNGI AND PLANT DISEASES. *List of Common British Plant Diseases*, 1944. (Cambridge University Press.)

PLANT VIRUSES AND VIRUS DISEASES (1957). *Rev. appl. Mycol.* **35**, Suppl. 1–78.

BACTERIA. Author's preferences in naming are at present accepted provided that the designation is unambiguous and conforms with the International Bacteriological Code of Nomenclature (1949; *J. gen. Microbiol.* **3**, 444) and the Opinions issued by the International Committee on Bacteriological Nomenclature. If desired, a synonym may be added in brackets when a name is first mentioned.

E. G. D. MURRAY

(*Facing p.* 1)

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Obituary Notice

E. G. D. MURRAY, 1890–1964

A stimulating teacher of outstanding ability, a research worker and philosopher of distinction, a linguist of no mean competence, a taxonomist endowed with an unusual share of common sense, above all a microbiologist and a friend of microbiologists; this was Everitt George Dunne Murray, an honorary member of this Society, who died suddenly on 6 July 1964, at his home in London, Ontario. Known to his English students, his friends, and his colleagues as Jo'burg, Murray enjoyed every moment of a full life, for he knew not only how to work hard but how great was the pleasure to be found outside the laboratory, in the company of his family and his friends.

EARLY LIFE IN SOUTH AFRICA

Murray was of the third generation born in South Africa. His great grandparents went there from England early in the nineteenth century and settled near Graafrienet, Cape Colony, where they built a fine homestead called Roodeblom and raised a large family. Their eldest son, Walter, carried on at the farm and became a stockbreeder. Of his five sons, two entered medicine; one died early in his career; the other, Jo'burg's father, George Everitt Murray, studied medicine at Bart's, took his F.R.C.S. and M.R.C.P., then married Kathleen Dunne, a sister at Bart's, and returned to South Africa, where, at Johannesburg, he practised for the rest of his life.

Jo'burg Murray was born there in 1890; one of the first British babies born in that collection of tin and wood houses that so soon became a great and wealthy city. During the Boer War while Johannesburg was in siege, the elder Murray was Surgeon-in-Chief to the British Forces and with his wife ran a large hospital for British casualties. Meanwhile, Jo'burg and his young brother were at Roodeblom safe in the care of the grandparents and it was there that he spent the happiest time of his young life. His independent spirit revelled in the freedom of the enormous farm and the huge areas of surrounding veldt. He had no fear, and, ranging afar on foot or on horseback he came to know the creatures of the veldt and studied their life habits. It was there that he laid the foundations of his deep and lifelong interest in zoology. His grandfather taught him efficiency in everything he tried to do; being a perfectionist himself, grandfather expected perfection and was ready to teach how it could be achieved. Jo'burg often said he owed much to the teachings and precepts of this wonderful man. After the war the boys returned to Johannesburg—and to school. Those were not such happy days, for the schools were grim and Jo'burg never got over his deep and justified dislike of them. But he treasured many good memories of holidays, when he could travel on the veldt with his father, camping and shooting game—or going off by himself on his horse for weeks on end—or swimming—or playing cricket. They had to ride 20 miles to swim, but in spite of this he became a fine swimmer and loved it all his life. Early in his boyhood his maternal grandfather, Captain J. J. Dunne, taught him to fish, to tie his own flies, and to master that most delicate art of fly-fishing. (Grandfather Dunne was a famous fly-fisherman and even today a make of fly-rod is named after him—the

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Hi-Regan; he was the Hi-Regan, being the direct descendant of an ancient Irish king, and he wore the heavy silver thumb-ring which was his ancestors' insigne.) Captain Dunne was well known as a soldier (he fought sundry battles in many parts of the world), explorer, writer and caricaturist, raconteur and wit.

At home Jo'burg met many interesting people, for the Murray home was a hospitable house for innumerable people. Some of the famous men he met in his boyhood continued their friendship in later life, among them General Smuts. Lord Baden-Powell, with whom he became great friends when he was in his early teens, had a standing invitation to tea every Wednesday afternoon and they used to discuss the possibilities of B.P.'s 'great idea' which later became the Boy Scout movement.

In one way and another Jo'burg did a great deal of travelling—on foot and on horseback, by Cape cart and ox-wagon; by train as an ordinary passenger and in his Great-Uncle Richard's private railway carriage (this was Sir Richard Southey, Premier of South Africa for a number of years). Also he made five or six journeys by ship between South Africa and England with his parents before he went to Cambridge. To one like Jo'burg, keenly interested in everything he saw, this was wonderful experience for a growing boy. Throughout his life he was never bored by travel—except, perhaps, on innumerable journeys to Washington in World War II!

TRAINING IN ENGLAND

Murray was about 15 when he and his two younger brothers were sent to Downside School in England. He had full responsibility for them—a heavy burden for one so young. Those school years were, again, not happy ones, but they all did well and, in turn, went to Cambridge. Holidays were spent with relations in England and in Ireland, and Murray was able to fish in wonderful trout waters with his Grandpa Dunne and proved an apt pupil. Then came the years at Cambridge, where he was entirely happy doing work he loved and excelling in intellectual freedom, the lack of which was one of the many things that irked him so much at school. He was a dedicated and indefatigable worker and after classes would spend long hours through the night working at the bench he set up in his sitting-room in Christ's College; one half resembled a laboratory with bench and microscope, and there were rows of shelves for the aquaria and other specimens. He found time for many outside activities—digging for fossils, cricket, swimming and tennis being only some of them—and he made many friends. Among them were some of the famous men who were teaching at Cambridge at that time—Sir Arthur Shipley (Master of Christ's College), Gowland Hopkins, Haddon, Sedgewick, Duckworth, Hardy, Brindley, and these friendships were a delight to him for many years and when he returned to Cambridge. During that period he spent vacations either fishing in Ireland, collecting specimens at various marine biological stations, or at his cousin's house at Oulton, which was only three miles from Blundeston, Suffolk, the home of Winifred Woods, whom he met during his first year at Cambridge. While there, near the east coast, he often went out in fishing trawlers on regular North Sea fishing trips, seeking specimens when the nets were hauled. It was hard living—mostly cold and wet but he loved it. One long vacation he spent on the west coast of Wales with a zoologist friend. They lived in Caldy Island entirely isolated from human contacts, for the only other inhabitants of the

island were a few cloistered monks in a tiny monastery. It was a marvellous place for collectors of zoological specimens.

At that period of his life he was, at first sight, a very serious and thoughtful young man, shy, until one knew him well, but underneath it all was that tremendous sense of fun, wit, and warm friendship which was a part of him all his life. Even as an undergraduate he made something of a name for himself in zoology, and he was asked to join the Scott Expedition to the Antarctic as a zoologist. He cabled his father asking permission to do so and received the answer 'No. Continue your studies' and that was that!

After taking his degree Murray went to Bart's, where he seems to have been very happy with medical training in general, though for 'lunacy' he always had a deep and ineradicable loathing. He had a tremendous admiration for the teachers of that time—especially Archibald Garrod and Frederick Andrewes. He very soon became great friends with Mervyn Gordon and it was through him that he developed his lifelong interest in pathology and bacteriology for, as time went on, he spent more and more hours in the pathology laboratories learning and working with Gordon. He knew, now, what his life's work was to be, and where his interest lay; but when war broke out in 1914 he joined up. But it wasn't long before he was recalled and was sent to the Central Cerebrospinal Fever Laboratories, where he joined Gordon, and they worked together on cerebrospinal fever throughout the epidemic which was raging at the time. Thus, before he was qualified, Murray was doing work that established him as an expert on the meningococcus, and he later wrote the chapter on that organism for the M.R.C. *System of Bacteriology.*

TIME IN THE R.A.M.C.

While doing the cerebrospinal fever work Murray must have worked under difficulties to complete his medical studies, but he qualified as a Licentiate of the Society of Apothecaries in 1916. He was then commissioned in the R.A.M.C. and worked at Millbank under Colonel David Harvey at a time when Sir David Bruce was Commandant of the R.A.M. College. Murray had a deep admiration and affection for Sir David and Lady Bruce. This was another very active and busy period. He did not have much time for recreation but made the most of what opportunities arose and sometimes he would spend a few days in Paris at the Institute Pasteur, or with an old friend, Charles Legalle at Chateau Tuloc. It was there that he laid the foundation of a taste for good wines—he had a fine and precise palate and claimed that he could tell the exact vintage of a good Port or Burgundy. M. Legalle's family lived in London and they were great friends of Murray's from his student days at Bart's. It was thus that he learned to speak French with ease and fluency.

Also at that time (1912–15) Murray met many of the most famous actors and actresses of the day. His mother's sister ('George Egerton', the writer) lived in London and her husband, Golding Bright, was a well-known impresario and it was at their house that he met these people of the theatre—for whom he never had much use. 'Mummers—just mummers' he would say. Nevertheless, he enjoyed the theatre as such—especially musicals, D'Oyly Carte and the music halls—so he was really quite normal in his tastes. He was a member of the Fly Fisher's Club in Piccadilly, where his grandfather Dunne had been well known for many years; and always enjoyed his meetings with fellow-piscators there. During the Second World War the Club roused his ire because they refused to let him use their library.

In 1916 Murray was sent to Mesopotamia to work on dysentery, which was causing enormous casualties in our troops in that theatre of war. He arrived there during the hot season—120–130° F. in the shade—and found that conditions were indeed very bad. Inadequate food, millions of flies, no refrigerators and barely sufficient apparatus to work with. His laboratory was a small canvas tent and he had to keep test tubes of specimens and thermometers in buckets of water so that the one should not cook and the other not burst! Nevertheless, he did a great deal of work there, collected more than a thousand cultures and got a lot of important information on dysentery before he, too, fell victim to the disease. He was seriously ill and nearly died in the hospital at Basra. When he was convalescent he was sent to India and went to Kasauli, where he found congenial friends in the Institute and spent much of his time there while he recovered his strength and health. It was at this time that a certain letter (which made him hopeful) caught up with him and was the prelude to a wild chase halfway round the world that took him to the Devonshire Hospital, Buxton, where Winifred Woods was nursing; the chase seemed fruitless for his proposal was refused. However, Murray never gave up and the chase ended, months later, in their marriage on 17 December 1917 at St Luke's, Kensington. Between the dash from India and his marriage he had been to Johannesburg to see his father and he had been Medical Officer in charge of troopships on both east and west coasts of Africa.

CAMBRIDGE PERIOD

In 1919 Murray was appointed Demonstrator in Pathology at Bart's and in 1920 he became an M.R.C. Research Bacteriologist, at first working in the Field Laboratories in Milton Road, Cambridge. In 1922 H. R. Dean went to Cambridge as Professor of Pathology, and Murray moved to the department in the following year when Dean, R. A. Webb, and he planned the Part II Pathology Tripos, which was first offered as a course in 1924–25. The class was held in the old pathology laboratory on the corner of Corn Exchange and Downing Streets. Graham Smith taught most of the systematic bacteriology but Murray did some, and introduced practical immunology, a revolutionary development in undergraduate teaching. Being a perfectionist in technique he imparted something of his critical ability of his students. The students in that first year were L. Foulds, Miss C. P. Giles, A. A. Miles, F. Smith, Miss M. P. Shackle, E. T. C. Spooner and Colonel Whitmore (of *Bacterium whitmori*, so spelt by Stanton and Fletcher). Murray was made a Fellow of Christ's College in 1923, and became Director of Medical Studies there in 1925; in 1926 he was appointed Lecturer in Pathology. It was during these Cambridge years that Murray, Webb and Swann started their work on *Bacterium monocytogenes* (which later became *Listerella monocytogenes* and finally *Listeria monocytogenes*), an organism that was infecting and killing some of their laboratory rabbits.

Murray left a permanent memorial to his time in Cambridge in the present Pathology Department in Tennis Court Road; during the building he played the big part of unofficial Clerk of Works and was responsible for much of the detailed planning. At the time, the design of the Department was regarded as something outstanding and it was described in the M.R.C. *System of Bacteriology*.

Murray was always at war with pomposity and humbug, and in this war his Cambridge students and friends were his allies, and he made them so. Part of his greatness

as a teacher and administrator lay in his persistent questioning of everything, even things that seemed to others to be unquestionable until he got to work on them. In his Cambridge days Murray had not grown his famous beard and without it his wide open eyes seemed even more penetrating. He would produce the most devastating comment on things and people in a soft, almost drawling voice that rose a semitone or two when he became really emphatic. He used to tell how Graham Smith reacted to the news that he was going to Canada by saying: 'It's going to be a hell of a shock for McGill; they don't know what they've let themselves in for.' He left Cambridge in 1930 and took with him the love and friendship of all his old students. One of them summarized this period in the sentence: 'He had a great capacity for loyalty and for friendship; he was a most lovable person and a wonderful man to have on one's side in a controversy.'

CANADA

Murray was appointed Professor of Bacteriology and Immunology at McGill University, Montreal, in 1930. Before his appointment there had not been any organized teaching of microbiology in the Faculties of Medicine or Arts–Science at McGill. It is true that as early as 1905 a Department of Agricultural Bacteriology had been established by Professor F. C. Harrison at Macdonald College, some twenty miles from the main Montreal campus, but the only teaching of bacteriology to medical students took the form of contributions made by Professor Harrison, the professors of Pathology and Hygiene and members of the staff of the Royal Victoria Hospital. Dr Harrison was appointed Professor of Bacteriology in the Faculty of Medicine in 1929 but within a year was forced to retire owing to ill health, and before time allowed for the organization of a department. When Murray reached McGill he found that he had been appointed the chairman of a department of which he was the sole staff member, in charge of a few small and poorly equipped laboratory rooms which had never been designed for bacteriological work, and he was to have the assistance of one trained technician. With the energy characteristic of the born pioneer and his usual determination and skill he undertook, without delay, the task of creating a new department within the Faculty of Medicine responsible for the teaching of medical students, undergraduate students in science and eventually graduate students in medicine and in the Faculty of Graduate Studies and Research. Before the term started he had organized a full and properly integrated course in bacteriology and immunology for the medical students. In a surprisingly short time he succeeded in creating a service in clinical diagnostic bacteriology and consultation which won the respect of the clinicians in the teaching hospitals in Montreal and which within a few years became the envy of his colleagues throughout Canada. To the credit of the University authorities and particularly the then Principal and Vice-Chancellor, Sir Arthur Currie, despite the fact that the University was suffering the financial deprivations of the depression years, they did not let a new man down and provided Murray with every support and encouragement for the onerous task with which they had charged him.

Murray came to McGill primarily with the reputation of a research man, although he had proven himself, in the planning and supervision of the construction of the Pathology Laboratory at Cambridge, an able builder of physical plant. This experience was to serve him well in building up and equipping his new Department of Bacteriology

at McGill. However, in the early years the task of establishing teaching and clinical diagnostic services involved a very real sacrifice in time and energy that he might otherwise have devoted to a continuing career in research. In 1931 he was fortunate in being able to recruit to his staff one of his former students at Cambridge, Frederick Smith (later to become Dean of Medicine at McGill), and from that time until the death of Dean Smith in 1949 the two shared in the responsibility of building up a department which, within not too many years, came to enjoy an enviable reputation in the fields of teaching, research and clinical diagnostic microbiology. The load was gradually lightened as Murray managed to attract to his staff young, able and enthusiastic recruits. While, up to the early war years, he unselfishly devoted his own energies to the creation and administration of a thriving and rapidly expanding department he tried, at all times, to provide his junior colleagues with the freedom and stimulus for research. He had a small but well-equipped laboratory leading directly from his office where he retired, when time allowed, to take up some much interrupted line of investigation, but the going was slow and the projects were rarely completed because of the day-by-day distractions of teaching, consultation and administration. When beset by some thorny administrative problem he could be found standing in his laboratory gazing longingly at the all too little used benches. Despite the pressure of administrative duties, however, he managed to participate in some, at least, of the research problems which his students and junior staff members had undertaken under his direction. One such project that particularly held his interest in his early years at McGill was a study of staphylococcal toxins, the development of improved methods for the production of staphylococcal toxoids, and for the evaluation of their antigenic, prophylactic and therapeutic efficacy.

Murray was a builder and an investor in talent. Indeed, the story of Murray at McGill is the saga of a university department that became one of the leading centres of microbiology on the North American continent. It was a department of which he was intensely proud and he sometimes fondly referred to it as his 'ship'; there was never any doubt in the minds of his colleagues as to the ability of the captain to hold a firm helm. His pride and enthusiasm were infectious and were shared by his colleagues and those who trained under him so that, wherever their careers might eventually take them, they never ceased to identify themselves with 'The Chief' and McGill. During his twenty-five years at McGill there was not a period during which things remained static in the department. A perfectionist in all things, he was never quite satisfied that things could not be better. The physical facilities were constantly being enlarged, remodelled or rebuilt and re-equipped. The teaching and diagnostic services continued to expand under his direction, and when Murray retired in 1955 the staff had grown to some sixty persons. The department developed along the diverse but related lines of teaching, graduate study and research, and medical diagnostic bacteriology.

Murray felt strongly that courses in microbiology must be designed, at all levels, to meet the specific needs and interests of the students. As a result of this attitude the department soon gained recognition for its invaluable services, not only to the faculty under which it had been instituted, Medicine, but to other faculties and schools within the university. With his personal participation in all courses, instruction was given in the schools of Medicine, Dentistry, Nursing and Arts–Science, with separate and appropriate courses for each. His special pride in undergraduate training lay in the development of a curriculum, leading to an Honours B.Sc. degree in bacteriology and

immunology, which brought the teaching of the subject to a level higher than had hitherto been attempted for undergraduates in North America.

GRADUATE TEACHING AT MCGILL

For some years the lack of space made it impossible for the Department to accept more than the occasional graduate student but immediately after World War II graduate training in microbiology became a major preoccupation of Murray and his teaching staff. The building up of a leading graduate school in microbiology was not the least of his contributions as an organizer. At the time of his retirement there were fifteen graduate students in the Department working towards the M.Sc. and Ph.D. degrees. In the relatively brief period between the end of the war and his retirement some forty-four postgraduate students went from his Department to take up professional positions in university, hospital, government and industrial laboratories in Canada and the United States. Indeed, virtually every university and major teaching hospital in Canada has or has had on its staff at least one Murray graduate. Murray was not one for large, integrated departmental research projects. He encouraged his colleagues to follow their own interests and as a result research in the Department developed along diverse lines relating, at one time or another, to almost every specialized branch of microbiology. New graduate students were encouraged to spend at least a couple of weeks talking with individual staff members about the research projects which they directed, and reading the theses and publications which had arisen from the investigations. They were then permitted to choose the general field of work and thus indirectly to choose their research director; it was rare, indeed, when the problems under investigation did not meet their interest and needs. Although Murray had his own students whom he personally directed, he made a point of taking a day-by-day interest in every graduate student in the Department and the students never failed to appreciate this gesture on the part of their departmental chairman. Upon arrival at his office in the morning he would turn a blind eye to the deep stack of mail which awaited his attention and, starting out while still buttoning his laboratory coat, he would make the Grand Tour. Starting at one end of the long corridor where the students were housed in a series of individual staff laboratories, he would stop for a while to chat informally with each student about his progress, his problems and to offer encouragement and advice from a vast storehouse of knowledge which was always a source of wonder and stimulus to his audience. He demanded from his students the highest standards of performance and refused to tolerate carelessly planned experiments or 'sloppy' thinking. Never hesitant to express positive and forceful criticism when it was needed, this sometimes couched in rather startling phraseology, he never withheld praise when it was merited. Murray was a raconteur without equal and in lectures and informal discussions with students he frequently spiced his dissertations with witty anecdotes relating to the great men of microbiology.

The post-war years were rewarding ones for Murray. The research activities of his Department and the output of worthwhile publications rapidly and steadily increased from year to year, and, as a result of his untiring administrative efforts of the earlier days, the Department ran as the efficiently designed machine he had made it, and he had time to turn more and more to his own research interests and writing. His research publications in co-operation with his students and colleagues ranged over a variety of

subjects in the fields of medical bacteriology, immunology, antibiotic therapy, etc. In his later years he gained particular recognition for his writings and lectures in the speculative and philosophical realms of science.

Because of his administrative abilities, his great energy and wide interests Murray served the University in many capacities. He was a member of the Faculties of Medicine, Arts–Science and Graduate Studies, for some years an elected member of the Senate from the Faculty of Medicine and served during the last six years of his tenure of office at McGill as one of the two University representatives on the Montreal City Council. Murray had the reputation of a man 'to be reckoned with' at Senate, Faculty or Committee meetings. He possessed a thorough working knowledge of the status of the University, of procedural methods and was a master in debate. He was never loath to object strenuously to the decisions of duly constituted authority when he felt them to be wrong. Two of his very close friends at McGill, T. H. Mathews, the then Registrar, and the late D. L. Thomson, Dean of the Faculty of Graduate Studies and Research, writing in the *McGill News* in 1954, described him thus: '...he is the best type of "no-man" for his instinctive opposition to most things (anything, in fact, which was not done in his day at Cambridge) is combined with a sense of humour and a trenchant flow of strong words devoid of rancour. Nothing brings a brighter light to his eye, a more electric crackle to his whiskers than the opening rounds of a promising argument with no holds barred and no insults excluded; but it is only fair to add that if there is one thing he enjoys more than delivering a bludgeon-stroke, it is receiving one that meets his high standards.'

In addition to his various academic posts, Murray actively served McGill's teaching hospitals. Until 1955 he was Bacteriologist-in-Chief of the Royal Victoria Hospital including the closely affiliated Montreal Maternity Hospital and the Montreal Neurological Institute, and an Honorary Consultant to the Royal Victoria, Montreal General, Children's Memorial, Jewish General, and Royal Edward Laurentian Hospitals. He was also Honorary Consultant to the Victorian Order of Nurses for Canada and a member of the Board of Governors of the Alexandra Hospital.

AN INTERNATIONAL FIGURE

Beyond the confines of McGill, Murray's talents were much in demand in national and international endeavours concerned with both microbiology and medicine. Recognition by his colleagues in microbiology for his contributions in the field of bacterial taxonomy, particularly for his studies of the meningococcus, of Shigella and *Listeria monocytogenes*, led to his appointment in his second year at McGill to the Committee of the Society of American Bacteriologists which had been formed to assist Professor D. H. Bergey in the preparation of his *Manual of Determinative Bacteriology*. Bacterial taxonomy was to become for Murray a field of major interest and intense endeavour for the rest of his life, and it was for his outstanding contributions in this area of microbiology that he became most widely known. Before his death in 1937, Bergey had requested that a Board of Trustees take over future editions of the *Manual* and Murray was appointed a member of the Trust. With R. S. Breed and A. P. Hitchens he was co-editor of the fifth (1939) and sixth editions (1948) of the *Manual* and, with Breed and N. R. Smith, of the seventh edition (1957). In addition to his editorial duties, to which he gave a very great deal of time and energy, he contri-

buted in one or other edition to the sections on Neisseriaceae, *Diplococcus*, *Streptococcus*, Corynebacteriaceae, Parvobacteriaceae and Spirochaetales.

Murray was well aware that *Bergey's Manual* was not an easy or even a practical book, and he voiced the opinion of many workers that the keys to the higher ranks were, when put to the test, useless, and that it was necessary for the user of the *Manual* to know enough bacteriology to identify his unknown organism down to the genus level. His description of *Listeria monocytogenes* was a model of what could be done and showed up the inadequacies of much of the rest of the *Manual*. Only the tape-recording made at the meeting of the Trustees in Washington in May 1964 remains of the flow of taxonomic wisdom, anecdotes, wit, leg-pulling (usually at the expense of his many American friends), and scorn at nomenclatural niceties and absurdities. A transcript of Murray's contributions would make fascinating reading but, alas, would be unpublishable. But behind the ribaldry there was the utmost common sense, and the other Trustees always sought and usually acted on Murray's advice in the discussions that must inevitably precede a new edition.

In the records of the International Committee on Bacteriological Nomenclature, Murray's name first appeared in a rare mimeographed document dated February 1939, 'Rules of Nomenclature Annotated with suggestions for Rules of Bacteriological Nomenclature prepared for the American–Canadian Committee on Compilation of Proposals for Consideration by the Third International Congress for Microbiology'. Other members of the Committee were R. E. Buchanan (chairman), R. S. Breed, J. H. Brown, I. C. Hall, W. L. Holman, O. Rahn and G. B. Reed. The joint committee produced the first 'Proposed International Rules of Bacteriological Nomenclature' in July 1939, and the Third International Congress for Microbiology agreed in Plenary Session to make them the basis of a Code to be presented at the next congress. At the Third Congress Murray was elected to the newly formed Judicial Commission. He was not at the Fourth Congress (1947) which approved the renamed International Bacteriological Code of Nomenclature, but in his absence he was appointed to an *ad hoc* Commission to act in an advisory capacity on the distribution of any funds that might become available for culture collections. This was an ill-fated Commission and was later replaced by a smaller committee to which Murray was appointed and on which he gave most valuable service, for he had the knack of picking out the most worthy from a large number of worthy collections for which there was inadequate financial support.

At the Sixth Congress (1953) Murray was a Vice-President of the Section on Bacterial Taxonomy; at this time he was also chairman of a Taxonomic Subcommittee on the Neisseriaceae, and had, as secretary, his old friend Sara Branham.

In 1939 Murray had been made the Canadian representative on the Permanent Commission for the Organization of Microbiological Congresses; and in 1958 he it was who conveyed the invitation of the Canadian Society of Microbiologists to hold the Eighth Congress in Canada. When this was accepted Murray was chosen by his colleagues to be President of the Congress.

At the end of the 1953 Congress Murray had been elected to succeed A. J. Kluyver as chairman of the International Committee on Bacteriological Nomenclature, and he presided at the meetings of the Committee in Stockholm in 1958. In the intervening period, and indeed until he announced his retirement as chairman at the beginning of the 1962 meeting (at which, as President of the Congress, he pleaded other business) he

was a great source of inspiration to, and a solid supporter of, the two secretaries. It was at the 1962 meeting, after he had resigned as chairman and indicated his retirement as a Canadian representative, that he was nominated and appointed a Life Member of the Committee (though this fact escaped record in the minutes of the meeting), an honour previously bestowed only on the two original secretaries of the committee (R. St John-Brooks and R. S. Breed).

Murray was called upon for active service in many capacities during the years of the Second World War. During the period from 1940 to 1945 he served as a member of the National Research Council Subcommittees on Infections and Shock and Blood Substitutes and was a member of the War-Time Prices and Trade Board Pharmaceutical Advisory Committee. He was Chairman of the Joint United States–Canadian Commission of the War Disease Control Station, Chairman of the Biological Warfare Committee and a Superintendant of Research of The Directorate of Chemical Warfare and Smoke at National Defence Headquarters in Ottawa. The latter three posts kept him away from the University almost continuously in the war years, during which time the Department continued to serve the University and teaching hospitals under the able direction of Dr Fred Smith. Murray's valued services to the joint U.S.–Canadian War Disease Control Commission and the Directorate of Chemical Warfare and Smoke were recognized by the award of the Medal of Freedom by the United States War Department and by his appointment following the war to the Advisory Board of the newly formed Canadian Defence Research Board.

Murray was an active member of many national and international scientific societies and committees and served many of these bodies in an executive capacity. In addition to his association with the older, well-established societies he gave freely time and energy to the development of new organizations and, indeed, the now thriving Canadian Society of Microbiologists virtually owes its existence to the organizing ability of two Murrays, E. G. D. himself and his son R. G. E. Murray, Professor of Bacteriology at the University of Western Ontario, and first President of the Society.

Murray was widely known at scientific meetings not only for his personal contributions but particularly for his always stimulating discussion of the papers presented. His pungent, discerning and witty comments, based on a wealth of experience covering many fields, were awaited with interest—and not infrequently with apprehension. His criticisms although forthright were delivered in a fatherly manner and in such good humour as not to breed resentment; they were received, rather, with the respect due to his obvious interest, authority and considered judgement.

HONOURS AND AWARDS

During his period of tenure at McGill, Murray was the recipient of many honours in recognition of his services as a teacher, scientist and administrator. These included Fellowship in the Royal Society of Canada in 1938, the award of the Medal of Freedom of the United States in 1947, the Flavelle Medal of the Royal Society of Canada in 1953 and the Coronation Medal of Queen Elizabeth II in 1953. On the eve of his retirement in 1955 the University of Montreal conferred upon him the Honorary Degree of Doctor of Medical Sciences and McGill an Honorary Doctor of Science degree. At the same time the University of Montreal appointed him an Honorary Member of the Executive Council of the Institute of Microbiology of that University

in recognition of the unfailing help and encouragement that he had so freely given in the early formative years of the Institute.

In the years following Murray's retirement from active duty at McGill other honours came his way. In 1955 he was elected an Honorary Fellow of the Montreal Medico-Chirurgical Society (of which he was a past president) and he received Honorary Life Membership in many associations including the Canadian Public Health Association (1957), the Canadian Society of Microbiologists (1957), this Society (1960), the American Society for Microbiology (1961) and the Canadian Association of Medical Microbiologists (1962). A special issue of the *Canadian Journal of Microbiology* containing contributions by his friends, colleagues and old students in Great Britain, the United States, Germany, Australia and Canada was dedicated to Murray in 1956 'in recognition of his contribution to science'.

THE RETIREMENT YEARS

Murray had not in any way slackened his pace with the approach of retirement; his colleagues could not conceive him in retirement except in the technical terms of the rules of tenure of the University and in this he did not disappoint them. He was appointed Guest Professor of Medical Research at the University of Western Ontario in September 1955, a post which brought him great delight in that he could, at long last, return to the satisfying quietude of work in the laboratory uninterrupted by a myriad of administrative details. He initiated most useful investigations dealing with the habitat and activities of his old friend *Listeria monocytogenes*; unfortunately unpublished at the time of his death but freely discussed with his colleagues in the field. He was particularly interested in finding improved methods of isolating Listeria from natural environments rich in other microbes and in epidemiological problems of listeriosis in wild life. In the last years of his life he struck up an effective partnership with K. K. Carroll of the Collip Laboratory at the University of Western Ontario on various projects concerning the isolation and characterization of the lipids of *L. monocytogenes*. This work is still very active and is being carried on by Carroll in association with R. G. E. Murray.

One of the most onerous of Murray's preoccupations in the first few of his post-retirement years involved the Bergey's Manual Trust, a service to his fellow bacteriologists that he took very seriously over the many years that he was a member, co-editor and contributor. The editing of the seventh edition was a task demanding a very great deal of work and there was much to do subsequently with the *Index Bergeyana*, published in 1966.

In addition to his research and editiorial duties he continued to serve on various committees and one particularly, which required continuing attention to detail and much travel, concerned his chairmanship of the very active Canadian Associate Committee on the Control of Hospital Infections. Canadian microbiologists elected him President of the VIII International Congress for Microbiology held in Montreal in 1962, a post which he filled with characteristic energy and distinction and a fitting crown to a truly great career in the service of science.

Although he had suffered a myocardial infarction and was forced to take things easier in the later years of his life, he lost none of his zest for living. He cheerfully continued to pursue his researches, to give the benefit of his wealth of experience to

committee affairs and to attend and take an active part in scientific meetings to the time of his sudden death on 6 July 1964.

RECOLLECTIONS

Jo'burg Murray was a very real person who will be remembered as a scientist, a teacher, a philosopher and a raconteur, but above all he will be remembered as a friend. He lived up to the highest Christian principles, but he was in fact an atheist, and he delighted to tell the story of how, as he lay in bed after his coronary, the hospital chaplain came to attend to his spiritual needs. Jo'burg put his viewpoint succinctly by saying that he worshipped devils and had no wish to have his soul commended to any other authority. The rest of the conversation, on other subjects, was very friendly. The chaplain often stopped by Jo'burg's bed to talk with him, and some weeks later, on one of these visits said: 'Are you still here? Your devils aren't doing much for you; you'd better change them.'

Jo'burg liked to do some leg-pulling, which he often combined with sarcasm. After hearing a paper in which a form of coding (numbers and letters) was suggested to replace names, he went to the microphone, stuck his chin out so that the beard reinforced the wagging and pointing finger, and said 'When Dr Cowan dies I hope they will put on his tombstone 606, 914...' (the rest was lost in laughter). Afterwards, he happily remarked 'and I meant to add G.P.I.'.

Tributes have been paid to the memory of Jo'burg Murray by colleagues from all over the world, and we cannot end without adding one more. He was a wonderful man to work with, but above all he was our friend.

ACKNOWLEDGEMENTS

It would have been impossible to compile so detailed a tribute without the help of many people; we are particularly grateful to Mrs Murray and Professor R. G. E. Murray for giving us some details known to them of Jo'burg's ancestry and life up to the time he was a Cambridge undergraduate.

J. W. STEVENSON

S. T. COWAN

Facsimile of *Curriculum vitae* compiled by E. G. D. M. and annotated in his own hand

Corrected Copy

NAME: MURRAY, Everitt George Dunne

NATIONALITY: (British) Canadian

PLACE AND DATE OF BIRTH: Johannesburg, South Africa, July 21, 1890.

Degrees:

B.A.—Cantab. (Hon. 1912)
M.A.—Cantab. (1918)
L.M.S.S.A.—(Lond. 1916) (Licentiate in Med. and Surg. of the Society of Apothecaries)
Specialists Certification as a Pathologist and Bacteriologist, Royal College of Physicians and Surgeons of Canada, 1946.

Honorary Degrees:

M.D.—Doctor of Medical Sciences, University de Montreal, 1955.
D.Sc.—Doctor of Science, McGill University, 1955.

University and Medical School:

Cambridge (Christ's College) England.
St. Bartholomew's Hospital, London.

Honours:

O.B.E. (Military Division) 1918
F.R.S.C. (Fellow of the Royal Society of Canada) 1938
Medal of Freedom awarded by the United States War Dept. 1947
Royal Society of Canada Flavelle Medal 1953.
Coronation Medal, Queen Elizabeth II, 1953.
Hon. Fellowship Montreal Medico-Chirurgical Society 1955.
Hon. Membership Canadian Society of Microbiologists 1957.
Hon Life Member Canadian Public Health Association 1957.
Honoured by dedication Special Number of the Canadian Journal of Microbiology Vol. 2 No. 3, May, 1956.

Present Appointments:

Visiting Professor in Medical Research, University of Western Ontario, September 1955.

Past Appointments:

Professor of Bacteriology and Immunology, McGill University, Montreal, and Chairman of the Dept. 1930 to 1955.
Member of Senate, McGill University 1943-53.
Honorary Consultant up to 1955.
Royal Victoria Hospital, Montreal
Montreal General Hospital, Montreal
Children's Memorial Hospital, Montreal
Alexandra Hospital, Montreal
Jewish General Hospital, Montreal
Victorian Order of Nurses for Canada
Royal Edward Laurentian Hospital, Montreal

Administrative Appointments: In Universities and Hospitals:

University of Cambridge, between 1923 *and* 1930—
Member of Board of Biology
Member Board of Medicine

Member Degrees Committee Board of Biology
Member University Building Syndicate
Examiner in Natural Sciences Tripos
Examiner in Pathology in Medical Examinations.

McGill University—

Faculty of Medicine
Faculty of Arts and Science
Faculty of Graduate Studies
Elected to represent the Faculty of Medicine on the University Senate 1943 to 1958.

Medical Board of Alexandra Hospital, Montreal, 1932 to 1955.

Member of Board of Governors of Alexandra Hospital, 1947 to 1955.

Academic and Teaching Appointments held previously:

London: (St. Bartholomew's Hospital) Senior Demonstrator in Pathology, 1919.
Cambridge: Fellow of Christ's College, 1923-31.
Cambridge: Lecturer in Pathology 1926-30.
Cambridge: Director Medical Studies, Christ's College, 1925-30.
Professor and Head of Department of Bacteriology and Immunology McGill University 1930-1955.
Bacteriologist-in-chief and member of the Medical Board of the Royal Victoria Hospital, 1931-46.
Bacteriologist-in-chief and member of the Medical Board of the Children's Memorial Hospital, 1938-48.
Member of the Medical Board of the Royal Edward Laurentian Hospital, 1940-46.
Member of the Medical Board of the Alexandra Hospital 1932 to 1955

SPECIAL POSITIONS HELD:

War Research and Service (1914-1918 inclusive)

Capt. Royal Army Medical Corps.
Research Staff of the War Office Central Cerebro-Spinal Fever Laboratory 1915 (Feb.) to 1916 (June).
Missions (War Office) to Pasteur Institute, Paris, on Meningococcus Serum (1915 and 1917).
Detailed for special duty on dysentry (3 B.G.H. Laboratory, Mesopotamia) 1916.
Staff of the War Office Vaccine Dept. R.A.M. College, 1917-1919.
One of the British representatives to Allies Medical Services Conferences in Paris (1917).
Member War Office Committee on Dysentery 1918.

Medical Research Council, Great Britain:

Research Bacteriologist 1920-26 (then given a Research Grant)
Committee on Meningococcus and Pneumococcus Serum.

5th International Botanical Congress

Recorder Bacteriology Section 1930.

International Association of Microbiological Societies

A Vice-President of Section VII for the Third Congress 1939. Member of Permanent International Commission for the Organization of Congress. Representative for Canada on the Permanent International Commission 1938-53. Member of Committee on Bacteriological Nomenclature (Chairman of this Committee at the 3rd

International Congress, Sept. 1939, New York).
Member of the Judicial Commission of the Permanent Committee of Nomenclature 1943-53 and re-elected 1953-63.
Vice-President, Section 1, (General Microbiology) V International Congress (Rio de Janeiro) 1950.
Vice-President Section (Taxonomy and Nomenclature) VI International Congress (Rome) 1953.
Elected Chairman of the International Committee on Bacteriological Nomenclature, 1953 to ~~date.~~ 1962
Appointed to "Committee of Honour" at VII International Congress of Microbiology, Stockholm, 1958.
President ~~Elect~~ VIII International Congress of Microbiology 1962, Montreal.
Life Member At-Large. International Committee on Bacteriological Nomenclature 1962

American-Canadian Committee on Bacteriological Nomenclature:

Member of Committee.

Society of American Bacteriologists:

Committee of Bergey's Manual of Determinative Bacteriology 1932-36
Member of Council 1940-42.
Member of the Committee on the Inter-American Society of Microbiology 1940.
Member of the Committee on Teaching Bacteriology 1944.
Member of the Nominating Committee 1947.

War Research and Service (1940-45 inclusive)

Member of N.R.C. Subcommittee on Infections.
Member of N.R.C. Subcommittee on Shock and Blood Substitutes.
Member of War-Time Prices & Trade Board Pharmaceutical Advisory Committee.
Canadian Chairman of the Joint U.S.-Canadian Commission (War Disease Control Station).
Chairman Biological Warfare Committee Directorate of Chemical Warfare and Smoke, N.D.H.Q. Ottawa.
Superintendent of Research D.C.W. & S., N.D.H.Q. Ottawa.
Member of an Advisory Board and Chairman of a Panel Under Defence Research Board, Ottawa, 1946-48.

Bergey's Manual of Determinative Bacteriology

Member of Trustee Board of Editors constituted in 1936 to date.
(SAB Committee on Bergey's Manual 1932-1936)

Canadian Public Health Association:

Chairman of the Laboratory Section 1937-38.
Chairman of the Programme Committee for the Laboratory Section meeting in Montreal, December 1946.
Member of Council 1952-53.

Montreal Medico-Chirurgical Society

Vice-President and Chairman of Finance Committee 1940-41.
President 1942.
Member of Council and Trustee 1946.

Special Conference on Gas Gangrene in United States (Harvard University)

Chairman by Invitation, April 1942.

Member Canadian Committee on Culture Collections of Microorganisms. 1953–

American Journal of the Medical Sciences

One of the Associate Editors (Progress of Medical Science, Bacteriology) 1940 to 1955.

National Research Council of Canada

Member of the Scientific Advisory Sub-Committee of the Institute of Parasitology 1947 to 1953.
Member of Medical Advisory Committee and its Executive Committee 1952-56.
Member of National Research Council 1953-56.
Member of the Standing Joint Committee on the Institute of Parasitology 1953 to ~~date~~. 1963
Member of the Associate Committee on Dental Research 1954-56.
Chairman Associate Committee on Control of Hospital Infections 1957.

University of Cambridge

Represented University of Cambridge at the installation of the Chancellor of the University of Toronto, Nov. 21, 1947.

City of Montreal:

Councillor (Class C) of the City of Montreal for three years to represent McGill University, 1947. Reappointed for four years, 1950.
Re-appointed for 3 years 1954.
Appointed member of the Board of Health of City of Montreal 1948, and several of its committees.
Member of the Board of Governors of Alexandra Hospital, 1948-1955.

Arctic Institute of North America

Charter Associate 1948 to date.

International Federation of Culture Collections of Microorganisms

Member of the Permanent Commission.

MEMBERSHIP OF SOCIETIES (SCIENTIFIC)

Member Pathological Society of G. B. and Ireland (and some time member of Committee).
Fellow Cambridge Philosophical Society.
American Association of Pathologists and Bacteriologists.
Society of American Bacteriologists (Member of Council 1940-42).
Montreal Medico-Chirurgical Society (President 1942-43, Hon. Fellow 1955).
Cambridge Natural History Society (President 1929)
McGill Chapter of Sigma XI
American Association for the Advancement of Science
American Association of Immunologists
Canadian Public Health Association (Chairman Laboratory Section 1938, Hon. Life Member 1957).
Montreal Physiological Society.
Biological Photographic Association.
Canadian Medical Association.
International Association of Microbiological Societies (Chairman and Member of various committees). President VIII Congress 1962.
Canadian Physiological Society
Royal Society of Canada (President of Section V. 1954), Flavelle Medalist 1953).

Institute of Food Technologists
Society de Biologie de Montreal.
Graduate Society of McGill University (Associate member).
Osler Society, McGill University, Honorary President 1950.
Canadian Society of Microbiologists (President 1956-57).
Societe' de Microbiologie de la Province de Quebec (President 1955)
(Hon. Life Member 1957).

PUBLISHED WORKS:

On Intercalary Vertebra—Journal of Anatomy and Physiology 1910.

The Identification of the Meningococcus (with M. H. Gordon, M.D., G.M.C., C.B.E., F.R.S.)—J of R.A.M.C. Oct.-Nov. 1918.

Attempt at Classification of B. dysenteriae, based upon the Agglutinating Properties of 53 strains—J. of R.A.M.C. Oct.-Nov. 1918.

Some Aspects of Meningococcus Virulence—J. of Hygiene, 22, No. 2, 1924, 175-207.

Observations on the Growth of Meningococci *in Vitro* in relation to Virulence, J. Hygiene, 23; No. 1, 1924, 23-63.

Staining Tubercle Bacilli in Formol-Fixed Tissues—J. of Path. & Bact. 27: 1924, p. 118, 119.

A Disease of Rabbits Characterised by a large mononuclear Leucocytosis, caused by a hitherto undescribed Bacillus Bacterium Monocytogenes (n. sp.) J. Path. & Bact. 29: 1926, 407-439.

Chapter "The Meningococcus" Chapter 5, Vol. 2, Med. Research Council System of Bacteriology 1929.

Monograph, The "Meningococcus" (M.R.C. Special Report Series 4, 124, 1929).

Chapter, Cerebro-Spinal Fever in Byam & Archibald "Practice of Medicine in the Tropics."

The Bacteriophage Phenomenon — Can. Med. Assn. J. 24: No. 5, 1931, 697-699.

Some Difficulties in Immunization with Vaccine — Can. Med. Assn. J. 25: 1931, 125-130.

Some General considerations indicating lines of Investigation of the Mechanism of Meningococcus Infection. — J. Bact. 23: 1932, No. 1.

The Titration of Dysentery (Shiga) Antitoxin — J. Bact. 25: No. 1. 1933, p. 85.

Therapeutic Immunization in Staphylococcus Infections — Can. Med. Assn. J. 31: No. 1, 1934, 66-68.

Observations by Dr. Murray — Discussions of papers by Dr. Rich and Menkin on Inflammation — Amer. J. of Path. 12: 1936, 728.

Co-editor (with Prof. R. S. Breed and Prof. A. P. Hitchens) of Bergey's Manual of Determinative Bacteriology (5th Ed.) and contributor of Sections: Neisseriaceae, Farvobacteriaceae, Diplococcus, Streptococcus, Listerella and Cornynebacterium.

Changes in the Outline of Classification introduced in the 5th Edition of Bergey's Manual of Determinative Bacteriology, Dr. R. S. Breed, E.G.D. Murray and A. P. Hitchens — Abstracts of Communications 3rd. Int. Congress for Microbiology, N.Y., Sept. 1939, p. 3.

The Place of Immunological Criteria in the Classification of Bacteria —Abstracts of Communications 3rd Int. Congress for Microbiology N.Y., Sept. 1939, p. 8.

Meningococcus Infections of the Male Urogenital Tract and the Liability to Confusion with Gonococcus Infections. Urologic and Cutaneous Review, Path. Number, 1939, 43: No. 11.

Outline of Classification Introduced in the 5th Edition of the Bergey's Manual of Determinative Bacteriology. Dr. R. S. Breed, E. G. D. Murray and A. Parker Hitchens, Osentralbl. fur Bakt. Parasit. & Infekt. 11.

"Public Influences of the Medical Profession". Address to Mtl. Med. Chi. Society as Retiring President of the Society — Med. Chi. Soc. Bulletin. August 1943, 11, 8, 76-82. Can. Med. Assn. J. Vol. 49, 417, Nov. 1943.

The Progress of Medicine — Can. J. of Comp. Med. Science, 1944, 8: 5, 135-138.

The Outline of Classification used in Burgey's Manual of Determinative Bacteriology (with R. S. Breed and A. P. Hutchins) Bacteriological Reviews 1944, 8 (2) 255.

Note on the new edition of Bergey's Manual of Determinative Bacteriology, Dr. E. G. D. Murray, R. S. Breed and A. Parker Hitchens, Chronica Botanica, 1945, 9: 226-227.

The value and function of Scientific Societies — Can. J. of Comp. Med., Feb. 1947, 47-52.

Bergey's Manual of Determinative Bacteriology, Co-Editor (with D. Bergey, R. S. Breed, A. P. Hitchens and N. R. Smith) 4th Edition, 1934: and 5th Edition, 1939 and 6th Edition 1948 and contributor of Sections: Neisseriaceae, Diplococcus, Streptococcus, Corynebacteriaceae, Parvobacteriaceae, Spirochaetales. 7th Edition 1957 and contributor to various sections.

Reflections on the Future of Veterinary Medicine — Can. J. of Comp. Med., 12: 283, Oct.1948.

Dubos' Bacterial and Mycotic Infections of Man. (Lippincott 1948) Chapter 1, Synopsis of the History of Medical Bacteriology. 1st 2nd and 3rd Editions.

Dubos' Bacterial and Mycotic Diseases of Man, 1948 (Lippincott) Chapter 37, (with Dr. G. G. Kalz) The cultivation and identification of Pathogenic Bacteria. 1st and 2nd Editions.

A Discussion of the Fuso-Spirochaetal Disease of the Mouth — J. of the Can. Dental Assn. Feb. 1949.

Plaster of Paris as a Source of Infection in Tetanus and Gas Gangrene, with Dr. G. D. Denton, Can. Med. Assn. J., April 1st, 1949.

"Listeria Monocytogenes as the cause of disease in man and animals and its relation to infectious mononucleosis from an etiological and immunological aspect." (with K. F. Girard) Amer. J. Med. Sci. 221: (No. 3) Mar. 1951, 343.

The Emergency for Research in Unexplored Fields of Public Health, Can. J. of Public Health 42: 485, Dec. 1951. Republished Can. Med. Assn. J. 66: 275-277, 1952.

Dubos' Bacterial and Mycotic Infections of Man, 2nd Ed., 1952 (Lippincott) Chapter 1. "A Synopsis of the History of Medical Bacteriology".

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Editor's note

This list of publications, although compiled by E. G. D. M., contains two mistakes and omits two papers.

The first paper should be:

'Note on a supernumerary vertebra' (with an appended note by W. L. H. Duckworth, M.D.), *Journal of Anatomy and Physiology*, 1913, vol. 47, 363–4.

The second paper should be:

'Identification of the Meningococcus' (with M. H. Gordon, M.D.), *Journal of the Royal Army Medical Corps*, 1915, Vol. 25, 411–423.

To this bibliography should be added:

'The story of Listeria', *Transactions Royal Society of Canada*, 1953, vol. 47, Series III, 15–21.

'The place of nature in man's world', *American Scientist*, January 1954, Vol. 42, 130–135.

and one posthumous publication

'The interaction of guanofuracin and *Listeria monocytogenes*' (with B. K. Gosh & R. G. E. Murray), *Canadian Journal of Microbiology*, 1966, vol. 12, 285.

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The Inhibition of the Growth of *Clostridium welchii* by Lipids Isolated from the Contents of the Small Intestine of the Pig

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SUMMARY

The growth of *Clostridium welchii* type A NCTC 8246 was inhibited by the contents of the small intestine of pigs from which food had been withheld for 24 hr; no inhibitory activity was observed by the contents of the stomach, caecum and large intestine. Inhibitory activity was absent from the contents of the small intestine during the early stages of digestion but first appeared about 6 hr after the pigs had been given food. This indicated that under normal feeding practice there were periods during each day when the contents of the small intestine would be inhibitory for *C. welchii*. The inhibitory activity was in the lipid fraction of the intestinal contents. Fractionation of the intestinal lipids showed that the inhibitory substances were linoleic and arachidonic acids, lysolecithin and an unidentified phospholipid. The poly-unsaturated fatty acids and lysolecithin appeared to be derived mainly from the action of pancreatic phospholipase A on biliary lecithin in the lumen of the small intestine. The possibility that these inhibitory lipids control to some extent the numbers of *C. welchii* in the alimentary tract of the pig is discussed.

INTRODUCTION

During an investigation of the microflora of the alimentary tract of the pig it was observed that the contents of the small intestine inhibited the growth of certain micro-organisms. A study of the factors responsible for this inhibition is now reported.

METHODS

Pigs. The animals used in this work were of the herd of Large Whites maintained at the National Institute for Research in Dairying. The pigs were weaned at 8 weeks and were then given a commercial pig diet until they were sent at the age of 4–7 months to the local slaughterhouse. As is normal practice, food was withheld from certain of the animals for 24 hr before they were killed. Other animals were killed at 4, 6, 15 or 21 hr after they had been given food. In all, some thirty pigs were examined.

Collection and treatment of material. The gastro-intestinal tracts, gall bladders and pancreatic tissues were collected at the slaughterhouse within 20 min. of death. The gastro-intestinal tract was divided into the stomach, small intestine, caecum and large intestine; as rapidly as possible thereafter the contents were removed from each section of the tract. To remove gross food residues, the samples of contents were centrifuged at 1250*g* for 30 min. The supernatant fluid was removed and the dry

matter content of each sample determined so that the concentration of the various components in the fluid phase of the gastro-intestinal contents could subsequently be calculated. The samples of intestinal fluid were either stored at $-20°$ or freeze-dried and then stored at $-20°$ until required for the determination of antibacterial activity or for chemical investigation. Samples of the small intestine wall, obtained from the upper, middle and lower regions, were washed free from contents with 0·85% (w/v) NaCl solution. Portions of each of the three sections were pooled and homogenized in 0·85% (w/v) NaCl solution. The homogenate was centrifuged at 1250*g* for 30 min. and the supernatant fluid removed and stored at $-20°$. Bile was removed from the gall bladders and was stored at $-20°$ until required. Acetone-dried powders of the pancreatic tissues were prepared as described by Laws & Moore (1963).

Sterilization of material. Initially, intestinal contents and extracts of intestinal tissues were sterilized by Seitz filtration but, after it had been shown that the inhibitory factors were heat stable, material was sterilized in an autoclave by allowing a pressure of 10 pounds/inch^2 (115°) to develop and then immediately removing the source of heat. In the case of lipid fractions, the chloroform treatment (see later) was sufficient to sterilize the sample.

Organisms tested. The microbial spectrum of inhibitory activity was determined as follows. A well cut in the centre of a yeast glucose agar plate was filled with contents of the small intestine and the contents were allowed to diffuse in the cold for 24 hr. The test organism was streaked up to the edge of the well, the plates incubated at 37° for 24 hr and then examined for inhibition. The organisms tested were *Clostridium welchii* type A, NCTC 8246; *Salmonella paratyphi B*, NCTC 5705; *S. cholerae suis*, NCTC 5735, and three strains of haemolytic *Escherichia coli* (isolated from cases of oedema disease by Mr W. J. Sojka, Central Veterinary Laboratories, Weybridge). Other organisms tested were *E. coli* I, *E. coli* III; *Streptococcus equinus*, *S. faecalis*, *S. liquefaciens*, *S. durans*, *S. faecium*, *S. bovis*, two unclassified streptococci; *Lactobacillus salivarius*, *L. acidophilus*, *L. brevis*, *L. fermenti*, *L. cellobiosus* and *L. plantarum* all isolated from the alimentary tracts of normal pigs as described by Fuller *et al.* (1960).

Test for inhibitory activity. Preliminary tests on a number of different organisms showed that *Clostridium welchii* NCTC 8246 and two others were the most sensitive to the inhibitory agent; *C. welchii* NCTC 8246 was adopted as the test organism. Cultures were grown in glucose Lemco broth (%, w/v: Evans peptone, 1; Lab. Lemco, 1; NaCl, 0·5; glucose, 1). Spore crops were produced by the method of Ellner (1956) and the remaining vegetative organisms killed by heating at 80° for 10 min.

To 0·9 ml. of the test material was added 0·1 ml. of an overnight culture of *Clostridium welchii* NCTC 8246 in glucose Lemco broth for vegetative organisms or in the medium of Ellner (1956) for spores. The number of viable vegetative forms added was between 5×10^6 and 5×10^7; the number of viable spores present in the spore inoculum was about 10^4. After 5 hr contact in an anaerobic atmosphere of 95% (v/v) H_2 + 5% (v/v) CO_2 colony counts were made on nutrient agar containing 5% (v/v) sheep blood and incubated anaerobically for 24 hr at 37°.

For the titration of inhibitory activity, dilutions were made in 0·1 M-phosphate buffer (pH 6·5) containing 0·1% (w/v) Tween 80 (polyoxyethylene sorbitan monooleate; obtained from Honeywill & Stein Ltd., London). The Tween 80 was purified by the method of Bier (1955) and was included to emulsify the lipid fractions in the test solutions. Stable emulsions of the various lipid fractions were prepared as follows.

A known amount of lipid dissolved in chloroform was placed in a glass-stoppered tube and the solvent removed under reduced pressure at room temperature by means of a rotary film evaporator attached to a supply of nitrogen. The requisite volume of a solution of Tween 80 in chloroform was then added and the solvent was again removed. When all traces of chloroform had disappeared the requisite volume of 0·1 M-phosphate buffer was added and the tubes were shaken vigorously. Solvent controls were prepared similarly. It was established that there was no inhibition due to the presence of Tween 80. To assess the minimum inhibitory concentrations of the various lipid fractions, the lowest concentration at which each fraction completely sterilized the inoculum was determined. In Table 1 the results are expressed as the % of the inoculum killed when the undiluted intestinal material was tested. Unless otherwise stated, all fractions separated from the intestinal contents were reconstituted and tested at the concentration at which they occurred in the fluid phase of the intestinal contents.

Extraction of lipids The lipids were extracted from the freeze-dried contents and cultures of *C. welchii* NCTC 8246 by a method similar to that described by Folch, Lees & Stanley (1957). The lipids were extracted from pig bile by a procedure adapted from that devised for plasma lipids by Nelson & Freeman (1959). The total lipid contents of the purified lipid extracts were determined gravimetrically.

Chromatography on columns of silicic acid. Total phospholipids were separated from the non-phospholipids by fractionating portions of the lipid extracts on 3 g. columns of silicic acid (100 mesh: A.R.; Mallinckrodt Chemical Works, New York). As described by Moore & Doran (1962) the non-phospholipids were eluted from the columns with chloroform and the total phospholipids with chloroform + methanol (1 + 4, by vol.) and then methanol. The unesterified fatty acids were separated from the other non-phospholipids (cholesterol, cholesterol esters, glycerides) by chromatography on columns of Florisil (Koch-Light Laboratories Ltd., Colnbrook, Buckinghamshire) according to the method of Carroll (1961). The individual phospholipids were fractionated on 5 g. columns of silicic acid. The columns were prepared by the method of Marinetti, Erbland & Kochen (1957). The individual phospholipids were then eluted with chloroform + methanol mixtures by the procedure of Hanahan, Dittmer & Warashina (1957) as modified by Moore & Doran (1961).

Chromatography on thin-layer plates of silica gel and on filter paper impregnated with silicic acid. The individual phospholipids were also separated by fractionation on thin-layer chromatoplates of silica gel (Camag A.G., Muttenz, Switzerland) with a solvent system of chloroform + methanol + acetic acid + water (25 + 15 + 4 + 2, by vol.) as described by Skipski, Peterson & Barclay (1964). After development the chromatograms were sprayed with a solution of dichlorofluorescein (0·1 %, w/v) in methanol + water (95 + 5, by vol.) and the positions of the various phospholipid bands located by viewing the plates under ultraviolet radiation. The various phospholipid fractions were eluted from the bands of silica gel by the procedure of Skipski *et al.* (1964). As additional aids in the identification of the various compounds, the phospholipids were chromatographed by the technique of Marinetti (1963) on filter paper impregnated with silicic acid and by the technique of Mangold (1961) on thin-layer plates of silica gel G (E. Merck A.G., Darmstadt, Germany) + ammonium sulphate (90 + 10, by weight) with a solvent system of chloroform + methanol + water (65 + 2 + 5, by vol.). Chromatography on thin-layer plates of silica gel G with a solvent system of chloro-

form + methanol + 14 N-ammonia (65 + 25 + 4, by vol.) was also used to establish the identity of certain of the phospholipids (Horrocks, 1963).

Fatty acid analysis. The fatty acids present in the various phospholipid and unesterified fatty acid fractions were converted to the corresponding methyl esters by the trans-esterification procedure of Stoffel, Chu & Ahrens (1959). The methyl esters were analysed by gas–liquid chromatography on both non-polar and polar columns (Moore & Williams, 1963, 1964). The non-polar (APL) columns consisted of 10% (w/w) Apiezon L grease on 100–120 mesh Celite and the polar (PEGA) columns consisted of 10% (w/w) polyethylene glycol adipate also on 100–120 mesh celite. Identification of the methyl esters of the fatty acids was made by comparison of their retention times on the two types of columns with those of known standard methyl esters and by plotting the logarithms of their retention times relative to methyl palmitate on PEGA columns against the logarithms of their retention times relative to methyl palmitate on APL columns (James, 1959). A check on the number of double bonds in each fatty acid was made by fractionating certain of the samples of methyl esters on thin-layer plates of silica gel G + silver nitrate (95 + 5, by weight) with a solvent system of light petroleum (b.p. 40–60°) + diethyl ether (85 + 5, by vol.). This technique (Morris, 1962) separated the methyl esters into saturated, mono-, di-, tri- and tetra-enoic acid esters. The separated zones were scraped from the plates and the methyl esters were eluted from the silica gel G + silver nitrate with hexane + diethyl ether (50 + 50, by vol.). The resulting methyl ester fractions were then analysed by gas–liquid chromatography. Thus, by these procedures, the number of carbon atoms and the number of double bonds in each fatty acid could be established.

Reference compounds. From egg yolk, phosphatidyl choline was prepared by the technique of Saunders (1957) and phosphatidyl ethanolamine by the technique of Rhodes & Lea (1957). The method of Saunders (1957) was also used to prepare pure lecithin from pig bile. Lysophosphatidyl choline and lysophosphatidyl ethanolamine were obtained after the corresponding pure diacyl compounds had been hydrolysed with the phospholipase A of snake (*Crotalus adamenteus*) venom. To prepare phosphatidic acid a sample of pure phosphatidyl choline was hydrolysed with the phospholipase D of cabbage by the method of Kates (1954). Phospholipase A and D and sphingomyelin were obtained from Koch-Light Laboratories Ltd. Diphosphatidyl glycerol (cardiolipin) was prepared from pig heart by the technique of MacFarlane (1961). Pure fatty acids and their methyl esters were obtained from Calbiochem Inc., New York.

Hydrolysis of phospholipids with phospholipase A. Phospholipase A solutions were prepared from snake venom by the method of Long & Penny (1957) or from an acetone-dried powder of pig pancreas by the method of Magee, Gallai-Hatchard, Sanders & Thompson (1962). The conditions of hydrolysis of lecithin and phosphatidyl ethanolamine were the same as those described in detail by Moore & Williams (1964, 1965). The reaction products, i.e. unesterified fatty acids and lyso-compounds, were separated on columns of silicic acid as described by Moore & Williams (1964).

Chemical analysis. Unesterified fatty acids were determined by the microtitration procedure of Albrink (1959) and lipid phosphorus was determined by the methods of Allen (1940) and Chen, Torribara & Warner (1956).

RESULTS

The location of inhibitory activity and the variation in the inhibitory activity of the contents of the pig small intestine with time after feeding

Inhibitory activity (this refers throughout to tests against *Clostridium welchii* type A, NCTC 8246) was tested for in the contents of the stomach, small intestine, caecum and colon. The activities of bile and of saline extracts of the wall of the small intestine were also examined. Significant inhibitory activity was found only in the contents of the small intestine.

It is commercial practice to withhold food from pigs on the day of slaughter so that animals may have been fasted for 20–24 hr when they are killed. Inhibitory activity was found in the contents of all of the small intestines obtained from twenty pigs that had been sent to the slaughterhouse in this way. However, it was important to investigate to what extent the inhibitory activity of the contents of the small intestine varied as the period of time after feeding was decreased. The results of such an investigation are shown in Table 1, from which it can be seen that no activity was detected in the contents of the small intestine of pigs that were killed 4 hr after they had been fed. Appreciable inhibitory activity was detected in the contents of the small intestines of animals killed 6 hr or more after they had been fed. The pigs in the Shinfield herd are given food twice a day and the periods between feeds are 7 hr during the day and 17 hr during the night. Thus it would appear that there are periods of about 1 and 11 hr in every 24 hr when the contents of the small intestine might be inhibitory for *Clostridium welchii*.

Table 1. *Variation in inhibitory activity against Clostridium welchii type A, NCTC 8246, of the contents of the pig small intestine with time after feeding*

Time after feeding (hr)	Pig no.	Proportion of *Clostridium welchii* inoculum killed (%)
4	1	0·0
	2	0·0
	3	26·0
6	4	99·9
	5	99·9
	6	99·9
15	7	93·9
	8	99·9
	9	100·0
24	10	100·0
	11	100·0
	12	100·0

Microbial spectrum of inhibitory activity

None of the Gram-negative organisms tested was inhibited by the contents of the small intestine, neither were *Streptococcus faecalis*, *S. durans*, *S. faecium*, nor *Lactobacillus plantarum*. The other streptococci and lactobacilli showed slight inhibition on one of the two occasions when they were tested. *C. welchii* NCTC 8246 was markedly inhibited and was by far the most sensitive organism of those tested.

Preliminary observations on the nature of the inhibitory factor

The inhibitory activity of the contents of the small intestine was destroyed when the contents were ashed but not when heated for 30 min. at 56° or for 10 min. at 120°. Inhibitory activity was unaffected when the pH value of the intestinal contents was increased to pH 10 but when decreased to pH 4 the active substance appeared to be precipitated. The observation that the inhibitory factor was extracted from the intestinal contents with diethyl ether suggested that the factor was present in the lipid fraction of the intestinal contents. The fact that the growth of *Clostridium welchii* was not inhibited by pig bile indicated that the inhibitory activity of the intestinal contents could not be attributed to the bile acids that would be present in them. Nevertheless, tests were made with pure bile acid salts, but solutions of sodium glycocholate (2%, w/v) and sodium taurocholate (8%, w/v) possessed no inhibitory activity.

The colony counts made at the end of the period of contact involved dilution beyond the point at which the inhibitory agent was active. In spite of this, organisms still present at this dilution did not grow when plated out on nutrient agar containing 5% (v/v) sheep blood. For this reason the substance was considered to be bactericidal.

Fractionation of the intestinal contents

During the investigation various samples of intestinal contents were fractionated and the inhibitory activities of the various fractions were determined. The fractionation of a sample of intestinal contents with high inhibitory activity is now described as an example of the type of result obtained. The pooled sample (no. 1) of intestinal contents used in this fractionation was obtained from four pigs from which food had been withheld for 24 hr before slaughter.

Freeze-dried material (30 g.), equivalent to 360 ml. intestinal fluid, was refluxed for 2 hr with 750 ml. chloroform, 750 ml. methanol and 120 ml. water. After the addition of a further 750 ml. chloroform the mixture was filtered through a funnel fitted with a sintered-glass plate. No antibacterial activity was found in the insoluble residue. To purify the crude lipid extract, 500 ml. water were added to the filtrate and the resulting mixture shaken and then allowed to stand at room temperature until separation between the lower chloroform layer and the upper aqueous methanol layer was complete. The purified lipid contained in the chloroform layer possessed inhibitory activity; the material in the aqueous methanol solution did not. A portion (59 mg.) of the purified lipid dissolved in a small volume of chloroform was placed on a 3 g. column of silicic acid. The non-phospholipids were eluted with 300 ml. chloroform and the phospholipids with 200 ml. chloroform + methanol (1 + 4, by vol.) followed by 100 ml. methanol. Both the non-phospholipid and phospholipid fractions contained inhibitory factors. Thin-layer chromatography showed the presence of cholesterol, cholesterol esters, triglycerides, diglycerides, monoglycerides and unesterified fatty acids in the non-phospholipid fraction. The non-phospholipid fraction was separated into unesterified fatty acids and neutral lipids by chromatography on a 5 g. column of Florisil. Antibacterial activity was found in the unesterified fatty acid fraction but none was detected in the neutral lipids (cholesterol, cholesterol esters, glycerides). The neutral lipid fraction was not investigated any further. The various steps in the fractionation procedure up to this stage are shown in Fig. 1, which also

shows the weights of the various fractions. Attention should be drawn to the relatively high concentration (12%) of total lipid in the dry intestinal contents and the very high proportion (55%) of unesterified fatty acids in the total intestinal lipids. The unesterified fatty acids were analysed by gas chromatography; the results are shown in Table 2, which also shows the calculated concentrations of each fatty acid in the

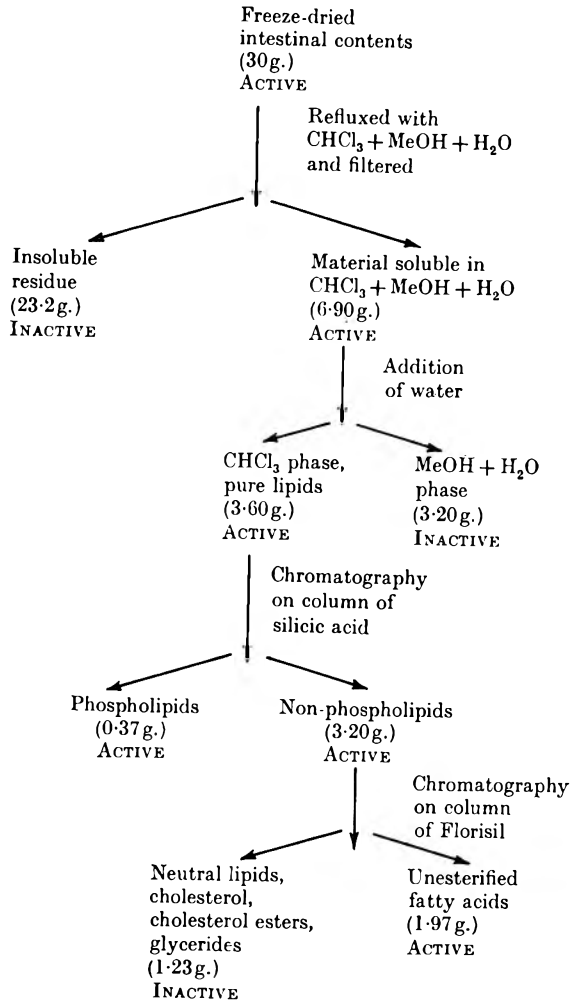

Fig. 1. Fractionation of pig intestinal contents sample no. 1. This sample was from a pool from four pigs having had food withheld for 24 hr before slaughter.

intestinal fluid. Unsaturated fatty acids constituted about 65% of the total fatty acids and linoleic acid was the major fatty acid. In view of the small amounts of fatty acids used in gas chromatographic separations it was not possible to test the inhibitory activities of individual fatty acids that were actually isolated from the unesterified fatty acid fraction of the intestinal contents.

The intestinal phospholipids were analysed by quantitative thin-layer chromatography (Skipsky *et al.* 1964); the results, together with the calculated concentrations

of individual phospholipids, are given in Table 3. Lysolecithin was the major phospholipid in the intestinal contents. Some doubt exists about the identity of one of the intestinal phospholipids, component A. When chromatographed on thin-layer plates of silica gel, either by the techniques of Skipski *et al.* (1964), Mangold (1961) or Horrocks (1963), component A moved with the solvent front. It also moved with the solvent front when chromatographed on filter paper impregnated with silicic acid

Table 2. *Composition of the unesterified fatty acids present in the pig intestinal contents (sample no. 1)*

Sample no. 1 was a pool of intestinal contents of 4 pigs from which food had been withheld for 24 hr before slaughter.

Fatty acid		Weight % of the total fatty acids	Calculated concentration of each fatty acid in the intestinal fluid (mg./100 ml.)
Myristic	(14:0)*	0·2	1·1
Pentadecylic	(15:0)	0·2	1·1
Palmitoleic	(16:1)	2·4	13·1
Palmitic	(16:0)	18·2	99·6
—	(17:0 br)	0·3	1·6
Margaric	(17:0)	1·6	8·8
Stearic	(18:0)	11·0	60·2
Oleic	(18:1)	25·7	141·0
Linoleic	(18:2)	30·8	169·0
Linolenic	(18:3)	2·2	12·0
Arachidonic	(20:4)	7·0	38·3

* Shorthand designation of Farquhar *et al.* (1959).

Table 3. *Composition of the phospholipids present in pig intestinal contents (sample no. 1)*

Sample no. 1 as Table 2

Phospholipid	Proportion of total phospholipid (%)	Calculated concentration of each phospholipid in intestinal fluid (mg./100 ml.)
Component A	22·9	23·6
Phosphatidyl ethanolamine	21·1	21·7
Lysophosphatidyl ethanolamine	3·0	3·1
Lecithin	18·0	18·5
Sphingomyelin	5·0	5·2
Lysolecithin	30·0	31·0

(Marinetti, 1963). Investigation by column chromatography revealed that component A was eluted from columns of silicic acid with chloroform + methanol (95 + 5, by vol.). Thus, the chromatographic behaviour of component A was similar to that described for diphosphatidyl glycerol by Skipski *et al.* (1964), Marinetti (1963) and MacFarlane (1961). A mixture of component A and diphosphatidyl glycerol prepared from pig heart was not separated by thin-layer chromatography. Thin-layer chromatography did not separate a mixture of component A and phosphatidic acid. However, in spite

of these similarities in chromatographic properties, evidence presented in a following section would seem to indicate that component A was neither diphosphatidyl glycerol nor phosphatidic acid.

In order that the antibacterial activities of the various intestinal phospholipids could be tested, a portion of the mixed phospholipids containing about 750 μg. lipid phosphorus was fractionated on a 5 g. column of silicic acid. Component A was eluted with chloroform + methanol (95 + 5, by vol.), phosphatidyl ethanolamine with chloroform + methanol (80 + 20, by vol.), lecithin with chloroform + methanol (60 + 40, by vol.) and lysolecithin with chloroform + methanol (20 + 80, by vol.). Examination of the four fractions by thin-layer chromatography revealed the presence of traces of lysophosphatidyl ethanolamine and sphingomyelin in the lecithin fraction. Traces of sphingomyelin were also observed in the lysolecithin fraction. Antibacterial activity was found in the fraction containing component A and in that containing lysolecithin and traces of sphingomyelin. Thin-layer chromatography (Mangold, 1961) of component A showed that this component was not contaminated with unesterified fatty acids. The lysolecithin fraction was rechromatographed on columns of silicic acid until traces of sphingomyelin were removed. Nevertheless, the inhibitory activity of the lysolecithin fraction was retained. It was concluded therefore that the antibacterial activity of the intestinal phospholipids was due to component A and to lysolecithin.

Table 4. *Minimum inhibitory concentrations against Clostridium welchii* NCTC *8246 of pure fatty acids and methyl esters in 0·1* M*-phosphate buffer (pH 6·5 or 7·5) containing 0·1% (w/v) Tween 80*

Fatty acid	pH 6·5	pH 7·5
	Minimal inhibitory concentration (mg./100 ml.)	
Caproic (6:0)	1160	5810
Caprylic (8:0)	721	3610
Capric (10:0)	172	862
Lauric (12:0)	1000	200
Myristic (14:0)	2280	457
Palmitic (16:0)	> 2560	> 2560
Stearic (18:0)	> 2850	> 2850
Oleic (18:1)	283	283
Erucic (22:1)	339	339
Linoleic (18:2)	5·61	5·61
Linolenic (18:3)	27·9	27·9
Arachidonic (20:4)	6·09	6·09
Methyl linoleate	146	733
Methyl linolenate	293	1460

The inhibitory activities of various unesterified and esterified fatty acids

The inhibitory activities of the pure fatty acids and methyl esters listed in Table 4 were determined at concentrations between 10^{-1} M and 10^{-5} M. Tests were carried out in 0·1 M-phosphate buffer containing 0·1% (w/v) Tween 80 at pH 6·5 and at pH 7·5. The minimum inhibitory concentration of each fatty acid is given in Table 4. An increase from pH 6·5 to pH 7·5 increased the minimum inhibitory concentrations of the short-chain acids (caproic, caprylic, capric) but decreased the minimum inhibitory

concentrations of the two medium-chain fatty acids (lauric, myristic). At pH 6·5 and 7·5 palmitic and stearic acids were inactive at 10^{-1} M. (When present in concentrations greater than 10^{-1} M, palmitic and stearic acids formed unstable emulsions under the test conditions and were therefore not tested at concentrations greater than 10^{-1} M.) The minimum inhibitory concentrations of the unsaturated fatty acids were unaffected by a change in pH value of the medium. Of the fatty acids tested, those with the lowest minimum inhibitory concentrations were the poly-unsaturated acids, linoleic, linolenic and arachidonic acids. However, the minimum inhibitory concentration of linoleic acid, with two double bonds, was somewhat less than that of linolenic acid, with three double bonds. At pH 6·5 and 7·5, the minimum inhibitory concentrations of methyl linoleate and methyl linolenate were greater than the corresponding minimum inhibitory concentrations of the free acids. An increase from pH 6·5 to pH 7·5 increased the minimum inhibitory concentrations of the methyl esters of linoleic and linolenic acids.

Comparison of the results given in Table 4 with those given in Table 2 shows that, of the major fatty acids present in the unesterified fatty acid fraction of the intestinal contents (sample no. 1), only linoleic and arachidonic acids occurred in the intestinal fluid at concentrations in excess of their respective minimum inhibitory concentrations. Thus it seemed reasonable to conclude that the inhibitory activity of the unesterified fatty acid fraction of the intestinal contents (sample no. 1) was due to linoleic and arachidonic acids.

The inhibitory activity of pure phospholipids

A number of pure phospholipids obtained from various sources were tested (in 0·1 M-phosphate buffer, pH 6·5, containing 0·1%, w/v, Tween 80) for antibacterial activity at concentrations which the phospholipids might be expected to attain in the intestinal fluid. Lecithin and phosphatidyl ethanolamine (from egg yolk) were inactive at concentrations as high as 10^{-3} M (i.e. lecithin, 77 mg./100 ml.; phosphatidyl ethanolamine, 73 mg./100 ml.). Sphingomyelin (Koch-Light Laboratories Ltd.) was also inactive at 10^{-3} M (75 mg./100 ml.). Lysolecithin, prepared from egg yolk lecithin, was active at 10^{-3} M (51 mg./100 ml.); dilution experiments showed that the minimum inhibitory concentration of lysolecithin was $0{\cdot}9 \times 10^{-4}$ M (4·5 mg./100 ml.). Owing to the small amount of lysophosphatidyl ethanolamine available, the highest concentration at which this compound was tested was 2×10^{-4} M (9·3 mg./100 ml.). At this concentration, lysophosphatidyl ethanolamine was inactive. The concentration of lysophosphatidyl ethanolamine in the intestinal fluid of sample no. 1 (Table 3) was only 3·1 mg./100 ml. Phosphatidic acid (prepared from egg-yolk lecithin) and diphosphatidyl glycerol (prepared from pig heart) were not active at 10^{-3} M (i.e. phosphatidic acid, 69 mg./100 ml.; diphosphatidyl glycerol, 143 mg./100 ml.).

Comparison of these findings with the results given in Table 3 showed that in the intestinal fluid of sample no. 1 the concentration of lysolecithin was 7 times the minimum inhibitory concentration. The fact that phosphatidic acid and diphosphatidyl glycerol exhibited no antibacterial activity at concentrations far in excess of the concentration of component A in the intestinal fluid (Table 3) would seem to indicate that component A was neither phosphatidic acid nor diphosphatidyl glycerol.

Experiments with biliary lecithin

It seemed possible that the lysolecithin and the unesterified unsaturated fatty acids in the intestinal contents might have resulted in part from the action of the phospholipase A secreted in the pancreatic juice on the lecithin in the bile. Accordingly, a sample of pure lecithin, prepared from pig bile, was hydrolysed by the phospholipase A extracted from an acetone-dried powder of pig pancreas. The products of hydrolysis were separated by silicic acid chromatography and the compositions and the antibacterial activities of the original bile lecithin and the resulting lysolecithin and fatty acids were determined. The fatty acid composition of the bile lecithin and lysolecithin,

Table 5. *Fatty acid compositions of biliary lecithin, the lysolecithin and fatty acids derived from the biliary lecithin, and the lysolecithin isolated from pig intestinal contents (sample no. 1)*

Sample no. 1 was from a pool from 4 pigs having had food withheld for 24 hr before slaughter.

Fatty acid	Lecithin from bile	Lysolecithin from bile lecithin	Fatty acids from β-position of bile lecithin	Lysolecithin from intestinal contents
	Fatty acid composition (%)			
Myristic (14:0)	0·3	0·3	0·4	0·2
Pentadecylic (15:0)	0·3	0·4	0·2	0·6
Palmitic (16:0)	23·0	40·2	5·9	38·1
Palmitoleic (16:1)	1·1	1·1	1·2	1·3
— (17:0 br)	0·4	0·3	0·5	0·2
Margaric (17:0)	0·9	1·5	0·3	1·6
Stearic (18:0)	19·3	34·9	3·7	36·7
Oleic (18:1)	24·6	18·6	30·5	16·5
Linoleic (18:2)	20·0	1·4	38·3	3·5
Linolenic (18:3)	1·1	< 0·2	2·3	< 0·2
Arachidonic (20:4)	7·4	< 0·2	14·9	< 0·2

with the composition of the fatty acids derived from the β-position of the lecithin, are shown in Table 5. The fatty acid composition of the lysolecithin isolated from the intestinal contents (sample no. 1) is also given in Table 5. The similarity in fatty acid composition of the two samples of lysolecithin shown in Table 5 should be noted.

The bile lecithin was inactive at the highest concentration tested (50 mg./100 ml.). The lysolecithin derived from the bile lecithin was active at the highest concentration tested (33 mg./100 ml.) and dilution experiments showed that, in 0·1 M-phosphate buffer (pH 6·5) and in the presence of 0·1% (w/v) Tween 80, the minimum inhibitory concentration of lysolecithin from this source was between 6·6 and 2·7 mg./100 ml. This compares quite well with the minimum inhibitory concentration found for the lysolecithin prepared from egg yolk lecithin (i.e. 4·5 mg./100 ml.). Since lysolecithin is to some extent water soluble, tests were also made with the lysolecithin derived from biliary lecithin, in 0·1 M-phosphate buffer (pH 6·5) but containing no Tween 80. In the absence of Tween 80, the minimum inhibitory concentration of lysolecithin was between 1·3 and 0·3 mg./100 ml. Thus the presence of Tween 80 appeared to increase the minimum inhibitory concentration of lysolecithin.

The unesterified fatty acids derived from the β-position of the biliary lecithin were

active and the minimum inhibitory concentration was between 18·2 and 7·3 mg. total fatty acid/100 ml. The calculated concentrations of the individual fatty acids corresponding to these two concentrations of total fatty acids in the test solutions are given in Table 6. It may be seen that the antibacterial activity of the total fatty acids at a concentration of 18·2 mg./100 ml. could well be accounted for by the fact that the minimum inhibitory concentration of linoleic acid (i.e. 5·6 mg./100 ml.) was exceeded. The minimum inhibitory concentration of linoleic acid was not exceeded in the test solution containing 7·3 mg. total fatty acids/100 ml. The minimum inhibitory concentration of arachidonic acid (i.e. 6·1 mg./100 ml.) was not exceeded in the test solutions containing either 18·2 or 7·3 mg. of total fatty acid/100 ml.

Table 6. *The concentration of individual fatty acids in a sample inhibitory for Clostridium welchii* NCTC *8246 (containing 18·2 mg. total fatty acid/100 ml.) and a non-inhibitory sample (containing 7·3 mg. total fatty acid/100 ml.) derived from the β-position of biliary lecithin by the action of pig pancreatic phospholipase A*

	Total fatty acids (mg./100 ml.)	
	18·2	7·3
Fatty acids	Individual fatty acids (mg./100 ml.)	
Myristic (14:0)	0·07	0·03
Pentadecylic (15:0)	0·04	0·01
Palmitic (16:0)	1·08	0·43
Palmitoleic (16:1)	0·22	0·09
— (17:0 br)	0·09	0·04
Margaric (17:0)	0·05	0·02
Stearic (18:0)	0·67	0·27
Oleic (18:1)	5·56	2·23
Linoleic (18:2)	6·98	2·80
Linolenic (18:3)	0·42	0·17
Arachidonic (20:4)	2·72	1·09

Experiments with samples of intestinal contents of differing inhibitory activities

An attempt was made to correlate the different inhibitory activities of various samples of intestinal contents with the concentrations of inhibitory factors present in each sample. Three samples of intestinal contents were chosen for this investigation. Pooled sample no. 2 (high inhibitory activity) was obtained from 4 pigs from which food had been withheld for 24 hr before slaughter; the inhibitory activity of sample no. 2 was retained even after a 25-fold dilution of the intestinal contents. Pooled sample no. 3 (medium inhibitory activity) was obtained from four pigs from which food had been withheld for 21 hr before slaughter; the inhibitory activity of sample no. 3 was lost after a 2-fold dilution of the intestinal contents. Sample no. 4 possessed no antibacterial activity and was obtained from one pig killed 4 hr after being given food; inhibitory activity was detected in sample no. 4 after a 2·5-fold concentration of the intestinal contents.

The lipid compositions of the three samples of intestinal contents are shown in Table 7. It may be seen that the total lipid content of the dry material was greatest in the sample with high antibacterial activity and lowest in the sample with no antibacterial activity. In the sample of high antibacterial activity the unesterified fatty acid content was about 5 times greater than that of the sample with no antibacterial

activity. The proportion of lysolecithin in the total phospholipids of the sample with high activity was considerably greater than in the phospholipids of the samples with medium or no activity.

Table 7. *Lipid compositions of samples of pig intestinal contents with different inhibitory activities against Clostridium welchii* NCTC *8246*

	Sample no. 2* (high activity)	Sample no. 3† (medium activity)	Sample no. 4‡ (no activity)
	Lipid composition (g./100 g. dry material)		
Total lipid	10·8	7·70	3·03
Neutral lipid	3·81	3·63	1·33
Unesterified fatty acids	6·10	3·41	1·25
Total phospholipids	0·893	0·665	0·448
	Phospholipid composition (g./100 g. total phospholipid)		
Component A	25·9	27·3	27·7
Phosphatidyl ethanolamine	18·5	19·8	22·3
Lysophosphatidyl ethanolamine	6·6	7·5	2·2
Lecithin	17·0	21·0	19·2
Sphingomyelin	9·0	11·0	16·4
Lysolecithin	23·0	13·3	12·1

* Sample no. 2, a pool from 4 pigs having had food withheld for 24 hr before slaughter.
† Sample no. 3, a pool from 4 pigs having had food withheld for 21 hr before slaughter.
‡ Sample no. 4, obtained from one pig killed 4 hr after being given food.

Table 8. *Compositions of the unesterified fatty acids and concentrations of each unesterified fatty acid in the fluid phase of pig intestinal contents of different inhibitory activities against Clostridium welchii* NCTC *8246*

	Sample no. 2 (high activity)		Sample no. 3 (medium activity)		Sample no. 4 (no activity)	
Fatty acid	Fatty acid in intestinal fluid %	(mg./100 ml.)	Fatty acid in intestinal fluid %	(mg./100 ml.)	Fatty acid in intestinal fluid %	(mg./100 ml.)
Myristic (14:0)	0·6	2·2	0·6	1·1	0·7	0·5
Pentadecylic (15:0)	0·3	1·1	0·7	1·2	1·1	0·7
Palmitoleic (16:1)	1·9	7·0	1·7	3·0	2·3	1·4
Palmitic (16:0)	20·4	75·5	28·4	49·7	36·3	23·7
— (17:0 br)	0·5	1·9	0·7	1·2	1·1	0·7
Margaric (17:0)	1·4	5·2	1·6	2·8	1·9	1·2
Stearic (18:0)	15·4	57·0	16·3	28·5	12·4	8·1
Oleic (18:1)	21·5	79·5	18·0	31·5	32·3	21·1
Linoleic (18:2)	28·6	106·0	26·1	45·7	8·3	5·4
Linolenic (18:3)	2·0	7·4	2·4	4·2	1·3	0·9
Arachidonic (20:4)	6·1	22·6	2·4	4·2	1·1	0·7
Total		370·0		175·0		65·2

The compositions of the unesterified fatty acids obtained from the samples of intestinal contents are given in Table 8, together with the calculated concentrations of the individual fatty acids in the intestinal fluid. In the sample of high activity (no. 2) the concentrations of linoleic and arachidonic acids in the intestinal fluid exceeded the respective minimum inhibitory concentrations of these two fatty acids (see Table 4).

In the sample of medium activity (no. 3) the concentration of linoleic acid in the intestinal fluid exceeded the minimum inhibitory concentration of this fatty acid. The respective minimum inhibitory concentrations were not exceeded either by the concentrations of linoleic or arachidonic acids in the intestinal fluid of the sample with no antibacterial activity. Dilution experiments with the total unesterified fatty acid fraction obtained from sample no. 2 showed that activity was lost when the concentration of unesterified fatty acids was reduced from 23·1 to 11·5 mg./100 ml. (Table 9). This decrease in concentration of total unesterified fatty acids corresponded

Table 9. *Effects of concentration of the unesterified fatty acid fractions, obtained from pig intestinal contents of different activities, on the inhibitory activities against Clostridium welchii* NCTC *8246*

	Concentration of total unesterified fatty acids (mg./100 ml.)	Recorded antibacterial activity	Calculated concentrations (mg./100 ml.)	
			Linoleic acid	Arachidonic acid
Sample no. 2 (high activity)	370	+	106*	22·6*
	185	+	53*	11·3*
	92·5	+	26·5*	5·6
	46·2	+	13·2*	2·8
	23·1	+	6·6*	1·4
	11·5	−	3·3	0·7
Sample no. 3 (medium activity)	175	+	45·7*	4·2
	87·5	+	22·8*	2·1
	43·7	+	11·4*	1·1
	21·8	−	5·6	0·6
	10·9	−	2·8	0·3
	5·4	−	1·4	0·1
Sample no. 4 (no activity)	65·2	−	5·4	0·7
	81·5	−	6·8*	0·9
	163	+	13·5*	1·8
	326	+	27·1*	3·6

* Greater than the minimum inhibitory concentration (see Table 4).

to a decrease in concentration of linoleic acid from 6·6 to 3·3 mg./100 ml. Part of the antibacterial activity of the unesterified fatty acids at concentrations above 92 mg./100 ml. would be due to arachidonic acid. Dilution of the unesterified fatty acid fraction obtained from sample no. 3 resulted in the loss of inhibitory activity when the concentration of unesterified fatty acids was decreased from 43·7 to 21·8 mg./100 ml., i.e. a decrease in the concentration of linoleic acid from 11·4 to 5·7 mg./100 ml. As expected, the total unesterified fatty acids obtained from sample no. 4 were not active when tested at the concentration at which they occurred in the intestinal fluid (65·2 mg./100 ml.). However, antibacterial activity was observed when the concentration of total unesterified fatty acids was increased to 163 mg./100 ml. At this concentration of total unesterified fatty acids the minimum inhibitory concentration of linoleic acid was exceeded. The calculated concentrations of the individual phospholipids in the intestinal fluid of the three samples are given in Table 10. The minimum inhibitory concentration of lysolecithin was considerably less than the concentration of lysolecithin in the intestinal fluid of the sample with high activity, but was about

the same as the concentration of lysolecithin in the intestinal fluid of the sample with medium activity. The concentration of lysolecithin in the intestinal fluid of the sample with no antibacterial activity was below the minimum inhibitory concentration of lysolecithin. The results of dilution experiments (Table 11) show that antibacterial activity was retained when the concentration of the total phospholipids obtained from sample no. 2 was reduced to 13·5 mg/100 ml. At this concentration of total phospholipids, the calculated concentration of lysolecithin was somewhat below the minimum inhibitory concentration of lysolecithin. However, the unidentified phospholipid would also contribute to the inhibitory activity of the total phospholipid fraction. Experiments with sample no. 3 showed that the loss of inhibitory activity that was observed on diluting the total phospholipids to 17·0 mg./100 ml. corresponded with a decrease in the calculated concentration of lysolecithin to 2·3 mg./100 ml. When the concentration of the total phospholipids obtained from sample no. 4 was increased, antibacterial activity was observed at 58·5 mg./100 ml. At this concentration of total phospholipids the calculated concentration of lysolecithin was greater than the minimum inhibitory concentration of lysolecithin.

Table 10. *Concentrations of individual phospholipids in the fluid phase of pig intestinal contents of different inhibitory activities against Clostridium welchii* NCTC *8246*

	Sample no. 2 (high activity)	Sample no. 3 (medium activity)	Sample no. 4 (no activity)
	Concentration (mg./100 ml.)		
Total phospholipids	54·2	34·1	23·4
Component A	14·0	9·3	6·5
Pnosphatidyl ethanolamine	10·0	6·8	5·2
Lysophosphatidyl ethanolamine	3·6	2·6	0·5
Lecithin	9·2	7·2	4·5
Sphingomyelin	4·9	3·8	3·8
Lysolecithin	12·5	4·5	2·8

Table 11. *Effect of concentration of phospholipids from pig intestinal samples 2, 3 and 4 on their inhibitory activities against Clostridium welchii* NCTC *8246*

	Concentration of total phospholipids (mg./100 ml.)	Recorded antibacterial activity	Calculated concentration of lysolecithin (mg./100 ml.)
Sample no. 2 (high activity)	54·2	+	12·5
	27·1	+	6·3
	13·5	+	3·1
	6·7	−	1·6
	3·3	−	0·8
	1·6	−	0·4
Sample no. 3 (medium activity)	34·1	+	4·5
	17·0	−	2·3
	8·5	−	1·1
Sample no. 4 (no activity)	23·4	−	2·8
	29·2	−	3·6
	58·5	+	7·3
	117·0	+	14·7

The fatty acid composition of the lipids isolated from Clostridium welchii NCTC *8246*

In view of the inhibitory action of linoleic, linolenic and arachidonic acids on the growth of *Clostridium welchii* NCTC 8246 it was of interest to determine the composition of the fatty acids synthesized by this organism when grown in a fat-free medium. *C. welchii*, NCTC 8246, was therefore grown in the defined medium of Boyd, Logan & Tytell (1948). The organisms were harvested by centrifugation, washed twice with 0·85% (w/v) sodium chloride solution and freeze-dried. The lipids were extracted from the organisms and the composition of the constituent fatty acids determined. The fatty acids listed in Table 12 amounted to about 94% of those present. The remaining 6% was accounted for by small amounts of several unidentified acids. The results in Table 12 show that saturated acids comprised over 80% of the fatty acids of *C. welchii*. An octadecadienoic acid was present in very low concentration (0·6%) but we have no evidence that this fatty acid was linoleic acid, i.e. $\Delta^{9,12}$ octadecadienoic acid. Oxidative degradation studies were not possible on the small amounts of octadecadienoic acid that could be isolated from the lipids obtained from the organism.

Table 12. *Composition of total fatty acids obtained from Clostridium welchii, type A,* NCTC *8246*

Fatty acid	%	Fatty acid	%
Lauric (12:0)	21·4	Linoleic (18:2)	0·6
Myristic (14:0)	18·6	— (19:1)	1·3
Palmitic (16:0)	12·6	Arachidic (20:0)	14·2
Palmitoleic (16:1)	1·3	— (20:1)	2·6
Stearic (18:0)	14·8	— (21:1)	2·9
Oleic (18:1)	3·3		

DISCUSSION

The results of the work now reported indicate that the antibacterial activity of the contents of the small intestine of pigs may be attributed to the presence of unesterified fatty acids, lysolecithin and an unidentified phospholipid. It has been well established that unsaturated fatty acids exert an antibacterial influence on Gram-positive micro-organisms. The subject was reviewed by Nieman (1954), who concluded that the inhibitory effects of unsaturated fatty acids increased as the number of double bonds in the molecule increased. Our findings (Table 4) support this conclusion in that the inhibitory effect of linoleic acid (18:2) was far greater than that of oleic acid (18:1). However, the minimum inhibitory concentration of arachidonic acid (20:4) was about the same as that of linoleic acid, whereas the minimum inhibitory concentration of linolenic acid (18:3) was somewhat greater than that of linoleic acid (Table 4). The mechanism of the antibacterial effect of unsaturated fatty acids has yet to be elucidated. As suggested by Kodicek & Worden (1945) the unsaturated fatty acids could form an adsorption layer around the bacterium and inhibit the absorption of essential nutrients. On the other hand, the unsaturated fatty acids might enter the bacterial cell and inhibit some essential metabolic process. In this respect it should be noted that unsaturated fatty acids do not appear to be synthesized to any extent by *Clostridium welchii* (Table 12). The results in Table 12 are in fair agreement with those of MacFarlane

(1962), who found that the fatty acids of *C. welchii* type A grown in a medium containing 3·0% peptone, 0·5% glucose and 0·45% sodium chloride were predominantly saturated and consisted mainly of lauric (12:0) and arachidic (20:0) acids.

Lysolecithin has been isolated from the contents of the small intestine of man (Borgström, 1957) and of sheep (Lennox, Lough & Garton, 1965). Hoffman (1961) and Lennox *et al.* (1965) suggested that lysolecithin, in addition to bile acids, facilitates the absorption of fat by forming soluble micelles with fatty acids and β-monoglycerides in the intestinal lumen. As far as we are aware, the only previous report drawing attention to the antibacterial properties of lysolecithin is that of Trager (1948), who found that the growth of *Lactobacillus casei* in a medium that contained suboptimal amounts of biotin was prevented by the presence of low concentrations of lysolecithin. At present it is difficult to put forward any suggestion about the nature or origin of the unidentified phospholipid component A that we found to inhibit the growth of *Clostridium welchii*.

It seems reasonable to contend that the lysolecithin present in the contents of the small intestine is derived from the hydrolytic cleavage of lecithin secreted in the bile. This view is supported by the similarity between the fatty acid composition of the lysolecithin isolated from the intestinal contents and that of the lysolecithin prepared from the lecithin isolated from pig bile (Table 5). The biliary lecithin is presumably hydrolysed in the lumen of the small intestine by the action of the phospholipase A which is secreted by the pancreas. The results in Table 5 show that the fatty acids liberated from the β-position of the biliary lecithin by phospholipase A were particularly rich in linoleic acid. This liberation of linoleic acid from the β-position of the biliary lecithin could account for the relatively high concentrations of linoleic acid observed in the unesterified fatty acid fraction of the contents of the small intestine of pigs which had not received any food for 20–24 hr. However, the fatty acids liberated from the α and α' positions of dietary or endogenous triglycerides by the hydrolytic action of pancreatic lipase might also contribute to the unesterified fatty acid fraction of the contents of the small intestine. Inhibitory activity was thus absent from the contents of the stomach, caecum and large intestine since little or no hydrolysis of lipids occurs in the stomach and most of the products of lipolysis are absorbed before the digesta reaches the caecum. Bernhart *et al.* (1952) showed that the inclusion of soya lecithin in the diet of rats decreased the numbers of *Clostridium welchii* in the faeces.

It is not clear why the concentration of inhibitory lipids in the contents of the small intestine increases as the time after feeding increases. Moore (1952) showed that the rate of passage of digesta from the stomach to the small intestine of the pig decreased very markedly between 4 and 6 hr after feeding. If there were no such decrease in the rate of secretion of bile during this period then there would be an increase in the concentrations of lipids of biliary origin in the fluid phase of the intestinal contents. This might explain why inhibitory activity is first detected in the contents of the small intestine 6 hr after feeding. Consistent with our findings are those of Smith (1961), who observed that it was possible to increase the numbers of *Clostridium welchii* in the intestinal contents of pigs by inducing the animals to consume large quantities of food after a period of enforced starvation. Subsequent periods of starvation resulted in a rapid decrease in the counts of *C. welchii* in the intestinal contents. Our results show that even under the conditions of normal feeding practice

there is a period during each day when the contents of the small intestine are inhibitory for *C. welchii*. It is thus tempting to suggest that the production of inhibitory lipids in the contents of the small intestine constitutes a mechanism whereby the numbers of *C. welchii* type A, the normal type in the alimentary tract of the pig, are controlled.

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Observations on the Weissensee G Strain of *Corynebacterium diphtheriae*

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SUMMARY

From a Park-Williams No. 8 strain (Weissensee G) of *Corynebacterium diphtheriae*, recently reported by Rajadhyaksha & Rao (1965) to be toxinogenic but *non*-lysogenic, two diphtherial strains have been isolated: 1, a typical phage-resistant, slow-growing, toxinogenic PW8 strain; 2, a fast-growing non-toxinogenic strain that is sensitive to diphtherial phages.

INTRODUCTION

The lysogenic conversion of sensitive, non-toxinogenic diphtheria bacilli to lysogeny and toxinogeny by the temperate bacteriophage β was first demonstrated by Freeman (1951). Since that time, there has accumulated a considerable body of evidence which suggests that the capacity of a given strain to produce the diphtheria toxin protein depends upon the phage genome (Groman, 1955; Groman & Eaton, 1955; Barksdale, 1959). However, expression of the toxinogenic character also depends upon certain peculiarities in host cell metabolism and the mere presence of the phage genome does not suffice (Miller, Pappenheimer & Doolittle, 1966). It still remains uncertain whether or not the β-phage DNA codes directly for the toxin protein.

Recently, Rajadhyaksha & Rao (1965), working with a variant of the classic PW8 strain (Weissensee G) reported, in this *Journal*, experiments which they interpreted as indicating that toxinogenicity depends upon an episomal cytoplasmic host factor, T^+. They suggested that the role of phage is merely to facilitate entry, by transduction, of the T^+ factor into sensitive T^- cells. According to the Indian workers, it is possible to remove the phage genome from lysogenic bacteria by treatment with low concentrations of acriflavine, without altering their capacity to produce toxin. They report that their Weissensee G strain is non-lysogenic and sensitive to phage despite its capacity to synthesize toxin in high yield.

Because of the important implications of these unexpected findings and because recent studies with our stock PW8 strain (Miller *et al.* 1966) are not in agreement with Rajadhyaksha & Rao, we decided to compare the Weissensee G strain with our own PW8 strain.

RESULTS AND CONCLUSIONS

A filter paper strip containing the dried Weissensee G organisms was kindly sent us by Dr S. S. Rao of the Haffkine Institute, Bombay. It was placed aseptically in a flask containing Casamino acid medium (Miller *et al.* 1966) and incubated overnight at 35° on a rotary shaker. The following day, the culture was streaked on chocolate

agar plates. After 48 hr of incubation, most of the colonies that grew out were tiny and resembled closely colonies of our own PW8 strain. There were, however, a few large colonies. After several subcultures and replatings, two distinct strains of diphtheria bacilli were separated from the Weissensee G specimen. The first gave small colonies on chocolate agar, grew slowly in liquid medium, did not require tryptophan for growth, was phage resistant and produced high yields of toxin. The second gave large colonies, grew rapidly on liquid medium containing tryptophan, was sensitive to phage β and its mutants and was non-toxinogenic. Table 1 summarizes the properties of the two strains separated from the Weissensee G culture and compares them with three stock strains from our own laboratory.

Table 1. *Properties of certain strains of Corynebacterium diphtheriae*

	Strains				
	PW8 (Weissensee G)				
Property			PW8*	P60 (β)*	C7 (β)*
Colony size (chocolate agar)	Small	Large	Small	Large	Large
Generation time (35°)	160 min.	60 min.	160 min.	60 min.	60 min.
Tryptophan requirement	−	+	−	+	+
Sensitivity to phages B and β	−	+	−	−	+
Toxin production (Lf/ml.)	$\geqslant 80$	0	80–130	15–20	0
Yield of β-phage particles/10^8 u.v.-irradiated organisms	1–2	0	*ca.* 5	*ca.* 10^9	0
Q_{O_2}† (mm.^{3}/mg./hr): Succinate	Not done	86	15–20	15–20	85–100
Glucose	Not done	58	15–20	15–20	40–60
Ethanol	Not done	104	35–40	35–40	100–150

* Strains marked with an asterisk are stock strains from this laboratory. The terminology is that of Miller *et al.* (1966). Strain P60(β) was formerly called PW8 (P) (Barksdale, 1959; Pappenheimer *et al.* 1962; Rajadhyaksha & Rao, 1965). Miller *et al.* have shown that the phage P is indistinguishable from β in host range, serological and other properties.

† See Pappenheimer *et al.* (1962).

The possibility, suggested by Rajadhyaksha & Rao (1965), that toxin production by diphtheria bacilli depends upon host cytoplasmic particles which are not controlled by the phage genome, cannot be rigorously excluded, as was indeed pointed out several years ago by Jacob & Wollman (1961). Nevertheless, evidence obtained in many laboratories makes such a possibility appear extremely unlikely. In any case, the results reported by the Indian workers cannot be considered as evidence since all of their observations can be readily explained by the presence of phage-sensitive fast-growing bacteria in their PW8 culture. On rich media such as are used to obtain phage plaques, the fast-growing sensitive strain would often predominate, thus accounting for the apparent phage sensitivity of the Weissensee G strain. Since PW8 strains do not absorb phage (Miller *et al.* 1966), only the fast-growing strain would be affected by treatment with β-phage and would be converted to a lysogenic strain similar to C7(β) which is fast-growing and gives low yields of toxin in comparison with PW8. Since the PW8 strains do not release phage particles except after induction with ultraviolet (u.v.) radiation (and then *less* than one β-particle/10^7 irradiated bacteria), a mixed culture of a few sensitive bacteria together with PW8 bacteria might be maintained through many transfers without conversion of the sensitive strain to lysogeny.

The findings reported by Rajadhyaksha & Rao (1965) are not in agreement with those of Saragea & Maximesco (1964), who included the Weissensee G strain in their survey of many diphtherial strains. In their hands, this PW8 variant was resistant to phages and yielded a few phage particles after u.v.-irradiation. Our own results with the small colonies isolated from the strain received from the Haffkine Institute agree with those of Saragea & Maximesco.

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Methionine synthesis in *Proteus mirabilis*

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SUMMARY

Sixty different isolates of methionineless auxotrophs of *Proteus mirabilis* were arranged in nine biochemical groups according to their growth responses to methionine or its precursors. These requirements suggested that *P. mirabilis* possesses a route for methionine biosynthesis which is similar to the pathway operating in *Escherichia coli* and *Salmonella typhimurium*. In contrast to findings with the latter organisms syntrophism was not observed between these mutants of *P. mirabilis* even with sonically disrupted potential feeder strains. Two methionineless auxotrophs of *E. coli* fed auxotrophs of *P. mirabilis* which had metabolic blocks earlier in this pathway. These results, which suggested an inability of methionineless auxotrophs of *P. mirabilis* to accumulate precursors of metabolic blocks, were confirmed by a quantitative comparison of methionine precursors in wild-type and mutant strains of *P. mirabilis* and *E. coli*. The presence of *S*-methylcysteine (SMC) was demonstrated in wild-type and methionineless auxotrophs of *P. mirabilis* and *E. coli*. The growth responses of methionineless auxotrophs of *P. mirabilis* to SMC supported a hypothesis for the participation of this amino acid in the synthesis of methionine via an alternative route.

INTRODUCTION

Syntrophism amongst methionineless auxotrophs of bacteria has been used to study the sequence of intermediates in the synthesis of methionine. The reports of Lampen, Roepke & Jones (1947) and of Davis & Mingioli (1950) dealt with such mutants of *Escherichia coli*; syntrophism occurred between auxotrophs with different nutrient requirements. Demerec *et al.* (1955) and Smith (1961) demonstrated syntrophism amongst different phenotypic groups of methionineless auxotrophs of *Salmonella typhimurium*. Clowes (1958) reported syntrophism among cysteineless mutants of *S. typhimurium*. Figure 1*a* represents the pathway of methionine formation by micro-organisms as revealed by the syntrophism tests of the above workers. It proceeds via cysteine and *O*-succinylhomoserine to form cystathionine, which is cleaved to homocysteine and then methylated to methionine. A second pathway was demonstrated by Wiebers & Garner (1963) who isolated from *Neurospora crassa* an enzyme which was capable of forming homocysteine from homoserine and H_2S. Ragland & Liverman (1956) suggested a third route, namely a transthiomethylation of the thiomethyl group of *S*-methylcysteine (SMC) to homoserine to yield methionine (Fig. 1*b*). These authors isolated SMC from extracts of *N. crassa* and reported that SMC could serve as sole sulphur source for certain strains. The results of Wiebers & Garner (1964) indicated the participation of SMC in the biosynthesis of methionine in

N. crassa by a pathway which excludes cysteine. The same may be true for yeasts (Maw, 1961). Wolff, Black & Downey (1956) showed the synthesis of SMC in bakers yeast. Roberts *et al.* (1955) reported that SMC could be utilized by *E. coli* in isotope competition experiments and also by a cysteine-requiring mutant of this organism.

The present paper deals with the pathway for methionine synthesis in *Proteus mirabilis*, the absence of syntrophism among methionineless mutants of *P. mirabilis* and the presence, in *P. mirabilis* and in *Escherichia coli*, of *S*-methylcysteine which had not previously been shown to occur in bacteria.

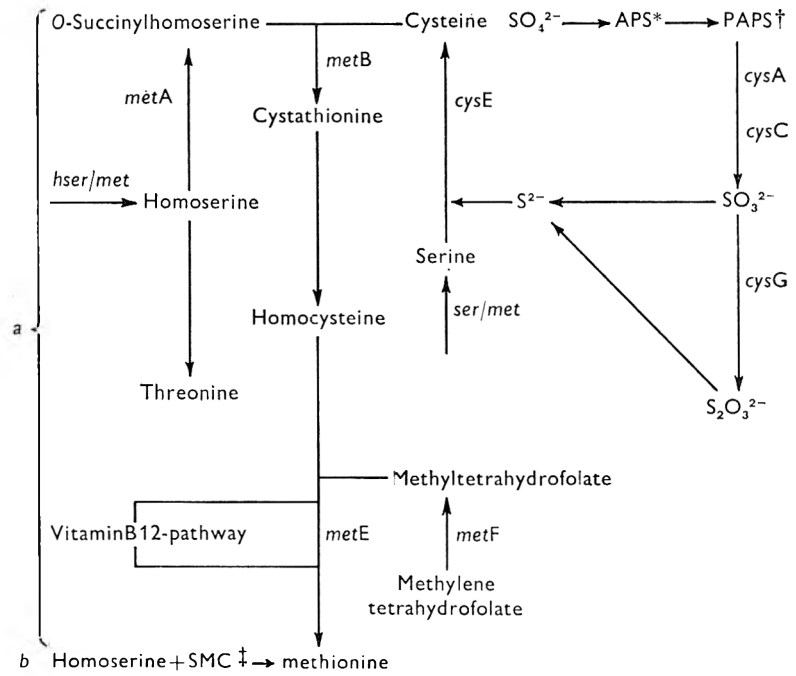

Fig. 1. Two possible pathways of methionine synthesis. *a*, Classical pathway; *b*, alternate pathway. * Adenosine-5′-sulphatophosphate; † 3′-phospho-adenosine-5′-sulphatophosphate; ‡ *S*-methylcysteine. *met*F, *cys*E, *hser/met*, etc., indicate metabolic blocks.

METHODS

Media. The minimal medium was a modification of that of Davis & Mingioli (1950) (g./l.): K_2HPO_4, 10·5; KH_2PO_4, 4·5; Na_3-citrate.$2H_2O$, 0·47; $(NH_4)_2SO_4$, 1·0; $MgSO_4.7H_2O$, 0·102; glucose (autoclaved separately), 2·5; agar 17·5; distilled water 1 l. The pH of this medium was 7·2. For the growth of *Proteus mirabilis* strains nicotinic acid (7·5 μg./ml., membrane filtered) was added. Top-layer agar contained 0·7% (w/v) agar. The sulphur-free medium had $(NH_4)_2SO_4$ and $MgSO_4.7H_2O$ replaced by equivalent amounts of NH_4Cl and $MgCl_2.6H_2O$, respectively.

Chemicals. Carrier-free ^{35}S-sulphate in aqueous solution (pH 6·5–7·5) was obtained from the Radiochemical Centre, Amersham, Buckinghamshire, England, and amino acids from the Nutritional Biochemicals Corporation, Cleveland, Ohio, U.S.A. Analytical grade sulphate, sulphite, thiosulphate and sulphide were used to test growth responses to inorganic precursors of cysteine (Postgate, 1963).

Organisms. Mutants of *Proteus mirabilis* strain 13 (Coetzee & Sacks, 1960) were obtained by treatment with ultraviolet radiation, manganese chloride or hydrogen peroxide (Demerec, Bertani & Flint, 1951). The penicillin method of Lederberg & Zinder (1948) and Davis (1948) was used to select auxotrophic mutants which were isolated by the replica-plating technique of Lederberg & Lederberg (1952). For the isolation of methionineless mutants of *Escherichia coli* strain CA-7 (obtained from Professor Fredericq, Liège) the more selective method of Gorini & Kaufman (1960) was used. The nutritional requirements of mutants were determined auxanographically (Lederberg, 1946); sulphur sources were applied in drops containing 3 mg. S/ml. The abbreviations *met*F, *cys*E, *hser*/*met*, *etc.*, are used to indicate specific metabolic blocks in the methionine pathway (Sanderson & Demerec, 1965) as shown in Fig. 1 and correspond to the growth requirements listed in Table 1. Organisms were maintained on nutrient agar slopes at 4°.

Disruption of organisms. Extracts of organisms were prepared from suspensions (30 ml.; equiv. 500 mg. dry wt. bacteria) by ultrasonic treatment in a Raytheon model S 102A oscilator (9 kcyc./sec. for 2 hr).

Syntrophism. This was determined by the following techniques. (*a*) Parallel streaking (Demerec *et al.* 1955; Smith, 1961; Clowes, 1958). Parallel streaks of mutants were made about 2–3 mm. apart on minimal medium enriched with either methionine (2 μg./ml.) or 0·2% (w/v) Difco nutrient broth powder. (*b*) Auxanographic technique (Clowes, 1958). Liquid cultures of organisms grown with limiting amounts of methionine (2 μg./ml.) were centrifuged down and the supernatant fluids and the deposits (after ultrasonic treatment) were tested auxanographically for their ability to support growth of other mutants. Testing of disrupted cell material for feeding ability eliminated the possible impermeability of feeding strains for accumulated metabolites. (*c*) Replica plating (Smith, 1961). Inocula of 6 methionineless mutants were spread over well-separated areas of about 1 cm. diameter on each of a number of Difco SS agar plates (Difco MacConkey agar for *Escherichia coli*), over a template. After overnight incubation these plates were replicated (Lederberg & Lederberg, 1952) to single enriched minimal medium plates on which different methionineless auxotrophs were spread. Syntrophism was evident as haloes of growth round replicated areas. (*d*) Turbidimetric technique (Lampen *et al.* 1947; Davis & Mingioli, 1950). Overnight broth cultures of test strains were washed twice with saline and resuspended in the original volume of saline. Tubes containing 40 ml. enriched minimal medium were inoculated separately with 0·5 ml. of these washed suspensions. Similar tubes were inoculated with 0·25 ml. of washed suspensions of each of two different strains to yield a total inoculum volume of 0·5 ml., analogous to that of tubes with single inoculun. After 24 hr incubation the turbidity of cultures was compared. Syntrophism was indicated by mixed inocula yielding heavier growth than single inocula. (*e*) Colonial syntrophism (Demerec *et al.* 1955). Pairs of mutants were plated in proportions so that one would form a background growth on the surface of slightly enriched minimal medium and might feed the small number of organisms of the other mutant present in the mixture. Apart from the turbidimetric technique, syntrophism experiments were observed for at least 7 days. Incubation temperature was 37°.

Isolation of free amino acids. Strains were grown for 20 hr on a sulphur-free medium supplemented with ^{35}S-sulphate (0·5 μc./ml.) as the sole sulphur source. For methionineless mutants the medium was enriched with DL-methionine (7 μg./ml.). After

20 hr incubation, by which time all free sulphur-containing amino acids would be metabolized and replaced by intermediates containing the isotope (Cowie, Bolton & Sands, 1950; Roberts *et al.* 1955) the organisms were harvested and a 30 ml. suspension (equiv. 500 mg. dry wt. bacteria) prepared in distilled water. Bacterial concentrations were also estimated by measuring the extinction at 630 mμ (E_{630}) and by determination of the protein content of disrupted extracts by the biuret method of Gornall, Bardawill & David (1949) with bovine albumin as standard. Disrupted cell material was centrifuged at 151,000 *g* in a Spinco Model L ultracentrifuge for 30 min. Proteins and peptides in the supernatant fluid were precipitated with a final concentration of 10% perchloric acid and centrifuged. The supernatant fluid was neutralized with 5 N-KOH followed by centrifugation and filtration. The filtrate was passed through a column (25 × 1 cm.) of cation exchange resin (Amberlite IR 120, H^+ form) followed by 100 ml. water to remove carbohydrates, salts, inorganic sulphur precursors of cysteine and excess ^{35}S-sulphate. The amino acids were then eluted with N-NH_4OH (50 ml.), concentrated to dryness under vacuum and one half of the material oxidized with performic acid (Leggett Bailey, 1962; Moore, 1963). Unoxidized sulphur-containing amino acids possess different R_F values than the oxidized derivatives, and experiments were done with both types of preparation (Wiebers & Garner, 1964; Roberts *et al.* 1955).

Chromatography of amino acid mixtures. Two-dimensional chromatograms on Whatman no. 3 MM paper were developed for 16 hr by the descending technique. The solvents used were: first dimension, *n*-butanol + formic acid + water (77 + 10 + 13, by vol.); second dimension, *n*-butanol + pyridine + water (1 + 1 + 1, by vol.). Amino acids were located by dipping into 0·2% (w/v) ninhydrin in acetone and heating at 100° for 5 min. The identity of amino acids was confirmed by paper electrophoresis, thin-layer chromatography and auxanography, using samples eluted from uncoloured chromatograms. Paper electrophoresis was done on Whatman no. 3 MM paper with a pH 1·85 buffer consisting of 2·5% (v/v) formic acid + 7·8% (v/v) acetic acid (Leggett Bailey, 1962). Thin-layer chromatography was done on activated silica-gel G layers according to Randerath (1964). For auxanographic identification crops of eluates from paper chromatograms were spotted on minimal medium plates seeded with auxotrophs with known nutrient requirements in top layers. Positive results were obtained after 24–48 hr incubation.

Quantitative comparison of sulphur-containing amino acids. The radio-activity of the sulphur-containing amino acids was determined by counting rectangular pieces (0·5 × 2·0 cm.) of the two-dimensional chromatograms in a Packard Model 3003 tricarb liquid scintillation spectrometer. The efficiency of the counter for ^{35}S was 62% and sufficient counts were recorded to give a statistical error of less than 2%. Counts were corrected for isotopic decay. The scintillation fluid was 0·4% (w/v) *p*-terphenyl + 0·04% (w/v) 1,4-*bis*-(4-methyl-5-phenyloxazol-2-yl)-benzene in toluene, + 1,4-dioxane (7 + 3, by vol.). Similar determinations were carried out after separation and identification by paper electrophoresis. The radioactivity of the sulphur-containing amino acids/mg. dry wt. organisms grown on ^{35}S-sulphate was used as standard for comparison of the amounts of these amino acids in mutant and wild-type strains of *Proteus mirabilis* and *Escherichia coli*. These estimates are minimal since no attempt was made to compensate for excretion of accumulated metabolites during growth or losses during isolation and chromatography.

RESULTS

Methionineless auxotrophs. Fifty-two isolation experiments yielded 2066 auxotrophic strains of *Proteus mirabilis* of which the largest group, 380, required methionine for growth; of these, auxotrophs responding to cysteine were especially abundant. These findings are analogous to those of Horowitz (1955) with mutants of *Neurospora crassa.* About 50% of our methionineless proteus auxotrophs were leaky. A final group of 60 auxotrophs was chosen on the basis of one phenotypically distinguishable type per isolation experiment. Mutant *ser*/*met*-544 was leaky but is included as the only representative of its type. The growth responses shown in Table 1 indicate that these

Table 1. *Grouping and growth requirements of methionineless auxotrophs of Proteus mirabilis*

Growth requirements were determined by applying possible precursors in drops of appropriate concentration on minimal medium containing auxotrophs in a top layer.

Auxotroph group	Growth requirements									
	SO_3^{2-}	$S_2O_3^{2-}$	S^{2-}	Ser	Cys	Hser	Cyst	Hcys	B12	Met
*met*F	−	−	−	−	−	−	−	−	−	+
*met*E	−	−	−	−	−	−	−	−	+	+
*met*A/B	−	−	−	−	−	−	+	+	−	+
hser/*met*	−	−	−	−	−	+	+	+	−	+
*cys*E	−	−	−	−	+	−	+	+	−	+
ser/*met*	−	−	−	+	+	−	+	+	−	+
*cys*G	−	+	+	−	+	−	+	+	−	+
*cys*C	+	+	+	−	+	−	+	+	−	+
*cys*A	+	−	+	−	+	−	+	+	−	+

Cys, Cysteine; Cyst, cystathionine; Hcys, homocysteine; Hser, homoserine; Met, methionine; Ser, serine; B12, vitamin B12; +, growth equivalent to that with methionine as supplement; −, no growth.

Table 2. *Methionineless strains of Proteus mirabilis*

Auxotrophic mutants were obtained by treatment of the wild-type strain with mutagenic agents, selected with penicillin and isolated by replica-plating. Sixty auxotrophs were chosen on the basis of one phenotypically distinguishable type per isolation experiment. The growth requirements for each group are listed in Table 1.

Auxotroph group	Auxotrophic strains
*met*F	286, 291, 974, 1445, 1710.
*met*E	244, 380, 452, 505, 572, 687, 855, 933, 1001, 1261.
*met*A/B	253, 1303, 1599.
hser/*met*	1430.
*cys*E	329, 448, 554, 663, 699, 811, 846, 1002, 1602, 1638.
ser/*met*	544 (leaky).
*cys*G	243, 306, 373, 435, 470, 523, 545, 576, 756, 845, 921, 1080, 1114, 1174, 1262, 1648, 1795, 1803.
*cys*C	484, 666, 697, 766, 865, 1148, 1796.
*cys*A	354, 832, 932, 1211.

mutants could be separated into nine biochemical groups. The nomenclature of each group corresponds to the metabolic block indicated in Fig. 1. Auxotrophs responding to methionine, homocysteine, cystathionine or homoserine are referred to as *hser*/*met*

(Table 2). It is not known where the metabolic block in the homoserine pathway is since the growth response of these mutants to possible precursors of homoserine was not tested. The same holds for the *ser/met* mutant which responds to methionine, homocysteine, cystathionine, cysteine or serine (Table 2). The strains of *P. mirabilis* used in these experiments will be referred to as methionineless strains and they are listed in Table 2. All the auxotrophs in Table 2 except *hser/met*-1430 and *ser/met*-544, when supplemented with *S*-methylcysteine (SMC) in minimal medium, show growth equivalent to that with methionine. The two exceptions grew very poorly with SMC. Prototrophic strains of *P. mirabilis* and *Escherichia coli* utilized SMC as the only source of sulphur in accordance with the findings of Roberts *et al.* (1955) with *E. coli*. *Proteus mirabilis* *met*E-1001 was classified as *met*E because it responded either to methionine or to vitamin B12 but not to homocysteine. However, this mutant was also found to grow when supplemented with threonine. The growth response of the auxotrophs to the possible intermediate *O*-succinyl-homoserine (Rowbury, 1964) was not tested and consequently *met*A and *met*B mutants were classed together in group *met* A/B which responded, to cystathionine, homocysteine or methionine. All *met*E auxotrophs, except *met*E-505, attained complete growth after 24 hr when supplemented with vitamin B12. *Met*E-505 was fully grown, with vitamin B12, only after 48 hr. When supplemented with methionine the *met*E mutants like all methionine auxotrophs showed full growth after 24 hr. This difference in response to vitamin B12 was also noted by Davis & Mingioli (1950) with similar mutants of *E. coli*.

Two methionine-requiring auxotrophs of *Escherichia coli* strain CA-7 were isolated and designated *met*F-1, -2. In minimal medium these mutants responded only to methionine and, in contrast to the *met*F mutants of *P. mirabilis*, did not grow with SMC.

Syntrophism among methionineless mutants of Proteus mirabilis and Escherichia coli. All syntrophism techniques gave positive results with several biochemically different arginine-requiring mutants of *P. mirabilis* (Coetzee, 1965). These experiments served as controls for the syntrophism techniques used. No syntrophism was detected between pairs of methionineless mutants of *P. mirabilis*. The only exception was *met*E-1001 which was fed by all the methionineless mutants of *P. mirabilis* and by the two *met*F auxotrophs of *E. coli*. These methionineless strains of *P. mirabilis* and the two *met*F mutants of *E. coli* also fed both threonine-requiring and glycine-requiring mutants of *P. mirabilis*, and it is possible that the ability to feed *met*E-1001 may be due to this mutant's growth response to threonine. This behaviour of *met*E-1001 was not further investigated.

The absence of syntrophism among methionineless auxotrophs of *Proteus mirabilis* pointed to an inability of these mutants to accumulate the precursors of metabolic blocks in a methionine pathway. Since syntrophism is known to occur among methionine-requiring auxotrophs of *Escherichia coli* (Lampen *et al.* 1947; Davis & Mingioli, 1950) the two *met*F mutants of this organism were tested for their ability to feed methionineless mutants of *P. mirabilis*. *Met*F mutants of *E. coli* fed all the methionineless mutants of *P. mirabilis* except those of groups *met*F and *met*E.

Quantitative comparison of sulphur-containing amino acids in wild-type and methionineless auxotrophs of Proteus mirabilis and Escherichia coli. The estimates of sulphur-containing precursors of methionine in wild-type and some methionineless auxotrophs of *P. mirabilis* and *E. coli* are listed in Table 3. These quantitative estimates confirmed

the results of syntrophism tests. Although *met*F-1710 and *met*E-855 contain about three times as much homocysteine and *met*A/B-1599 contains almost twice as much cysteine as the wild-type organism, this small accumulation of precursors of metabolic blocks is possibly not enough to produce syntrophism. In contrast, *E. coli met*F-1 contained about forty times as much homocysteine and more than thirty times the cystathionine content of the wild-type strain. Similar results were obtained with all representatives of each group of *P. mirabilis* mutants. As would be expected from its early block, *P. mirabilis cys*C-697 only contained traces of sulphur-containing precursors of methionine.

Table 3. *Sulphur-content of amino-acid precursors of methionine in wild-type and methionineless auxotrophs of Proteus mirabilis and Escherichia coli*

Organisms were grown on sulphur-free medium containing limiting amounts of ^{35}S-sulphate as sole sulphur source, harvested after 20 hr and sonically disrupted. Free amino acids from equivalent amounts of organisms (equiv. 500 mg. dry wt.) were isolated and separated by paper chromatography. The radioactivity of each amino acid/mg. dry wt. bacteria was taken as quantitative standard of comparison. Counts were corrected for isotopic decay which ranged between 9 and 22% when the measurements were made.

	Sulphur source					
Organism	Methionine	Homo-cysteine	Cysta-thionine	Cysteine	SMC*	Total activity
	Radioactivity (counts/min./mg. dry wt. bacteria)					
Escherichia coli						
Wild type	136·0	124·0	96·0	128·0	118·0	602·0
*met*F-1	5·8	5800·0	3600·0	246·0	4400·0	14051·8
Proteus mirabilis						
Wild type	124·0	106·0	90·0	122·0	102·0	544·0
*met*F-1710	4·2	302·0	196·0	166·0	3·8	672·0
*met*E-855	3·6	294·0	192·0	158·0	3·8	651·4
*met*A/B-1599	3·8	3·4	2·8	214·0	4·2	228·2
*cys*C-697	4·0	3·6	3·0	3·8	3·2	17·6

* SMC, *S*-methylcysteine.

The chromatographic studies of methionine precursors led to the discovery of *S*-methylcysteine (SMC) in wild-type and mutant strains of *Proteus mirabilis* and *Escherichia coli* (Table 3). The identity of SMC was confirmed by two-dimensional paper chromatography, paper electrophoresis and thin-layer chromatography of oxidized and unoxidized samples of amino acids isolated from these organisms. SMC isolated from strains grown on ^{35}S-sulphate as sole sulphur source contained the ^{35}S isotope. Mutant strains of *P. mirabilis* which attained growth equivalent to that with methionine when supplemented with SMC contained negligible amounts of SMC themselves. The two *met*F mutants of *E. coli* which did not respond to SMC accumulated SMC in large quantities (Table 3); this suggested that they should feed *met*F and *met*E mutants of *P. mirabilis* which do respond to SMC. However, no such syntrophism was observed.

DISCUSSION

The growth requirements of the methionineless auxotrophs of *Proteus mirabilis* (Table 1) indicate that the route of methionine synthesis (Fig. 1*a*) is similar to that found in *Escherichia coli*, *Salmonella typhimurium* and *Neurospora crassa* (Meister, 1965; Cauthen, Foster & Woods, 1966). The absence of syntrophism between methionineless auxotrophs of *P. mirabilis* contrasts sharply with similar mutants of *E. coli* and *S. typhimurium*. According to growth requirements the *met*F, *met*E and *met*A/B mutants of *P. mirabilis* appear to be similar to auxotrophs of *S. typhimurium* with which Smith (1961) and Demerec *et al.* (1955) obtained syntrophism. Clowes (1958) showed cross-feeding with cysteineless mutants *cys*D, *cys*C, *cys*E and *cys*A of *S. typhimurium* whose growth requirements are much the same as the *cys*G, *cys*C, *cys*E and *cys*A mutants of *P. mirabilis*. Lampen *et al.* (1947) and Davis & Mingioli (1950) reported syntrophism between several methionineless strains of *E. coli* with similar blocks to the *P. mirabilis* strains studied here. The absence of syntrophism caused by impermeability of the cell membranes to intermediates in methionine synthesis was ruled out, furthermore the syntrophism techniques appeared to be reliable and all previous studies on methionine metabolism have indicated that these intermediates are stable. The explanation for this behaviour appears to lie in the inability of *P. mirabilis* mutants to accumulate sufficient quantities of methionine precursors to produce syntrophism. Evidence for this possibility was contributed by the finding that methionineless auxotrophs and wild-type strains of *P. mirabilis* contained equivalent amounts of methionine precursors. In contrast, a *met*F mutant of *E. coli* contained more than twenty times the methionine precursors than the wild-type strain.

The observation that all our mutants which require cysteine, homoserine and serine respond to methionine is noteworthy. This may mean that the methionine–cysteine pathway is reversible in *Proteus mirabilis* and that methionine can serve as a precursor for these amino acids. However, cysteineless mutants of *Escherichia coli* (Lampen *et al.* 1947) and *Salmonella typhimurium* (Demerec *et al.* 1955) were also found to respond to methionine although the pathway is unidirectional in these organisms (Delavier-Klutchko & Flavin, 1965). In contrast to reports on the latter two bacterial organisms the methionine–cysteine pathway is reversible in various fungi (Delavier-Klutchko & Flavin, 1965). The possible reversibility of this pathway in *P. mirabilis* has not been investigated but in the light of the above-mentioned reports on *E. coli* and *S. typhimurium* the growth response of cysteine, homoserine and serine requiring mutants to methionine does not provide any proof in this respect.

The quantitative estimates of *S*-methylcysteine (SMC) in methionineless auxotrophs of *Proteus mirabilis* and *Escherichia coli* are in agreement with the growth responses of these strains to SMC. The *met*F mutants of *E. coli* which do not grow with SMC accumulate it in large quantities. The *met*F mutants of *P. mirabilis* which do grow with SMC contain negligible amounts of this amino acid. This difference in the *met*F metabolic blocks of *P. mirabilis* and *E. coli* cannot be explained. Previous reports of the occurrence of SMC in micro-organisms have been confined to *N. crassa* and yeasts. SMC and its sulphoxide occur in the non-protein nitrogen fraction of various plants (Morris & Thompson, 1955; Synge & Wood, 1956; Thompson, Morris & Zacharius, 1956). Bolton, Cowie & Sands (1952) did not detect it in *E. coli* strain B,

nor did they find homocysteine and cystathionine, although this may be due to the severity of the hydrolytic techniques used. In the present investigation the organisms were not hydrolysed and only free amino acids were examined.

The inability of auxotrophs to accumulate precursors of metabolic blocks has not been reported for other amino acid synthetic pathways in micro-organisms. The existence of more than one pathway leading to the same end-product may possibly influence the accumulation of intermediates in metabolic routes, although this has not been reported. The present findings support the hypothesis that methionine may be synthesized by more than one pathway. Ragland & Liverman (1956) suggested that methionine was synthesized alternatively by the transthiomethylation of the thiomethyl group of SMC to homoserine. According to Wolff *et al.* (1956) SMC is synthesized from serine and methanethiol in yeasts. The restricted growth of the homoserine-requiring methionineless mutant of *Proteus mirabilis* (*hser*/*met*-1430) when supplemented with SMC in minimal medium supports these findings since this amino acid is a precursor of methionine in the pathway *via* SMC. The ability of *met*F, *met*E and *met*A/B mutants of *P. mirabilis* to grow with SMC also supports the participation of SMC in the formation of methionine *via* a route which excludes cysteine (see Fig. 1). These results agree with the findings of Wiebers & Garner (1964) who encountered a cystathionineless mutant of *Neurospora crassa* which responded to SMC.

Maw (1961) reported the ability of *S*-methylcysteine (SMC) to annul the inhibition of yeast growth by L-ethionine. This methionine analogue is a competitive antagonist of methionine in reactions subsequent to the synthesis of the latter, e.g. incorporation into protein (Gross & Tarver, 1955) and conversion to *S*-adenosylmethionine (Parks, 1958). According to Maw (1961) the apparent absence of SMC from proteins argues against the possibility that it may annul the action of ethionine by substituting for methionine in one or more reactions essential for growth which have been blocked by ethionine. It is also doubtful whether SMC is an intermediate in the major metabolic reactions of methionine, such as protein synthesis and *S*-adenosylmethionine formation. Maw (1961) concluded that the most tenable explanation is that SMC gives rise to methionine by a separate pathway unaffected by ethionine. Even if SMC participates in the synthesis of methionine by an alternative route, the formation of methionine remains obscure since Wiebers & Garner (1964) presented data which indicate that neither SMC nor cystathionine are obligate precursors for methionine in *Neurospora crassa*. They proposed that SMC and cystathionine contribute to sulphur metabolism in general as alternative sources of reduced sulphur, or function as regulatory compounds. The fact that conventional methionineless auxotrophs can be isolated suggests that methionine synthesis by an alternative route does not satisfy the requirements of the organism except when exogenous SMC is present. This may mean that methionine synthesis *via* SMC is in turn dependent on the synthesis of methionine by the classical pathway or on the presence of methionine or a source of methyl groups. The role of SMC in the synthesis of methionine by *P. mirabilis* and *E. coli* is at present being investigated. This may yield information to explain the failure of methionine auxotrophs of *P. mirabilis* to accumulate precursors of metabolic blocks.

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Incorporation of ^{3}H-Uridine into RNA during Cellular Slime Mould Development

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SUMMARY

Dictyostelium discoideum wild-type amoebae incorporate ^{3}H-uridine into RNA at a linear rate during the developmental sequence until the terminal stage of fruiting body construction. However, 3 aggregateless strains perform in similar fashion (except for the final decrease). A sensitive criterion of bacterial contamination, making use of MAK-column chromatography of the labelled RNA, is described.

INTRODUCTION

The RNA content of *Dictyostelium discoideum* vegetative amoebae is about 15% of the total dry weight and the absolute amount of RNA decreases during fruiting body construction in proportion to the overall loss of cell material (White & Sussman, 1961). Previous experiments involving pulse labelling with ^{3}H-uridine and ^{32}P-phosphate have shown that appreciable RNA synthesis occurs during at least part of the development of a mutant strain (FR-17) of *D. discoideum*. Sucrose density gradient centrifugation of the labelled material revealed it to be distributed primarily in 4, 16, and 23 S components, but also to a small extent in regions other than these (Sussman & Sussman, 1965; M. Sussman, 1966). The present communication indicates that, in the wild type, RNA synthesis as reflected by ^{3}H-uridine incorporation proceeds at a significant rate throughout morphogenesis and decreases to a low value only at the terminal stage of fruit construction. However, three aggregateless mutants of *D. discoideum* yielded incorporation patterns essentially like that of the wild type (except for the final decrease in rate). Thus, the gross aspect of RNA synthesis does not of itself accurately reflect developmental events though differential transcription assuredly does (Sussman & Sussman, 1965).

METHODS

Organisms and experimental conditions. Wild-type *Dictyostelium discoideum* strain NC-4 (haploid) and aggregateless mutant derivatives were grown in association with *Aerobacter aerogenes* on SM agar (Sussman & Lovgren, 1965). The amoebae were harvested at room temperature in SM broth and streptomycin sulphate (0·5 mg./ml.), spun at 1000 *g* for 2–3 min. and resuspended in the SM + streptomycin broth at a concentration of 2×10^{7} amoebae/ml. After incubation for 1 hr on a shaker at 22°, the amoebae were harvested by centrifugation, washed once with cold water and

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resuspended in water at 2×10^8 amoebae/ml. Samples (0·1 ml.) were dispensed on quarter pieces of 2 in. black Millipore filters, resting on absorbent pads saturated with 0·05 M-phosphate (pH 6·5) + streptomycin sulphate (0·5 mg./ml.) within 60 mm. Petri dishes and were incubated at 22° in a humid atmosphere. Under these conditions a very high degree of morphogenetic synchrony was achieved (Sussman & Lovgren, 1965).

Incorporation experiments. The amoebae were labelled by adding ^{3}H-uridine to the fluid saturating the support pads (4 mc./μmole; 5 μc./ml. pad fluid). After the desired periods of incorporation, the amoebae were harvested in cold water, spun at 7000 ***g*** for 5 min. and the pellets frozen. After thawing and brief homogenization with a Vortex mixer, 10% (w/v) trichloroacetic acid (TCA) was added along with 200 μg. DNA as a carrier. After 30 min. in the cold the precipitates were collected on Millipore filters, washed with cold 5% (w/v) TCA and placed in vials with a toluene base scintillation fluid for counting. A Nuclear-Chicago counter was used at an efficiency of 16%.

RNA fractionation. RNA was extracted from the harvested amoebae by treatment with 3% Duponol and purified by the method of Gierer & Schramm (1956). The material was then adsorbed to a methylated albumin + kieselguhr (MAK) column and eluted in a linear NaCl gradient (Mandell & Hershey, 1960). The extinction at 260 mμ (E_{260}) was monitored in a Gilford recording spectrophotometer and 1 ml. samples were precipitated in 10% TCA for counting.

RNAse controls. In some experiments duplicate TCA precipitates were suspended in 0·075 M-phosphate (pH 7·5) with 30 μg. of 2 × recrystallized pancreatic RNAse. After 30 min. at 37° the material was reprecipitated with TCA, 10% (w/v), washed, and counted. 80–90% of the originally precipitable counts were rendered soluble by this treatment.

RESULTS

Cumulative and pulsed uridine incorporation

Wild-type amoebae, treated as described in the Methods section, were dispensed on Millipore filters and incubated at 22° for 4 hr. The Millipore filters were then shifted to new support pads containing ^{3}H-uridine and the organisms harvested at 2 hr intervals thereafter to determine the incorporation into TCA-insoluble material. As Fig. 1 indicates, the rate of incorporation remained constant over a 6 hr period but then declined. However, this represented equilibration, not cessation of RNA synthesis, since a second set of Millipore filters shifted to ^{3}H-uridine for 2 hr incubation periods between 8 and 14 hr continued to incorporate uridine at the original rate. Thus the apparent decline simply represented a resultant between the breakdown of previously synthesized RNA and the fabrication of new. To avoid this complication, the complete time course of RNA synthesis was followed by pulsed incorporation studies. Duplicate Millipore filters were exposed to ^{3}H-uridine between 2 and 4 hr and then harvested; two other Millipore filters between 4 and 6 hr; two more between 6 and 8 hr and so forth. In this manner a succession of 2 hr pulses was administered over a 26 hr period. Figure 2 shows the results. The counts incorporated during each pulse were added to the previous ones to provide a measure of the cumulative incorporation. The net rate is seen to have remained constant for about 20 hr and then to have declined at the end of fruit construction.

Three aggregateless strains of *Dictyostelium discoideum* yielded the same general

pattern of incorporation except that they continued to increase at a high rate over the period in which the wild-type rate declined. The data for one mutant, Agg-204, are summarized in Fig. 2.

The problem of bacterial contamination

When one examines the synthesis of developmentally regulated cell components which are unique to the slime moulds, i.e. certain enzyme activities, antigenic determinants, etc., the presence of the bacterial associate in small quantities creates no

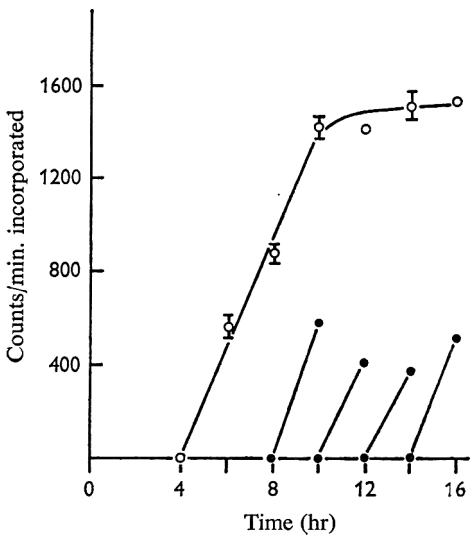

Fig. 1

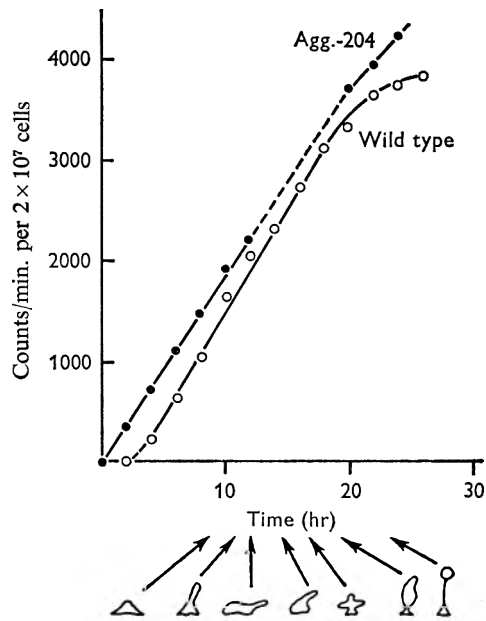

Fig. 2

Fig. 1. -○-, Cumulative incorporation of ^{3}H-uridine into TCA-insoluble material. The uridine was added 4 hr after the amoebae were dispensed on Millipore filters. -●-, ^{3}H-uridine incorporated during 2 hr exposures starting at 8, 10, 12, and 14 hr, respectively.

Fig. 2. Pulsed incorporation of ^{3}H-uridine by *Dictyostelium discoideum*: -○-, wild type; -●-, mutant Agg-204. Each point (the mean of duplicate samples), represents the counts/min. incorporated during exposure of the amoebae to ^{3}H-uridine for the preceding 2 hr. Each successive 2 hr pulse was added to the previous ones to provide a measure of cumulative incorporation. Under these conditions aggregation was completed in 10 hr, pseudoplasmodia formed by 12 hr, fruiting body construction started at 15–16 hr and was completed by 23–24 hr.

ambiguities. When, however, the component is common to both, as is true in the present instance, the possibility of bacterial contributions must be eliminated. In the case of RNA synthesis, a sensitive criterion does fortunately exist, namely the comparative elution patterns of ribosomal RNA from bacteria and that from slime mould cells during MAK-column chromatography. The former is distributed in two well-defined peaks corresponding to the 16 and 23 S fractions (Hayashi, Hayashi & Spiegelman, 1963). The latter is eluted as a single peak and is released by a salt

concentration different from those for either of the bacterial fractions. Figure 3A shows the elution of RNA synthesized by the slime mould cells prepared as described in the Methods section during 20 hr incubation on Millipore filters in the presence of ^{3}H-uridine. Unlabelled carrier RNA from wild-type amoebae was added for purposes of comparison. The labelled RNA is seen to have been eluted coincidentally with the carrier RNA. The degree of bacterial contamination in this experiment, as revealed

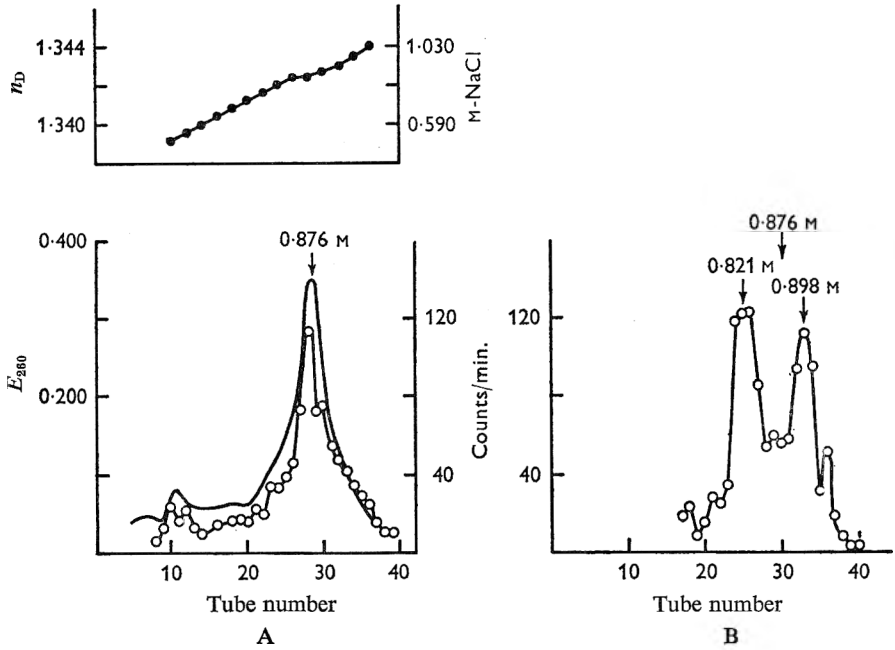

Fig. 3. A: MAK-column chromatography of RNA from amoebae prepared as in Methods and labelled with ^{3}H-uridine during 20 hr incubation on Millipore filters. Extraction E_{260} was measured in a continuous flow cell with the Gilford recording spectrophotometer. Radioactivity (–O–) measured in TCA-precipitable material from 1 ml. samples of the eluate. Upper curve shows the gradient of NaCl concentration in the eluate measured by a calibrated refractometer. B: Chromatography of RNA from amoebae not pretreated in SM + streptomycin medium and labelled with ^{3}H-uridine between 14 and 20 hr during incubation on Millipore filters.

by colony counts, is given in Table 1. Figure 3B shows the extent to which the presence of the bacterial associate even in relatively low concentration affected the elution pattern of the RNA. The amoebae were not pre-incubated in SM + streptomycin medium but instead were washed three times in cold water by 5 min. centrifugation at 1200 g and were dispensed directly on to Millipore filters. The ^{3}H-uridine was administered over a 6 hr period (14–20 hr of incubation). The elution pattern is typical of *Aerobacter aerogenes* RNA with the exception of the barely significant peak at the refractive index of 1·3425 (similar to that of the slime mould RNA).

Table 1 shows that under these conditions the degree of contamination at the time of exposure to uridine was 3×10^5 bacteria/2×10^7 amoebae or about 1 part in 10^4 by weight.

DISCUSSION

The uridine incorporation data indicate that RNA synthesis was not confined to any particular stage of slime mould development but continued at an appreciable role throughout the morphogenetic sequence. However, it should be noted that no absolute rate estimates can be made until determinations of the specific activity of the UTP pool are available.

Table 1. *Extent of bacterial contamination during the incubation of amoebae Dictyostelium discoideum on the Millipore filters*

Period of incubation on Millipore filters (hr)	Viable bacteria/2×10^7 amoebae	
	A*	B
0	5×10^3	5×10^7
5	—	3×10^5
15	2×10^2	3×10^5

* A, amoebae treated as described in Methods; B, amoebae harvested from growth plates, washed three times by centrifugation (1200*g* for 5 min.) in cold water and dispensed on Millipore filters. After incubation the amoebae were harvested in 10 ml. sterile cold water and samples (0·05 ml.) were spread on SM agar plates and incubated at 37° overnight in order to make colony counts.

In addition, the net loss of RNA content (White & Sussman, 1961) and the evidence for equilibration seen in Fig. 1 suggest an appreciable turnover, both of the RNA that was part of the vegetative amoebae and the material synthesized during development. Parallel studies with another species, *Polysphondylium pallidum* grown in axenic medium, indicate that ribosomal RNA contributed the major portion of this turnover, such that at the end of fruit construction at least 50% of the ribosomal RNA then present was synthesized during the morphogenetic sequence (R. R. Sussman, private communication). The results of MAK-column chromatography shown in Fig. 3 indicate that appreciable amounts of ribosomal RNA were also synthesized by *Dictyostelium discoideum*. The question then arises as to why amoebae which are starving and are in the process of losing a significant proportion of their dry weight during development find it necessary to synthesize new ribosomes.

At present, numerous developmental studies are being made with phagotrophs like the slime moulds and ciliated protozoa and with forms like sea urchins, sponges and Acetabularia which are contaminated with bacterial parasites and symbionts. The danger that the bacteria contribute to the data is always serious. For RNA synthesis, MAK-column chromatography offers a sensitive and convenient criterion of such contamination.

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The Metabolism of Triglycerides by Spores of *Penicillium roqueforti*

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SUMMARY

The ability of suspensions of washed spores of *Penicillium roqueforti* to oxidize triglycerides was markedly stimulated by L-proline, L-alanine and L-serine, although all the sources of nitrogen tested were effective over extended periods to different degrees. The rate of oxidation was further increased by the addition of certain sugars, although these were without effect in the absence of a nitrogen source. The same compounds that stimulated methylketone formation from triglycerides also promoted the rapid germination of spores, suggesting that common reactions were involved. The maximum yield of methylketone obtained was 25% from trioctanoin and trihexanoin at pH 6·0, with lower yields from tridecanoin, tributyrin and trilaurin. The addition of sodium azide, and to a lesser extent of 2,4-dinitrophenol and certain organophosphorus compounds, inhibited the oxidation of triglycerides. The possible role of fungal esterases in spore germination is discussed.

INTRODUCTION

Growing cultures of *Penicillium glaucum* have been shown to oxidize up to 15% of synthetic triglycerides containing C_4 to C_{12} fatty acids to the corresponding methylketones with one less carbon atom (Acklin, 1929; Thaler & Eisenlohr, 1941). The first step in the oxidation is presumably the hydrolysis of the triglyceride, since the formation of extracellular esterases is well documented (e.g. Morris & Jezeski, 1953; Alford, Pierce & Suggs, 1964). It is not clear however from these investigations whether the initial hydrolysis of the triglyceride and the subsequent metabolism of the liberated fatty acid is the result of spore activity or of mycelial activity. Gehrig & Knight (1958, 1963) considered that spores of *Penicillium roqueforti* are able to oxidize fatty acids but that the capacity to form methylketones disappears rapidly and progressively as the spores germinate. On the other hand Lawrence (1965*a*) found that the slow rate of formation of heptan-2-one from octanoic acid by washed spore suspensions of *P. roqueforti* was markedly increased by the addition of the same specific amino acids and sugars that have been shown to stimulate fungal germination.

The germination of sporangiospores of *Rhizopus arrhizus* (Weber & Ogawa, 1965), *R. stolonifer* (Weber, 1962) and the conidia of *Aspergillus niger* (Yanagita, 1957; Miller, 1962) is strongly stimulated by L-proline and less effectively by some other amino acids. For *A. niger* alanine is also stimulatory, but for *R. arrhizus* proline can be replaced by ornithine, arginine or asparagine only when phosphate is also present. Cochrane, Cochrane, Simon & Spaeth (1963) showed that, in addition to glucose

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and a nitrogen source, the macrospores of *Fusarium solani* required a factor present in yeast extract which could be partially replaced by several amino acids but not by carbohydrates.

Germination has also been associated with the decrease in fungal spore lipid which occurs during incubation (Farkas & Ledingham, 1959; Cochrane, Cochrane, Collins & Serafin, 1963; Jack, 1964). Lipids are a prominent constituent of several species of fungi (Allen, 1965) and the activation or synthesis of an esterolytic system and its subsequent attack upon the lipid of the spore coat may be related to the swelling and increase in permeability of fungal spores which precedes germination (Yanagita, 1957; Ekundayo & Carlisle, 1964).

The present paper describes the effect of amino acids and sugars upon the ability of suspensions of washed spores of *Penicillium roqueforti* to metabolize triglycerides containing short-chain fatty acids. The possibility of a relationship between the hydrolytic activity of the spores and their subsequent germination was also investigated.

METHODS

General. Penicillium roqueforti ATCC 6989 (American Type Culture Collection, Washington, D.C.) used in previous work on the oxidation of fatty acids (Lawrence, 1965*a*, 1966), was grown on slopes of Czapek-Dox agar or malt agar (Oxoid) at 22°. After incubation for 7–10 days the spores were gently dislodged with an inoculation needle and quickly washed in sterile water by centrifugation. The washed spores were resuspended in water or phosphate buffer to give an extinction of 0·6 at 650 mμ which corresponded to about 5×10^8 spores/ml. as determined from a calibration curve of haemocytometer counts of spores against extinction.

Samples of these spore suspensions (10 ml.) were incubated at 30°, the optimal temperature for oxidation, with equimolecular amounts of triglycerides (normally 30 μmoles) which were dispersed by shaking in stoppered bottles (100 ml.). Streptomycin (0·25 mg./ml.) was added to prevent bacterial growth. Germination (appearance of the germ tube) was determined by microscopic observation of samples of the spores stained with dilute carbol fuchsin.

Cell-free extracts of spores. Sufficient 0·05 M-phosphate + 0·05 M-sucrose buffer (pH 7·0) was added at 4° to spores (1 g. wet wt) and acid-washed sand (2 g.) in a pre-cooled mortar to form a thick paste. This was ground for 5 min., more buffer added to a final volume of 5 ml. and the suspension centrifuged at 1200 *g* for 10 min., the supernatant fluid being used.

Measurement of oxygen uptake. Manometric measurements of respiration were made by the direct Warburg method at 30°. The total volume in each Warburg flask was usually 3·0 ml. spore suspension + substrate, with 0·2 ml. 20% (w/v) KOH in the centre well.

Estimation of methylketones. Methylketone concentrations were determined at intervals during the experiments by adding 1 ml. of the contents of a 100-ml. stoppered bottle containing spore suspension and substrate into 2 ml. 2,4-dinitrophenyl hydrazine solution (2 g./l. 2 N-HCl) in a stoppered test-tube. After 30 min. the 2,4-dinitrophenyl hydrazone was extracted and the methylketone subsequently estimated by the method previously described (Lawrence, 1965*b*).

Estimation of esterase. Esterase activity was determined by a quantitative thin-layer

agar diffusion method at 30° (Lawrence, Fryer & Reiter, 1966) by measuring the zones of clearing (recorded in mm.) of a 0·1% emulsion of tributyrin in agar buffered with 0·05 M phosphate (pH 8).

Hydrolysis of *o*-nitrophenylbutyrate was measured by the spectrophotometric estimation of the liberated *o*-nitrophenol at 410 mμ with 1 cm. cuvettes and total volumes of 3·2 ml. Assay mixtures contained 100 μmoles tris+HCl buffer and 7·5 μmoles *o*-nitrophenyl butyrate (added as a solution in 0·2 ml. methanol). In preliminary experiments *p*-nitrophenyl acetate was also used, but the very low esterase activities of the cultures with this substrate were of the same order as the rate of spontaneous hydrolysis of the substrate at pH 7. The more stable *o*-nitrophenylbutyrate did not spontaneously hydrolyze although non-enzymic hydrolysis in presence of certain nitrogen compounds, e.g. proline and ammonium sulphate, had to be taken into account. When the rates of hydrolysis (change in extinction/min.) were appropriately corrected for spontaneous hydrolysis and non-enzymic hydrolysis, the esterase from *Penicillium roqueforti* appeared to be equally active against *o*-nitrophenylbutyrate and *p*-nitrophenylacetate. In the present work it appeared that only one enzyme was involved in the hydrolysis of the emulsified tributyrin and of the soluble *o*-nitrophenylbutyrate; the general term esterase has therefore been used to cover both types of activity.

Chemicals. Chromatographically pure glycerides were obtained from Sigma Chemical Co. (St Louis, Mo., U.S.A.), Tween 20 (polyoxyethylene sorbitan monolaurate) from Honeywill-Atlas Ltd. (Carshalton, Surrey), *p*-nitrophenyl acetate and *o*-nitrophenyl butyrate from British Drug Houses Ltd. Bactopeptone, Casamino acids (Difco), amino acids and sugars (British Drug Houses Ltd.) were either autoclaved or Seitz-filtered (Ford's Sterimats, A. Gallenkamp and Co. Ltd.). Chloramphenicol was obtained from Parke, Davis and Co. (Detroit, Mich.). Di-isopropylfluorophosphate (DFP) and diethyl *p*-nitrophenylphosphate (E 600) were gifts from Dr G. C. Cheeseman and Dr P. Andrews (N.I.R.D.), respectively.

RESULTS

Effect of shaking spore suspensions

Although shaking the spore suspensions increased the oxygen uptake and methylketone formation, the spores tended to clump together and replicate experiments showed considerable variation. Reproducible results were obtained by using 100-ml. medical flat bottles, sloped to give the maximum surface area of solution.

Activation of spores

Spores from 5-day cultures were harvested, washed and suspended in phosphate buffer (pH 6·5). These spores oxidized trioctanoin extremely slowly, whereas equivalent amounts of octanoic acid were readily oxidized, indicating that the hydrolytic activity of the spores for triglycerides was extremely low. The addition of Casamino acids or specific amino acids to the spore suspensions markedly increased both oxygen uptake (Fig. 1) and methylketone formation (Fig. 2) after an initial lag of at least 5 hr. This lag was shorter in the presence of Casamino acids than with any single amino acid, but L-proline stimulated methylketone formation to a greater extent over a period of 24 hr. Even small amounts of L-proline (1 μmole/10 ml.) were effective;

about 100 μmoles/10 ml. were optimal. Whereas the lag before oxidation of fatty acids occurred was less than 2 hr, no mixture of stimulatory compounds was able to decrease the lag period with triglycerides below 5 hr, presumably because the additional step of esterase formation or activation was involved. L-alanine was nearly as effective as Casamino acids followed by L-serine, L-arginine, L-glycine, L-leucine, L-glutamic acid, L-asparagine, L-glutamine, L-aspartic acid, L-ornithine, in decreasing order of effectiveness. Although other sources of nitrogen tested did not stimulate the oxidation

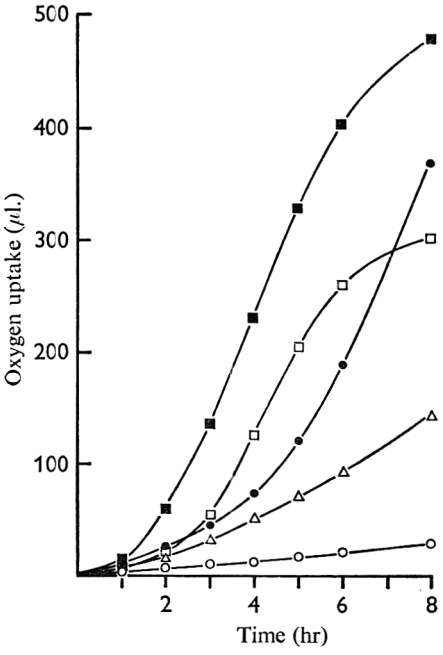

Fig. 1

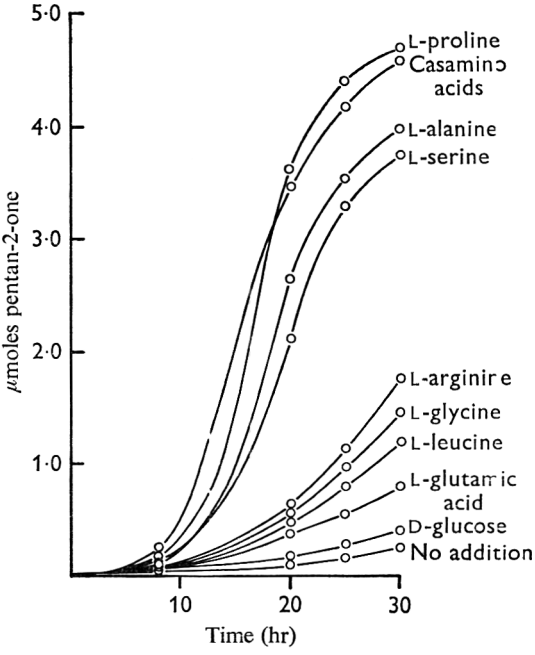

Fig. 2

Fig. 1. The oxidation of octanoic acid (9 μmoles) and trioctanoin (3 μmoles) by washed 6-day spores ($1{\cdot}5 \times 10^9$) of *Penicillium roqueforti* at pH 6·0 (100 μmoles phosphate buffer). Each flask contained either octanoic acid (□—□), octanoic acid + 0·05 g casamino acids (■—■), trioctanoin (○—○), trioctanoin + casamino acids (●—●) or casamino acids only (△—△). Total volume 3 ml. Endogenous respiration deducted.

Fig. 2. The effect of amino acids (200 μmoles) on the formation of pentan-2-one from trihexanoin (30 μmoles) by washed 7-day spores (3×10^9) of *Penicillium roqueforti* at pH 6·0 (200 μmoles phosphate buffer). Total volume 10 ml.

of triglycerides in the first 24 hr of incubation, oxidation was markedly increased after 48 hr, to various extents, by L-valine, ammonium sulphate, potassium nitrate, L-hydroxyproline, L-phenylalanine, L-lysine, L-threonine, L-methionine, L-histidine, in that order. After 72 hr the yield of pentan-2-one from trihexanoin in the presence of potassium nitrate was significantly greater than with L-proline (Fig. 3).

In the absence of a source of nitrogen, none of the sugars added to the spore suspensions was stimulatory in the first 24 hr. In the presence of a nitrogen source, however, some sugars were remarkably effective even when the nitrogen compound was not stimulatory by itself. Thus D-galactose, D-glucose or D-xylose in the presence of potassium nitrate markedly activated the oxidation of trihexanoin to pentan-2-one;

sucrose and maltose were less effective; D-ribose and lactose were virtually without effect (Fig. 4).

The time of harvesting the spores had no significant effect upon the rate of oxidation of triglycerides, in marked contrast to the oxidation of fatty acids (Lawrence, 1966). The hydrolytic step appeared to be a function of spore germination and might therefore be expected to be independent of the age of the spore. However, although a source of nitrogen was essential for formation of mycelium, spores in the absence of stimulatory compounds oxidized triglycerides very slowly to methylketone (up to 5% after 6 days) without any obvious morphological change.

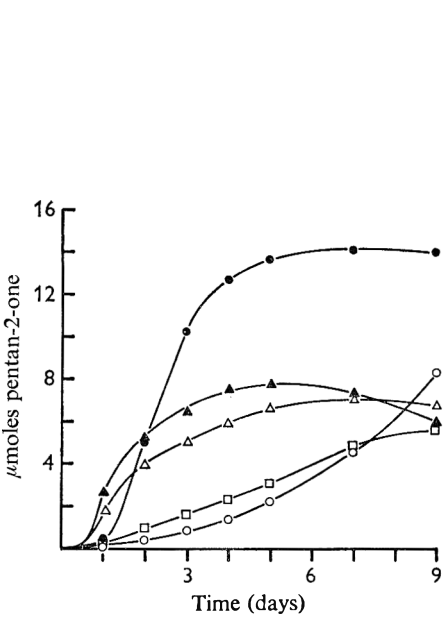

Fig. 3

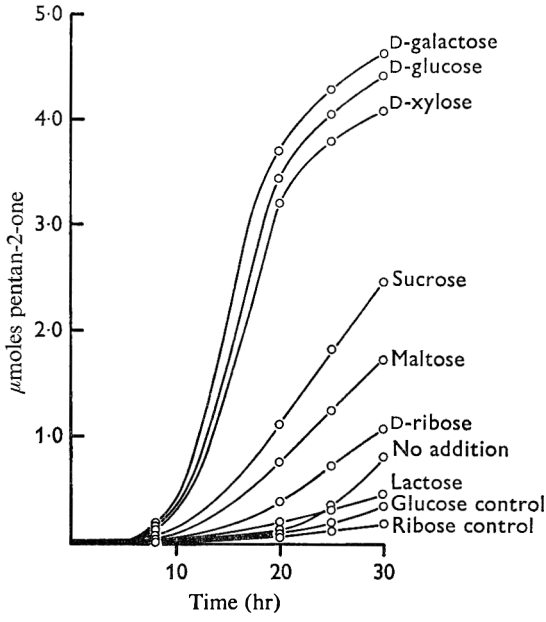

Fig. 4

Fig. 3. The formation of pentan-2-one from trihexanoin (30 μmoles) over extended periods by washed 7-day spores (3×10^9) of *Penicillium roqueforti* at pH 6·0 (200 μmoles phosphate buffer) in the presence of 100 μmoles glucose (□—□), alanine (△—△), proline (▲—▲), KNO_3 (●—●), and in the absence of stimulating compounds (○—○). Total volume 10 ml.

Fig. 4. The effect of sugars on the formation of pentan-2-one from trihexanoin by spores of *Penicillium roqueforti* in the presence of KNO_3. Each flask contained 3×10^9 spores, 200 μmoles phosphate buffer (pH 6·0), 200 μmoles of each sugar, 100 μmoles KNO_3. Total volume 10 ml. Control flasks containing glucose or ribose, respectively, but not KNO_3, are also shown.

Effect of Seitz filtration on amino acid solutions

Solutions of amino acids which had been Seitz-filtered were considerably more active than were autoclaved solutions in stimulating the oxidation of triglycerides by spores (Fig. 5). Distilled water that had been Seitz-filtered was also stimulatory after an extended lag period but only in the presence of a nitrogen source. This confirmed the results of earlier work that Seitz-filtered solutions may contain a factor which is stimulatory to the germination of fungal spores (Farkas & Ledingham, 1959; Lawrence, 1966) and which may be identical with a partially characterized factor extracted from

cotton which permits the rapid germination of rust uredospores (Atkinson & Allen, 1966). Since, however, the effect of Seitz-filtered solutions on the oxidation of triglycerides varied, autoclaved solutions were used in the experiments reported in this paper even although the rate of oxidation was considerably lower.

Esterase activity

The extremely low esterase activity found in the spore suspensions was unexpected since *Penicillium roqueforti* was strongly esterolytic when grown on standard tributyrin agar or butterfat + Victoria Blue agar. However, this difference in activity as between solid and liquid media has also been observed with bacterial lipases (O'Leary & Weld, 1964). Fodor & Chari (1949) found that lipolytic activity of *P. roqueforti* in liquid cultures was very weak even under optimal conditions.

Table 1. *Penicillium roqueforti: the effect of stimulatory compounds on the esterase activity of spores, formation of heptan-2-one from trioctanoin and growth of mycelium*

Each flask contained 3×10^9 spores, 30 μmoles trioctanoin, 200 μmoles phosphate buffer (pH 6·0), 200 μmoles amino acid or 0·1 g. Casamino acid, 200 μmoles glucose, distilled water to 10 ml.: time of incubation 20 hr. Esterase activity for substrate *o*-nitrophenylbutyrate (*o*-NPB) is expressed as rate of change of extinction/min. X is the diameter of the zone of clearing of tributyrin emulsion (0·1 %) minus the diameter of the well.

Addition	Esterase activity		Heptan-2-one (μmoles)	Mycelium*
	o-NPB	Tributyrin (X^2)		
—	0·01	0·8	0·14	−
Glucose	0·01	0·9	0·15	−
Alanine	0·04	1·2	0·37	+
Alanine + glucose	0·07	2·6	0·79	+
Ammonium sulphate	0·02	0·8	0·21	−
Ammonium sulphate + glucose	0·02	0·8	0·40	−
Casamino acid	0·10	2·9	0·90	+
Casamino acid + glucose	0·10	2·6	1·32	+

* +, Nearly all spores germinated; −, no spores germinated.

Suspensions of *Penicillium roqueforti* spores in water, phosphate buffer or solutions of sugars did not germinate. Such suspensions were only weakly active against tributyrin and *o*-nitrophenylbutyrate but nevertheless oxidized triglycerides very slowly during a longer period of incubation. The addition of stimulatory compounds such as Casamino acids and L-alanine resulted in a slight increase in the esterase activity of the suspension, although methylketone formation from triglycerides (Table 1) and the rapid germination (i.e. within 24 hr) of the spores was greatly stimulated. No increase in esterase activity was detected until after a lag of at least 6 hr; the activity reached a maximum after about 24 hr. Attempts to increase esterase production by germinating the spores in complex media such as Bactopeptone and yeast extract were unsuccessful. The addition of butterfat or tributyrin to growing cultures of *Penicillium roqueforti* did not enhance esterase production, in contrast to earlier reports (Goodman, 1950; Imamura & Kataoka, 1963) that fungal lipases are adaptive enzymes.

Effect of phosphate

Although suspensions of spores harvested from growth on malt agar did not require added phosphate for the initiation of oxidation of triglycerides in the first 24 hr, the rate of oxidation by spores harvested from Czapek-Dox agar was markedly stimulated by small amounts of phosphate (0·1 μmole/ml.) and was further increased by higher concentrations (Fig. 6). After 48 hr, however, considerable amounts of methylketone were formed in the absence of phosphate and after 6 days a maximum of 18% of the trihexanoin had been oxidized to penta-2-one. At no stage was the yield of methylketone as great as with added phosphate.

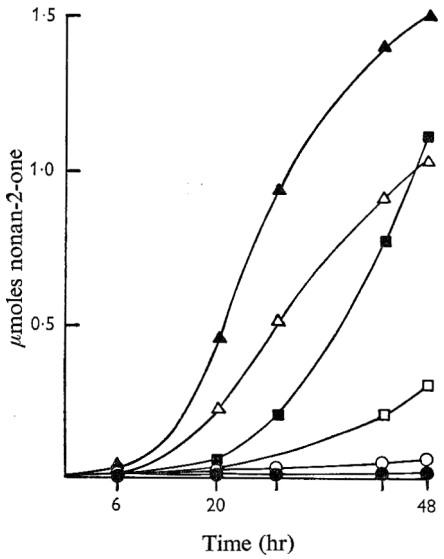

Fig. 5

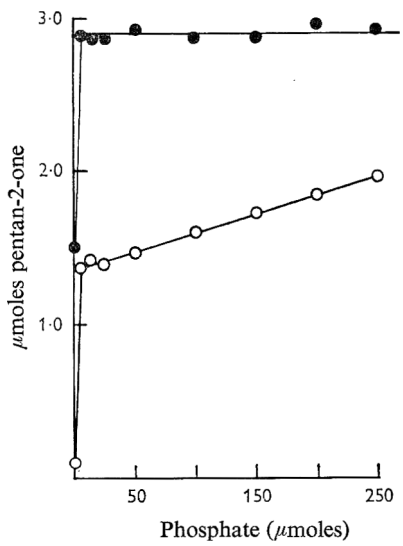

Fig. 6

Fig. 5. The effect of Seitz-filtered solutions on the formation of nonan-2-one from tridecanoin by spores of *Penicillium roqueforti* in the presence of proline. Each flask contained 6 ml. spore suspension (3×10^9 spores), 30 μmoles tridecanoin, 2 ml. phosphate buffer (pH 6·0; 200 μmoles), 1 ml. autoclaved proline solution (200 μmoles) and 1 ml. autoclaved water (□—□) or Seitz-filtered water (■—■); glucose (200 μmoles) solution (△—△) or Seitz-filtered glucose solution (▲—▲). Control flasks containing Seitz-filtered water (○—○) and Seitz-filtered glucose solution (●—●) but not proline are also shown.

Fig. 6. Effect of phosphate concentration on the formation of pentan-2-one from trihexanoin (30 μmoles) by washed 7-day spores (3×10^9) after growth on Czapek-Dox agar slopes, at pH 6·0 (50 μmoles maleateb uffer) in the presence of Casamino acids (0·1 g.). Total volume 10 ml. Times of incubation 24 hr (○—○) and 48 hr (●—●).

There was a slight but significantly greater difference in the esterase activity of spores harvested after growth on malt agar than of those from Czapek-Dox agar. The two media contain different sugars which may have affected the relative amounts of available phosphate in the spores and their esterolytic behaviour. Grover (1964) found that the characteristics of spores of *Aspergillus flavus*, including their requirements for germination, were apparently greatly influenced by the cultural conditions during spore formation.

Effect of pH value

The rate at which methylketones were formed from triglycerides by spores activated by 0·1% Casamino acids increased rapidly below pH 7·0. Trihexanoin and trioctanoin were most readily oxidized at pH 6·0 (Fig. 7), approximately 25% of each triglyceride being oxidized to the corresponding methylketone after 6–10 days. Lower yields of methylketone were obtained from tributyrin (8% at pH 5·5), tridecanoin (14% at pH 6·0) and trilaurin (2% at pH 6·8). Only the corresponding methylketone with one less carbon atom was detected in each case. The oxidation of these triglycerides followed the same pattern as that of trihexanoin (Fig. 3), i.e. methylketone formation increased after an initial lag of 6–12 hr to a maximum after 6–10 days; after which the methylketone itself was slowly metabolized.

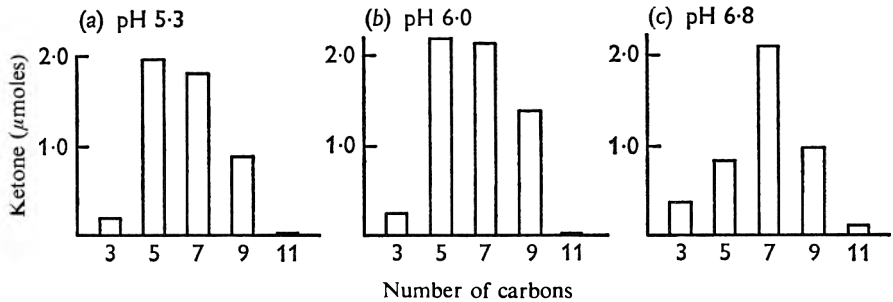

Fig. 7. Formation of methylketones from triglycerides (30 μmoles) by washed 7-day spores (3×10^9) of *Penicillium roqueforti* at (*a*) pH 5·3, (*b*) pH 6·0, (*c*) pH 6·8 (200 μmoles phosphate buffer) in presence of Casamino acids (0·1 g). Total volume 10 ml.

The ability of amino acids to stimulate the oxidation of trihexanoin to pentan-2-one by spores was most pronounced at pH 5·5 (Table 2) although only the basic amino acids arginine and lysine showed markedly decreased stimulatory activity at pH 6·5.

Trilaurin is solid at 30°, the temperature used in this work, which may have accounted for the low rate at which it was oxidized (Fig. 7). The water-soluble Tween 20 (polyoxyethylene sorbitan monolaurate), which contains approximately 60% lauric acid, was not, however, rapidly oxidized by spores in the presence of Casamino acids, the maximum yield of undecan-2-one obtained being less than 2% at pH 6·8.

Effect of some enzyme inhibitors

Sodium azide and 2,4-dinitrophenol, which are considered to disrupt the development of transport systems by uncoupling oxidative phosphorylation mechanisms (Quastel, 1964), also inhibited the formation of pentan-2-one from trihexanoin (Table 3) and from hexanoic acid by spores activated by Casamino acids. Sodium azide was the more effective inhibitor and it also completely inhibited the growth of mycelium. On the other hand high concentrations (10^{-3}M) of 2,4-dinitrophenol did not completely suppress the oxidation of triglycerides and was without effect upon mycelial growth.

Low concentrations (10^{-5}M) of the organophosphorus compound (E 600), which specifically inhibits hydrolytic mechanisms, partially suppressed the oxidation of

triglycerides to methylketones but did not inhibit the growth of mycelium from spores activated by Casamino acids (Table 3). Compound E 600 was considerably more effective than di-isopropylfluorophosphate (DFP) against triglycerides, but neither compound inhibited to any significant extent the oxidation of fatty acids to methylketones. Chloramphenicol has been reported to inhibit bacterial esterases (Smith, Worrell & Swanson, 1949) but even high concentrations were without effect on the oxidation of triglycerides or fatty acids by spores.

Table 2. *Penicillium roqueforti: the effect of pH value on the ability of amino acids to stimulate the formation of pentan-2-one from trihexanoin by washed spores*

Each flask contained 3×10^9 spores, 200 μmoles phosphate, 30 μmoles trihexanoin, 200 μmoles amino acid or 0·1 g. Casamino acid, distilled water to 10 ml. Time of incubation 22 hr.

	pH value of spore suspension		
	pH 5·5	pH 6·5	pH 7·5
Addition	Pentan-2-one formed (μmoles)		
—	0·21	0·01	0·03
Proline	3·80	2·51	0·24
Alanine	2·41	2·56	0·39
Arginine	1·91	0·21	0·07
Lysine	0·31	0·03	0·03
Glutamic acid	0·43	0·21	0·06
Aspartic acid	0·49	0·25	0·04
Casamino acids	3·47	3·16	0·37

Table 3. *Penicillium roqueforti: the effect of inhibitors on the formation of pentan-2-one from trihexanoin by spores activated by Casamino acids at pH 6·0*

Each flask contained 3×10^9 spores, 200 μmoles phosphate buffer (pH 6·0), 30 μmoles trihexanoin, inhibitor as below, distilled water to 10 ml. Times of incubation as below.

		Time of incubation (hr)		
		20 hr	44 hr	20 hr
		pentan-2-one (μmoles)		mycelium*
Inhibitor				
None		3·07	5·22	+
2,4-dinitrophenol (M)	1×10^{-4}	1·70	4·90	+
	5×10^{-4}	1·44	3·81	+
	1×10^{-3}	0·19	0·30	+
Sodium azide (M)	1×10^{-4}	Nil	Nil	−
	5×10^{-4}	Nil	Nil	−
Chloramphenicol (μg./ml.)	500	3·17	5·40	+
	1000	3·21	5·74	+
E 600 (M)†	10^{-4}	1·61	3·34	+
	10^{-5}	2·65	4·22	+
DFP (M)‡	10^{-4}	2·40	4·77	+
	10^{-5}	3·14	4·96	+

* +, Nearly all spores germinated; −, no spores germinated.
† E 600, diethyl-*p*-dinitrophenyl phosphate.
‡ DFP, di-isopropyl fluorophosphate.

Cell-free extracts of spores of Penicillium roqueforti

A thick paste of spores and sand ground with buffer (see Methods) yielded an extract with slight esterase activity and which also slowly oxidized low concentrations of trioctanoin (3 μmoles/10 ml.). Disintegration of a concentrated spore suspension by ultrasonic treatment for times up to 45 min. resulted in inactive extracts.

DISCUSSION

Suspensions of washed spores of *Penicillium roqueforti* oxidized triglycerides extremely slowly but the addition of Casamino acids or certain amino acids (particularly L-proline or L-alanine) greatly stimulated oxidation after an initial lag of about 6 hr. The addition of other amino acids did not increase the effectiveness of L-proline, indicating that the pathway of activation was the same for all amino acids. The specificity of the stimulatory amino acids was very marked; L-hydroxyproline and L-phenylalanine had no effect during the first 24 hr of incubation. Over extended periods, however, the formation of methylketones from triglycerides by spores was stimulated by the addition of a variety of sources of nitrogen, but to different degrees. The rate of oxidation was further increased by certain sugars (particularly D-galactose, D-xylose, D-glucose) although these were effective only in the presence of a nitrogen source. It may be significant that these three sugars all possess the same spatial configuration about carbon atoms 1, 2 and 3.

A source of nitrogen was essential for triglyceride oxidation by spores and for their germination, but not for the oxidation of fatty acids (Lawrence, 1966). Since cell-free extracts of spores possessed esterase activity it seems likely that the utilization of certain nitrogen compounds resulted in the transport of hydrolytic enzymes across the spore wall. One of the essential prerequisites to the formation of germ tubes is considered to be the accumulation of amino acids in the spore (Yanagita, 1957). The enhancement of the stimulatory affect of amino acids by certain sugars on spore germination and on triglyceride oxidation may be due therefore to a greater rate of transport of amino acids across the cell wall in the presence of an external source of energy; this has been shown to occur in bacterial and mammalian systems (Kepes & Cohen, 1962; Scholefield, 1964). Similarly, 2,4-dinitrophenol and sodium azide may inhibit triglyceride oxidation by spores in the presence of Casamino acids by preventing the accumulation of exogenous amino acids in the spores, as has been shown for *Escherichia coli* (Kepes & Cohen, 1962). Sodium azide, but not 2,4-dinitrophenol, also prevented the growth of mycelium. The differential sensitivity of various phosphorylation steps toward uncouplers is well established (e.g. Malkov & Suprunenko, 1958) and 2,4-dinitrophenol is presumably more inhibitory to the phosphorylation processes involved in the metabolism of triglycerides than to other phosphorylations of vital importance in germ-tube growth.

The ability of fungal esterases to synthesize triglycerides has recently been shown (Iwai, Tsujisaka & Fukumuto, 1964) but the results of the present work support the view that lipid metabolism is predominant during the early stages of germination (Farkas & Ledingham, 1959). Factors which greatly stimulated the formation of methylketones from triglycerides by spores of *Penicillium roqueforti* also promoted the rapid germination of these spores, suggesting that similar mechanisms may be

operating. Methylketone formation involves two hydrolytic steps: the initial liberation of fatty acid and the hydrolysis of the subsequently formed β-oxo acyl ester to the unesterified β-oxo acid. The insensitivity of fungal de-acylases to high concentrations of organophosphorus compounds observed in the present work confirms similar findings with de-acylases from mammalian tissues (Srere, Seubert & Lynen, 1959). These inhibitors only partially suppressed methylketone formation from triglycerides and were without effect upon either fatty acid oxidation to methylketones or the germination of the spores. While such evidence is not conclusive it is consistent with the view (Lawrence, 1966) that de-acylation may play an important part in spore germination.

I would like to thank the Director of the National Institute for Research in Dairying for affording facilities for doing this work and to colleagues in the Bacteriology Department for helpful discussions and assistance.

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The Spore-Surface Depsipeptide of *Pithomyces sacchari*

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SUMMARY

The spore-surface spicules of *Pithomyces sacchari* contain the cyclotetradepsipeptide, angolide.

INTRODUCTION

Spores of species of the genus *Pithomyces* (Ellis, 1960, 1965) are covered with minute easily detached spicules. In *P. chartarum*, *P. maydicus* and *P. cynodontis* (IMI101184; Ellis, 1965) these spicules are composed largely or entirely of cyclodepsipeptides (Russell, 1966), the chief components being sporidesmolide I, sporidesmolide IV, and angolide, respectively (Bertaud, Morice, Russell & Taylor, 1963; Bishop *et al.* 1965). The spore-surface spicules of *P. sacchari* (Speg.) M. B. Ellis have not hitherto been examined chemically, because the isolate available, IMI102686, formed very few spores when grown under the same conditions as the other three species (Bishop *et al.* 1965). We now report experiments, with this and two other isolates of *P. sacchari*, which show that the spicules in this species contain angolide. A preliminary account has already been given (Riches & Russell, 1965).

METHODS

Organisms

Isolate IMI102686 of *Pithomyces sacchari* was obtained from the Commonwealth Mycological Institute. Dr M. O. Moss (Tropical Products Institute, Grays Inn Road, London, W.C.1) sent us an isolate, TPI122, which was also disposed (Dr M. B. Ellis, personal communication) as *P. sacchari*. This isolate, which spored sparsely on potato glucose agar at 25°, was designated TPI122a. From it, by single-spore isolation from a vigorously sporing sector, was obtained TPI122b (see p. 83). The two isolates were distinguishable only by their different sporulation intensities. Mycelial colonies growing on potato glucose agar at 25° are superficial, olive green when young becoming black later. Hyphae are of two types, those in contact with the substratum consisting of main branches with laterals radiating as if from a common origin, and aerial hyphae forming a loose irregular network. Conidiophores form from modified aerial hyphae, usually where several of these approximate and form many anastomoses. Aerial hyphal elements are $3{\cdot}1 \times 19{\cdot}5\ \mu$; conidiophores, 2–5 per hyphal element, are born randomly; conidia are born singly, with 2–5 transverse septa; the distal septum is occasionally oblique. Spores with 2 septa are 14–18 μ long; with 3, 18–22 μ; with 4, 22–28 μ; with 5, 27–36 μ; all spores are 6–8 μ wide at the widest point.

The *Pithomyces chartarum* used was a new isolate, obtained from a high-sporing

sector of a culture of isolate 'c' (Done, Mortimer, Taylor & Russell, 1961) which during repeated subculture on potato glucose agar had lost its capacity to spore freely. Grown in surface culture on potato + carrot broth at 25° it spored profusely and produced the typical mixture of sporidesmolides (Bishop *et al.* 1965) in a yield of more than 300 mg./l.

Cultivation of organisms

The isolates were maintained by subculture on potato glucose agar plates at 25°.

Agar cultures. Each isolate of *Pithomyces sacchari* was grown on the salts + glucose + asparagine medium of Ross (Ross & Thornton, 1962; Butler, Russell & Clarke, 1962) to which was added 2% (w/v) agar. This medium, in 100 ml. portions, was distributed into 1 pint milk bottles (44 mm. internal neck diameter) which were plugged with cottonwool, autoclaved (20 min. at 120°), allowed to cool in a horizontal position and inoculated with a mycelial suspension (2 ml.) prepared as described for *P. chartarum* (Russell, Sturgeon & Ward, 1964). Cultures were incubated at 25° under a standard light regime (Bishop *et al.* 1965) for 2–3 weeks.

Liquid surface cultures were grown in 1 l. Roux bottles as previously described (Bishop *et al.* 1965) on Ross medium containing 0·1% (w/v) yeast extract (Difco).

Shake-flask cultures were grown in plugged 2 l. conical flasks each containing sterile Ross + yeast extract medium (200 ml.), which were inoculated with a spore suspension (0·1 ml. containing 10^5 spores) and incubated on a Gyrotory Incubator Shaker (New Brunswick Scientific Inc., New Brunswick, N.J.) at 26° and 250 strokes/min.

Harvesting and extraction

Agar cultures. Chloroform (50 ml.) was rocked gently over the surface of each culture for 5 min. and decanted through Whatman no. 1 silicone-treated filter paper (H. Reeve Angel and Co. Ltd., London) to remove entrained water. The filtrates from three successive extractions were mixed and evaporated to dryness *in vacuo* at 50°. To the residue was added diethyl ether (2·5 ml. for each culture extracted). The depsipeptides were isolated from the ether-insoluble material and recrystallized from ethanol to constant melting point and specific rotation.

Liquid cultures were harvested at intervals, and spore counts and dry-weight determinations made; depsipeptide analyses were done, using ether saturated with angolide.

In one experiment, five 2-week liquid cultures of *Pithomyces chartarum* were mixed with five similar cultures of *P. sacchari* TP1122b in a Waring Blendor, and the chopped felts collected, washed, dried and coarsely powdered. The powder (10·4 g.) was stirred with chloroform (20 ml.) in a sintered-glass Büchner funnel (porosity 4) for 15 sec. and the extract removed by suction. Three more similar extractions were made. The powdered felts were then completely extracted with chloroform in a Soxhlet extractor. From each of the five extracts so obtained, total depsipeptides were isolated by using ether saturated with respect to both angolide and the total sporidesmolide fraction of *P. chartarum* (Done *et al.* 1961).

Spore profiles were examined in the electron microscope as before (Bishop *et al.* 1965).

Analytical methods

Except where otherwise stated, details of or references to the analytical methods used in this work were given by Bishop *et al.* (1965).

Isoleucine + alloisoleucine in cyclodepsipeptide hydrolysates were determined by paper chromatography in a solvent system that did not separate them. Spots (10 μl.) of an acid hydrolysate of authentic angolide (Russell, 1965*a*) or of a presumptive angolide sample, reconstituted to contain the material from 4·26 mg. cyclodepsipeptide/ml., were applied to Whatman no. 3MM paper. The paper, bearing three spots each of sample and standard arranged alternately, was chromatographed at 25° in the descending sense for 16 hr with the solvent t-butyl alcohol + 4·25N-ammonia (4 + 1, by vol.; Vining & Taber, 1957) and dried overnight in a stream of air at room temperature. It was then dipped in a solution of ninhydrin (0·1%, w/v) in acetone and heated at 105° for 15 min. All samples gave a single spot corresponding exactly with that given by the standard. The spot intensities were measured by reflectance, on both sides of the paper, with a Chromoscan densitometer (Joyce, Loebl and Co. Ltd., Gateshead-on-Tyne), with the dark green filter no. 5025 and the slit no. 5007 supplied for the instrument, which was operated at maximum sensitivity and with a chart speed:strip speed ratio of 1:1. The six readings for the sample were averaged (T) as were those for the standard (S). The total amount of isoleucine + *allo*isoleucine, in mmoles/426 mg., was $2T/S$, since 1 mole of angolide yields 1 mole of each amino acid on hydrolysis. In view of the close correspondence of the sample and standard readings no standard curve was used. In separate experiments it was established that the densitometer reading was proportional to the amount of amino acid in the range 0·02–0·2 μmole.

Separation of isoleucine from *allo*isoleucine in the system t-amyl alcohol + acetic acid + water (20 + 1 + 20, by vol., lower phase; Gray, Blake, Brown & Fowden, 1964) was insufficient for this method to be used for their separate determination, but enabled a visual estimate of the relative amounts to be made.

Partial hydrazinolysis was performed, and the products examined, as described by Russell (1965*a*, *b*).

RESULTS

Profile electron micrographs of spores of *Pithomyces sacchari* TPI122a and TPI122b showed the presence of spore-surface spicules similar to those observed earlier in IMI102686 (Bishop *et al.* 1965) (Pl. 1, figs. 1, 2). Spicules were absent from spores which had been rinsed with chloroform on the grids before being shadowed.

Isolation and identification of angolide

All three isolates of *Pithomyces sacchari* spored on the agar medium. Chloroform that had been left for a short time in contact with the surface of such sporing cultures contained depsipeptides, which were isolated from the chloroform extracts by an established technique (Bishop *et al.* 1965) and shown to be homogeneous by thin-layer chromatography. The yields (mg./l. culture fluid) were 48 and 18 for IMI102686, 146 for TPI122a and 241 for TPI122b. The materials so obtained, after being purified by recrystallization, were identified as angolide, the cyclotetradepsipeptide previously (Bishop *et al.* 1965) isolated from *P. cynodontis* IMI101184. The physical and chemical

Table 1. *Physical properties of pure depsipeptides from isolates of Pithomyces sacchari*

	Value determined for: Depsipeptide isolated from			
Property	IMI102686	TPI122a	TPI122b	Angolide
M.p.	261–262°	261–262°	261–262°	261–262°
Mixed m.p.*	261–262°	261–262°	261–262°	
R_F†	0·61	0·60	0·61	0·60
$[\alpha]_D$‡	−82°	−81°	−82°	−83°
M§	431	433	409	443

* With authentic angolide.

† In thin-layer chromatography on Kieselgel G, and solvent chloroform + ethyl acetate (4 + 1 by volume).

‡ Specific rotation, for the sodium D line, in chloroform solution.

§ Molecular weight, micro-Rast method. Calculated molecular weight of angolide = 426.

Table 2. *Elemental analysis of depsipeptides from isolates of Pithomyces sacchari*

	Percentage found in depsipeptide from:			$C_{22}H_{36}N_2O_6$
Element	IMI102686	TPI122a	TPI122b	requires
Carbon	61·5	61·6	61·6	61·9
Hydrogen	9·1	8·8	8·8	9·0
Nitrogen	6·7	6·85	6·65	6·6

Table 3. *Properties of the products of chemical degradation of depsipeptides from isolates of Pithomyces sacchari*

	Depsipeptide isolated from:			
Degradation product	IMI102686	TPI122a	TPI122b	Angolide
Components of a total acid hydrolysate				
α-Hydroxyisovaleric acid*	+	+	+	+
Isoleucine*	+	+	+	+
*Allo*isoleucine*	+	+	+	+
Isoleucine + *allo*isoleucine†	2·02	2·07	2·03	2·00
Properties of hydrazinolysis products‡				
U_{REL}	1·02	0·98	0·98	1·00
R_F (pic)	0·61	0·61	0·61	0·61
R_F (TLC)§	0·41, 0·48	0·39, 0·46	0·40, 0·47	0·40, 0·48

* In the hydrolysate of authentic angolide, the amounts of the degradation products are given arbitrarily as +; in the hydrolysates of the other samples, the symbol + indicates that no difference in the amount of the degradation product could be detected by visual inspection of the chromatograms of standard and sample.

† In millimoles released on hydrolysis of 426 mg. of the sample.

‡ U_{REL} = electrophoretic mobility on paper at pH 2 relative to the products from angolide; R_F (pic) = R_F of the picryl derivatives on Whatman 3 MM paper with solvent 15% (w/v) dipotassium hydrogen phosphate in water; R_F (TLC) = R_F in thin-layer chromatography with Kieselgel G and solvent benzene + ethanol (2 + 1, by vol.).

§ Two spots.

evidence on which this identification is based in presented in Tables 1–3. In addition, the *P. sacchari* depsipeptides and authentic angolide had identical infrared spectra and optical rotatory dispersion curves. The latter were kindly measured for us by Dr V. T. Ivanov as a part of his study (to be published) of conformation in cyclic peptides and cyclodepsipeptides.

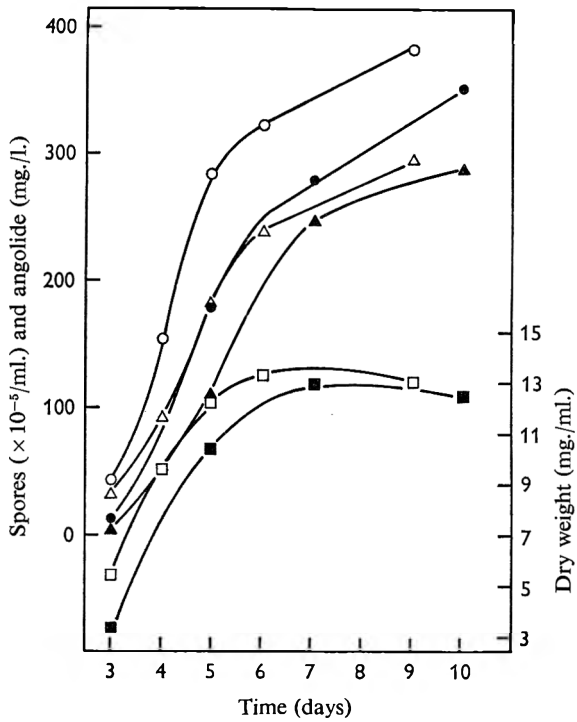


Fig. 1. Dry weight, spores and angolide production in *Pithomyces sacchari* TPI122b grown in liquid surface culture at 25°. Results are given for two separate experiments, open symbols being used for one and full symbols for the other. ● and ○, spore count $\times 10^{-5}$/ml.; ▲ and △, angolide (mg./l.); ■ and □, mycelial dry weight (mg./ml. culture).

Morphological location of angolide in Pithomyces sacchari

Having thus established that three isolates of *Pithomyces sacchari* produced angolide, we wished to determine whether, as in other species, the depsipeptide was present in the spore-surface spicules. Only one of the isolates, TPI122b, spored in liquid surface culture. Under such conditions it also formed angolide, the amount formed being approximately proportional to the number of spores present in a culture at any given time, but not to the total dry weight (Fig. 1). The same isolate, grown in submerged liquid culture, formed abundant vegetative mycelium but no spores, and only traces of angolide were isolated from such cultures. Similar results have been recorded for sporidesmolide production by *P. chartarum* (Dingley *et al.* 1962). This evidence, taken with the previous failure to obtain more than traces of depsipeptide from feebly sporing liquid surface cultures of *P. sacchari* IMI102686 (Bishop *et al.* 1965), established that the angolide from *P. sacchari* is associated with the spores.

The experiments of Bertaud *et al.* (1963) showed that the bulk at least of spori-

desmolides in *Pithomyces chartarum* was located on the spore surface, and Bishop *et al.* (1965) found sporidesmolides to be completely removed from dried sporing felts by three successive brief chloroform washes. To compare the rates of removal of sporidesmolides from dried *P. chartarum* felts and of angolide from dried *P. sacchari* felts, liquid surface cultures of the two species were mixed before harvesting. Very brief extractions of the mixed, dried and powdered felts were made with a limited volume of chloroform; after four such extractions the residual depsipeptides were completely removed by a fifth extraction in order to determine the total amount present. The depsipeptides present in each extract were isolated and weighed, and the proportions of sporidesmolides and angolide calculated from the measured specific rotations of the mixtures. Rather more than 60% of both depsipeptides was removed by the first two extractions. The third and fourth extracts, however, contained a rather smaller proportion of angolide, and after the fourth extraction about 20% of the total angolide remained unextracted, as compared with only 10% of the total sporidesmolides (Table 4). It was concluded that most of the angolide in *P. sacchari* was present on the spore surface but that, as compared with the sporidesmolides of *P. chartarum*, a larger proportion was relatively inaccessible. Whether, in either species, any of the depsipeptide is intracellular is uncertain. Conceivably some might be located within the matrix of the thick spore wall.

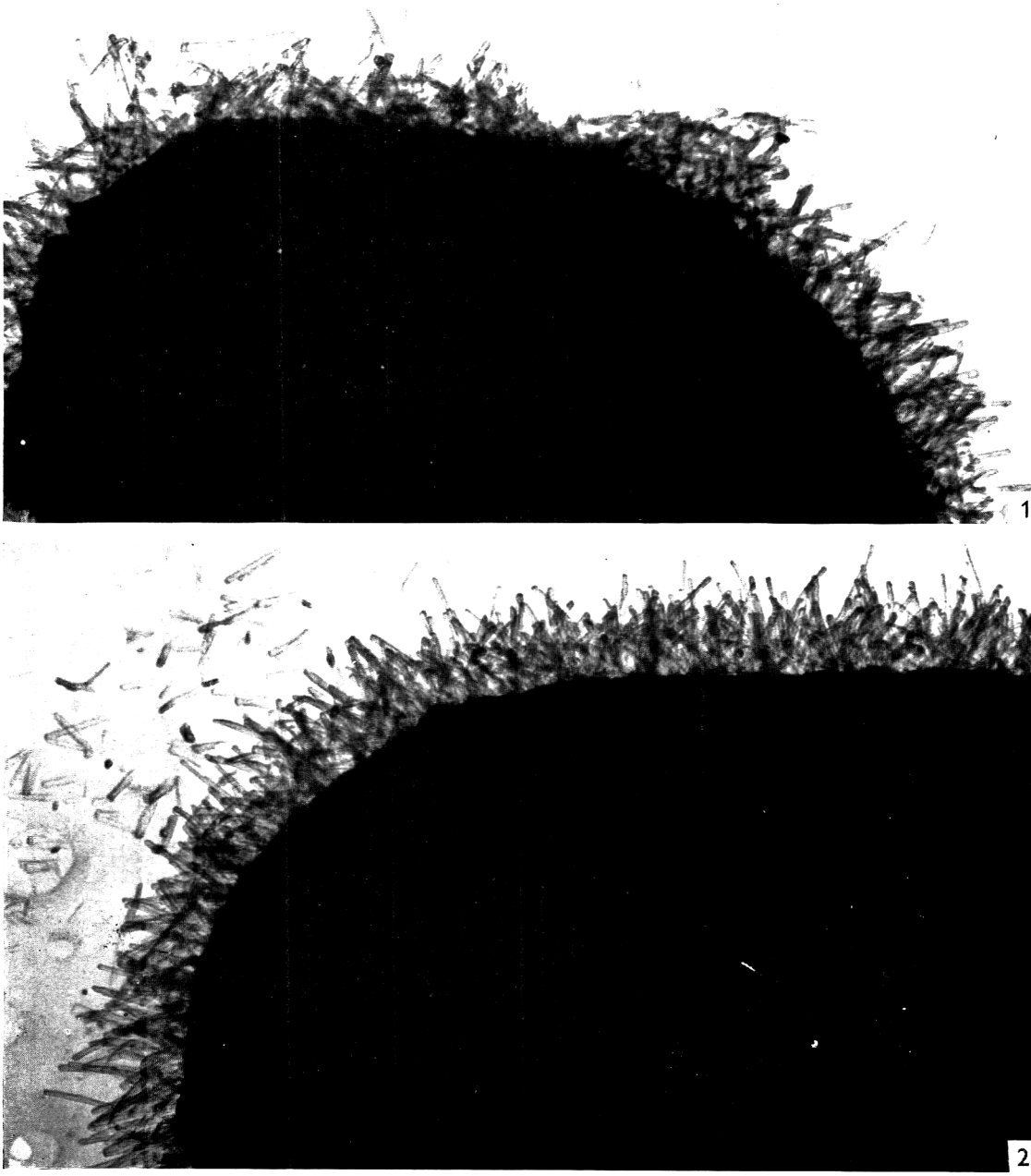
Table 4. *Extraction of sporidesmolides and angolide from mixed powdered dried felts of Pithomyces chartarum and P. sacchari*

Each extraction was performed by stirring mixed dried powdered felts with chloroform for 15 sec. After four extractions, the remaining depsipeptide was removed by continuous extraction with chloroform.

Extraction no.	Depsipeptide remaining unextracted (% of total)	
	Sporidesmolides	Angolide
1	72·9	67·3
2	37·9	38·3
3	19·3	27·3
4	10·8	21·3

DISCUSSION

Although many species of the genus *Pithomyces* are described and figured by Ellis (1960, 1965), only *P. chartarum*, *P. maydicus*, *P. cynodontis* and *P. sacchari* are available in culture. It may therefore be useful at this stage to summarize the state of our knowledge of the spore-surface spicules. As to their nature, it has been established that a major component in each case is a cyclodepsipeptide (whose chemical nature has been defined and which can serve as a taxonomic character). This does not exclude the possibility that other substances, such as lipids, may enter into the spicule structure. As to the location of the spore-surface spicules, there can be no doubt that the major portion of the spicular depsipeptides is located on the surface of the spores. We cannot exclude the possibility that small numbers of spicules, or small amounts of spicular depsipeptides, may occur elsewhere than on the spore surface. As to the genesis of the spore-surface spicules, it is known (Butler *et al.* 1962) that sporidesmolide I can be derived from exogenous amino acids, but biochemical details of cyclodepsipeptide biosynthesis, where it takes place, and how the insoluble products come



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(*Facing p.* 83)

to be located on the spore surface, are all unknown. As to the significance of the surface spicules, they appear unlikely to confer a selective advantage by antimicrobial action; no such action was demonstrable by Bishop *et al.* (1965). Their contribution to spore water-repellancy, and their possible biochemical implication in the process of sporogenesis, remain open questions. Of the oustanding problems, the last appears to be the most fundamental. All the Pithomyces spore-surface cyclodepsipeptides are composed of residues which may be derived from branched-chain aliphatic α-keto acids. This suggests that such acids may accumulate at the time of sporulation. Whether such accumulation does occur, and whether it is causally related to sporogenesis, are questions which it is hoped to explore further.

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EXPLANATION OF PLATE

Profile electron micrographs of portions of spores of two *Pithomyces sacchari* isolates, shadowed with carbon; ×20,000. Fig. 1. Strain TPI122a. Fig. 2. Strain TPI122b.

NOTE

Isolates TPI 122a and TPI 122b have been allocated the accession numbers IMI 120724 and IMI 120725 respectively.

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Associated Diploids Involving Penicillinase Plasmids in *Staphylococcus aureus*

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SUMMARY

Staphylococcus aureus penicillinase plasmid diploids of the type (α.i$^+$p^+_A/β.i$^-$pC$^+$), when constructed in strain 147, normally segregated one of the two parental genotypes at a frequency of about 1/5000 divisions, and recombinants were rare. In about 2% of the diploid clones, however, segregation occurred at a much higher frequency and in these clones both plasmids might be lost together. Examination of these 'unstable' diploids suggests that their two plasmids are associated to form a single structure, so that they are subsequently lost or transduced together. The ease of formation of associated diploids suggests that the penicillinase plasmids may be circular.

INTRODUCTION

The genes responsible for penicillinase synthesis in *Staphylococcus aureus* are usually carried on a plasmid, an extrachromosomal piece of DNA (Novick, 1963). Apart from the penicillinase-controlling and structural genes, a number of other genetic markers have been located on the penicillinase plasmids, and a number of distinct plasmid types can be identified on the basis of the markers they carry (Novick & Richmond, 1965; Richmond, 1965*a*; Dyke & Richmond, 1967).

Transduction of a penicillinase plasmid to a strain already containing another, leads to one of two situations, depending on the pair of plasmids involved. With some pairs, recombination occurs and is followed by elimination of one of the parental plasmid types. With other pairs, however, diploids are formed (Richmond, 1965*b*; Novick & Richmond, 1965). Normally, these diploids—such as the diploid 147(α.i$^+$p$^+$/β.i$^-$p$^+$) which is formed by transducing strain 147(β.i$^-$p$^+$) with phage propagated on strain 8325(α.i$^+$p$^+$)—segregate to the two possible parental haploid states, 147(α.i$^+$p$^+$) and 147(β.i$^-$ p$^+$), at a frequency close to the rate at which the relevant plasmids are lost from a haploid cell, and recombination is rare.

The use of a special dye reagent (see later) allows this process to be seen by observing stained colonies growing on the surface of agar, constitutive segregants appear as dark sectors in a pale purple colony (see fig. 4 in Novick & Richmond, 1965). When the dye is used in this way, however, a few colonies are found in which segregation appears to occur at a much higher rate. Further examination of these 'unstable' diploids shows that, in addition to a high rate of segregation of the α-plasmid, a very high rate of loss of both the α- and β-plasmids occurs, to give rise to penicillin-sensitive organisms. This high rate of loss to the α-plasmid in a small proportion of the clones was found to be characteristic of the α-plasmid when present in *Staphylococcus aureus*

strain 147, whether alone or accompanying another plasmid (Richmond, 1966*a*). However, the β-plasmid (the one carried by strain 147 in nature) shows an abnormally high rate of loss only in the presence of an 'unstable' α-plasmid. The instability of the β-plasmid in the presence of α is almost certainly due to association of α and β so that they act as a single unit whose stability is controlled by the α-component. Furthermore, examination of the behaviour of such α/β 'associated' diploids shows that recombinants are common among the segregants obtained from this diploid, whereas segregants from 'stable' diploids are almost invariably parental in type. This suggests that a genetic interaction between plasmids is possible in the 'unstable' diploids which is not possible when the diploids are in the stable state. The simplest mechanism whereby two plasmids can interact to form an associated diploid, from which recombinants can arise easily, is one in which the plasmids are circular.

METHODS

Organisms. The strains of *Staphylococcus aureus* used in these experiments were derived from three naturally occuring strains—NCTC 8325, 147 (Segalove, 1947; Richmond, 1965*c*) and 258 (Mitshuhashi, Morimura, Kono & Oshima, 1963). The following nomenclature has been used throughout. The host strains and the plasmids are distinguished by keeping the original strain numbers of the hosts and inserting the genotype of the plasmid in parenthesis. The Greek letter refers to the plasmid as a whole. It is followed by letters and subscript figures indicating the markers carried by the plasmid and which are relevant to the cross under consideration. The symbol *p* refers to the structural gene for penicillinase, p_A being the gene for the A-type and p_C for the C-type penicillinase (Richmond, 1965*c*). The symbol *i* refers to an inducibility gene and i^+ is the induced state since this is dominant to the constitutive (i^-) in diploids (Richmond, 1965*b*). The symbols Hg^R and em^R refer to markers conferring mercury and erythromycin resistance, respectively.

Strains carrying no penicillinase plasmid fall into two categories: those that are naturally occurring penicillinase-less strains and those that have lost their penicillinase plasmid to become penicillin sensitive. The former are designated by their strain number alone: the latter have the suffix (N), e.g. 147(N), for emphasis. In cases where recombination has occurred between different plasmids, the designation indicates which of the markers in the recombinant comes from each of the parent plasmids—as far as can be ascertained. Thus 8325(α.i$^+$p$^+$Hg^R...γem^R) designates strain 8325 carrying a recombinant plasmid, the penicillinase and mercury regions being those from α and the erythromycin being from plasmid γ. In plasmid diploids the plasmid genotypes are separated by an oblique stroke, e.g. 147(α.i$^+$p$^+$/βi$^-$p$^+$).

Diploid 147(α.i$^+$p$^+$...γ.em^R/β.i$^-_{223}$/p$^+$.em^S) was constructed by transducing strain 147(β.i$^-_{223}$/p$^+$.em^S) with phage obtained by u.v.-irradiating strain 8325 (α.i$^+$p$^+$...γ.em^R). Similarly, strain 147 (α.i^{-1}p$^+$...γ.em^R/β.i$^+$p$^+$.em^S) was made by transducing strain 147(β.i$^+$p$^+$.em^S) with phage raised on strain 8325(α.i^{-1}p$^+$...γ.em^R); and diploid 147(α.i$^+$p$^+$.Hg^S...γ.em^R/β.i$^-_{223}$ p$^+$.Hg^R.em^S) was made with phage from strain 8325(α.i$^+$p$^+$.Hg^S...γ.em^R) using strain 147(β.i$^-_{223}$ p$^+$.Hg^R.em^S) as recipient. In all cases the transductants were selected on plates containing 10 μg. erythromycin/ml. and the diploid character of the transductants detected by examining their segregation patterns. The method whereby strains 8325(α.i$^+$p$^+$...γ.em^R),

8325(α.i$^-$p$^+$...γ.em^R) and 8325(α.i$^+$p$^+$.Hg^S...γ.em^R) are obtained from strains 8325(α.i$^+$p$^+$.em^S), 8325(α.i$^-$p$^+$.em^S) and 8325(α.$^+$p$^+$.Hg^S.em^S), respectively, is described by Richmond (1966*b*). Strain 147(N) is the penicillinase-less variant obtained spontaneously from strain 147(β.i$^+$p$^+$) (Novick & Richmond, 1965).

Media. The composition of the CY medium used was described by Novick (1963). When colonies were to be stained with the *N*-phenyl-naphthylamine-azo-*o*-carboxybenzene (PNCB) dye reagent, they were grown on A^1 agar. This medium has the composition of Andrade agar (Kogut, Pollock & Tridgell, 1965) but omits the indicator. The test for mercury resistance or sensitivity was done on the peptone agar described by Moore (1960).

Staining of colonies. The production of penicillinase by colonies was normally detected by the penicillin + iodine method (Novick & Richmond, 1965) and the PNCB dye reagent was reserved for detecting sectored colonies. Staining with the dye was done as described by Novick & Richmond (1965). The test is qualitative but, in general, colonies producing much penicillinase stain dark purple, those producing less are pale purple and those producing no enzyme are orange. The presence of constitutive segregant sectors in diploid colonies thus appear as dark purple wedges round the edge of pale purple colonies when the diploids are plated out on agar lacking inducer. Any penicillinase-less sectors appear as orange wedges under similar conditions.

Resistance markers. Erythromycin-resistant strains were detected by using discs containing 10 μg. erythromycin base/disc; resistance to $HgCl_2$ was tested by the method of Green (1962).

Materials. *N*-phenyl-1-naphthylamine-azo-*o*-carboxybenzene was obtained from British Drug Houses (Poole, Dorset, England). Benzylpenicillin was obtained from Glaxo Ltd., and the methicillin used for induction experiments was part of a generous gift from the Beecham Research Laboratories.

RESULTS

'Stable' and 'unstable' diploids

The diploid 147(α.i$^+$p$^+$...γ.em^R/β.i$^-$p$^+$.em^S) was grown exponentially in CY medium and about 500 colony-forming units plated on A_1 agar. The plates were incubated overnight, stained with the PNCB dye reagent, and developed with penicillin as described in Methods. Two types of sectored colony were observed. (1) Colonies with one or two fine dark (constitutive) sectors; these will be called 'stable' clones. (2) Colonies with many dark (constitutive) flecks, mostly towards the periphery of the colony. In these colonies the rim was often pale purple or even orange (penicillin-less) and substantial orange sectors were often seen. These colonies will be called 'unstable' clones.

The appearance of these two types of colony suggested that segregation was occurring at different rates and, since the α-plasmid was known to enter an unstable state in strain 147 (Richmond, 1966*a*), the proportion of the segregants in each type of clone was measured.

For this purpose, six entire colonies of each type were picked from the surface of agar after treatment with PNCB + benzylpenicillin and subcultured in 2 ml. CY medium containing 5000 units sterile staphylococcal penicillinase to destroy excess penicillin.

The tubes were shaken for 4 hr at 35° and about 15,000 colonies from each tube plated on CY + starch agar. After incubation overnight, the plates were stained with the penicillin/iodine reagent and a differential count made to determine the proportion of constitutives and negatives on each plate. Although this method allowed ready scoring of these two segregant classes, it did not allow inducible segregants, such as 147(α.i^+p^+...γ.em^R), to be distinguished from the parent diploid and, for the purposes of this experiment, the constitutives and negatives are quoted as a proportion of all colonies (Table 1). As expected from previous work, the constitutive segregant—147(β.i^-p^+.em^S)—was present at a frequency of about 1/5000 colonies in the relatively stable diploid clones and all those picked (24/24) were erythromycin-sensitive. Penicillinase-less colonies were extremely rare; in fact, only one was found among the 40,000 or so colonies screened from the stable diploids.

Examination of the unstable diploids showed a very different picture. Constitutive segregants occurred at a very variable frequency but the lowest was about 1/600 (Table 1). Among these, erythromycin-resistant recombinants were relatively common, amounting to between 10 and 20% of all the constitutives isolated from the six parent clones. However, it was the incidence of penicillinase-less cocci in these clones

Table 1. *Staphylococcus aureus: proportion of constitutive and negative segregants obtained from stable and unstable clones of the diploid 147(α.i^+p^+...γ.em^R/β.$i^-p^+em^S$)*

Stable diploids	Total number of clones examined (approx.)	Number of constitutive segregants	Constitutives as % of total colonies	Number of i^-.em^R segregants	Number of penicillinase-less segregants	Penicillinase-less as % of total colonies
1	24,000	6	0·025	0	1	0·004
2	14,000	6	0·043	0	0	—
3	9,000	2	0·022	0	0	—
4	11,000	4	0·036	0	0	—
5	10,000	2	0·020	0	0	—
6	15,000	4	0·026	0	0	—
Unstable diploids						
1	11,000	18	0·16	2	107/11,000*	0·97
2	16,000	41	0·26	4	47/2,000*	2·35
3	8,000	23	0·29	3	407/2,000*	20·3
4	10,000	107	1·07	19	681/2,000*	34·0
5	10,000	61	0·61	8	127/2,000*	6·4
6	10,000	44	0·44	5	69/2,000*	3·5

* Smaller total samples screened because of the high incidence of negatives.

that was most surprising. In all cases the proportion of these cocci was far greater than the proportion of constitutives, and in one case they appeared as frequently as 1:3 (Table 1). All were erythromycin-sensitive.

The higher incidence of penicillinase-less than constitutive segregants in the last experiment could be due to a number of causes. For example, the unstable α-plasmid (carrying the i^+ gene) might destabilize the β-plasmid by competing for an attachment site in the cell. Alternatively, the α-plasmid might join the β to form a double plasmid whose stability was determined by the α component. If the second possibility were correct, a plasmid of this type might be transduced *en bloc* and this possibility is supported by the finding that co-transduction of two plasmids occurs, as a rare

event, in certain other diploid strains (Novick, 1965).

If the coordinate loss of both the α- and β-plasmid from the unstable version of the diploid 147(α.i$^+$p$^+$...γ.em^R/β.i$^-$p$^+$.em^S) was due to the association of the two plasmids, then the frequency with which the diploid culture formed penicillinase-less segregants at a given point in its growth would be related to the frequency with which transducing phage raised on these unstable diploids at that time could co-transduce both plasmids to a suitable recipient. There are certain technical difficulties, however, against determining the frequency with which a clone is undergoing segregation to the negative state, at a given moment in its history. In any segregating culture the number of segregants appearing *de novo* during a single generation is a small proportion of the total number of segregants, since most of the negatives present will have arisen by division of those formed earlier in the life of the culture. As a result, to obtain an accurate measure of the co-ordinate rate of loss of both plasmids during a single generation, a very large number of colonies would have to be examined to obtain an estimate of worth-while precision. As an alternative, it was decided to compare the rate at which both components of the diploid were co-transduced with the proportion of penicillinase-less organisms present at the time the transducing phage was grown on the strain. Under these circumstances, there should be an approximately linear relationship between these two quantities, particularly since the unstable state of the α-plasmid in strain 147 changes to the stable relatively infrequently (Richmond, 1965*b*).

To test this possibility, the diploid 147(α.i$^-{}_1$p$^+$...γ.em^R/β.i$^+$p$^+$.em^S) was grown on the surface of agar, and 4 stable and 10 unstable colonies picked after staining with PNCB + penicillin, as described above. This diploid, rather than the one used in the previous experiment, was chosen to simplify the identification of haploid and associated diploid transductants (see later). After transfer to 3·0 ml. fresh CY medium, the colonies were grown to a concentration of about 10^9 organisms/ml. and the culture then divided into two parts. One part (1·0 ml.; culture 1) was used to provide serial dilutions for plating about 10,000 colony-forming units on CY + starch plates to determine the proportion of cocci in the clones which were 'negatives' at this stage: the other part (2·0 ml.; culture 2) was centrifuged, the cocci resuspended in 2·0 ml. physiological saline and u.v.-irradiated to induce the carried phages. Transducing phage, prepared in this way, was then used to infect strain 147(N) at a multiplicity of 1·1 p.f.u./c.f.u. and the transductants selected with erythromycin. Selection by this means allowed isolation of any transduced diploids, together with the α...γ haploid—i.e. 147(α.i$^-$p$^+$...γ.em^R)—and any recombinants of the type 147(β.i$^+$p$^+$...γ.em^R).

The strain 147(α.i$^-$p$^+$...γ.em^R) was constitutive and could be readily distinguished therefore from the transduced diploid which was inducible. To distinguish the inducible diploid transductants from the inducible recombinant 147(β.i$^-$p$^+$...γ.em^R), colonies were streaked on CY + starch agar lacking inducer and the presence of constitutive segregants sought by staining with penicillin + iodine. The frequency of segregation of the diploids, and the characteristically large amounts of penicillinase synthesized by the constitutive segregants, ensured that the diploids were distinguished easily from haploid transductants such as 147(β.i$^+$p$^+$...γ.em^R).

The proportion of negatives present in each of the 10 unstable diploid clones and the proportion of *em*^{R} transductants which were diploids is shown in Table 2. By and large there was an approximate correspondence between the proportion of negatives found in an unstable diploid clone and the ability of that clone to act as a source of

transducing phage capable of transferring both components of the diploid. As far as the 4 stable clones are concerned, a single penicillinase-less segregant was obtained in one of the clones tested. Furthermore, no examples of transduced diploids were found among the 50 or so em^R transductants examined from each clone (Table 2). In all clones the em^R transductants were all identical with the stock strain 147(α.i$^-$p$^+$...γ.em^R).

These results suggested that the two plasmid components of an unstable diploid associated to form a structure capable of being transduced by a single phage and liable to be lost as a single event. The absence of the phenomenon of co-transduction from stable clones and their property of segregating the two component plasmids separately suggested that association of the two plasmids is rare in stable diploids.

Table 2. *Staphylococcus aureus: comparison of the behaviour of stable and unstable versions of the diploid 147(α.i$^-$p$^+$...γ.em^R/β.i$^+$p$^+$.em^S), (a) as a source of penicillinase-less variants, and (b) as a source of phage capable of transducing both plasmids from the diploid to strain 147(N)*

Stable diploids	Total number of colonies examined	Number of penicillinase-less variants	Penicillinase-less variants as % of total colonies	Total number of em^R transductants	Total number of constitutive em^R transductants	Number of diploid transductants
1	11,000	1	0·009	946	922	0/50
2	12,000	0	—	654	636	0/50
3	10,000	0	—	962	944	0/50
4	8,500	0	—	922	914	0/50
Unstable diploids						
1	628	45	7·1	841	607	7/25
2	714	192	27	888	240	20/25
3	396	87	22	619	301	16/25
4	841	302	35	712	81	20/25
5	627	81	13	397	233	7/25
6	388	70	18	581	239	9/25
7	912	218	24	577	209	14/25
8	902	180	20	828	322	12/25
9	688	337	49	618	37	21/25
10	703	232	33	714	238	18/25

Recombination in stable and unstable diploids

The results shown in Table 1 suggest, on the basis of very small samples, that recombination between the β- and γ-plasmids (i.e. the appearance of (β.i$^-$...γ.em^R) recombinants) was more common in unstable than in stable plasmid diploids. To test this, constitutive segregants from stable and unstable clones of the diploid 147(α.i$^+$p$^+$...γ.em^R/β.i$^-$p$^+$.em^S) were picked and screened to see whether they were erythromycin resistant or sensitive. In all, 67 constitutives were picked from the stable diploid and only one was erythromycin resistant, whereas of the 81 constitutives picked from unstable diploids, 13 were resistant to erythromycin. The twelve-fold greater abundance of erythromycin-resistant recombinants among the unstable diploids shows that recombination, at least between the β and γ plasmids, was more common in the unstable than in the stable colonies.

Similarly, in a separate experiment, examination of the constitutive segregants from the diploid 147(α.i$^+$p$^+$Hg^S...γ.em^R/γ.i$^-$p$^+$Hg^R.em^S) showed that recombinants involving the Hg marker also occurred more frequently in unstable than in stable diploid clones.

DISCUSSION

The behaviour of the two plasmids which comprise the diploids studied here was very different, depending whether they were present in a stable or in an unstable clone. In the stable state, each component of the diploid segregated to leave a haploid coccus containing the other plasmid, and penicillinase-less clones only arose by the step-wise loss of both plasmids. Propagation of phage on a stable diploid followed by transduction to a suitable penicillinase-less recipient allowed transfer of either plasmid (but not both) and recombinants were only formed rarely between the plasmid components of a stable diploid. Among the unstable diploids, however, three characters appeared to be correlated with the unstable state: (1) a high frequency of co-ordinate loss of both plasmids from the diploid; (2) a high frequency of co-transduction of both plasmids to a suitable penicillinase-less recipient when the diploid is used as a source of transducing phage; (3) the appearance of recombinants at a relatively high frequency among the segregants from an unstable diploid. The first two of these characters suggest that in unstable diploids the two plasmids behave as a single unit. These properties, taken in conjunction with the relatively high frequency of recombination found among the segregants from such diploids, suggests that the connexion formed between the two plasmids may be similar to that formed on integration of an F-factor into the *Escherichia coli* chromosome. Campbell (1962) has suggested that F-factors may be circular and experiments on the mechanism of integration of the F-factor into the *E. coli* chromosome (Broda, Beckwith & Scaife, 1964) are consistent with the idea that a circular F-particle forms an 'open-eight' structure with the circular *E. coli* chromosome. Similarly, if it be postulated that penicillinase plasmids are circular, an associated α/β plasmid could arise by a single cross-over between α and β to form an 'open-eight' structure (Fig. 1). Such a structure could then act as a single unit both with respect to the loss of α and β from the cell or co-transduction of α and β by transducing phage. Furthermore, segregation of a single plasmid from such a diploid would require a return to the unassociated state as a first step and this would involve a cross-over between the two components of the 'open-eight' structure in the opposite sense to the one that established the structure. This second cross-over would re-establish single plasmids wherever it occurred on the opposed circles of the open-eight structure, and plasmids arising by this process would therefore be expected to contain recombinants in proportion to the distances which separate the markers on the two parental plasmids. The relatively high proportion of recombinants among the segregants from associated diploids in these experiments, when compared with segregants from stable clones, is entirely consistent with associated diploids being structures of the 'open-eight' type.

The appearance of associated plasmids in the unstable but not the stable diploid suggests that it is only in the unstable clones that the plasmids come into close enough contact within the cell for direct genetic interaction to occur. Jacob, Brenner & Cuzin (1963) postulated that there is a membrane attachment site for each distinct genetic element within the bacterial cell, and Novick (1966) produced evidence that sites of this type may exist for the penicillinase plasmids of *Staphylococcus aureus*. The

facility with which genetic interaction occurs in the unstable diploids, as compared with the behaviour of the stable diploids in the experiments reported above, suggests that the stable and unstable states might be a reflexion of two different membrane sites of attachment for the α-plasmid in strain 147: one which allows genetic interaction with β and the other which prevents it.

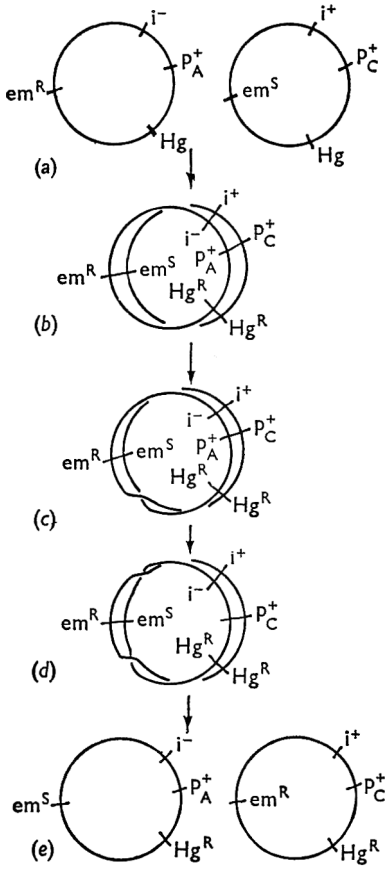

Fig. 1. A possible mechanism for the formation and separation of associated plasmid diploids in *Staphylococcus aureus*. (*a*) Normal diploids. (*b*) First stage of formation of associated diploids: apposition of the two circles. (*c*) Formation of the associated diploid by a cross-over. (*d*) Re-formation of two circles by a cross-over in the opposite sense from the first. (*e*) Final form: note new position of em^R marker in relation to pC^+.

If genetic interaction between plasmids can only occur via the associated state in an unstable diploid, it is necessary to account for the presence of any recombinants among the segregants from the stable diploids (Table 1). Previous work (Richmond, 1966) has shown that the α-plasmid can move from the stable to the unstable state (and vice versa) in *Staphylococcus aureus* strain 147, and every colony of a stable diploid will therefore contain some cocci carrying the α-plasmid in the unstable state. The incidence of unstable plasmids in a predominantly stable clone (Richmond, 1966*a*) is sufficient to account for the number of recombinants among the segregants from stable plasmid diploids.

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Studies on the Effects of Aerosolization on the Rates of Efflux of Ions from Populations of *Escherichia coli* Strain B

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SUMMARY

Potassium and phosphate were found to be the most suitable of the common ions for investigations into a possible correlation between ion movements and survival in populations recovered from aerosols of *Escherichia coli* strain B. Except at high relative humidities and short holding times, populations of [^{43}K]-labelled *E. coli* lost practically all the radioisotope within a very short time after recovery from bacterial clouds. Very little [^{43}K] was removed from labelled bacteria by agitation in water or buffer solutions under conditions which simulated some of the stresses arising from the generation and collection of aerosols. Loss of potassium appeared to be a sequel of aerosolization, but was not in itself immediately lethal to the organisms. However, the results indicated that organisms which had been recovered from aerosols could not be regarded as unchanged rehydrated forms of the original bacteria. The pattern of phosphate efflux from [^{32}P]-labelled organisms was quite different from the corresponding loss of potassium. A great deal of phosphate loss was due merely to the violent 'washing' procedures involved in the generation and collection of aerosols. Damage to ion-retention mechanisms may contribute to the decreased viability of organisms recovered from bacterial aerosols.

INTRODUCTION

Lethal processes in bacterial aerosols are of interest since the causative organisms of many diseases are transmitted in aerosols. Possible causes of loss of infectivity of aerosols of pathogenic bacteria may be inferred from the results of studies of factors which affect the survival of non-pathogenic organisms. Since the internal ionic environment of a bacterium is of fundamental importance in maintaining normal metabolic functions, the rate of efflux of various ions was measured from populations of *Escherichia coli* strain B both before aerosolization and immediately after recovery from aerosols. The purpose of these measurements was to detect changes in ion permeability or rates of exchange of ions and to establish whether any correlation existed between such changes and changes in the applied stress and survival of bacteria. Although many factors may affect survival in bacterial aerosols, this investigation was mainly concerned with the effects of relative humidity and holding time on aerosols maintained in the dark in a suitable apparatus (Anderson, 1966).

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METHODS

Organism. Cultures of *Escherichia coli* strain B were maintained as described by Anderson (1966).

Media. Tryptone agar medium and a standard liquid tryptone medium (concentration of main ions in μg./ml.: K, 400; Na, 2480; total PO_4, 1110; inorganic PO_4, 800; Cl, 2860; total SO_4, 250; inorganic SO_4, 135) were described by Anderson (1966). For certain radiotracer experiments, isotopic uptake was increased by modification of the standard liquid tryptone medium to give smaller concentrations of either potassium (cations in μg./ml.: K, 90; Na, 2500), sodium (cations in μg./ml.: Na, 1100; K, 1610), phosphate (total phosphate 750 μg./ml.; inorganic phosphate 475 μg./ml.) or sulphate (84 μg./ml. total SO_4; 20 μg./ml. inorganic SO_4).

Phosphate+alginate buffer. KH_2PO_4, 0·45%; $(NH_4)_2SO_4$, 0·05%; NH_4Cl, 0·05%; sodium alginate (Manucol SS/LH by Alginate Industries Ltd., London W.C.2), 0·25%; NaOH to pH 7·2, approximately 0·1%. Apart from a few special cases, this fluid was used for the recovery of organisms from aerosols and for serial dilution of most bacterial samples. The alginate was incorporated to decrease losses of organisms by physical processes (Henderson, 1952).

Gelatine saline. NaCl, 0·5%; gelatine, 0·1%; pH 7·2. This fluid was used as an alternative to phosphate+alginate buffer for serial dilutions and the collection of certain aerosol samples.

Radiochemicals. These were supplied by The Radiochemical Centre, Amersham, Buckinghamshire. Carrier free [^{43}K] was obtained as the chloride in approximately 0·001 N-hydrochloric acid. [^{24}Na] was provided as unprocessed irradiated sodium chloride of specific activity 350 mc./g. [^{32}P] was obtained as the orthophosphate in dilute hydrochloric acid solution; specific activity 41·5 c./mg. Carrier-free [^{35}S] was supplied as the sulphate in aqueous solution.

Measurement of radioactivity. Solutions containing [^{35}S] and [^{32}P] were determined by a 'coincidence' scintillation counting technique by using apparatus which had previously been used for [^{14}C] assay (Anderson & Smith, 1965). The isotopes [^{24}Na] and [^{43}K] were assayed in a solid crystal scintillation counter (bench scintillation counter no. 6006 and scaler no. 1700 by Isotope Developments Ltd., Reading, Berkshire). Suspensions or solutions (3 ml.) were placed in 5 ml. disposable polythene pots with snap-on lids (catalogue no. XT 1580; X-Lon Products Ltd., London, S.W.1).

Determination of cations by flame photometry. Sodium and potassium were estimated with an E.E.L. flame photometer (Evans Electroselenium Ltd., Halstead, Essex). Bacterial suspensions were heated at 100° for 10 min. before ion estimation. The apparatus was calibrated with standard solutions which contained appropriate amounts of ions which might interfere with determinations.

Determination of phosphorus, chloride and sulphate. Phosphorus was determined by the method of King (1932), chlorides by titration with silver nitrate and potassium thiocyanate and sulphates by gravimetric estimation as barium sulphate.

Dry-weight determinations. A measured volume (about 5 ml. containing 10^{11} organisms) of bacterial suspension was centrifuged and the deposit washed once with formol saline (sodium chloride, 0·85% (w/v); formalin 3% (v/v)). After one wash with water, the bacterial pellet was dried (100° for 16 hr) and weighed.

Growth of radiolabelled suspensions of Escherichia coli, strain B. All suspensions

were grown by shake culture in liquid media at 37° and stored at room temperature for 2–5 hr before use. An inoculum of 5×10^7 organisms/ml. provided a resting phase suspension containing about 10^{10} organisms/ml. after overnight (16 hr) growth. [^{43}K]-labelled bacterial suspensions of low specific activity were obtained by overnight growth in the standard tryptone medium supplemented with 40 μc./ml. of radiotracer. [^{43}K]-labelled organisms of high specific activity were prepared by overnight growth in standard tryptone medium; the organisms were separated from the residual medium by centrifugation then resuspended as a dense suspension (2×10^{10} organisms/ml.) in a 'low potassium' tryptone medium containing the radiotracer (30 μc./ml.; 90 μg.K/ml.). This suspension was then re-incubated in a shake flask (90 min. at 37°) and stored for 1–5 hr at room temperature before use. [^{24}Na]-labelled organisms were prepared by overnight growth in normal or 'low sodium' media supplemented with radiotracer (10 μc./ml.).

[^{32}P]-labelled bacterial suspensions were obtained by overnight growth in normal or 'low phosphate' media containing radiotracer 10 μc./ml. Overnight growth in a 'low sulphate' medium containing radiotracer 10 μc./ml. provided [^{35}S]-labelled bacterial suspensions.

Determination of bacterial survival values. Viability was defined as the ability to produce a visible colony on tryptone agar in 18 hr. Total bacterial numbers recovered from aerosols were calculated from the radiotracer content of impinger samples and the viable and radiotracer content of unsprayed suspensions. Unsprayed suspensions were given a nominal viability of 100%. Plate counts and radiotracer determinations were so arranged that survival estimates generally had a 95% confidence belt of better than $\pm 10\%$ of observed values.

Apparatus for the study of bacterial aerosols. The technique and apparatus used have already been described (Anderson, 1966). Aerosols were stored in a rotating drum (Goldberg, Watkins, Boerke & Chatigny, 1958) and were recovered over a period of 1 min. into sonic impingers (raised Porton impinger; May & Harper, 1957) containing either phosphate+alginate buffer or gelatine saline (12 ml.; in duplicate). Impinger samples contained about 3×10^6 organisms/ml. (equiv. 0·15 μg. dry wt organism/ml.)

Preparation of bacterial samples for aerosolization and ion analysis. The bacterial pellet obtained by centrifugation of cultures was quickly washed with water by centrifugation and prepared as a suspension in water containing about 4×10^9 organisms/ml. Radiotracer and ion determinations were made on appropriate suspensions and supernatant fluids to determine the extent of incorporation of the various ions and the amounts removed on washing.

Determination of [^{32}P] *and* [^{43}K] *loss from populations of washed radiolabelled Escherichia coli strain* B. Bacterial suspensions recovered from aerosols were quickly filtered through a membrane filter (Millipore PHWP) 1 min. after the conclusion of sampling and at intervals of 14 and 29 min. thereafter (nominal 2, 15 and 30 min. samples). The efflux of radiotracer at each sampling time was obtained by subtraction of the radiotracer content of the filtrates from that of the original unfiltered bacterial suspension. Suspensions removed from the spray pot before and after each experiment were diluted 1/1000 in the appropriate collecting fluid to measure the rate of loss of tracer from non-stressed organisms at a bacterial concentration similar to that of the samples recovered from aerosols. After 2, 15 and 30 min., portions of these diluted

suspensions were filtered to measure the rate of efflux of radiotracer. Estimates of the [^{43}K] and [^{32}P] content of labelled bacteria had 95% fiducial limits of the order of ±5%.

RESULTS

Survey of the suitability of common ions and growth procedures for permeability studies with Escherichia coli strain B

The small bacterial samples (10^6–10^7 organisms/ml.; equivalent to about 10^{-6}–10^{-7}g. dry wt. organisms/ml. suspension) which may be recovered from aerosols in conventional apparatus prevented the use of ordinary assay methods and limited investigations to those ions with suitable radioactive isotopes. In a preliminary survey, the rates of efflux of a number of common ions from dense (10^{10} organisms/ml.) suspensions of *Escherichia coli* was measured by radiotracer methods. Ions were identified which were normally retained in non-stressed organisms and which would

Table 1. *Radioactive ion uptake and loss of suspensions of various cultures of Escherichia coli strain B*

Period of incorporation of isotope	Growth medium	Ion	Uptake of labelled ion (% amount added to growth medium)	Amount of labelled ion remaining in organisms after one wash with water (% amount added to growth medium)
16 hr	Standard tryptone	[^{24}Na]	Below measurable limits	—
		[^{43}K]	7·6	3·4
		[$^{32}PO_4$]	33	27
		[$^{35}SO_4$]	Below measurable limits	—
16 hr	'Low sodium' tryptone	[^{24}Na]	2·4	0·3
90 min.	16 hr growth in normal tryptone followed by incubation for 90 min. in 'low potassium' tryptone	[^{43}K]	64	55
16 hr	'Low phosphate' tryptone	[$^{32}PO_4$]	52	43
16 hr	'Low-sulphate' tryptone	[$^{35}SO_4$]	2·6	0·2

therefore be suitable indicators of changes in permeability or rates of internal–external ion exchange. Considerations of cost and safety limited investigations to [^{43}K], [^{24}Na], [$^{32}PO_4$] and [$^{35}SO_4$]. Results summarized in Table 1 show that the uptake of the various radiotracers could be improved by modification of the standard liquid tryptone medium. Sodium and sulphate were obviously unsuitable for study since they were easily washed out from non-stressed bacteria. Phosphate efflux studies were all made with organisms grown on a medium of decreased phosphate content. Uptake of [^{43}K] by organisms which had been grown overnight in normal tryptone medium was extremely low. The low specific activity of organisms which had been grown in this manner made radiotracer determinations inaccurate and the waste of large quantities of [^{43}K] in the residual medium was expensive and hazardous. However,

uptake of the radiolabel was improved, and the potassium content of the organism increased from 0·12 to 0·58% (dry weight) by re-incubation of a concentrated suspension of washed organisms in fresh [^{43}K]-labelled medium of decreased potassium content.

Effect of relative humidity and holding time in the aerosol on the rate of loss of [^{43}K] by Escherichia coli strain B

As mentioned above, this work was largely done with bacterial suspensions which had been re-incubated in fresh medium to increase the specific activity of the radiolabel. Aerosols generated from washed bacterial suspensions were stored at various relative humidities. At each relative humidity value, bacterial samples were recovered from the aerosol cloud 1·2 sec., 5 min. and 30 min. after generation of the cloud. For

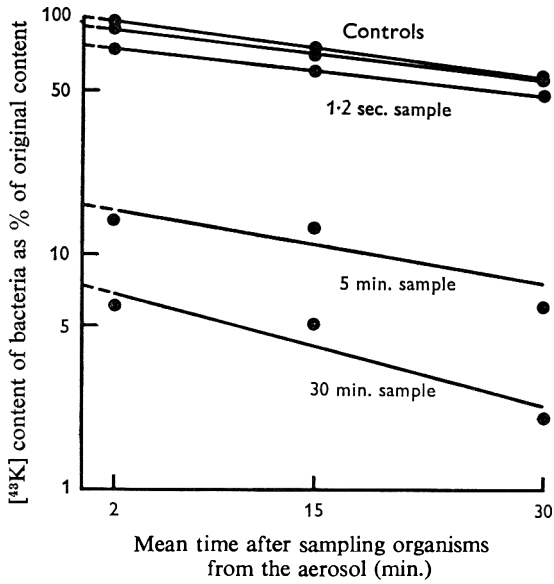

Fig. 1. The efflux of [^{43}K] from labelled populations of *Escherichia coli* strain B as a function of time after generation of aerosol, and of time after sampling of the bacterial population from an aerosol held at a relative humidity of 95%. The plots represent (reading downwards) efflux from 'control' samples removed from the spray pot before, then after the experiment, then samples recovered from aerosols at 1·2 sec., 5 min. and 30 min. after generation.

each bacterial sample collected from the aerosol the [^{43}K] content of the organisms was determined at 2, 15 and 30 min. after the mean time of sampling. The plot of log [^{43}K] content against time for these three points (2, 15, 30 min.) showed excellent linearity in nearly all instances. Figure 1 illustrates the results of a single typical experiment. A Ferranti Meteor-Mercury computer was programmed to return the slopes (log % [^{43}K]-content organisms against time) and intercepts for 13 experiments. Since the radiotracer content of the bacterial populations was calculated from the difference between the observed radiotracer values of filtered and non-filtered bacterial suspensions, the statistically acceptable scatter in radiotracer determinations sometimes resulted in apparent losses of over 100% of the original radiotracer.

The [^{43}K] content of bacteria which had been recovered from aerosols could not

generally be conveniently measured at less than 2 min. after the mean time of collection. However, values for the [^{43}K] content of bacterial populations 30 sec. after collection from the aerosol were similar to values obtained at 2 min. Likewise the [^{43}K] content of non-sprayed suspensions was similar at 10 sec. and at 2 min. after dilution with collecting fluid. The intercept or 'initial' value of potassium efflux was thus a measure of the extent of loss or exchange of internal potassium with the collecting fluid in a period of probably less than 10–30 sec. after recovery from the aerosol. Increases in 'initial' potassium efflux values imply increases in bacterial permeability or damage to other ion retention mechanisms.

The 'initial' loss of [^{43}K] from aqueous bacterial suspensions which were merely diluted in phosphate + alginate buffer collecting fluid was only 5·8% (mean of thirteen determinations). The corresponding figure (8·3%) for organisms recovered from the spray pot at the conclusion of experiments, and which had suffered violent agitation in water for 3 min., was similar. Agitation of washed bacterial suspensions

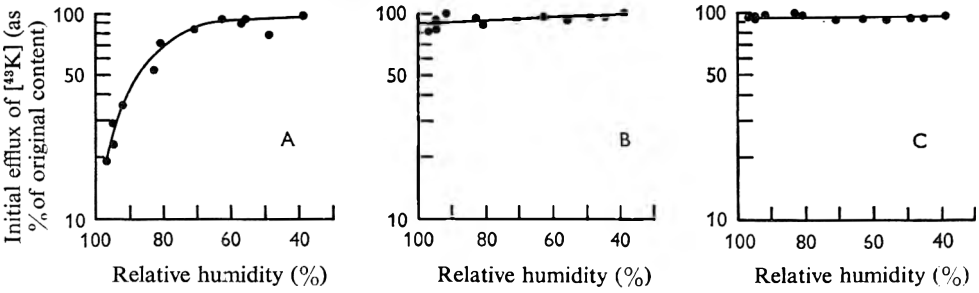

Fig. 2. The effect of relative humidity and holding time in the aerosol on the 'initial' efflux of [^{43}K] from labelled populations of *Escherichia coli* strain B. A, B and C represent, respectively, results obtained with populations recovered from aerosols at 1·2 sec., 5 min. and 30 min. after generation.

in water thus had little effect on the initial [^{43}K] efflux from this organism. Figure 2 shows that organisms recovered from aerosols at relative humidities ranging from 39 to 97% suffer a much larger loss of [^{43}K] than the controls (5·8% and 8·3%). For samples recovered from aerosols 1·2 sec. after generation there was a highly significant negative correlation (P = 0·1%) between relative humidity and initial potassium loss (correlation coefficient = −0·92). For bacterial populations recovered from aerosols 5 min. and 30 min. after generation the corresponding correlation coefficients were still negative, although smaller (−0·26 and −0·16, respectively) and not significant (P > 10%). 'Initial' [^{43}K] losses in samples recovered from aerosols after 1·2 sec. were much higher than the two controls in all 12 experiments; losses after 5 and 30 min. were invariably greater than after 1·2 sec. 'Initial' [^{43}K] losses in samples recovered from aerosols after 5 and 30 min. were mainly in the 90% region and differences between losses at these two sampling times were therefore not pronounced.

The large initial loss of radiotracer from populations recovered from aerosols could not have been due to mechanical stresses involved in the generation and collection of aerosols since the extent of the effect was dependent upon the relative humidity and holding time in the aerosol cloud. The negligible increase (1·8%) in the 'initial' loss of radiotracer when organisms recovered from the spray pot were diluted in phosphate +

alginate buffer and then violently aerated in an impinger for 1 min. provided further evidence that these mechanical stresses were unimportant.

If loss of [^{43}K] were a controlled exchange process instead of a net loss, then increases in [^{43}K] efflux would not be of such significance as a measure of the failure of ion control mechanisms. This possibility was disproved by collecting a representative array of aerosol samples into gelatine saline; this collecting fluid is free from potassium and gives survival values only slightly inferior to those obtained with phosphate + alginate buffer. Results summarized in Table 2 show that [^{43}K] efflux into gelatine saline was only a little lower than into the normal collecting fluid. This confirms that, following recovery of this organism from aerosols, there was a loss of control over mechanisms responsible for potassium retention.

Table 2. *The effect of relative humidity and holding time in the aerosol on the loss of* [^{43}*K*] *by labelled populations of Escherichia coli strain* B, *sprayed into air and collected into gelatine saline*

Relative humidity (%)	'Initial'* loss of [^{43}K] (as % original radiotracer content) from bacterial populations recovered from aerosol clouds at various times after generation:		
	1·2 sec.	5 min.	30 min.
45	91 (95)	96 (98)	93 (94)
71	84 (84)	94 (94)	85 (92)
94	23 (29)	68 (89)	86 (94)

* Comparable figures for populations collected into phosphate + alginate buffer are shown in parentheses. Mean values for 'initial' [^{43}K] efflux from non-aerosolized populations sampled from the spray pot before and after each experiment were 6·4 % (5·8) and 6·5 % (8·3), respectively.

Table 3. *The effect of relative humidity and holding time in the aerosol on the loss of* [^{43}*K*] *by labelled populations of Escherichia coli strain* B, *sprayed into nitrogen and collected into phosphate alginate buffer*

Relative humidity (%)	'Initial'* loss of [^{43}K] (as % original radiotracer content) from bacterial populations recovered from aerosol clouds at various times after generation:		
	1·2 sec.	5 min.	30 min.
48	91 (95)	92 (98)	89 (95)
66	83 (88)	90 (95)	91 (95)
92	26 (32)	98 (100)	102 (98)

* Comparable figures for populations recovered from an atmosphere of air are shown in parentheses. Mean values for 'initial' efflux from non-aerosolized populations sampled from the spray pot before and after each experiment were 5·7 % (5·8) and 7·6 % (8·3), respectively.

The enhanced survival of aerosols of *Escherichia coli* strain B, and of other organisms, in an atmosphere of nitrogen has been attributed to a toxic action of oxygen (Ferry, Brown & Damon, 1958; Hess, 1965; Cox, 1966). The rate of efflux of [^{43}K] was determined from organisms which had been aerosolized into an atmosphere of nitrogen, in order to detect whether ion efflux was affected by the absence of oxygen. Results given in Table 3 show that the extent of the 'initial' [^{43}K] efflux from bacteria recovered from aerosols which had been stored in nitrogen was similar to that from aerosols stored in air.

Small variations in growth conditions may often have a marked effect on bacterial survival in aerosols. 'Resting phase' populations were used for the phosphate efflux studies described below and for past investigations of the biochemical properties of organisms recovered from bacterial aerosols (Anderson, 1966). To prove that [^{43}K]-efflux phenomena described for 're-incubated' organisms were not unique to the particular growth conditions, comparative studies were made of resting-phase populations over a representative array of holding times and relative humidities. The low specific activity of the resting-phase organisms widened the usual fiducial limits for [^{43}K] loss from $\pm 5\%$ to about $\pm 20\%$ in these particular experiments. Results summarized in Table 4 show that although [^{43}K] loss from non-stressed resting-phase organisms was greater than from 're-incubated' bacteria under similar conditions, the large initial loss of tracer, and the general dependance of this initial loss on relative humidity and holding time, was similar for populations grown under either condition.

Table 4. *Effect of relative humidity and holding time in the aerosol on the loss of* [^{43}*K*] *by labelled populations recovered from aerosols generated from resting-phase suspensions of Escherichia coli strain* B

Relative humidity (%)	'Initial'* loss of [^{43}K] (as % original radiotracer content) from bacterial populations recovered from aerosol clouds at various times after generation: 1·2 sec.	5 min.	30 min.
34	103 (94)	120 (102)	111 (96)
36	96 (94)	101 (101)	99 (96)
70	97 (94)	100 (94)	102 (95)
71	97 (84)	95 (94)	80 (95)
89	62 (55)	—	107 (95)
92	53 (36)	92 (90)	86 (98)

* Comparable figures for populations derived from 're-incubated' cultures are shown in parentheses. Mean values for 'initial' efflux from non-aerosolized populations sampled from the spray pot before and after each experiment were 19·9% (5·8) and 39·4% (8·3), respectively.

Effect of relative humidity and holding time in the aerosol on the survival of [^{43}*K*]*-labelled populations of Escherichia coli strain* B

Figure 3 summarizes the effect of relative humidity and holding time in the aerosol on the survival of *Escherichia coli* strain B aerosols generated from washed suspensions of cultures which had been re-incubated for 90 min. in a medium of decreased potassium content. The corresponding figures for the survival of lag-phase organisms were generally lower and similar to those of Anderson (1966). There does not appear to be any simple correlation between changes in viability and the extent of [^{43}K] efflux shown in Fig. 2.

The effect of relative humidity and holding time in the aerosol on the rate of loss of [^{32}*P*] *by Escherichia coli strain* B

Aerosols were generated from washed suspensions of resting-phase cultures of *Escherichia coli* strain B which had been grown in a medium of decreased phosphate content. The 'initial' or 'intercept' values for [^{32}P] efflux were calculated in a similar way to the comparable figure for [^{43}K] efflux. The loss of [^{32}P] from populations recovered from aerosols, and from non-stressed populations, in a single typical ex-

periment is shown in Fig. 4 (note the enlargement of the y scale as compared to Fig. 1). The pattern of loss of phosphate was obviously quite different to that of loss of [^{43}K].

Results summarized in Table 5 show that the 'initial' [^{32}P] loss appeared to be independent of the relative humidity of the bacterial cloud, and only showed a small increase with increasing aerosol age. The large increase in the value of the 'initial'

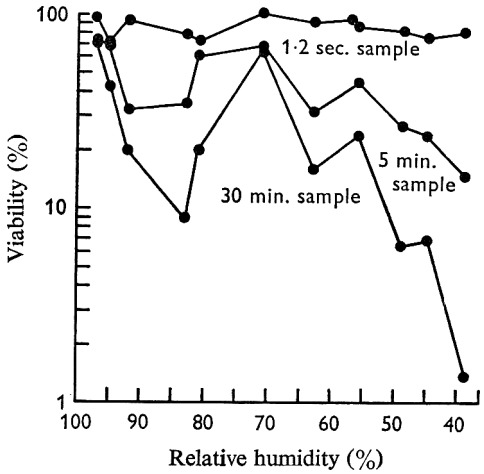

Fig. 3

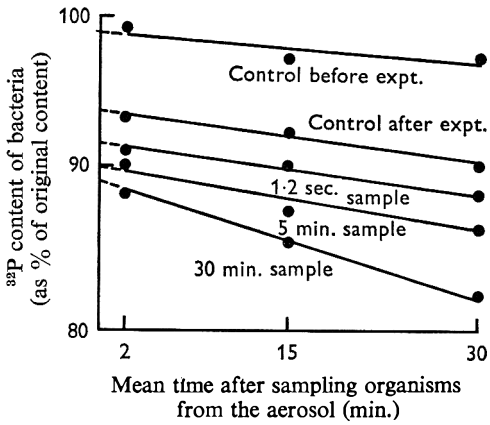

Fig. 4

Fig. 3. Viability of populations of *Escherichia coli* strain B recovered from aerosols at 1·2 sec., 5 min. and 30 min. (reading plots downwards) after generation of aerosol.

Fig. 4. The efflux of [^{32}P] from labelled populations of *Escherichia coli* strain B, as a function of time after generation of aerosol and of time after sampling of bacterial population from an aerosol stored at a relative humidity of 70%. The plots represent (reading downwards) efflux from 'control' samples recovered from the spray pot before, then after each experiment, then samples recovered from aerosols at 1·2 sec., 5 min. and 30 min. after generation.

Table 5. *The effect of relative humidity and holding time in the aerosol on the efflux of* [^{32}P] *from labelled populations of Escherichia coli strain* B

Relative humidity (%)	'Initial'* loss of [^{32}P] (as % original radiotracer content) from bacterial populations recovered from aerosol clouds at various times after generation:		
	1·2 sec.	5 min.	30 min.
36	11	11	13
44	12	10	14
53	8	8	9
55	10	17	7
70	9	10	11
78	6	10	13
84	10	16	14
89	11	14	12
93	14	14	16

* Mean values for 'initial' [^{32}P] efflux for non-aerosolized populations sampled from the spray pot before and after each experiment were 1·2% and 7·4%, respectively.

phosphate loss following agitation of the bacterial suspension in the spray device during the course of each experiment, suggests that a great deal of this ion was removed from the organism merely by agitation with water. Agitation of organisms for 1 min. in phosphate + alginate buffer in an aerosol collection device caused an exchange or loss of [^{32}P] approaching that from samples which had been recovered from the aerosol 1·2 sec. after its generation. A high proportion of [^{32}P] efflux can thus be accounted for as a sequel of the vigorous 'washing' processes involved in the generation and collection of aerosols.

The effect of relative humidity and holding time in the aerosol on the survival of [^{32}P]-labelled populations of Escherichia coli strain B

The response of [^{32}P]-labelled populations of *Escherichia coli* strain B to variations of relative humidity and holding time in aerosols was similar to that described by Anderson (1966) for non-labelled populations. There was no simple correlation between survival and the extent of [^{32}P] efflux (Table 5).

DISCUSSION

The high and generally complete loss of potassium by populations of *Escherichia coli* strain B immediately after recovery from aerosol clouds obviously prevents any attempt to establish a simple correlation between potassium loss and survival. Organisms recovered from bacterial clouds may, under certain conditions, lose most of their potassium and yet be substantially viable. Loss of control of potassium metabolism is not therefore in itself immediately lethal to the organisms provided that these are eventually returned to a medium containing adequate potassium. However, these potassium efflux studies do suggest that after recovery from aerosols *E. coli* is biochemically, and probably physically, quite different to the non-stressed organism. Therefore one cannot regard organisms which have been recovered from aerosols merely as unchanged rehydrated forms of the original bacteria. Furthermore, loss of control of potassium implies a possible derangement of bacterial processes which control the metabolism of other ions and substrates.

Populations of *Escherichia* strain B show a severe decrease in ability to synthesize inducible protein immediately after recovery from aerosol clouds (Anderson, 1966). This decreased rate of protein synthesis might well be due to the disruption of potassium ion control, since there is evidence that potassium ion is responsible for normal ribosomal function in a mutant of *E. coli* (Ennis & Lubin, 1965) and in *Aerobacter aerogenes* (Tempest, Dicks & Hunter, 1966). The decreased rate of protein synthesis might also be explained by the finding that a particular ratio of monovalent ions to magnesium is necessary for the correct functioning of ribosomes from *E. coli* (Cammack & Wade, 1965). Besides affecting protein synthesis, disturbances of potassium ion metabolism might affect the many enzymes for which this ion is a co-factor, especially those involved in carbohydrate and aromatic compound metabolism.

The rates of efflux of phosphate and potassium ions following stress in the aerosol are fundamentally different. A great deal of the [^{32}P] present in *Escherichia coli* is bound to high molecular weight organic compounds and would obviously be unable to leave the organism before extensive damage had occurred. Although potassium efflux appears to be a direct sequel of aerosolization, much of the loss of phosphate

is due to a mere 'washing out' of this anion. No attempt was made to determine the chemical nature of [^{32}P] lost from organisms recovered from aerosols, because of technical difficulties. If suitable techniques could be developed, a knowledge of the effect of various stresses in the aerosol on the efflux of organic phosphorus compounds might provide further information on the changes in metabolism and ion control resulting from aerosolization.

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Some Observations on the Envelope of an Influenza Virus

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SUMMARY

Micrographs of the envelope of fowl-plague virus revealed an arrangement of subunits each one of which was surrounded by either five or six others. The similarity of this arrangement to that of a cubic virus capsid has led us to consider which components of morphologically different viruses fulfil similar functions.

INTRODUCTION

A virus particle consists of two parts, the essential nucleic acid and a means of protecting this from the environment. It was predicted that the protective covering would be built up of identical repeating subunits (Crick & Watson, 1956). Early X-ray diffraction studies suggested that these units would be arranged symmetrically (Hodgkin, 1949) and that the symmetry would be one of two types, helical or cubic. The application of negative-staining to virus particles shortly after this revealed pictorially both types of symmetry (Horne & Wildy, 1961). Viruses which had by previous techniques been considered to be spherical were now seen to be icosahedral, i.e. belonged to the cubic group, and particles which had been described as rod-shaped were now seen to have helically-arranged subunits. The cubic arrangement was found for DNA and for RNA viruses and can be found among bacterial, plant and animal viruses. The simple helical arrangement, on the other hand, seems to be confined mainly to RNA viruses, and, of even greater importance in the present connexion, has not been found in the simple form among animal viruses. Helical symmetry, however, is frequently present among animal viruses, the simple RNA and protein arrangement (ribonucleoprotein) being found enclosed within an additional lipoprotein envelope. The overall form of this outer envelope is usually irregular, and viruses such as influenza are usually described as being pleomorphic. The lipoprotein covering of such viruses seems to contain both viral and host material (Cruickshank, 1964). Until now it has not been looked on as conforming to the requirements of virus structure—that is, made up of identical repeating subunits identically arranged in a helical virus, or with each subunit surrounded by either six or five others in a cubic virus (Caspar & Klug, 1962).

RESULTS

While working with fowl-plague virus, an influenza A virus (Almeida, Himmelweit & Isaacs, 1966), we obtained micrographs which suggested that, although not conforming to the arrangement found in either helical or cubic viruses, the envelope of pleomorphic viruses such as the myxoviruses may bear a much closer resemblance to these arrangements than was previously suspected.

Plate 1, fig. 1., shows a negatively-stained fowl-plague virus particle with a completely regular array of subunits on the surface. Particles showing such complete regularity of arrangement were found only with difficulty and probably resulted from a fortunate combination of staining conditions and particle orientation. On the other hand, particles showing regular subunit arrangement over small areas could be found frequently and confirmed that the phenomenon was a real one and not an artifact formed by local conditions (Pl. 1, fig. 2). Like the cubic capsid (Pl. 1, fig. 3) the fowl-plague envelope illustrated in Pl. 1, fig. 1, is built up of subunits surrounded by either five or six other subunits. The difference between them lies in the fact that while the distribution of 'fives' and 'sixes' is fixed for the adenovirus capsid, it is random for the fowl-plague envelope. (Pl. 2, fig. 5)

This finding, that the envelope of a myxovirus resembles more closely than had been supposed the capsid of a cubic virus, led us to consider which components of morphologically distinct viruses—for example, measles and poliovirus—correspond most closely with regard to structure and function. Of course, there is no doubt that the helical ribonucleoprotein which constitutes the virion (the entire infective virus) in plant viruses, such as tobacco mosaic virus, bears a marked similarity to the internal component of a virus such as measles. They both consist of RNA and protein: in both the protein is arranged helically and the diameter of both is the same. The morphology of the ribonucleoprotein of measles, though usually seen as a herringbone pattern indicative of an extended helix, can nevertheless sometimes be seen in a much more straight and compressed form, very similar to the tobacco mosaic virus rod (Pl. 1, fig. 4).

However, when the two are compared biologically, the roles of the two proteins are markedly different The tobacco mosaic virus protein is in direct contact with the environment and is apparently capable of protecting the nucleic acid from it, whereas the measles helix is separated from the environment by the envelope, and it seems improbable that the ribonucleoprotein should be capable of survival in the environment as an infective entity when not surrounded by this envelope. Again, when compared in their capacity as antigens, the tobacco mosaic virus protein will cause the production of antibodies which neutralize the virus, but the internal myxovirus protein will not (Schäfer, 1957). This means that, when compared biologically, the simple cubic virus bears the same relationship to the helical protein of tobacco mosaic virus as it does to the outer envelope of animal viruses with an internal helical component.

It follows that that, in terms of biological function, if not of morphology alone, it is the coverings presented to the environment that correspond, and the flexible helical ribonucleoprotein of the myxoviruses, inside its envelope, corresponds with the contents of the simple cubic virus capsid. This leads to the question of whether cubic capsids contain an internal protein in association with the nucleic acid. The absence of such a protein is by no means certain, and its presence would of course heighten the resemblance. One virus with a regular capsid (vesicular stomatitis virus) has now been shown to contain within its capsid a ribonucleoprotein helix (Brown, Cartwright & Almeida, 1966); also, an arbovirus (Venezuelan equine encephalomyelitis), which may have a symmetrical cubic capsid, has been shown to contain a helix (Klimenko *et al.* 1965).

A further corollary is that the myxovirus envelope and the cubic capsid have more in

common with each other than has been realized hitherto (Pl. 1, figs. 1, 3). Admittedly the myxovirus envelope contains lipid and carbohydrate, which are lacking from the cubic capsid, and is asymmetrical. On the other hand, this envelope is the basic means of protecting the internal nucleic acid, it is in contact with the environment, and it, rather than protein of the ribonucleoprotein, is the immunogen. In all of these respects the myxovirus envelope parallels the function of the cubic virus capsid.

It appears therefore that animal viruses may be regarded as having two components: first, the nucleic acid, which in some cases, e.g. the myxoviruses and vesicular stomatitis virus, has helically-arranged protein associated with it; secondly, the capsid or envelope, which consists essentially of protein units arranged as hexamers or pentamers, and which surrounds and protects the contained nucleic acid or nucleoprotein. This protective apparatus of 'fives' and 'sixes' can be arranged in either a symmetrical or a pleomorphic manner and can be built solely of protein or may have additional lipid and carbohydrate. The term 'nucleo-capsid' (Caspar *et al.* 1962) used for both the virion of simple cubic viruses and the ribonucleoprotein of myxoviruses is therefore misleading, being applied to two functionally different entities. If this reasoning is valid, then the distinction into cubic and helical would not be as meaningful as a division into cubic and pleomorphic. This would then compare analogous structures that appear to be constructed in a similar manner.

We are indebted to Dr H. G. Pereira for supplying us with the fowl-plague virus.

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EXPLANATION OF PLATES

PLATE 1

Electron micrographs of negatively-stained preparations, all at ×415,000.

Fig. 1. An unusual micrograph of a fowl-plague virus particle, showing the arrangement of subunits on its surface. The majority of these subunits are surrounded by six others, but a small, randomly located number have only five others round them. The arrow points to a location where a subunit surrounded by five others can be clearly seen. The centre-to-centre spacing of the subunits, about 70 Å, in this particle is almost identical with that of the capsomeres of the adenovirus particle shown in fig. 3.

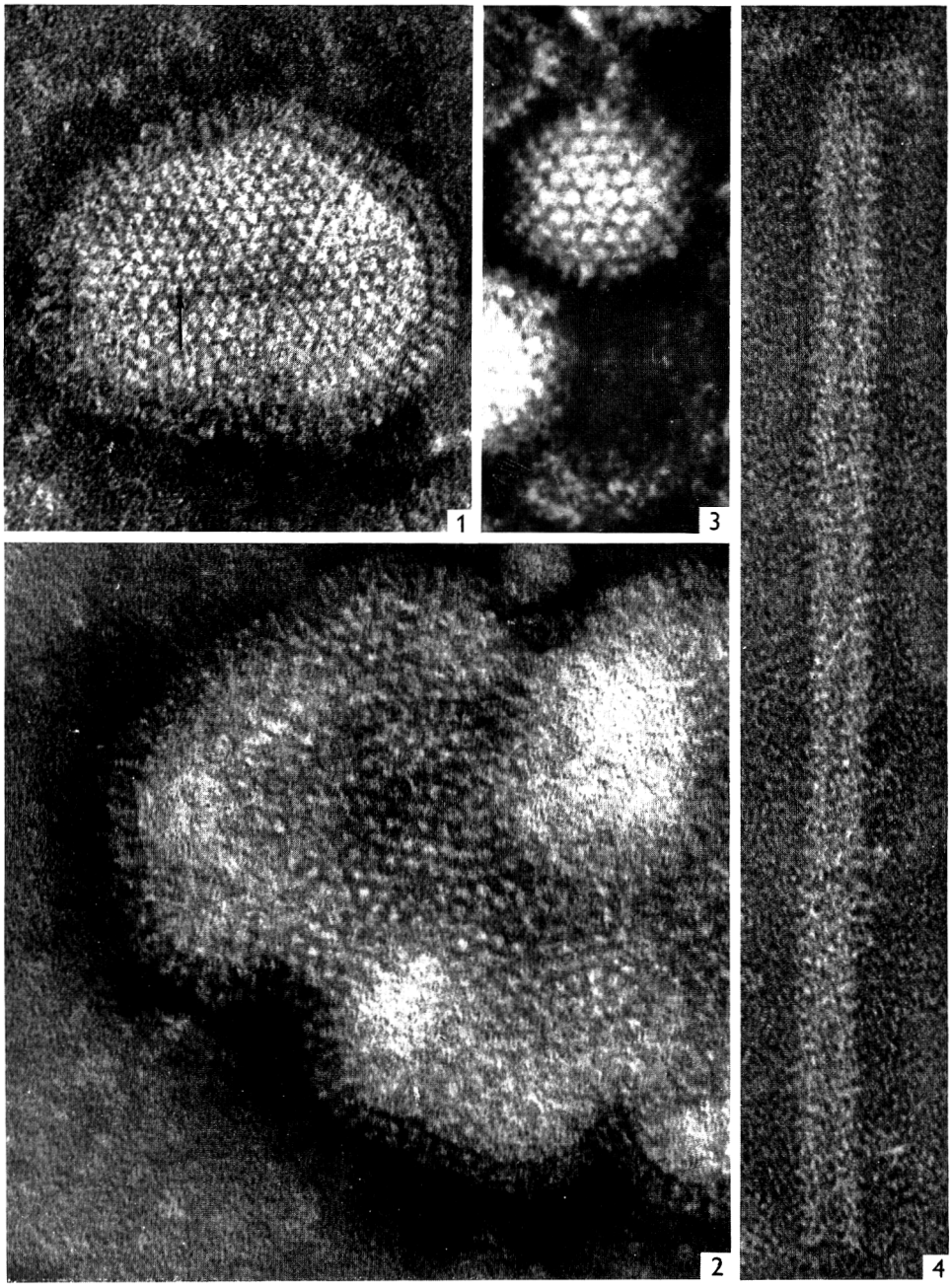
Fig. 2. Subunit arrangements of the kind shown here are frequently encountered in preparations of influenza-type viruses. Although not as regular as the subunit array in fig. 1, it is still possible to see a geometric arrangement on the surface.

Fig. 3. An adenovirus particle viewed along a two-fold axis. It is similar to fig. 1 inasmuch as the capsid is composed of subunits surrounded by either five or six others, but dissimilar in that the distribution of these is rigidly fixed.

Fig. 4. A length of the ribonucleoprotein component of measles virus. More usually, micrographs of this structure show a herringbone arrangement, but in this case the helix has remained compressed and shows a strong resemblance to helical plant viruses (e.g. tobacco mosaic virus).

PLATE 2

Fig. 5. Using 5- and 6-sleeve connectors, model (*a*) was built to fulfil icosahedral requirements, that is, a 5-sleeve connector at each apex and 6-sleeve connectors in between, to give a total of 92 subunits. Model (*b*) is built of randomly distributed 5- and 6-sleeve connectors. Although the overall appearance of the two models is quite different, the basic arrangement of each is the same and differs only in the presence or absence of symmetry.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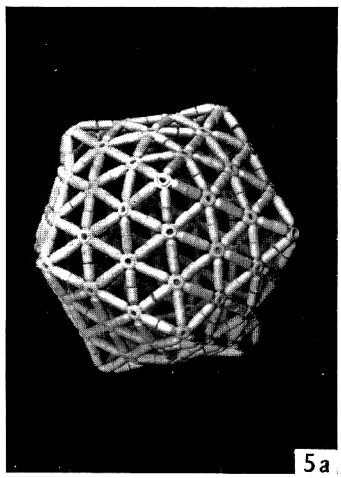

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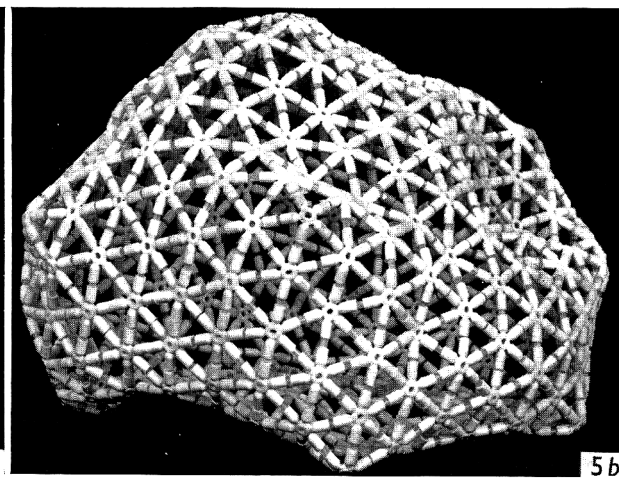

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The Aliphatic Acylamide Amidohydrolase of *Mycobacterium smegmatis*: Its Inducible Nature and Relation to Acyl-Transfer to Hydroxylamine

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SUMMARY

Mycobacterium smegmatis NCTC 8159 grew well in a minimal medium with succinate or acetamide as sole carbon source. Washed bacteria or cell-free extracts hydrolyzed 15 monocarboxylic amides, but not 4 related *N*-substituted amides. Formamide was the best substrate, followed by *n*-butyramide. Extracts of bacteria grown on acetamide hydrolyzed formamide about 60 times and butyramide about 20 times as rapidly as bacteria grown on succinate. Other short-chain fatty acylamides were also more rapidly hydrolyzed, but benzamidase activity was not similarly induced by growth on acetamide. Extracts of bacteria grown on succinate transferred acyl groups from propionamide, butyramide and nicotinamide to hydroxylamine, to form hydroxamates. Transferase activity, unlike aliphatic amidase activity, was decreased in extracts of bacteria grown on acetamide. Amidase activity in extracts was purified twofold and freed from transferase activity. Formamidase and butyramidase activities were not separated, and were similarly affected by heat and dithio-*bis*-nitrobenzoic acid. The amidase was induced by growth on acetate and on butyramide, but not on propionate, butyrate or benzamide, all of which were good growth-substrates. *N*-methylacetamide and *N*-acetylacetamide were non-substrate inducers of amidase for bacteria growing on succinate.

INTRODUCTION

That mycobacteria are able to hydrolyze amides has been recognized by implication since Sauton (1912) described a chemically defined medium for *Mycobacterium tuberculosis*, which contained asparagine. Grossowicz & Halpern (1956*a*, *b*, 1957) and Halpern & Grossowicz (1957) found that bacteria and bacterial extracts from *M. phlei* and *M. tuberculosis* strain BCG hydrolyzed several amides, and they used inhibitors and heat-treatment to differentiate nicotinamidase, glycinamidase and asparaginase activities. Pershin & Nesvab'da (1960) showed by heat-treatment that acetamidase and nicotinamidase were separate activities of a saprophytic mycobacterium. Kimura (1959*a*, *b*, *c*, *d*) studied *M. avium* and purified a nicotinamidase from it, as well as measuring the ability of *M. avium*, *M. smegmatis and M. phlei* to hydrolyze several amides, and to form hydroxamates from salts or amides of organic acids and hydroxylamine.

The characteristic patterns of amide-hydrolyzing activity were used by Bönicke

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(1960) for classifying mycobacteria; the method has been discussed by Juhlin (1960) and Schneidau (1963), who also extended the range of amides used and included members of the genus *Nocardia*. Nagayama, Konno & Oka (1961) and Urabe, Takei & Saito (1965) investigated the formamide-hydrolysing ability of various mycobacteria, though they disagreed as to its usefulness in classification.

Bönicke (1960) found that acetamide was hydrolyzed by *Mycobacterium smegmatis* after incubation for 24 hr, but not in a shorter period. He attributed this to very slow hydrolysis, but the possibility of enzyme synthesis induced by acetamide was not specifically excluded. Several inducible enzymes have been found in mycobacteria: penicillinase (Bönicke & Dittmar, 1957); enzymes for the oxidation of benzoic acids and catechol (Fitzgerald, Bernheim & Fitzgerald, 1948; Gale, 1952); enzymes for the oxidation of inositol (Ottey & Bernheim, 1956). Inducible permeases were found in *M. smegmatis* NCTC 8159 by Ellard & Clarke (1959). The observation that increased oxygen uptake occurred only after a lag when acetamide was added to organisms of the same mycobacterium grown on succinate led to the discovery, described in this paper, that the hydrolysis of some amides by *M. smegmatis* NCTC 8159 is an adaptive process. A preliminary report has been presented (Draper, 1965).

METHODS

Organism. Mycobacterium smegmatis NCTC 8159 was obtained from the National Collection of Type Cultures, subcultured monthly on slopes of Lemco agar (Clarke & Meadow, 1959) and stored at 4°.

Media. Bacteria for inoculation were grown overnight at 37° with shaking in 5 ml Lemco broth containing 0·05% Tween 80. This culture was used to inoculate experimental media at the rate of 4 ml. inoculum/l. The experimental medium was based on the minimal medium of Kohn & Harris (1941) without glucose, having trace-elements provided in 5 ml./l. of the solution described by Kelly & Clarke (1962) and containing 0·05% Tween 80. It was sterilized at 121° for 15 min. Solutions of carbon sources (10%, w/v), sterilized separately at 115° for 5 min. or by filtration through Oxoid membranes, were added at the rate of 20 ml./l. The medium (50 ml., 200 ml. or 1 l.) was contained in conical flasks (250 ml., 1 l., 5 l. respectively). Cultures were incubated at 37° with vigorous shaking for 40 hr.

Suspensions of bacteria and cell-free extracts. Mycobacterium smegmatis NCTC 8159 was collected by centrifugation, washed twice with 0·05 M-sodium potassium phosphate buffer (pH 7·2) containing 0·05% Tween 80, and suspended in the same buffer. The dry weight was measured either by drying a known volume of bacteria washed with 0·05% Tween 80, or by measuring the extinction of the suspension at 540 mμ (E_{540}) and comparing it with that of suspensions of known dry weight. To prepare extracts the bacteria were washed twice with cold 0·1 M-tris adjusted to pH 7·2 with concentrated HCl (tris buffer), suspended in the same buffer, and disrupted either by ultrasonic treatment in a 60 W. ultrasonic device (M.S.E.) with a cooled cell containing 2 ml. suspension, or with a bacterial press modified from the apparatus described by Milner, Lawrence & French (1950). The extract was centrifuged at 25,000 *g* for 20 min. at 4° to remove debris, and was stored at −20°.

Further treatment of extracts. All these manipulations were carried out on ice-cooled solutions. Streptomycin sulphate was added to the extract (1 g./40 ml.), and the pre-

cipitated material, containing nucleic acids, was removed by centrifugation at 16,500*g* for 20 min. Solid ammonium sulphate was added to the clear supernatant fluid until it was 50% saturated; the precipitated material was collected after 15 min. by centrifugation at 16,500*g* for 20 min. The sediment was carefully drained and dissolved in a small volume of tris buffer. The supernatant fluid was vacuum-dialyzed against tris buffer to remove $(NH_4)_2SO_4$ and streptomycin, and to concentrate the proteins.

The concentrated supernatant solution from $(NH_4)_2SO_4$ fractionation was further dialyzed against tris buffer containing 0·15 M-KCl and applied to a $40 \times 1{\cdot}5$ cm. column of DEAE-Sephadex A-50. The column was eluted with 1 l. tris buffer containing KCl in a linear concentration gradient from 0·15 to 0·45 M. The effluent was collected in 5 ml. fractions, and the extinction was measured at 280 mμ. Protein and amidase activity were also measured in the fractions. Fractions containing amidase activity were combined and vacuum-dialyzed, to give the material described below as partially purified enzyme.

Estimation of amide hydrolysis. The hydrolysis of amides was measured at 37° in 0·05 M-phosphate buffer (pH 7·2) for organisms, or 0·1 M-tris buffer (pH 7·2) for extracts. In the general method, samples (1 ml.) of a mixture containing 0·04 or 0·02 M-amide and organisms or extract were taken at appropriate times, and ammonia determined in Conway units (Conway, 1947) by the method described by Kelly & Clarke (1962) but allowing diffusion of ammonia to occur for 90 min. at 37°. Amounts of cells or protein were chosen to give measurable hydrolysis in 30 min.–1 hr; typically, 5 mg. adapted cells (0·2 mg. for formamide) and 0·5 mg. protein were used in 10 ml. buffer. Of the amides used only formamide, pyruvamide and glutamine were significantly hydrolyzed by the alkali in the Conway units. Hydrolysis of formamide was alternatively followed by measuring the formamide remaining in the reaction mixture after incubation with extract, by using a modification of the hydroxamate reaction (Snell & Snell, 1954). To 1 ml. of incubation mixture containing bacterial extract in tris buffer (pH 7·2), and 5 μmoles formamide, were added 2 ml. of a 1+1 (by vol.) mixture of hydroxylamine hydrochloride (15%, w/v) and NaOH (15%, w/v) cooled in ice. Amounts of protein used in the incubation mixture were adjusted to hydrolyze about half the formamide present in 5–10 min., and were typically 50–100 μg./5 ml. buffer. After 5 min. or more (formation of the hydroxamate is very rapid, and it is stable under these conditions) at 37°, 2 ml. of a solution of $FeCl_3$ (5% (w/v) in 1·4 N-HCl) were added. The mixture was shaken vigorously for about 30 sec., and its extinction measured at 500 mμ. The colour faded, so that it was necessary to read at a fixed time after mixing, conveniently 1 min. The colours were compared in each experiment with standards containing formamide but no enzyme. Phosphate interfered with the reaction.

Estimation of transferase activity. The ability of bacteria and extracts to transfer acyl groups from amides or fatty acid salts to hydroxylamine, to form hydroxamates, was measured as by Brammar & Clarke (1964). Since the activity in *Mycobacterium smegmatis* was low, incubation times of up to 1 hr were used, and corrections were made for the non-enzymic formation of hydroxamates which occurred in this time.

Heat and chemical denaturation. The enzyme preparation was heat-treated at 60° for various periods in tris buffer (pH 7·2). The mixture was cooled and used for amidase assay in the usual way. The thiol reagent dithio-*bis*-nitrobenzoic acid was used at $1{\cdot}5 \times 10^{-6}$ M in tris buffer (pH 7·2) at 37° or at $1{\cdot}5 \times 10^{-5}$ M in tris buffer (pH 8) con-

taining 10^{-3} M-ethylene-diaminetetra-acetate, at room temperature (about 22°). The reaction with thiol groups was measured at 412 mμ, with cysteine as an internal standard. After use of the more concentrated reagent the enzyme was dialyzed against tris buffer (pH 7·2) before the amidase activity was measured.

Ultracentrifugal analysis. The partially purified enzyme preparation was concentrated by vacuum dialysis and further dialyzed against tris buffer containing 0·2 M-KCl. The non-diffusible material was clarified by centrifuging, and examined in a Spinco model E ultracentrifuge at 10°. Sedimentation coefficients were calculated from the observed rates of migration of protein boundaries.

Disc electrophoresis in acrylamide gel. Electrophoresis of extracts of bacteria and of the partially purified enzyme on columns of acrylamide gel was done by using the system of Ornstein (1964) and Davis (1964). Zones of protein were stained with 0·1% naphthalene black in methanol + acetic acid + water (4 + 16 + 1, by vol.), and the gels were de-stained electrolytically in the same solvent.

Protein estimation. Protein in extracts was measured by the modified Folin phenol method of Lowry, Rosebrough, Farr & Randall (1951).

Chemicals. Pyruvamide was kindly prepared by Dr C. A. Vernon by the method of Claisen (Claisen & Shadwell, 1878; Claisen & Moritz, 1880) using acetyl cyanide (Tschelinzeff & Schmidt, 1929). β-Hydroxypropionamide was made from β-propiolactone (Gresham *et al.* 1951), glycolamide from ethyl glycolate by ammoniolysis, and lactamide from lactide (Schmuck, 1924). Other chemicals were from commercial sources. Acetamide was recrystallized from acetone, sodium succinate from aqueous ethanol and tris from aqueous methanol.

RESULTS

Hydrolysis of amides by washed bacteria

Figure 1 shows typical curves of ammonia production from amides by washed *Mycobacterium smegmatis* grown on acetamide. Liberation of ammonia was linear with time during the period of measurement (30 min.). The rates at which washed

Table 1. *Rates of hydrolysis of aliphatic amides by washed Mycobacterium smegmatis* NCTC *8159*

Carbon sources were present in the growth medium at 0·2%. Amide hydrolysis was measured by following release of ammonia using Conway units (see Methods), and is expressed as μmoles/hr/mg. dry weight. Figures in parentheses are numbers of experiments, and dashes that measurements were not made.

Hydrolysis substrate	Growth substrate: Succinate	Acetate	Propionate	Butyrate	Acetamide	Butyramide	Benzamide	Nicotinamide
	Ammonia (μmoles/hr/mg. dry wt.)							
Formamide	13 (2)	741	—	—	803 (4)	—	—	—
Acetamide	0·7	—	—	—	9·2 (6)	—	—	—
Propionamide	0·7	—	—	—	13 (2)	—	—	—
Butyramide	1·7 (9)	11.8	2·6	1·9	26 (11)	4·8*	1·2	0·7†
Valeramide	1·0	—	—	—	17 (2)	—	—	—

* Butyramide added at 0·02% after 24 hr growth on succinate gives 10 units.
† Nicotinamide supported growth very poorly.

suspensions of bacteria grown on various carbon sources hydrolyzed aliphatic amides are shown in Table 1. Discrepancies up to twofold between rates of hydrolysis of a given amide in different preparations grown on the same carbon source were observed, but the relative rates were similar for each preparation. Formamide was the most rapidly hydrolyzed amide, followed in order by butyramide, valeramide, propionamide, acetamide.

Mycobacterium smegmatis grew equally well on succinate or acetamide as sole carbon source. Bacteria grown on acetamide hydrolyzed formamide 60 times and the other amides 13–19 times as rapidly as those grown on succinate. Bacteria grown on acetate as carbon source showed enhanced rates of amide hydrolysis, almost as great as acetamide-grown bacteria, but propionate and butyrate, though equally able to support growth, yielded bacteria with butyramidase activity only slightly greater than that obtained after growth on succinate. Butyramide, another good growth substrate, gave only slight enhancement, but was a more effective inducer when added after 24 hr to bacteria growing on succinate. Benzamide was a good carbon source for growth, but induced no amidase activity; nicotinamide, a poor carbon source, was also inactive as an inducer.

Table 2. *Hydrolysis of formamide and butyramide by cell-free extracts of Mycobacterium smegmatis* NCTC *8159*

Rates are given in μmoles of ammonia produced or of formamide destroyed/hr/mg. protein. Range of results and number of experiments are given in parentheses.

	Growth substrate	
Amide	Succinate	Acetamide
Formamide	188 (82–460; 6)	16980 (3240–27000; 7)
Butyramide	18 (10–29; 6)	210 (73–343; 8)

Hydrolysis by cell-free extracts

When a suspension of bacteria grown on acetamide was treated ultrasonically the rates of hydrolysis before and after breakage were, respectively (μmoles/hr/mg. dry weight organisms originally present) 29 and 26 for butyramide, and 1360 and 1700 for formamide. Centrifuged extracts of bacteria hydrolyzed amides at the rates shown in Table 2, where extracts from bacteria grown on succinate or acetamide are compared. Large variations occurred in the rates of amide hydrolysis among different preparations, especially those grown on acetamide, but the trend was clear. Formamide was hydrolyzed 90 times and butyramide 12 times as fast by extracts from bacteria grown on acetamide as by extracts from bacteria grown on succinate. The ratio of formamide hydrolysis to butryramide hydrolysis was 77 ± 24 S.D. (7 experiments) for extracts of acetamide-grown, and 11 ± 6 S.D. (5 experiments) for extracts of succinate-grown *Mycobacterium smegmatis*. The enzymic activity was slowly lost during storage of frozen extracts: in one sample it was decreased to 40% of the starting value after 3 months at $-10°$. It was not protected by mercapto-ethanol (10^{-4} M).

Hydrolytic activity for other amides

Washed suspensions or cell-free extracts of *Mycobacterium smegmatis* also hydrolyzed benzamide, phenylacetamide, nicotinamide, malonamide, fumaramide, gluta-

mine, asparagine. Of these, nicotinamide was the best substrate; it was hydrolyzed at about 20% of the rate for butyramide. Benzamide supported growth, but did not induce benzamidase or butyramidase activity when compared with succinate. In bacteria grown on acetamide the ability to hydrolyze benzamide was only about 20% of that of bacteria grown on succinate. Nicotinamide was a poor growth substrate and not an inducer of benzamidase (or butyramidase) activity.

Effect of amides which were not growth-substrates

The *N*-substituted amides *N*-methylformamide, *N*-methylacetamide, *N*-dimethylformamide and *N*-dimethylacetamide did not support growth of *Mycobacterium*

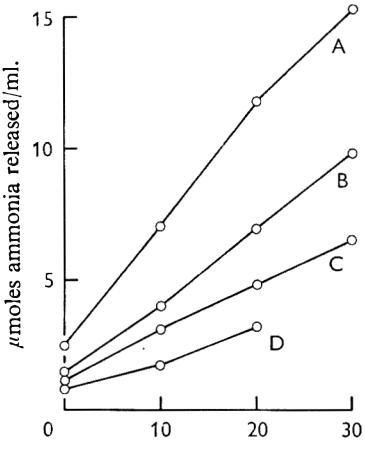

Fig. 1

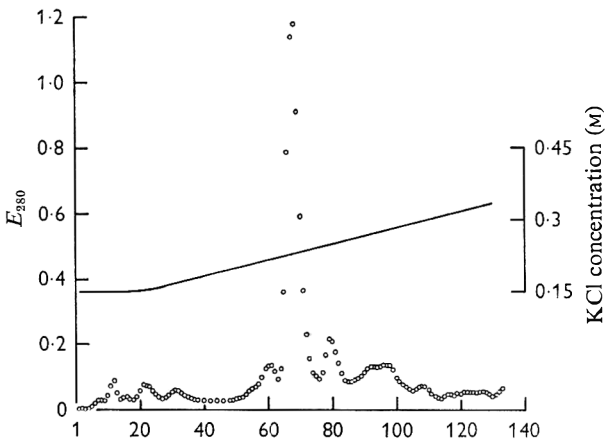

Fig. 2

Fig. 1. Rate of hydrolysis of amides by washed *Mycobacterium smegmatis* NCTC 8159. The bacteria were grown on minimal medium + acetamide, and washed as described under Methods using 4·5 mg. of cells/10 ml. buffer. Production of ammonia from amides (0·02 M) was measured by using Conway units. Curve A, butyramide; B, valeramide; C, acetamide; D, hexanoamide.

Fig. 2. Elution of protein from DEAE Sephadex column. Cell-free extract of *Mycobacterium smegmatis* NCTC 8159, grown on acetamide, was freed from nucleic acids with streptomycin. The material soluble in 50% saturated ammonium sulphate was applied to a column of DEAE Sephadex A 50 in 0·1 M-tris buffer (pH 7·2) containing 0·15 M-KCl, and was eluted with a linear gradient (0·15–0·45 M-KCl). Concentration was measured by conductivity; the protein spectrophotometrically at 280 mμ in the eluate.

Table 3. *Non-substrate inducers of amidase activity in Mycobacterium smegmatis NCTC 8159*

Rates of amide hydrolysis by cell-free extracts of bacteria grown on minimal medium, containing the additions shown, are given relative to the rates for succinate-grown bacteria. Values for succinate-grown bacteria in μmoles/hr/mg. protein are given in parentheses. Rates for acetamide are given for comparison.

Growth medium	Activity measured: Formamidase	Activity measured: Butyramidase
Succinate	1 (100)	1 (10)
Succinate + formamide	2·1	1·3
Succinate + *N*-acetylacetamide	50	10·5
Succinate + *N*-methylacetamide	2·2	0·9
Acetamide	108	16·5

smegmatis; *N*-dimethylacetamide inhibited growth on succinate. None of these substances was hydrolyzed. Formamide was a substrate of the enzyme, but did not support growth. The effects of some of these compounds on the amidase activity of extracts of bacteria grown on succinate in their presence (10mM) are shown in Table 3. The effect is also shown of *N*-acetylacetamide, which was extensively used by Brammar & Clarke (1964) as a non-substrate inducer of the amidase of *Pseudomonas aeruginosa*; the activities of extracts from bacteria grown on succinate alone or acetamide are included for comparison. Formamide (which presumably survived for only a short time in the medium) and *N*-methylacetamide caused a doubling in the formamidase activity but no change in the butyramidase activity. *N*-Acetylacetamide was a somewhat less effective inducer of both activities than acetamide.

Partial purification of an amidase from Mycobacterium smegmatis

An extract from *Mycobacterium smegmatis* grown on acetamide, having a specific activity of 3250 μmoles/hr/mg. dry weight (formamidase) lost 10% of its activity after

Table 4. *Butyramidase, formamidase and protein content of fractions from DEAE-Sephadex column*

Material from a cell-free extract of *Mycobacterium smegmatis* NCTC 8159, freed from nucleic acids and soluble in 50% satd. $(NH_4)_2SO_4$ was applied to a column of DEAE-Sephadex and eluted with a linear gradient of KCl (0·15–0·45 M). Fractions analysed absorbed at 280 mμ. Protein was measured by the method of Lowry *et al.* (1951), butyramidase activity by following release of ammonia in Conway units, and formamidase by estimating residual formamide by the modified hydroxamate method (see Methods).

Fraction number	51	55	60	65	68	70	75	79	85	95
E_{280}	0·033	0·065	0·141	0·360	1·18	0·592	0·102	0·215	0·084	0·130
μg. Protein/ml.	10	20	40	120	410	210	32	60	27	35
Butyramidase (μmole/hr/mg.)	< 100*	< 50*	< 25*	507	507	486	138	20	< 37*	< 28*
Formamidase (μmole/hr/mg.)	1800	500	1250	$3{\cdot}4 \times 10^4$	$5{\cdot}9 \times 10^4$	4×10^4	> 8400	2000	1700	450

* Rate below sensitivity of assay. Maximum values calculated from known sensitivity.

streptomycin precipation. (Specific activity not determined since streptomycin interfered with the protein estimation.) After fractionation of nucleic acid-free material with 50% satd. $(NH_4)_2SO_4$ the insoluble material had a specific activity of 332 and the soluble protein of 7240. The latter contained 62% of the original total activity, and the purification achieved in these two steps was 2·2. Butyramidase activity paralleled formamidase.

When the 50% satd. $(NH_4)_2SO_4$ soluble fraction was chromatographed on DEAE-Sephadex, only one major peak of material absorbing at 280 mμ was obtained (Fig. 2). Rates of hydrolysis of formamide and butyramide by some of the fractions, and the protein present in them, are shown in Table 4. Both hydrolytic activities were associated with this major protein peak. The material in fractions 63–76 was collected and concentrated by ultrafiltration *in vacuo*, and constituted the partially purified extract referred to below. Recovery in the main protein peak of material added to the column was almost complete, and little apparent purification of the material was effected by its use.

Ultracentrifugal analysis of partially purified extract

Two peaks were observed in the ultracentrifuge, which separated sufficiently well for the migration of each to be measured. The faster, which was considerably the larger, had a sedimentation coefficient of 8·3 and the slower of $4{\cdot}2 \times 10^{-13}$ sec. Svedberg units (corrected for temperature and viscosity of KCl but not viscosity of tris buffer). There was no indication of any other component present in comparable amount.

Disc electrophoresis

The pattern of bands obtained by disc electrophoresis of the partially purified extract is shown in Fig. 3. For comparison are shown bands of the original extract after the removal of nucleic acids with streptomycin, and also bands of an extract from bacteria grown on succinate. The partially purified material contained only two major bands, and a trace of a third component. Of the major bands one was much larger than the other.

Table 5. *Rates of hydrolysis of amides by partially-purified amidase preparation Mycobacterium smegmatis* NCTC *8159*

Rates are in μmoles/hr/mg. protein in enzyme preparation, which contained 5·1 mg. protein/ml. Hydrolysis of amides other than formamide was followed as liberation of ammonia. Residual formamide was measured as its hydroxamate after action of the enzyme.

	Rate		Rate
Formamide	$2{\cdot}4 \times 10^4$	Glycolamide	232
Acetamide	240	Lactamide	64
Propionamide	302	β-Hydroxypropionamide	88
n-Butyramide	613	Pyruvamide	5·3*
n-Valeramide	340	Benzamide	6·7
n-Hexanoamide	140	Phenylacetamide	124
iso-Butyramide	122	Nicotinamide	9·0
Acrylamide	376	*N*-Methylacetamide	4·3*

* Amounts of ammonia present doubtfully significant.

Substrate specificity of the partially purified material

In Table 5 are shown the rates of hydrolysis of 16 amides by the partially purified enzyme. Apart from formamide, butyramide was the most rapidly hydrolyzed, and the rate decreased as the alkyl chain was lengthened or shortened, branched, or substituted by an hydroxyl group. Acrylamide was hydrolyzed more rapidly than its saturated analogue, propionamide. Pyruvamide was scarcely hydrolyzed.

The activity of the partially purified enzyme at various pH values

The rates of hydrolysis of formamide and butyramide by the partially purified enzyme preparation in tris buffer at various pH values are shown in Fig. 4. The two activities followed similar curves. In phosphate buffer the rates were a little lower but the shapes of the curves remained the same.

Inhibition of activity

Taking the original rates of hydrolysis of formamide and butyramide by the partially purified enzyme as 100, the rates after heating at pH 7·2 at 60° for various times

were as follows: for 10 min., 77 (formamide), 82 (butyramide); for 20 min., 70 and 78; for 30 min., 58 and 68. Dithio-*bis*-nitrobenzoic acid had no effect in 60 min. at pH 7·2, but when used at the higher concentration at pH 8 for 24 hr the hydrolysis of both substrates by the enzyme was decreased to 10% of its former value. However, the spectrophotometric assay showed that even after 24 hr the reagent had reacted with not more than 1 mole of thiol in 10^7 g. enzyme-protein.

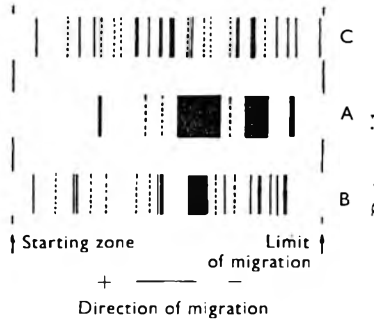

Fig. 3

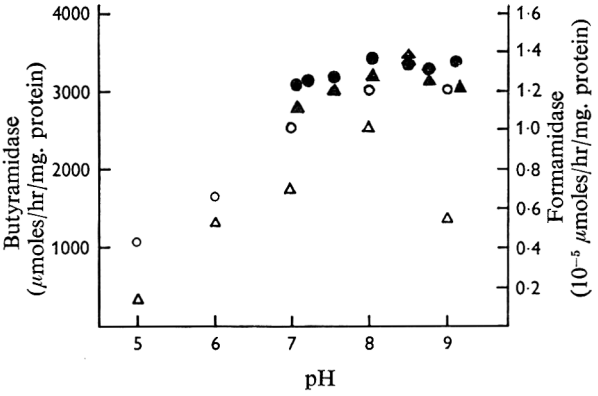

Fig. 4

Fig. 3. Disc electrophoresis of cell-free extracts of *Mycobacterium smegmatis* NCTC 8159. Extracts were analysed on colums on acrylamide gel by using the system of Ornstein (1964) and Davis (1964); the bands were stained with naphthalene black. Pattern A, partially-purified amidase from acetamide-grown bacteria; B, cell-free extract of acetamide-grown bacteria (nucleic acids removed with streptomycin); C, cell-free extract of succinate-grown bacteria. Approximately 50 μg. protein in pattern A, 25 μg. in patterns B and C.

Fig. 4. Variation of amidase activity with pH value. Rates were measured in 0·1 M-tris buffer or 0·05 M-phosphate buffer, as described in Methods. Ammonia release was followed in Conway units for butyramidase. The modified hydroxamate reaction was used to measure unhydrolyzed formamide for formamidase. Open symbols, phosphate buffer; solid symbols, tris buffer; △, ▲, butyramidase; ○, ●, formamidase.

Table 6. *Rates of formation of hydroxamates from amides and hydroxylamine by cell-free extracts of Mycobacterium smegmatis* NCTC *8159*

Rates are in μmoles hydroxamate formed/hr/mg. protein. Figures in parentheses are rates of hydrolysis of amides in μmoles/hr/mg.

Amide	Extracts from bacteria grown on: Acetamide	Extracts from bacteria grown on: Succinate	Washed bacteria grown on acetamide, per mg. dry wt.
Formamide	*	*	—
Acetamide	2·6	—	0·5 (9·2)
Propionamide	11·6	25	1·5 (13)
n-Butyramide	2·8 (210)	7·7 (18)	0·6 (26)
n-Valeramide	0·7	—	—
Benzamide	0·9	—	—
Nicotinamide	22	59	—

* Formamide reacts non-enzymically under the conditions of the assay.

Transfer of acyl groups to hydroxylamine

The rates of formation of acyl-hydroxamates from amides and hydroxylamine by cell-free extracts and suspensions of *Mycobacterium smegmatis* are shown in Table 6.

Material from bacteria grown on acetamide catalyzed transfer only very slowly as compared with the rates of hydrolysis of amides; among *n*-alkanoamides the rate was maximal for propionamide rather than butyramide. Nicotinamide was a better substrate than propionamide. Higher rates were obtained with bacteria grown on succinate—the reverse of the situation for amide hydrolysis.

Formation of hydroxamates from salts of acids was slower still; from propionate it was 0·9 and 1·9 μmoles/hr/mg. protein, respectively, for extracts from bacteria grown on acetamide or on succinate. Rates of hydrolysis of acylhydroxamates by extracts of acetamide-grown and succinate-grown bacteria were, respectively (μmoles/hr/mg. protein); propionohydroxamate 0·6 and 1·9; butyrohydroxamate, 1·1 and 2·9; acetohydroxamate, not detectable.

An ammonium sulphate fraction (precipitated between 60 and 80% of saturation) containing most of the amidase activity of the original extract from acetamide-grown bacteria effected no transfer from butyramide or propionamide to hydroxylamine. The presence of transferase activity in other fractions was not investigated.

DISCUSSION

Apart from adding some more amides to the list of those known to be hydrolyzed by mycobacteria, the experiments reported here show that in *Mycobacterium smegmatis* NCTC 8159 several of these amidase activities are simultaneously induced by growth on acetamide as carbon source, and are much lower in bacteria grown on succinate. An inducible amidase rather than a permease is indicated since the increased activity is found with cell-free extracts as well as with whole organisms. There is some evidence that benzamidase activity varies according to growth conditions, independently of the aliphatic amidase. These findings bear on the use of amidase activities as a means of classifying species in the genus *Mycobacterium*. The presence or absence of an inducer in the growth medium will affect the observed amidase activity, while prolonged incubation of the bacteria with an inducer amide may change their hydrolytic activity from negative to positive for that amide. It is possible that differences in the inducing activity in the growth media used may account for the discrepancies between reports of formamidase activity in mycobacterium species (Nagayama *et al.* 1961; Urabe *et al.* 1965).

There are two possible explanations of the observed substrate specificity of the aliphatic amidase. Formamide may be hydrolyzed by a separate enzyme from the other aliphatic amides, or there may be a single enzyme with an anomalously high activity for formamide. The ratio uninduced : induced activity is much smaller for formamidase than for butyramidase in bacteria grown on acetate or acetamide, as compared with those grown on succinate. The difference is less marked when *N*-acetylacetamide is the inducer, while *N*-methylacetamide and formamide do not alter butyramidase activity but double formamidase activity. These facts might be explained either if the activities are separate but have common inducers or if they are activities of one enzyme, but a constitutive (in this system) butyramidase activity masks the small increase in the inducible enzyme caused by formamide or *N*-methylacetamide.

The two activities showed the same variation with pH value. Attempts to denature or to inhibit the activities selectively failed, and the attempted purification did not separate them. This last piece of evidence is compelling only if a substantial purification of the amidase was achieved, and the actual increase of specific activity obtained

was only 2·2 times, suggesting that the purification was slight. However, both the electrophoretic and ultracentrifugal results showed that the partially purified amidase contained only two major components, although the crude cell-free extract contained many components. The possibility arises that the amidase in induced cells constitutes a major part of the protein extracted by the present method, and this is supported by the appearance of a very intense band of protein, migrating in the same position as one of the components of the partially-purified enzyme, in the disc electrophoresis pattern of the crude extract from induced cells. This band was absent in extract from succinate-grown cells.

With other saprophytic mycobacteria Pershin & Nesvab'da (1960) showed that the acetamidase differed from the nicotinamidase, and Nagayama *et al.* (1961) that the formamidase differed from the benzamidase; in both cases this was shown by differential heat-denaturation. The result of Nagayama *et al.* is confirmed by the present observation that benzamidase did not increase when formamide was induced.

It is interesting to compare the mycobacterial amidase with that isolated from *Pseudomonas aeruginosa* NCTC 8602 by Kelly & Clarke (1962). The pseudomonad enzyme had a much sharper substrate specificity, and both crude extracts and a purified enzyme preparation hydrolyzed acetamide and propionamide, but scarcely at all formamide or butyramide. Neither enzyme hydrolyzed *N*-substituted amides. Both enzymes were induced by acetamide, *N*-methylacetamide and *N*-acetylacetamide (Kelly & Clarke, 1962; Brammar & Clarke, 1964), and also during growth on acetate (Kelly & Kornberg, 1962*a*); in *P. aeruginosa* the kinetics of induction of amidase by acetate and acetamide differed markedly. The significance of this apparently useless induction of amidase by acetate is not understood, and it is of interest that it occurs in two unrelated species of bacteria, both of which are notable for their ability to grow on a wide variety of simple carbon compounds. W. J. Brammar (personal communication) has suggested that sufficient acetamide is formed chemically in a medium containing acetate and ammonia to induce the amidase in *P. aeruginosa*. Insufficient is known of the concentrations of acetamide needed to induce the mycobacterial amidase to decide the likelihood of this explanation.

Data reported here show that the adaptive amidase of *Mycobacterium smegmatis* NCTC 8159 is not responsible for the acyl transferase activity of crude extracts, since the activity disappeared from the ammonium sulphate fraction which contained the amidase (it was not sought in other fractions), while the rates of transfer were higher in succinate-grown bacteria than in acetamide-grown bacteria, which is the reverse of the situation with the formamidase. It is possible that the transferase is itself inducible: the conditions of its induction remain to be investigated. In *Pseudomonas aeruginosa* the enzyme appears to possess both activities (Brammar & Clarke, 1963; Kelly & Kornberg, 1962*b*), but in other mycobacteria transferase and amidase are separate. Kimura & Sasakawa (1956) and Kimura (1959*c*) found that hydroxamate formation from fatty acids and their amides by *Mycobacterium avium* was not catalyzed by the corresponding amidase, while Grossowicz & Halpern (1957) identified in *M. phlei* separate enzymes for transferring aspartyl residues from asparagine to hydroxylamine, for hydrolyzing asparagine and for hydrolyzing asparto-hydroxamate.

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Effect of Food Intake on Growth and Survival of Salmonellas and *Escherichia coli* in the Bovine Rumen

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SUMMARY

When salmonella organisms were put into the rumen of cattle their subsequent growth or elimination depended on the dietary intake before and after the organisms were ingested. When the animals were receiving a regular daily ration of 6·8 kg. lucerne hay the organisms were rapidly eliminated from the rumen and viable organisms in the faeces were rarely detected. Decreasing the daily food intake to 2·3 kg. or interruption of feeding for one or more days retarded the elimination of salmonellas and *Escherichia coli* or permitted their growth in the rumen. Growth of salmonellas and *E. coli* type I occurred during starvation, and resumption of feeding after starvation caused further multiplication. Starvation for 2 or 3 days was generally followed by infection of the intestine, with salmonellas persisting in the faeces for at least a week.

INTRODUCTION

In an earlier study Grau & Brownlie (1965) showed that at five abattoirs in south-eastern Queensland salmonella organisms occurred in the rumen contents of 45% of healthy cattle at slaughter. *Escherichia coli* type I at about 10^5 organisms/ml. were also found in the rumen fluid, in agreement with other reports on the occurrence of *E. coli* (Mann, Masson & Oxford, 1954).

The work described in the present paper was undertaken to ascertain the fate of known numbers of salmonella organisms put into the rumen, and to determine concomitant changes in the population of *Escherichia coli*. The effect of deprivation of foodstuff was also examined, as it is common for cattle to be given little or no food for several days while being moved long distances or held at abattoirs. Deprivation of food may be a predisposing condition for infection in ruminants (Salisbury, 1958; Seddon, 1953).

METHODS

The salmonella organisms used were *Salmonella chester*, *S. typhimurium*, *S. oranienburg*, *S. muenchen*, *S. anatum* and *S. adelaide*, these having been isolated from the rumens of cattle at slaughter and identified by Dr K. F. Anderson (Salmonella Reference Centre, Adelaide). Cultures were grown in 0·8% (w/v) nutrient broth (Difco) for two 24 hr subcultures at 37°, and after suitable dilution in 0·1% (w/v) peptone (Oxoid) water the salmonellas were inoculated into the rumens of the animals.

The salmonellas were introduced into the stomach tube used for sampling (Raun & Burroughs, 1962) and flushed into the rumen with 50 ml. rumen liquor previously taken from the animal. The number of salmonellas given was estimated by serial dilution of the inoculum with peptone water and pour-plating with plate-count agar (Oxoid); colonies were counted after incubation at 37° for 24 hr. In some experiments the organisms were introduced into the rumen as a peptone water suspension of air-dried faeces contaminated with salmonellas, the numbers of which were estimated by the most-probable-number technique described below.

The experimental cattle were Polled Hereford females from 18 months to 3 years of age. In the experiments reported the animals were confined in a cattle crush, to prevent their ingesting faecal material. They were usually fed 6·8 kg. of lucerne hay, presented at the same time daily. In some experiments the animals were deprived of food, but not water, for various periods; while in others food was supplied in a limited amount (2·3 kg.) at the normal times. On the completion of an experiment the animals were treated with sulphadimidine (Mezobols, Parnell Laboratories, Carlton, N.S.W.) daily until faecal samples were consecutively negative. An animal was fed lucerne hay for a minimum of 2 weeks before an experiment was begun, or was grazed for about 6 weeks.

Samples of rumen fluid were obtained by the stomach-tube method, and after their transfer to the laboratory suitable samples of fluid or of peptone water dilutions of it, were subjected to enrichment as described by Grau & Brownlie (1965). Tetrathionate Tergitol brilliant green broth enrichments were incubated at 37° for 24 hr and streaked on the surface of brilliant green sulphadiazine agar and aged bismuth sulphite agar. The selective media were incubated overnight at 37° and colonies suspected of being salmonellas were suspended in saline and tested by slide agglutination with somatic antigens. The enrichments proven to contain salmonellas were eliminated from further incubation, those negative at the 24 hr streaking were retained in incubation for a total of 72 hr before restreaking. The enrichments positive for salmonellas after 24 hr incubation were counted cumulatively with those found positive at the 72 hr streaking. The most-probable-number of salmonellas, after 72 hr enrichment, was estimated from the number of positive enrichments in ten tubes at each dilution (McCoy, 1962). *Escherichia coli* type I was estimated by serial dilution in peptone water and pour-plating with eosin methylene blue agar (Oxoid), counting the lactose-positive colonies greater than 0·5 mm. in diameter after incubation at 44·5° for 48 hr. Only 2 of 200 such colonies tested did not give typical reactions of *E. coli* type I. Faecal samples were examined for salmonellas by enrichment of two 5 g. samples, each in 100 ml. of tetrathionate Tergitol brilliant green broth, following the procedure used for rumen samples. Representative isolates from faeces and rumen were identified by the Salmonella Reference Centre, Adelaide, and proved to be of the serotype inoculated.

pH measurements of the pH values of rumen fluids were made with a Radiometer pH meter and glass electrode after the bacterial samples were taken. The measurements were made about 30 min. after removal from the animal and no precautions were taken to maintain carbon dioxide in solution. Volatile fatty acids were determined in a 20 ml. sample of clarified rumen liquor by the methods outlined by Neish (1952) for clarification and distillation. Ammonia was determined on suitable samples of clarified rumen liquor by vacuum distillation from borate buffer pH 10 (Varner,

Bulen, Vanecko & Burrell, 1953) into a boric acid trap, titrated by the technique of McKenzie & Wallace (1954). Paper chromatography and thin-layer chromatography of acidified ether extracts of clarified rumen fluid were done by the methods of Gordon, Thornburg & Werum (1962) and Ting & Dugger (1965).

RESULTS

The effects of regular daily feeding with 6·8 kg. lucerne hay/day

Four experiments were made to test the effect of inoculation of salmonellas into the rumens of animals fed 6·8 kg. lucerne hay daily. These experiments involved three cattle and three different salmonella serotypes. The results of a typical experiment are shown in Fig. 1. In this experiment, air-dried faeces obtained from an animal shedding *Salmonella chester* was inoculated as a peptone water suspension. The inoculum contained $2{\cdot}8 \times 10^5$ salmonellas, estimated by the most-probable-number technique. Salmonellas in the rumen decreased rapidly from 300/100 ml., and were not detectable 48 hr after inoculation. In this experiment salmonellas were present in the faeces 24 hr after inoculation, but not at later samplings. In three other experiments in which a similar inoculum of salmonellas, grown in nutrient broth, was used, the organisms were not detected in the rumen at or after 24 hr and were not detected at any sampling of the faeces.

The effects of regular daily feeding with 2·3 kg. lucerne hay/day

Three experiments with three animals and two salmonella serotypes were made to test the effect of feeding 2·3 kg. lucerne hay every day on changes in the numbers of salmonella and *Escherichia coli* type I organisms.

In the first experiment the inoculation with $3{\cdot}5 \times 10^5$ salmonellas was on the first day of the smaller feed and the numbers decreased from 161/100 ml. rumen liquor until they were undetected at 48 hr when the experiment was terminated. In the second experiment the animal was given $1{\cdot}4 \times 10^6$ salmonellas on the third day of decreased feeding. The numbers decreased from 693/100 ml. to 1/100 ml. 72 hr after inoculation, when the experiment was terminated. Salmonellas were not detected in the faeces in either experiment.

In the experiment illustrated in Fig. 2, a larger inoculum of *Salmonella oranienburg* ($9{\cdot}8 \times 10^7$) was used to follow changes in the salmonella numbers in the rumen over a longer period of time. Initially the salmonella numbers in the rumen decreased, but from about 35 to 40 hr after inoculation (i.e. 110 hr from last 6·8 kg. ration) there was a gradual increase until the animal was returned to a feed of 6·8 kg. lucerne hay/day; then the number decreased abruptly. The *Escherichia coli* type I numbers increased for about 70 hr after inoculation, then stabilized, and decreased when the animal was returned to 6·8 kg. lucerne hay/day. The period of salmonella growth during feeding with 2·3 kg. hay/day may be related to that period when the volatile fatty acids value was low and the pH value high. When the volatile fatty acids value increased and the pH value decreased on feeding 6·8 kg. lucerne/day, the numbers of salmonellas and *E. coli* decreased. A plot of pH value against the concentration of volatile fatty acids showed no points deviating from the approximate linear relation (Briggs, Hogan & Reid, 1957), in contrast to the later experiments which involved a starvation period

(Fig. 3). The faeces contained salmonellas from 24 hr after inoculation until after remedial treatment with Mezobols.

The effect of regular feeding on alternate days with 6·8 kg. lucerne hay

Three experiments were made to test the effect of alternate-day feeding; three animals and two salmonella serotypes were used. The results of one experiment are shown in Fig. 4: the inoculum was $2{\cdot}1 \times 10^8$ *Salmonella muenchen* and was introduced during the second starvation period. The numbers decreased from an initial value of $5{\cdot}1 \times 10^5$/100 ml. rumen liquor to $1{\cdot}2 \times 10^4$/100 ml. at 24 hr after inoculation. The numbers then tended to be maintained, with some fluctuations. From 24 hr after inoculation, salmonellas were detected in the faeces for the duration of the experiment.

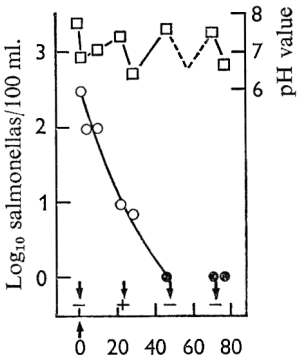

Fig. 1

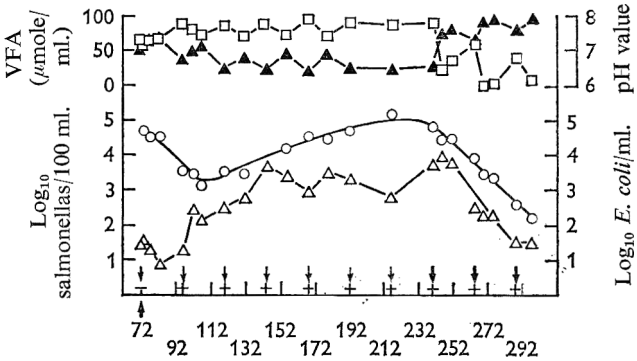

Fig. 2

Fig. 1. The effect on inoculated *Salmonella chester* (○) of regular daily feeding 6·8 kg. lucerne hay (↓). ●, Salmonellas not detected in 10×10 ml. rumen liquor samples; +, detected in faeces; −, not detected in faeces. ↑, Time of inoculation with faeces suspension containing $2{\cdot}8 \times 10^5$ salmonellas. □, pH value.

Fig. 2. The effect on *Salmonella oranienburg* (○) and *Escherichia coli* type I (△) on inoculation (↑) of $9{\cdot}8 \times 10^7$ salmonellas into the rumen of an animal regularly fed (↓) 2·3 kg. lucerne hay/day. The animal was inoculated on the third day of diminished feeding. +, Salmonellas detected in the faeces; −, not detected in faeces. □, pH value; ▲, VFA, concentration of volatile fatty acids.

The *Escherichia coli* type I count increased during the second starvation period and also for 6–11 hr after each feed; in the period between feeds the numbers tended to decline. The *E. coli* numbers appeared to cycle roughly with the changes in volatile fatty acids. The plot of pH value against volatile fatty acids for this experiment (Fig. 3) shows two points off the approximate straight-line relation; these two points corresponded to the only two samples taken at 6 hr after feeding. Most of the *E. coli* growth occurred within this 6 hr period. The results of the other experiments were similar. In one experiment the salmonella fluctuations were greater and tended to parallel the *E. coli* fluctuations, except for a growth during the third starvation period.

The effects of withholding food for 2 to 3 days

Inoculation of salmonellas before feeding lucerne hay and before withholding food for 2 days. Salmonella chester (2×10^8) was inoculated into the rumen of one animal

immediately before feeding (Fig. 5). Food was then withheld for 2 days before the animal was given a single feed of 6·8 kg. lucerne hay. The number of salmonellas in the rumen was initially 3×10^5/100 ml. liquor, decreased to 916/100 ml. in 22 hr, remained constant for about 24 hr, and then increased a thousandfold at 48 hr after the last feed. Only a slight increase occurred on feeding after this starvation. On withholding food for a further 3 days the numbers declined about one log unit per day. On a subsequent feed the number increased about 30-fold.

The faeces contained salmonellas at 24 hr after inoculation and then for the duration of the experiment.

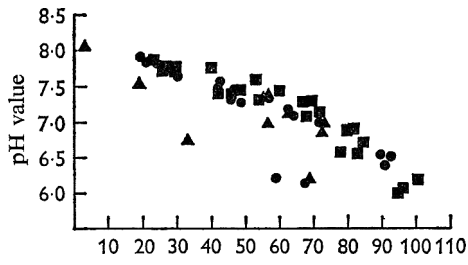

Fig. 3

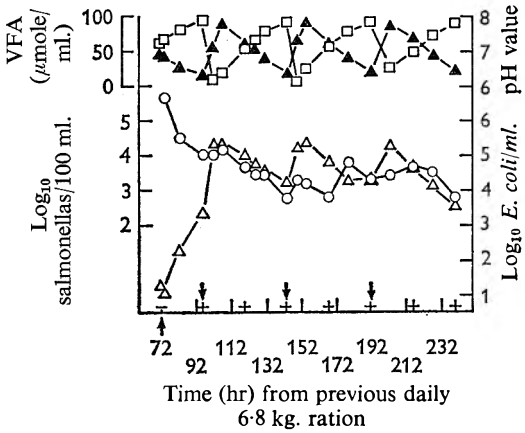

Fig. 4

Fig. 3. The relation between pH value and volatile fatty acids in rumen liquor. ▲, Two days without food (see Fig. 7); ●, fed 6·8 kg. lucerne hay every alternate day (see Fig. 4); ■, fed 2·3 kg. lucerne hay daily (see Fig. 2).

Fig. 4. The effect on *Salmonella muenchen* (○) and *Escherichia coli* type I (△) on inoculation (↑) of $2{\cdot}1 \times 10^8$ salmonellas into the rumen of an animal fed (↓) 6·8 kg. lucerne hay every alternate day. The animal was inoculated during the second starvation period. +, Salmonellas detected in faeces; −, not detected in faeces. □, pH value; ▲, VFA, concentration of volatile fatty acids.

From low numbers *Escherichia coli* type I increased during starvation by over 10^5 times. A further increase of 30-fold occurred on feeding, but the numbers decreased during the second starvation. A further increase was obtained on giving the final feed.

Inoculation of salmonellas 24 hr after feeding lucerne hay and before withholding food for 2 days. In two experiments the animals were inoculated 24 hr after feeding and then deprived of food for 2 days; the results of one experiment are shown in Fig. 6. The inoculum contained $3{\cdot}8 \times 10^5$ *Salmonella oranienburg* and the initial concentration of salmonellas in the rumen liquor was 300/100 ml. The salmonella count initially decreased, but at about 54 hr from the regular 6·8 kg. feed the salmonellas began to grow and continued to increase for 24 hr after the next feed, the total increase being about 3·5 log units; on continued feeding the salmonella concentration fell daily about one log unit.

The *Escherichia coli* type I numbers in the rumen increased at about 35 hr from the regular 6·8 kg. feed and showed a further sharp increase on feeding after the 2-day starvation. Salmonellas were detectable in the faeces 24 hr after inoculation and then

for the duration of the experiments. The results of the second experiment were similar except that *E. coli* began to increase in numbers at about 50 hr from the last feed and the count paralleled the changes in the numbers of salmonellas.

Inoculation of salmonellas before feeding and after food was withheld for 2 days. Two experiments using 2 animals and 2 salmonella serotypes were done to test the effect on inoculated salmonellas of feeding 6·8 kg. lucerne hay to cattle after food had been withheld for 2 days. Each inoculum was about 1000 salmonellas, and was given immediately before feeding the animals after starvation for 2 days. The results of one experiment are shown in Fig. 7. The numbers of *Salmonella anatum* increased over 24 hr

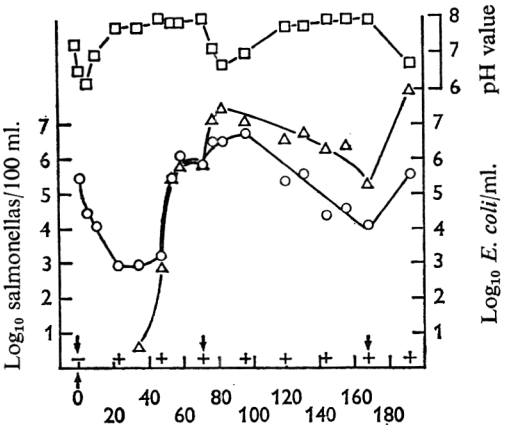

Fig. 5

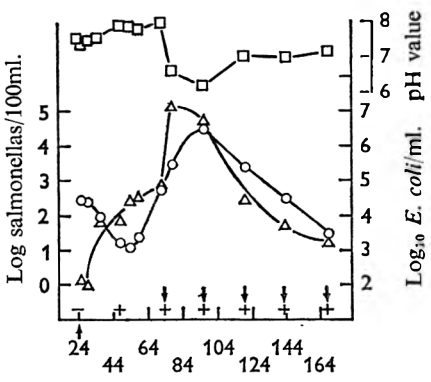

Fig. 6

Fig. 5. Changes in the numbers of *Salmonella chester* (○) and *Escherichia coli* type I (△) on inoculation (↑) of $2{\cdot}07 \times 10^8$ salmonellas into the rumen of an animal before feeding 6·8 kg. lucerne hay (↓) and before 2 days without food. +, Salmonellas detected in the faeces; −, not detected in the faeces. □, pH value.

Fig. 6. Changes in the numbers of *Salmonella oranienburg* (○) and *Escherichia coli* type I (△) on inoculation (↑) of $3{\cdot}8 \times 10^5$ salmonellas into the rumen of an animal 24 hr after feeding and before 2 days without food. ↓, Fed 6·8 kg. lucerne hay; +, salmonellas detected in faeces; −, salmonellas not detected in faeces. □, pH value.

from an undetectable number to a maximum of 1610/100 ml. rumen liquor; most of the salmonella growth occurred in the first 6 hr. On subsequent feeding the salmonella numbers decreased by about 0·8 log units/day until undetectable. Salmonellas were detected in the faeces at 24 hr after inoculation and for 6 days subsequently; they were not detected after 8 days. Similar results were obtained in the other experiment.

The *Escherichia coli* type I count increased by about four log units during the starvation period. After feeding, the *E. coli* count increased rapidly about 2·5 log units to 8·45 log units/ml. and decreased again on further feeding.

The plot of pH value against volatile fatty acids for this experiment (Fig. 3) showed two points well below the approximate straight-line relation; these two points (at pH 6·8 and 6·3) corresponded to the samples taken at 6 and 11 hr after the feed which followed starvation. It was during this period that rapid growth of *Escherichia coli* and salmonellas occurred. It appeared that there were considerable amounts of non-volatile acids present. The major acids detected by chromatography of these rumen

liquors were lactic, succinic and pyruvic acids, none of which were detected in other rumen samples from the same experiment.

Inoculation of salmonellas before withdrawal for 3 days from lucerne hay or grazing. Three experiments to test the effect of no food for 3 days on the fate of inoculated salmonellas used two animals and three salmonella serotypes. In two of these experiments the animals were inoculated during grazing and then starved for 3 days, whilst in the other experiment the animal was fed the normal lucerne hay ration then inoculated and starved for 3 days.

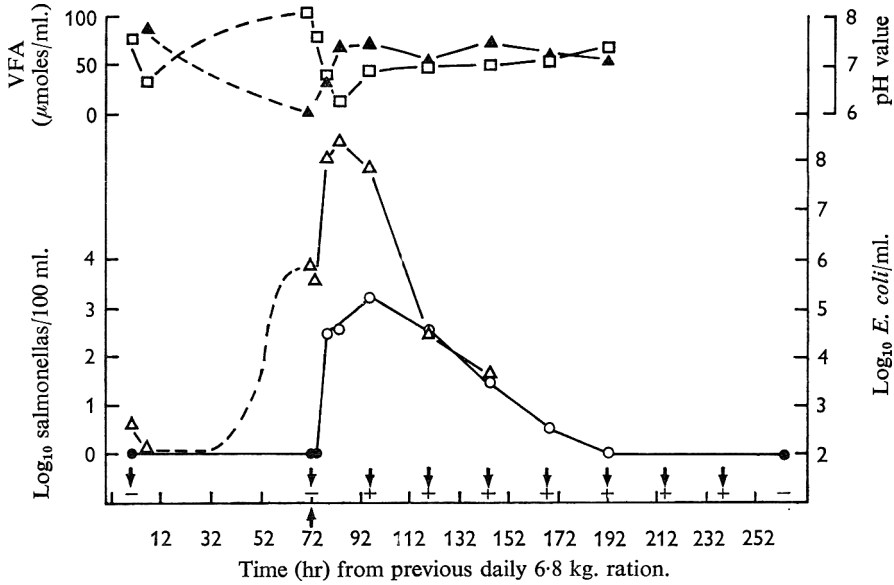

Fig. 7. The effect on inoculated *Salmonella anatum* (○) of feeding 6·8 kg. lucerne hay/day (↓) after 2 days without food. ●, Salmonellas not detected in 10 × 10 ml. rumen liquor samples; +, detected in faeces; −, not detected in faeces. ↑, Time of inoculation with 1130 nutrient broth-grown salmonellas. △, *Escherichia coli* type I count; □, pH value; ▲, VFA, concentration of volatile fatty acids.

Figure 8 shows the results of one grazing experiment in which the inoculum was $4{\cdot}3 \times 10^8$ *Salmonella oranienburg*, the initial salmonella concentration in the rumen then being $1{\cdot}61 \times 10^5$/100 ml. rumen liquor. The animal was returned to grazing for 30 hr after inoculation and was sampled once during this period. The salmonella concentration in the rumen decreased rapidly during grazing and at the beginning of starvation was 10·5/100 ml. rumen liquor. During the next 18 hr the salmonella concconcentration decreased to 1·05/100 ml. rumen liquor. From this time on the salmonella and *Escherichia coli* type I numbers increased to 200–300/100 ml. and $5{\cdot}7 \times 10^5$/ml., respectively. On feeding 6·8 kg. lucerne hay the numbers of salmonellas and *E. coli* increased rapidly to maxima of $3{\cdot}75 \times 10^5$/100 ml. and $3{\cdot}4 \times 10^7$/ml., respectively. The animal took 24 hr to consume the 6·8 kg. lucerne hay, which would normally have been consumed in 2–3 hr. The salmonellas and *E. coli* decreased from the maximum concentrations during a further 3 days of food deprivation. Salmonellas were detected in the faeces at 24 hr after inoculation and for the duration of the experiment.

The results of the second grazing experiment were similar except that the time at

which growth occurred during the starvation period was 40 hr after removal from grazing, as against 18 hr in the previous experiment. In these two experiments the conditions of grazing were different; the experiment illustrated (Fig. 8) being on young green pasture and the second experiment on dry standing pasture.

The results of the experiment with lucerne hay were similar to those of the second experiment (dry standing pasture); the growth of salmonellas and *Escherichia coli* type I occurred during the starvation period at 48 hr from the last feed.

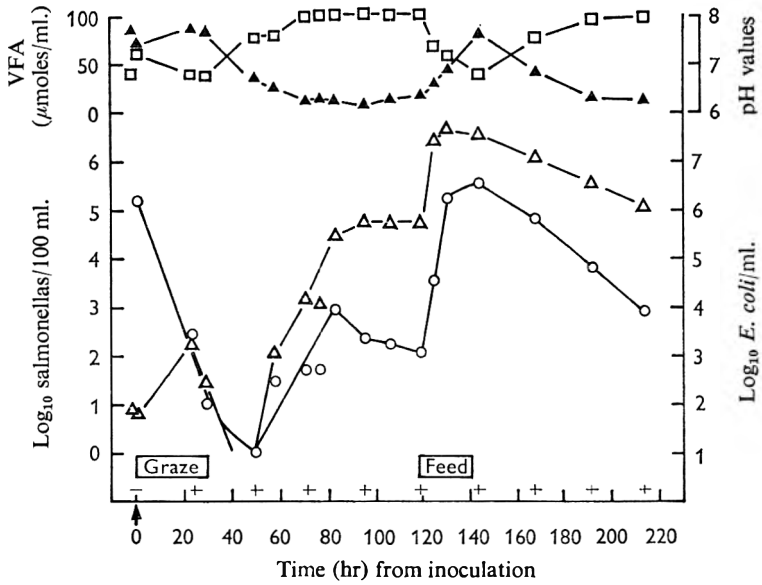

Fig. 8. Changes in the numbers of *Salmonella oranienburg* (○) and *Escherichia coli* type I (△) on inoculation (↑) of $4{\cdot}3 \times 10^8$ salmonellas into the rumen of an animal allowed to graze for 30 hr ('Graze'), then 3 days without food, and then fed one lot of 6·8 kg. lucerne hay ('Feed'). +, Salmonellas detected in the faeces; −, not detected in the faeces. □, pH value; ▲, VFA, concentration of volatile fatty acids.

DISCUSSION

It is apparent that when the animals were fed 6·8 kg. lucerne hay/day, or were allowed to graze freely, salmonellas were rapidly eliminated from the rumen. The *Escherichia coli* type I concentrations were usually under 10/ml. rumen liquor when the animals on this feeding regime were restrained in a cattle-crush to prevent ingestion of faecal material. Decrease of the food intake to 2·3 kg. hay/day, or deprivation of food for 1 or more days, resulted in maintenance or increases of the salmonella and *E. coli* numbers.

The period for the growth of salmonellas and *Escherichia coli* to take place during starvation varied with the type of feed. With animals fed lucerne hay salmonellas grew 48–54 hr after feeding and 20 or 40 hr after grazing; the *E. coli* increases were detected earlier. On feeding after this starvation further increases in numbers of salmonellas and *E. coli* were obtained; the period of most rapid growth was when non-volatile fatty acids were present in the rumen. The average net increases obtained during starvation and subsequent feeding were about 3×10^4-fold for the salmonellas

and 10^7-fold for *E. coli*, but the total increase in numbers of organisms within the rumen would have been greater considering dilution and passage. Further starvation or feeding caused the concentrations of these organisms to decrease by about one log unit/day.

Feeding on alternate days (i.e. starvation for one day) tended to stabilize the salmonella concentration after several cycles, while *Escherichia coli* type I increased from an initial low concentration to between four and five log units/ml. There was some evidence of cycling of *E. coli* and possibly of the salmonellas. Increased numbers of *E. coli* were detected within 6 hr after feeding when non-volatile acids were present in the rumen.

Decrease of the feed to 2·3 kg. lucerne hay/day after a period of 4–5 days on reduced feed led to a slow increase in salmonellas and *Escherichia coli*; a return to 6·8 kg. lucerne hay/day caused an immediate decrease in the numbers of these organisms. This contrasted with the observation that when the animal was starved entirely and then fed 6·8 kg. hay the numbers increased.

There was general agreement in the trends of the numbers of *Escherichia coli* type I and the salmonellas, although the methods of enumeration were independent. The method of estimating salmonellas by the most-probable-number technique (McCoy, 1962) although laborious, yielded consistent results.

Bergeim, Hanszen, Pincussen & Weiss (1941) established that volatile fatty acids at low pH values were bactericidal or bacteriostatic for several genera including salmonellas. Meynell (1963) discussed the findings of Bergeim and others on undissociated volatile fatty acids in relation to the inhibition of *Salmonella typhimurium* in the normal mouse gut. Wolin (1966) has shown *in vitro* that the concentration of volatile fatty acids and pH values found in the bovine rumen inhibit the growth of *Escherichia coli*. Although the concentration of volatile fatty acids and pH value may be correlated with changes in numbers of salmonellas in the bovine rumen in conditions of full feed and 2·3 kg. feed, our experiments involving starvation followed by a single feed and further starvation indicated that volatile fatty-acid changes may not be the full explanation. During the first starvation period growth of *E. coli* type I and salmonellas occurred at a pH value and volatile fatty-acid concentration which were attained again during the second starvation period, when the numbers of the bacteria were actually decreasing (e.g. Fig. 8). There did not seem to be any obvious relation between ammonia concentration in the rumen and growth or death of the organisms.

The growth or death of salmonellas and *Escherichia coli* type I in the rumen in relation to feeding or starvation may be important in the development of salmonellosis and infections with enteropathogenic *E. coli*, as well as in influencing the incidence of salmonellas found in cattle at slaughter, especially since a low dose of the order of 1000 salmonellas resulted in substantial growth in the rumen and in salmonellas being shed in the faeces for at least a week after dosage. Other experiments have shown that when soil from an abattoir holding-pen was added to the food of a starved animal the salmonella concentrations in the rumen were some hundreds/100 ml. rumen liquor and salmonellas were present in the faeces. Conditions which obtain during the transport and holding of cattle before slaughter would appear to predispose these animals to salmonella and *E. coli* infection.

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The Fine Structure of the Mature Zoosporangium of *Nowakowskiella profusa*

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SUMMARY

The fine structure of the mature sporangium of *Nowakowskiella profusa* Karling (Chytridiales, Phycomycetes) is described. Deductions are made about the dehiscence mechanism and the nature of the zoospore. A striking feature of the zoospore is a conspicuous fibrous body immediately adjacent to the refractive globule. This body possibly represents a primitive photoreceptor organelle.

INTRODUCTION

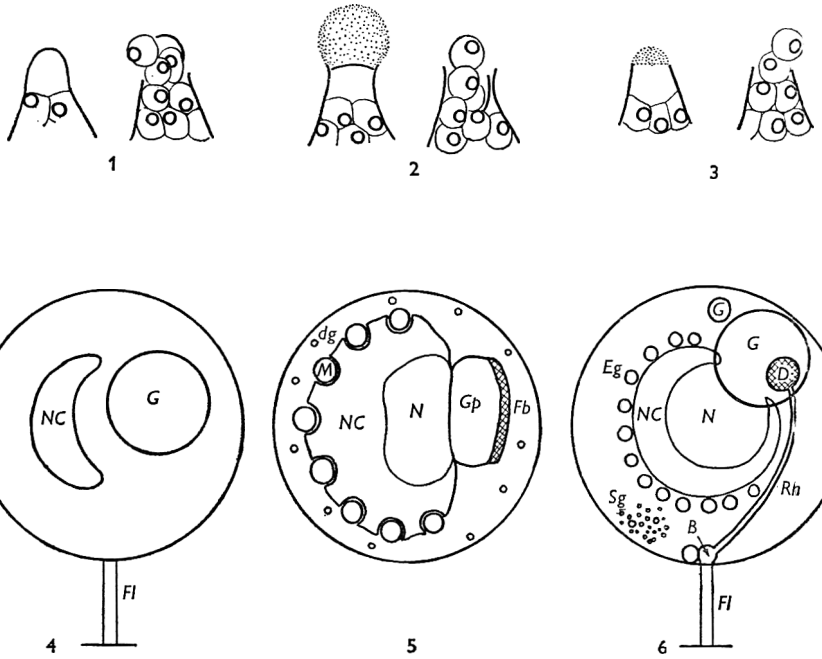
A previous study on the fine structure of lower fungi (Chytridiales, Phycomycetes) featured *Rhizophlyctis rosea*, a monocentric species the whole thallus of which is converted into a single sporangium at maturity (Chambers & Willoughby, 1964). The present paper is concerned with *Nowakowskiella profusa*, a polycentric species of the same order, the thallus of which takes the form of a rudimentary mycelium with rhizoids bearing a potentially unlimited number of sporangia. In both *R. rosea* and *N. profusa* dehiscence of the sporangia results in the release of large posteriorly uniflagellate zoospores.

METHODS

Taxonomic identity of the material studied. According to Sparrow (1960) *Nowakowskiella profusa* is only doubtfully distinct from *N. elegans*. However, examination of much material of both species in ecological studies indicates that a true separation can be made. In his original descriptions of *N. profusa*, Karling (1941, 1944) described exo-operculate material only, but our own observations (Willoughby, 1961) tend to suggest that the species comprises an overlapping series of morphological forms in which endo-operculate and even inoperculate dehiscences may also occur (see Figs. 1–3.) The material studied in the present work exhibited dehiscences of the latter two types only. Typical Nowakowskiella zoospores with a laterally orientated nuclear cap and large refractive globule were liberated on dehiscence (Fig. 4). Zoospores bearing several smaller globules in addition were also observed.

Origin and treatment of the material studied. The material was derived from an uncultivated soil sample from Rutherglen, Victoria, Australia. The sample was flooded with water and baited with small squares of MXXT/S 300 (waterproofed) Cellophane, a good cellulose source for the lower aquatic fungi. After 2 weeks of incubation at 20° numerous young colonies of *Nowakowskiella profusa* bearing sporangia were present on the Cellophane, particularly along the free edges. Marginal strips of the sub-

stratum were cut away, checked for the presence of the fungus, washed vigorously in clean water, and fixed in 2% $KMnO_4$ at 2° for 25 min. Attempts to fix in various osmium tetroxide solutions were unsuccessful. Dehydration, infiltration with methacrylate and orientation during embedding followed the methods previously described (Chambers & Willoughby, 1964). Sections were cut with glass knives in a Porter–Blum microtome, stained with uranyl acetate for 90 min. (Gibbons & Grimstone, 1960) and examined in a Siemens Elmiskop I at 80 kV. Plate numbers with the figure explanations refer to plates stored in the electron-microscope laboratory, Botany School, University of Melbourne.



Figs. 1–6. Figs. 1–3: drawings of three types of dehiscence observed in *Nowakowskiella profusa* showing portions of mature and dehisced sporangia for each type: Fig. 1, exo-operculate; Fig. 2, endo-operculate; Fig. 3. inoperculate. Gelatinous plug material at the exit tube apex is indicated by fine stippling in Figs. 2 and 3. Figs. 4, 5, 6: diagrammatic representations of a living zoospore of *Nowakowskiella profusa* as observed for Rutherglen material, a zoospore unit as deduced from the sections of mature sporangia, and the Nowakowskiella zoospore type as reconstructed by Koch, respectively. Fig. 6 is redrawn (by permission) from Koch's original papers, and for comparative interest the disc with grid substructure he reported for a *Chytridium* sp. is transferred to it, in its correct position. *B*, blepharoplast; *D*, disc with grid substructure; *dg*, dark granule; *Eg*, extranuclear granule (presumptive mitochondrion); *Fb*, fibrous body; *Fl*, flagellum; *G*, refractive globule; *Gp*, presumptive refractive globule; *M*, mitochondrion; *N*, nucleus; *NC*, nuclear cap; *Rh*, rhizoplast; *Sg*, side granules.

RESULTS

The mature sporangium is divided into zoospore units, the approximate limits of which are readily apparent from the symmetrical groupings of the various organelles present (Pl. 1, fig. 1). In a typical median section of a sporangium between 20 and 25 zoospores are seen in various planes of sectioning, implying a total complement of about 40. Zoospores are approximately 3 μ across and their irregularity in outline is

due to their packing within the sporangium. This is in accord with a slightly larger diameter of the zoospores after release from the sporangium, when they are perfectly spherical.

In section the sporangium wall, about 0·35 μ wide overall, is composed of three distinct zones (Pl. 2, figs. 2, 3), the most conspicuous being the middle dense one which is about 0·2 μ wide. The outer zone, at least 0·07 μ wide, is rather diffuse and has the appearance of being easily sloughed off. It may well only represent the zone of partly dissolved cellulose bait. The inner zone is also about 0·07 μ wide. It can be distinguished because there is a distinct layer of spongy material on its inner side. This spongy material, in addition to running beneath the sporangium wall, forms a continuous matrix between the cleaved zoospores and their flagella and extends in considerable quantity into the base of the dehiscence papilla. The particular dehiscence papilla illustrated in section (Pl. 2, fig. 3) is of the inoperculate type (see Fig. 3). The spongy material at its base is capped by a sharply defined dome of dense granular material. It appears that this effectively plugs the exit pore opening.

Provided the section is in a suitable plane each zoospore unit shows its conspicuous nucleus, bounded by a typical nuclear membrane (Pl. 4, fig. 5). Dense irregular masses of granular material are visible within it. Depending on the plane of the section, there is a tendency for the nucleus to have a flattened or even concave face and associated with this is often a very conspicuous clear region which may be up to 1·5 μ across (Pl. 1, fig. 1; Pl. 3, fig. 4). This is presumed to correspond to the refractive globule of the living zoospore (Fig. 4). On the side of the clear region furthest from the nucleus there is a striking fibrous structure (Pl. 3, fig. 4; Pl. 4, fig. 5). In longitudinal section this structure which we are designating the fibrous body is seen to be made up of a series of cylindrical tubular elements each bounded at one end and on the sides by a clearly defined electron-dense margin—perhaps a membrane. The ends of the tubes on the outer side of the body against the cytoplasm are without such a defined margin (Pl. 5, fig. 6). Cross-sections of this fibrous structure indicate that the tubes are closely packed and circular in outline so that there are small inter-tubular spaces (Pl. 5, fig. 7). Each tube is in contact with six neighbouring ones with a centre-to-centre distance of 45 mμ. On the side of the nucleus opposite to that occupied by the presumptive refractive globule there is a large densely granular mass which has considerable lateral extension. In certain sections, in fact, the nucleus appears to be totally embedded in it. This granular mass is taken to correspond to the nuclear cap of the living zoospore (Fig. 4). The nuclear cap, frequently reported for the zoospores of aquatic fungi, is generally assumed to represent a dense accumulation of ribosomal material (Blondel & Turian, 1960).

Closely associated with the periphery of the nuclear cap and typically partially embedded in it are the mitochondria. Also associated with the periphery of the nuclear cap are occasional strands of endoplasmic reticulum and sometimes groups of small membrane-bounded electron-lucent regions. The nuclear cap is typically bounded from the cytoplasm by a definite double membrane (Pl. 3, fig. 4), and this is particularly apparent where the mitochondria abut on to it. In regions of the nuclear cap where the mitochondrial investment is lacking, however, so too may be the nuclear cap membrane (Pl. 4, fig. 5). The general cytoplasm of the zoospore is a loosely packed region, mottled in appearance. Embedded in it are Golgi dictyosomes, membrane-bounded dark granules and occasional short profiles of endoplasmic reticulum. The

enveloping cell membrane is a very thin electron-dense structure sometimes appearing as two fine lines (Pl. 4, fig. 5). Between the zoospores, and suspended in the spongy material discussed above, are the flagella. These are seen in longitudinal section in Pl. 4, fig. 5 and in transverse section in Pl. 5, fig. 8.

DISCUSSION

Comparison of the fine structure of the mature sporangium of *Nowakowskiella profusa* with that of *Rhizophlyctis rosea* shows a common basic organization. Individual zoospores are distinguished together with their flagella, the whole compact structure being pervaded by the spongy ground material. In the case of *N. profusa* the disposition of this material in the dehiscence papilla region is especially interesting. It can be assumed that the dome of dense granular material observed capping it in the sections corresponds to the gelatinous plug material (Fig. 3) observed in living material. With the disappearance of this gelatinous plug material, which is diffluent, dehiscence ensues promptly. Clearly the zoospores emerge with (some even within) the spongy material of the papilla and their subsequent brief collective swarming before they finally disperse takes place in a vesicle transformed from it. Our observations then tend to suggest that the papillar material forming the physical basis for collective swarming is not some special tissue formed only in this region; rather it is merely an extension of similar material which is present throughout the whole sporangium.

In the mature sporangium of *Rhizophlyctis rosea* we reported a complex presumptive centriole described as 'extra-nuclear strands' immediately adjacent to the nucleus of the zoospore but in the present work with *Nowakowskiella profusa* no trace of any similar structure was observed. Instead we have to report another quite different and equally conspicuous structure closely associated with the nucleus (but separated from it by the refractive globule), namely the fibrous body. This is equated with the disc with grid substructure reported by Koch (1956) for zoospores of *Chytridium* sp. (Figs. 5, 6) from a study of shadowed electron-microscope preparations. Its possible function must be largely a matter for speculation, but since Koch demonstrated a rhizoplast thread joining it to the base of the flagellum, a connexion with the motility of the zoospore is strongly implied. In a search of the literature for anything similar, even remotely so, we have considered published figures of bands of cylindrical fibres connecting flagellar bases to the plastids in *Prymnesium parvum* (Manton & Leedale, 1963), extensions of pharyngeal rods in *Peranema trichophorum* (Roth, 1959), and the honeycomb membrane between the shell and the cytoplasm in *Gromia oviformis* (Hedley & Bertaud, 1962). A mechanical function is implied in the first two instances. Such a possibility is attractive; it might partly explain the origin of the peculiarly jerky and erratic movements of the chytrid zoospore flagellum. However, we have been advised (A. V. Grimstone, personal communication) that a direct connexion with motility is unlikely and that our fibrous body is reminiscent of a brush border. Bearing in mind that receptor organelles are commonly formed from such surface membranes our attention has been directed to the fine structure of the rhabdom in the eye of the lobster (Rutherford & Horridge, 1965). This consists of enormous numbers of microvillar tubules, 60–100 mμ in diameter. Microvillar receptors are apparently widely encountered in the invertebrates. Their arrangement in dense arrays is probably to give a large surface area to the photoreceptor and it is generally assumed that the

photosensitive pigment lies in this region. If then we consider our fibrous body as a possible photoreceptor organelle, reports of Chytridiales zoospores which show phototactic reactions (cited in Sparrow, 1960) can be examined with renewed interest. The refractive globule of *N. profusa* might conceivably function as a lens. However, light microscopists have traditionally regarded it as lipid and primarily as providing a convenient high-energy source for the motile spore. Hints that this traditional view may be erroneous derive from the general conclusions of Koch (1958) that the refractive globule may have some direct role in the action of the flagellum, and from studies on *Blastocladiella emersonii* (Blastocladiales) by Cantino & Lovett (1964). The latter have observed that zoospores of this species which swim and eventually disintegrate without the possibility of encystment and growth do not suffer depletion of their refractive globules. Carbohydrate available in the zoospore rather than the lipid is therefore proposed as the energy source. A past observation of our own may also bear recapitulation: when *N. profusa* zoospores germinate on nutrient agar their refractive globule is not lost; rather it persists apparently unchanged. In this case, however, it must be borne in mind that soluble carbohydrate from the medium would clearly constitute a more readily available energy source than a lipid zoospore inclusion. If the fibrous body in *N. profusa* is indeed a photoreceptor organelle its fine structure is of a much more definite and complex nature than the photoreceptor in *Euglena spirogyra* which is reported merely as a body of uniform electron density, except for some indication of fine striations (Leedale, Meeuse & Pringsheim, 1965).

A point of interest concerning the nuclear cap is its close investment by the mitochondria, as reported by Koch (1958, 1961) for a *Nowakowskiella* sp. (Fig. 6) and also by Turian & Kellenberger (1956) for *Allomyces macrogynus*. The accentuation of membranes where the mitochondria are in direct contact with the nuclear cap (there are actually eight consecutive parallel membranes across a line of 83 mμ in the rectangle marked in Pl. 3, fig. 4) may be because of their importance in the intercellular translocation of small molecules (Frey-Wyssling, 1964). Finally the membrane-bounded dark granules are noted. These figured in our account of *Rhizophlyctis rosea* and were previously reported by Blondel & Turian (1960) for *A. macrogynus*.

When our previous paper was written very few published studies on the fine structure of Phycomycete fungi were available. Studies by Cantino, Lovett, Leak & Lythgoe (1963), Chapman & Vujičić (1965), Fuller & Reichle (1965), Goldstein, Moriber & Hershenov (1964) and Renaud & Swift (1964) must now be considered in any attempt to relate our findings to the development of the subject. It has already become commonplace to report the basic eucaryotic organelles such as membrane-bounded nuclei, mitochondria, etc., for these fungi. Accordingly, in the investigations cited above, emphasis has been placed on features of special interest, the uniqueness or otherwise of which it is difficult to evaluate at the present time. Cantino *et al.*, for example, reported that in the zoospore of *Blastocladiella emersonii* there is a single huge mitochondrion attached by at least one banded rootlet to the flagellum. Chapman & Vujičić, for *Phytophthora erythroseptica*, showed how the sporangium wall developed a prominent vesicular layer when the sporangium was destined to dehisce, but not when destined to germinate. Fuller & Reichle, with *Rhizidiomyces apophysatus*, selected the presence of two centrioles in the zoospore as an unexpected attribute; apparently the single flagellum developed from only one of these. They also drew attention to membrane-bounded groups of fibres which may be essential for the development of wall material

as the naked reproductive cell encysts and begins to germinate. Goldstein *et al.*, for *Thraustochytrium aureum*, described peculiar mitochondria with concentric membranes enclosing cristae-free regions. Finally Renaud & Swift, with *Allomyces arbusculus*, also demonstrated a paired centriole condition in the zoospore and made a detailed study of the ontogeny of the flagellum. In the present work with *Nowakowskiella profusa*, as in that on *Rhizophlyctis rosea*, some light has been thrown on the dehiscence mechanism in the Chytridiales and it seems unlikely that the dramatic preliminary fine structure changes reported for *Phytophthora erythroseptica* are involved. The extra-nuclear strands (centriole?) observed in *Rhizophlyctis rosea* cannot be equated very readily with the structures seen in *N. profusa*. The structural feature of special interest for the zoospore of *N. profusa* is the fibrous body; its true significance will only become apparent when studies on the fine structure and the biology of Phycomycete fungi are expanded further.

Our thanks are due to Professor J. S. Turner for encouragement to persevere with this project and to Mrs. C. Myers for photographic assistance. Dr A. V. Grimstone kindly advised us by correspondence. One of us (L. G. Willoughby) is indebted to the University of Melbourne for the award of a 1962–63 Senior Research Fellowship.

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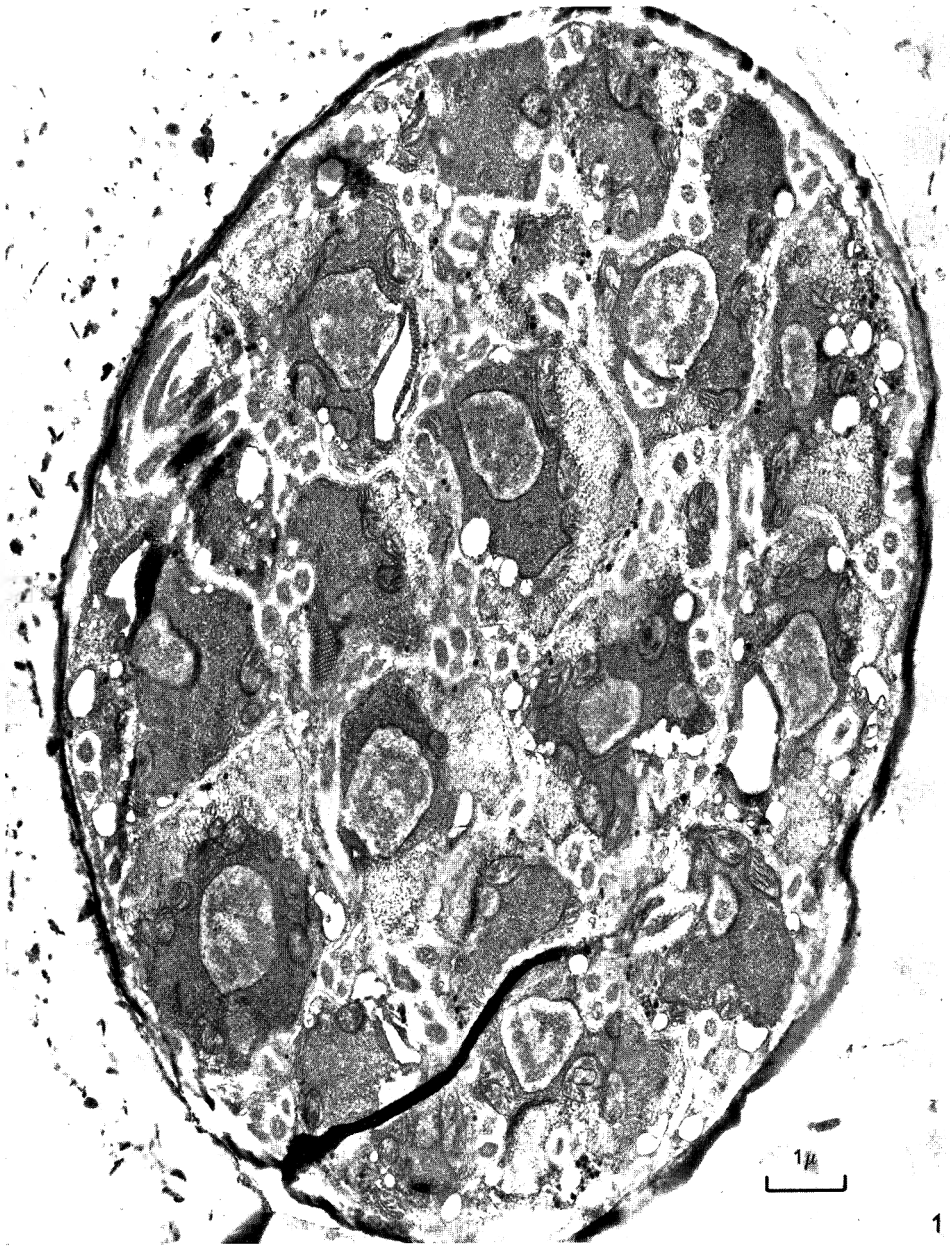

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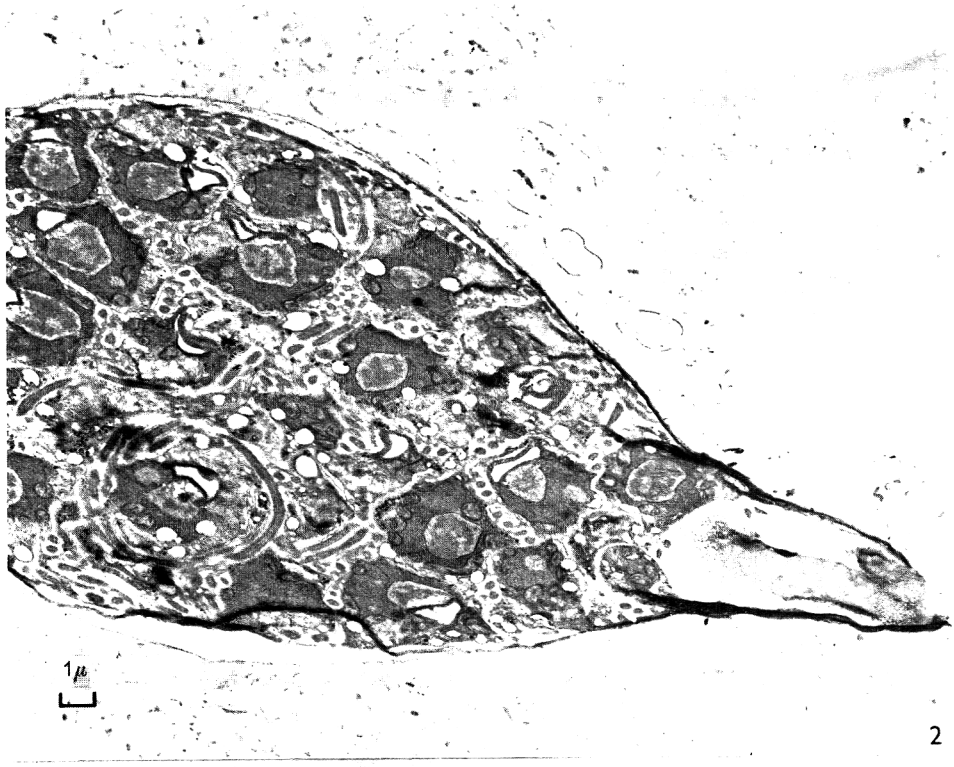

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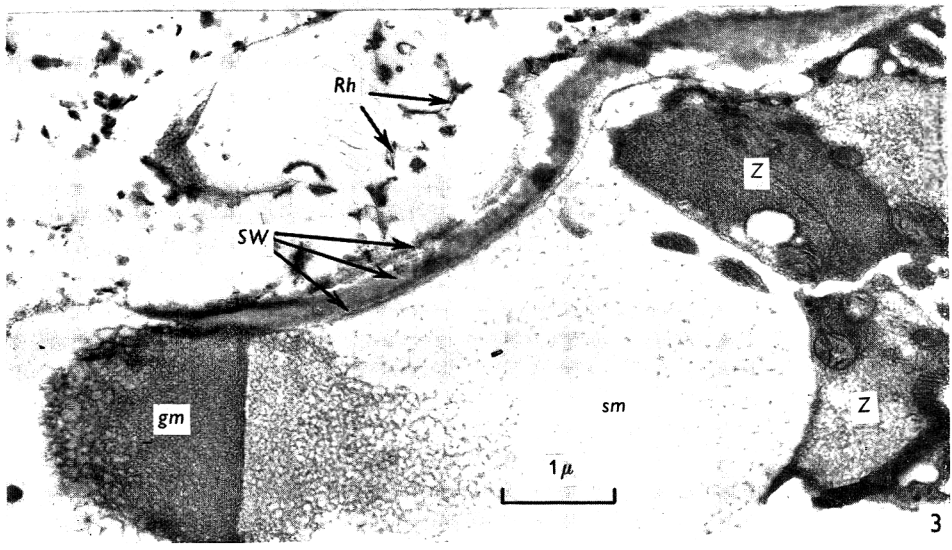

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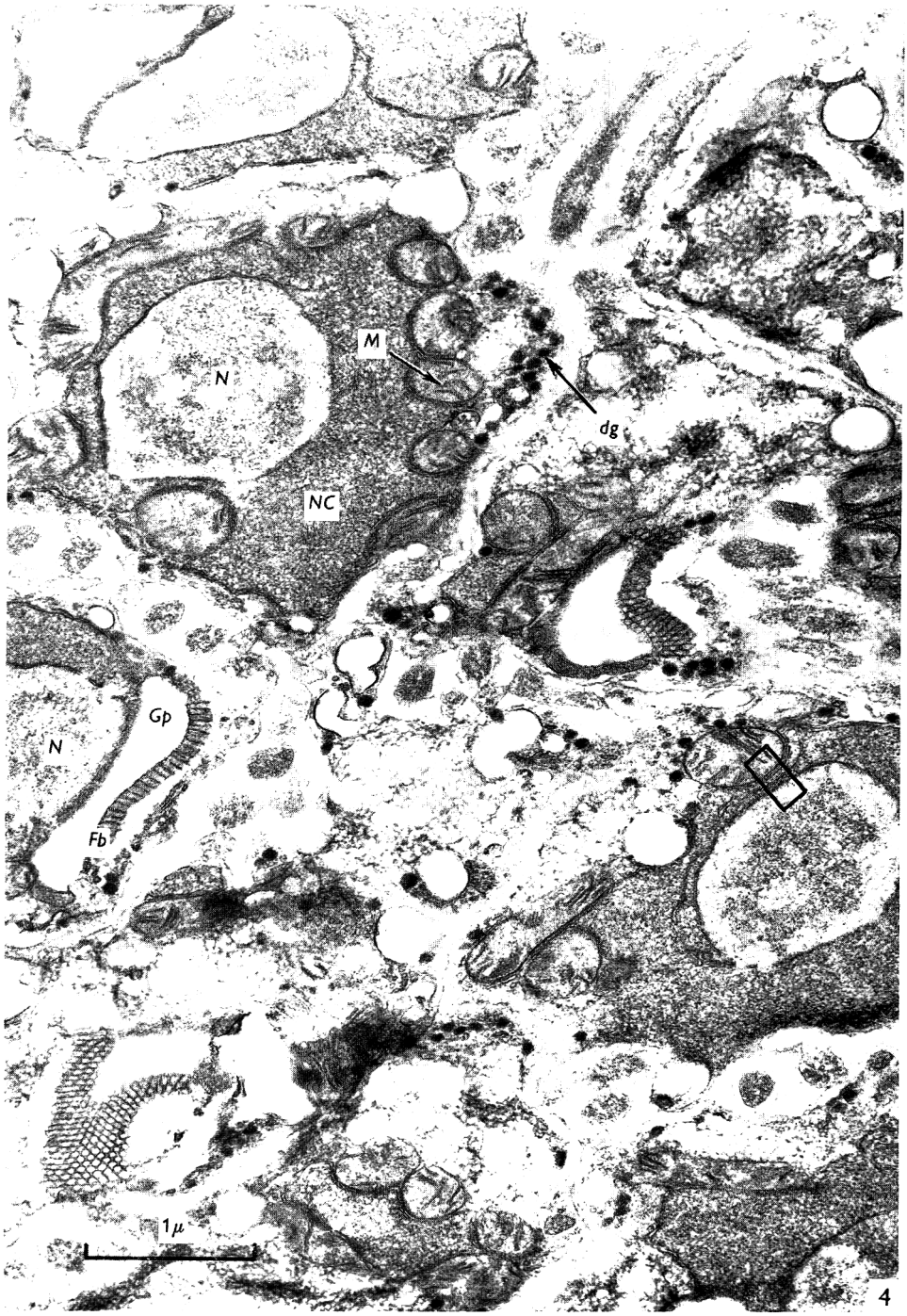

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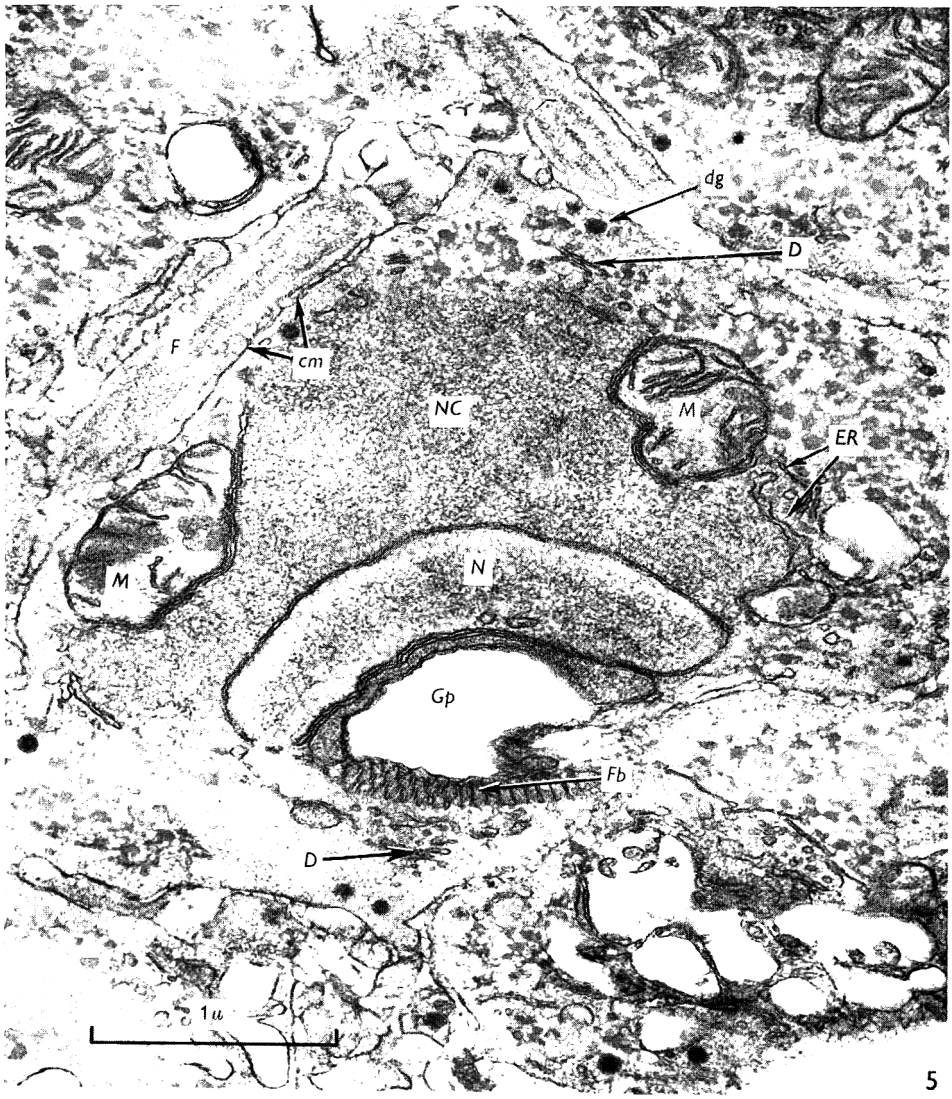
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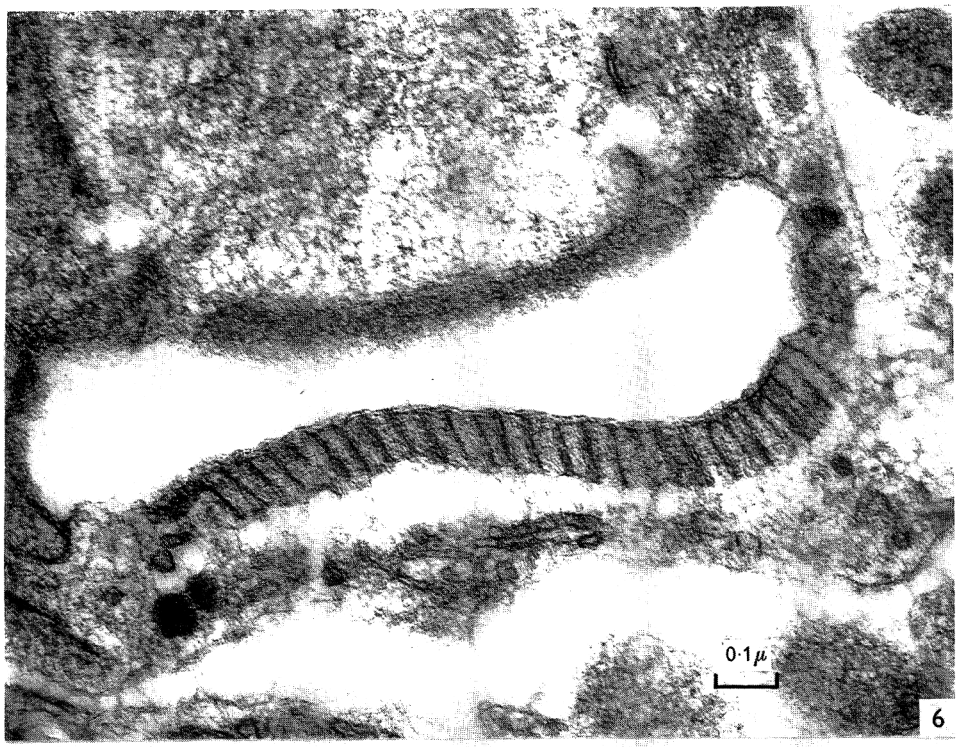
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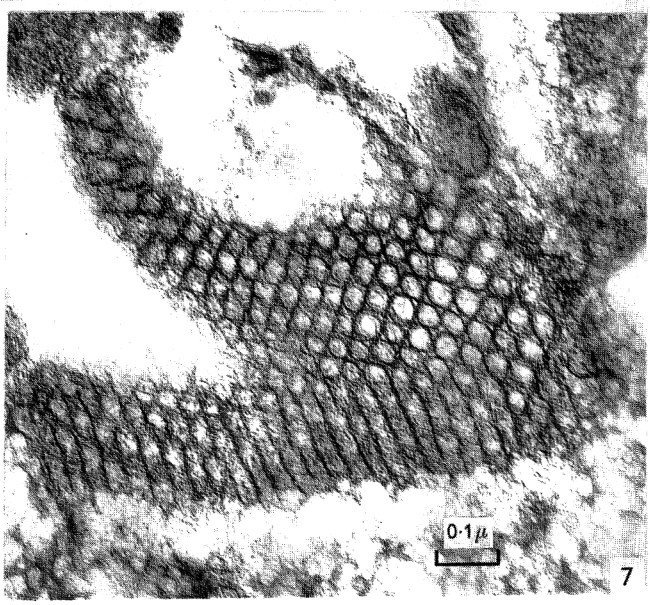
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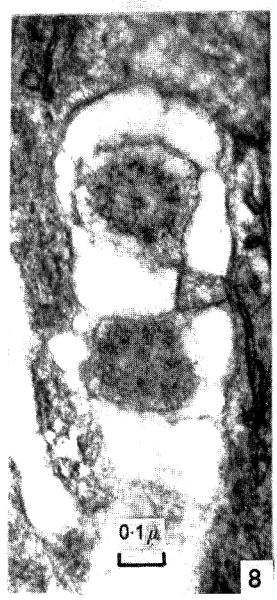
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EXPLANATION OF PLATES

PLATE 1

Fig. 1. Median transverse section of a mature sporangium of *Nowakowskiella profusa*. The zoospores are each associated with a nucleus and nuclear cap material and in some the fibrous body is visible. The flagella, cut longitudinally, transversely and obliquely are wrapped around each zoospore and lie outside the cell membranes along the cleavage spaces between them. Fragments of poorly preserved rhizoid material are embedded in the surrounding Cellophane substratum. × 11,000 (4000 × 2·75). Pl. 7880.*

PLATE 2

Fig. 2. Section of a sporangium of *N. profusa* in the plane of a dehiscence papilla. × 4400 (2000 × 2·2). Pl. 7885.

Fig. 3. *N. profusa*. Details of a dehiscence papilla. Dense granular material (*gm*) caps the spongy material (*sm*) which is in contact with the zoospores (*Z*). The sporangium wall (*SW*) shows outer, middle and inner zones. Poorly preserved rhizoid material (*Rh*) is embedded in the Cellophane substratum. × 15,000 (6000 × 2·5). Pl. 7927.

PLATE 3

Fig. 4. *N. profusa*. A small portion of a sporangium, showing details of several zoospores cut in different planes. Dark granules (*dg*), fibrous bodies (*Fb*), presumptive refractive globules (*Gp*), mitochondria (*M*), nuclei (*N*), and nuclear caps (*NC*) are distinguished throughout the section. The marked rectangle, referred to in the text, delimits an area with particularly numerous membranes. × 24,000 (8000 × 3). Pl. 7888.

PLATE 4

Fig. 5. *N. profusa*. A single zoospore with portions of others closely associated with its periphery. Flagella (*F*) cut in longitudinal section are outside the cell membrane (*cm*). The crescent-shaped nucleus (*N*) lies above a membrane-bounded electron-lucent region, the presumptive refractive globule (*Gp*), and this in turn adjoins a concave plate of tubules, the fibrous body (*Fb*), sectioned longitudinally in this preparation. Above the nucleus is a zone of densely granular material, the nuclear cap (*NC*), and embedded in its periphery are the mitochondria (*M*). Between the nuclear cap and the cell membrane is a cytoplasmic zone containing a few Golgi dictyosomes (*D*), some short profiles of endoplasmic reticulum (*ER*) and a few membrane-bounded dark granules (*dg*). This cytoplasmic zone has a mottled appearance. × 32,500 (10,000 × 3·25). Pl. 2833.

PLATE 5

Figs. 6, 7. *N. profusa*. The fibrous body in longitudinal and transverse section, showing structural details. × 80,000 (40,000 × 2). Pl. 7895, 7893.

Fig. 8. *N. profusa*. Detail of transverse sections of flagella and their surrounding spongy ground material. × 60,000 (30,000 × 2). Pl. 7897.

* This and similar numbers attached to the figure explanations refer to the numbers of the photographic plates stored in the electron-microscope laboratory, Botany School, University of Melbourne.

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Cereolysin: Production, Purification and Partial Characterization

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SUMMARY

A method is described for production of the extracellular haemolytic growth-product of *Bacillus cereus*. The lytic substance, called 'cereolysin', can be purified by fractional precipitation with ammonium sulphate, density-gradient electrophoresis and gel filtration. The purified material is a labile protein of molecular weight about 52,000. It has a very high order of haemolytic activity, is lethal for mice, and resembles streptolysin O in being inhibited by cholesterol, but differs from streptolysin O in other respects.

INTRODUCTION

Broth cultures of *Bacillus cereus* contain a powerful extracellular haemolysin which appears to have been little studied. We have investigated the nature and properties of this lytic product of bacterial growth in the belief that it might prove a useful reagent in studying the biochemistry of cell membranes. We were also curious to know whether the *B. cereus* lysin was related to other bacterial haemolysins which have been characterized, particularly those of Gram-positive cocci and clostridia.

METHODS

Organism. Bacillus cereus P2, a derivative of ATCC 12137, was kindly supplied by Professor M. R. J. Salton.

Measurement of haemolytic activity. The capacity of cereolysin to lyse washed rabbit red cells was measured as for staphylococcal alpha toxin (Bernheimer & Schwartz, 1963), but with 0·1% (w/v) gelatin instead of bovine serum albumin. One unit of haemolysin is defined as the smallest amount which liberates half the haemoglobin in the test red cell suspension in 30 min. at 37°.

Protein. Protein was estimated by reading extinction at 280 mμ in a Zeiss PMQ II spectrophotometer with optical cells of 10 mm. light path, and also by use of the Folin–Ciocalteu reagent (Colowick & Kaplan, 1957).

Specific activity. The specific activity of cereolysin is expressed as units of haemolysin per unit extinction at 280 mμ.

Inhibition tests. The capacity of lipids to inhibit the haemolytic activity of cereolysin was assayed by mixing decreasing concentrations of test material with a fixed amount (3 haemolysin units) of cereolysin, allowing the mixtures (1 ml. each) to stand for 10 min. at 20°, and adding 1 ml. 0·7% (v/v) washed rabbit red cells. After 30 min. at

37° the mixtures were centrifuged briefly, and the haemoglobin in the supernatant fluids estimated colorimetrically at 545 mμ. The tests were done in the presence of M/13-NaCl, M/15-phosphate (pH 7·0) and 0·05% (w/v) gelatin; 50% haemolysis was used as the endpoint of the titrations. The same conditions were used to estimate the inhibitory capacity of sera except that complete inhibition of haemolysis was used as endpoint.

Reagents. Crystalline trypsin and papain were purchased from Worthington Biochemical Corp., Freehold, N.J.; crystalline chymotrypsin from Armour and Co., Chicago, Ill.; pronase (grade B) from Calbiochem, Los Angeles, Calif.; phosphatidylcholine and diphosphatidylglycerol from Sylvana Co., Millburn, N.J.; phosphatidylserine and sphingomyelin from Applied Science Laboratories, Inc., State College, Pa.; cholesterol from Matheson Coleman and Bell, East Rutherford, N.J.

Production of cereolysin. Preliminary experiments showed that in spite of heavy growth *Bacillus cereus* P2 produced very little cereolysin (< 100 haemolysin units/ml.) when growing in chemically defined media, either the 16 amino acid medium of Proom & Knight (1955) or medium 687 of Puziss & Wright (1954). The same was true of a broth diffusate medium. In contrast, relatively high titres (500–3000 haemolysin units/ml.) were consistently obtained in meat-infusion peptone broth. To avoid using broth culture supernatant fluids as starting material for purification, and with the knowledge that substantial amounts of cereolysin were not formed during growth in defined media, the following technical compromise was evolved after much experimentation.

Each of eight 2-l. Erlenmeyer flasks, containing 300 ml. 1% (w/v) Neopeptone meat-infusion broth (Difco) per flask, was inoculated with 0·05–0·1 ml. broth culture of extinction 0·1 (650 mμ), and incubated for 16–17 hr at 37° on a rotary shaker operating at 180 cyc./min. After centrifugation the culture supernatant fluids were discarded, the deposited bacilli washed in 1200 ml. 0·025 M-phosphate (pH 6·0) and then suspended in 2400 ml. of solution containing 0·025 M-phosphate (pH 6·0), 1% (w/v) ammonium sulphate, 0·33% (w/v) glucose, and 17% (v/v) meat infusion. The suspension was distributed among eight 2-l. Erlenmeyer flasks and returned to the 37° rotary shaker for 3–5 hr. In these secondary cultures, very little growth occurred, less than a twofold increase in turbidity, but cereolysin appeared to the extent of 2500–5000 haemolysin units/ml., and occasionally more.

RESULTS

Purification of cereolysin

Cereolysin was routinely produced in the secondary cultures described in the preceding section. After centrifugation, the deposited bacilli were discarded and the supernatant fluid chilled, passed through a Millipore filter (0·45 mμ porosity) and the filtrate adjusted to pH 6·0 with (about 10 ml.) N-NaOH (stage 2, Table 1). All subsequent steps were done in the cold. The filtrate was approximately half-saturated with ammonium sulphate by dissolving in it 780–790 g. of solid $(NH_4)_2SO_4$. After standing overnight the very small amount of precipitate that had formed was recovered by centrifugation at 13,000 rev./min. for 30–45 min. and was collected in 32 ml. 0·4 saturated ammonium sulphate (Table 1, stage 3).

The suspended precipitate was centrifuged at 13,000 rev./min. for 15 min., the super-

natant fluid discarded, and the residue dissolved in 2 ml. 5% (v/v) glycerol in 0·025 M-phosphate, (pH 6). A small amount of insoluble material was removed by centrifugation and discarded (Table 1, stage 4).

The preparation was fractionated by electrophoresis in a linear density gradient (0–50%, v/v, glycerol) in 0·005 M-phosphate (pH 6·0), in the apparatus designed by Svensson (1960) and with the general conditions described earlier (Bernheimer, 1962). The distribution of material absorbing at 280 mμ along the length of the column is shown in Fig. 1. All the recoverable haemolytic activity (50–80% of the input activity) was present in the fractions comprising the narrow symmetrical peak. These fractions were pooled, and 4·5 g. ammonium sulphate added, with stirring, to each 10 ml. After standing for 1 hr. the precipitate was collected by centrifugation and dissolved in 2 ml. 5% (v/v) glycerol in 0·025 M-phosphate (pH 6·0; Table 1, stage 5).

Table 1. *Purification and recovery of cereolysin*

Stage		Volume (ml.)	Total haemolytic activity (units of haemolysin)	Specific activity (units of haemolysin per unit extinction at 280 mμ)	Recovery of activity (%)
1	'Secondary' culture supernatant fluid	2320	$10{\cdot}5 \times 10^6$	—	100
2	Filtrate	2290	$10{\cdot}3 \times 10^6$	2,400	98
3	Ammonium sulphate precipitate	32	$7{\cdot}2 \times 10^6$	89,000	69
4	Precipitate extracted with 0·40 saturated ammonium sulphate, dissolved in 5% glycerol in 0·05 M-phosphate (pH 6); supernatant fluid	2	$6{\cdot}1 \times 10^6$	220,000	58
5	Pooled fractions from density-gradient electrophoresis, precipitated with ammonium sulphate and dissolved in 5% glycerol at pH 6	2	$4{\cdot}8 \times 10^6$	1,500,000	46
6	Pooled active factcions of Sephadex G-100 effluent, precipitated with ammonium sulphate and dissolved in 5% glycerol at pH 6	2·3	$2{\cdot}8 \times 10^6$	1,900,000	27

The sedimentation coefficient of stage 5 (Table 1) cereolysin was estimated by the method of Martin & Ames (1961) by using a 5–25% (v/v) linear glycerol gradient with crystalline bovine serum albumin and crystalline beef liver catalase as standards. From the position of the peak of activity of cereolysin an $S_{20,w}$ of 3·7 was obtained; from this value the molecular weight of cereolysin was estimated to be about 49,000.

In density gradient electrophoresis, haemolytic activity in the narrow symmetrical peak (Fig. 1) was never congruent with the 280 mμ absorption curve but always fell along the right limb of the absorption peak while little or no activity was found on the left side. It follows that the sharp peak was not homogeneous; this conclusion was supported (*a*) by the results of acrylamide gel electrophoresis (analytical disc method) which revealed two major amido-black staining components, (*b*) by the schlieren patterns obtained in the analytical ultracentrifuge in which the material had resolved into two rather polydisperse components having approximate $S_{20,w}$ values of 3·4 and

1·8. The former value is in reasonably good agreement with that obtained by density gradient centrifugation, and it may be presumed to represent the active material.

Stage 5 (Table 1) material was further purified either by recycling in the density gradient electrophoresis apparatus or by filtration through cross-linked dextran beads (Sephadex G-100). Both procedures yielded preparations having the same, very high, specific activity ($1{\cdot}9 \times 10^6$ haemolysin units per unit of 280 mμ extinction) and the product is considered to be cereolysin in a substantially pure state. The data of Table 1 represent the best of a considerable number of purifications, in some of which the recovery of activity was lower than that shown.

Nature and properties of cereolysin

Cereolysin is a highly unstable substance. The half-life of haemolytic activity of fresh culture supernatant fluid was 1–3 min. at 50°, 45 min. at 40° and 1–3 days at 5°. It was labile over a wide range of pH values but lost activity less rapidly between

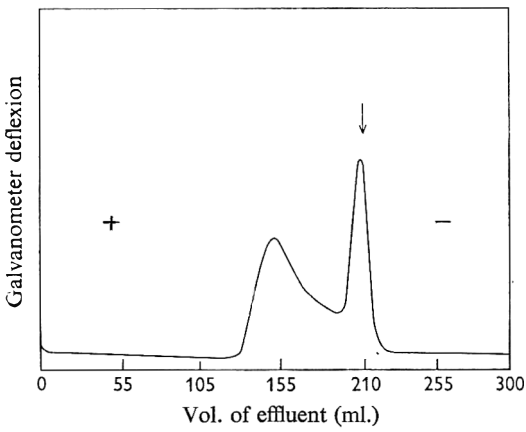

Fig. 1

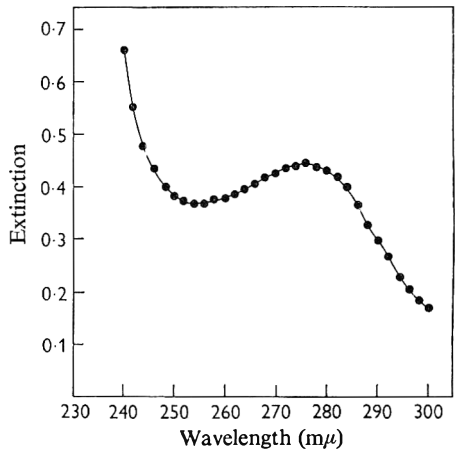

Fig. 2

Fig. 1. Ultraviolet absorption of column effluent after electrophoresis of stage 4 cereolysin (Table 1) in glycerol density gradient for 21 hr. at 400 V and 4 mA. Arrow indicates position of starting zone.

Fig. 2. Ultraviolet absorption spectrum of stage 6 cereolysin (Table 1).

pH 5·0 and 7·0 than outside this range. It was not stabilized by ethylenediamine tetra-acetate, cysteine or mercaptoethanol, nor could it be freeze-dried without loss of activity. However, in the presence of 5–20% (v/v) glycerol in phosphate buffer (pH 6·0) solutions maintained full activity for at least several days at 5° and for at least several weeks when they were slow-frozen and stored at −20°. For these reasons, cereolysin was handled and stored in the presence of glycerol (usually 5%, v/v), whenever practical.

The ultraviolet absorption spectrum of the purified haemolysin is typical of a protein (Fig. 2). The ratio of extinction at 280 mμ to protein, as estimated by phenol colour (Colowick & Kaplan, 1957) with crystalline bovine serum albumin as standard, was 1·8 extinction units/mg. protein. In spite of its lability, cereolysin was inactivated little or not at all by trypsin, chymotrypsin or papain, but appreciable loss of activity accompanied treatment with pronase (Table 2).

The molecular weight of cereolysin was estimated by gel-filtration according to the method of Andrews (1964) by using Sephadex G-100 (superfine) in a 9 × 405 mm. water-jacketed column, with bovine serum albumin and soy trypsin inhibitor as standards. The standard proteins were estimated in the effluent spectrophotometrically while cereolysin was estimated spectrophotometrically and by haemolytic assay. The results are shown in Table 3, which includes for comparison the molecular weights,

Table 2. *Effect of proteolytic enzymes on cereolysin activity*

Approximately 10,000 haemolysin units of cereolysin in 0·9 ml. 0·025 M-phosphate (pH 6) containing 0·5% (v/v) glycerol, *plus*	Percentage of control haemolytic activity after 30 min. at 20°
0·1 ml. 0·025 M-phosphate (pH 6)	100
10 μg. crystalline trypsin	100
100 μg. crystalline chymotrypsin	74
100 μg. crystalline papain in 0·1% (w/v) cysteine	100
100 μg. pronase	20
10 μg. pronase	35

Table 3. *Estimations of molecular weight of various haemolysins by gel-filtration*

	Molecular weight found	Property measured in order to localize peak
Cereolysin	54,000	haemolytic activity
Staphylococcal α-toxin	41,000	280 mμ absorption
Phospholipase C (*Clostridium welchii*)	31,000	egg-yolk turbidity

Table 4. *Lethality of cereolysin to mice*

Amount of cereolysin injected (units haemolysin)	Number of mice dead / Number of mice injected	Survival time of mice dying (min.)
10,000	3/4	2 to 7
5,000	4/4	17 to 25
2,500	1/4	52
1,250	0/4	—
None*	0/4	—

* Diluent control.

obtained by the same means, of two other haemolytic proteins. The agreement between the values for cereolysin of 54,000 by gel-filtration and 49,000 calculated from the sedimentation coefficient are well within the experimental error of the methods.

A sample of stage 6 cereolysin, estimated from its 280 mμ absorbance to contain 0·52 mg. protein, was dialysed against distilled water and analysed for amino acids by the method of Piez & Morris (1960) with the following results, expressed as g. amino acid residue/100 g. protein: cysteic acid 2·15; aspartic acid 12·65; threonine 4·78; serine 4·43; glutamic acid 10·2; proline 8·42; glycine 9·7; alanine 5·08; valine 4·47; half-cystine 0·31; methionine 1·38; isoleucine 3·74; leucine 4·47; tyrosine 3·12; phenylalanine 3·01; ammonia 1·30; lysine 5·73; histidine 1·55; arginine 3·48; hydroxyproline 9·10; glucosamine (?) tr; hydroxylysine 0·73; ornithine (?) 0·20. The total weight of amino acids detected in the chromatogram was 0·414 mg.

Lethality. Swiss mice of the Webster strain weighing about 25 g. were injected intravenously with 0·1 ml. stage 5 cereolysin diluted in 0·9% (w/v) NaCl containing 0·1% (w/v) gelatin. The results (Table 4) showed that about 5000 haemolysin units, or 1–2 μg. toxic protein, constituted a mouse lethal dose. The mice appeared to die of respiratory difficulty and their response to cereolysin resembled that occuring in mice injected with streptolysin O.

Inhibition by serum. The capacity of human sera to inhibit cereolysin haemolysis of rabbit red cells is shown in Table 5. Different serum specimens inhibited to different degrees, and no correlation with capacity to inhibit streptolysin O was evident.

Inhibition by lipids. Several lipids were tested for capacity to inhibit cereolysin haemolysis. Only cholesterol inhibited in low concentration (Table 6).

Table 5. *Inhibition of cereolysin haemolysis of rabbit red cells by human sera*

Serum	Highest dilution of serum completely inhibiting cereolysin haemolysis	Highest dilution of serum completely inhibiting streptolysin O (Todd units)
E	< 15	12
F	15	125
W	250	250
A	< 15	625
G	80	1250

Table 6. *Effect of lipids on cereolysin haemolysis of rabbit red cells*

Lipid	Concentration required to inhibit 2/3 of test-amount of cereolysin (μg./ml.)
Phosphatidylcholine (egg)	> 500
Phosphatidylcholine (beef)	100
Phosphatidylserine (brain)	250
Diphosphatidylglycerol (beef)	> 250
Sphingomyelin (brain)	> 500
Cholesterol	10

DISCUSSION

The results show that the cereolysin produced by *Bacillus cereus* P2 is one of the most potent *in vitro* haemolytic agents. In purified form and under the conditions described it causes gross lysis of test red cells in a concentration of about 1 $\mu\mu$g./ml. Other haemolytic agents known to have a comparable potency are streptolysin S with a specific activity calculated to be 2×10^6 haemolysin units/mg. dry weight (Koyama & Egami, 1963) and staphylococcal β-toxin having a specific activity of $1{\cdot}2 \times 10^6$ haemolysin units/mg. protein (Wiseman, 1965). The latter lysin has been shown (Doery, Magnusson, Gulasekharam & Pearson, 1965) to be a phospholipase C with a preference for sphingomyelin and lysophosphatidylcholine. During the present work we have been aware of the possibility that cereolysin might be a similar enzyme, but purified cereolysin produced no turbidity in diluted egg yolk and therefore appears to be completely free from phospholipase C activity. This finding is in agreement with those of Johnson & Bonventre (1966) who reported that *B. cereus* phospholipase C is neither haemolytic nor lethal.

The general behaviour of cereolysin indicates that it is a protein, and the results of ult racentrifugal and gel-filtration experiments show that its molecular weight is close to 50,000. However, the presence in our most active material of 9·1% hydroxyproline, as well as a little hydroxylysine, is an unexpected finding, and implies either (*a*) that cereolysin is a protein of unusual amino acid composition somewhat resembling that of collagen, or (*b*) that our most active preparation is still grossly impure and consists largely of gelatin-like material derived from meat infusion present in the medium in which the cereolysin was originally produced. If conditions can be found for preparing cereolysin in adequate amounts in the absence of meat infusion, it should be possible to decide which explanation is correct.

Cereolysin is similar to streptolysin O in being inhibited by cholesterol in low concentration, in giving rise to 'rapid' haemolysis (as distinct from lysis preceded by an induction period of the kind seen with streptolysin S, for example), and in its effects on mice; its mode of action probably resembles very closely that of streptolysin O. Nevertheless it differs from streptolysin O in not being activated by cysteine and other sulphydryl compounds. Although human sera inhibited cereolysin-induced haemolysis to various degrees, there was no correlation between the cereolysin-inhibiting and the streptolysin O-inhibiting capacities of the sera, and therefore there is no suggestion of an immunological relationship between the two lytic agents.

The function that cereolysin may fulfil for the organism which produces it is almost wholly obscure, but as much can be said for many other extracellular products of microbial growth. A clue might perhaps be provided by an understanding of the determinants of its production.

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Sensitivity of Streptomycetes to Antibiotics as a Taxonomic Character

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SUMMARY

A procedure for testing the sensitivity of streptomycetes to antibiotics is described. Eight antibiotics, at various concentrations, were incorporated into filter-paper discs and by selection of suitable concentrations results of diagnostic value were obtained. Streptomycetes previously grouped together by using other criteria had identical or very similar antibiotic sensitivity patterns. It is considered that the reactions of streptomycetes to a range of antibiotics would be of value for description of species and studies of overall similarity.

INTRODUCTION

The ability of species of streptomycetes to produce a certain antibiotic or antibiotics is usually recorded in their descriptions. Krassilnikov (1958, 1960) emphasized the importance of antibiotic production as a taxonomic character and considered that it was one of the most stable attributes of streptomycetes; other workers, such as Waksman (1961), did not regard it as a significant criterion. Its value is questioned because some species can produce several different antibiotics and different species can sometimes produce the same one. In contrast, the sensitivity of streptomycetes to antibiotics has not often been considered in taxonomic studies, although Waksman (1961) included it in a list of characters which he regarded as important in the taxonomy of the genus *Streptomyces*.

Filter-paper discs impregnated with antibiotics at known concentrations have been used to test the sensitivity of streptomycetes and other actinomycetes. Waksman & Lechevalier (1953) found nearly all the streptomyces species they tested were sensitive to the antibacterial antibiotics used, but none was inhibited by antifungal antibiotics. Okami (1956), in a study of strains in the *Streptomyces lavendulae* group, tested their sensitivity to five antibiotics, each incorporated into paper discs at a concentration of 100 μg./ml. The results allowed a distinction between some of the closely related strains of this group. By the same method Okami, Hashimoto & Suzuki (1960) tested the reactions of a wide range of streptomycetes to 22 antibiotics. They noted that antibiotic producers generally resisted their own antibiotics and showed a characteristic spectrum of sensitivity. It was considered that the test could be used as a guide to the identification of species of the genus *Streptomyces*. Burkholder, Sun, Anderson & Ehrlich (1954), considering criteria for the description of species in the genus, concluded that antibiotic-sensitivity results could provide useful information for the characterization of strains and perhaps species. The sensitivity of other genera of actinomycetes to antibiotics has occasionally been considered. Lechevalier, Solo-

torovsky & McDurmont (1961), in a study of the genus *Micropolyspora*, included sensitivity to several antibiotics in a range of tests used to describe new species. Similar tests were used by Cross, Lechevalier & Lechevalier (1963) for the description of Microellobosporia species. In an investigation of overall similarity of actinomycetes, which included many streptomycetes, Hill & Silvestri (1962) allocated 16 code marks to antibiotic sensitivity tests. Six antibiotics incorporated into paper discs at various concentrations were used. The results obtained with four of the antibiotics were considered to be of diagnostic significance. Those obtained with neomycin at 125 μg./ml. were used in the construction of a diagnostic key. These authors considered that a similar reaction by different strains to an antibiotic indicated that they shared part or all of a limiting metabolic pathway.

Methods other than impregnated paper discs have occasionally been used to test the sensitivity of actinomycetes. An investigation of the overall similarity of oral actinomycetes, mainly in the genus *Actinomyces*, was made by Melville (1965). The reactions of strains to five antibiotics, each at a concentration of 10 mg./100 ml., was tested by placing antibiotic solutions in ditches cut in the solid medium. All strains were inhibited by each antibiotic, so that the results were of no diagnostic value. Krassilnikov & Agre (1964) cross-streaked known antibiotic-producing strains of streptomycetes with Thermopolyspora strains and noted the inhibition of the latter. Strains previously grouped together on their morphological characteristics generally had similar patterns of inhibition. A similar approach was made by Pridham & Lyons (1965), who examined the sensitivity of streptomycetes to streptomycin by cross-streaking them with a *Streptomyces griseus* strain known to produce this antibiotic.

Although sensitivity of streptomycetes to antibiotics has occasionally been used as a taxonomic criterion, little information about its reliability or significance is available. The present work was designed to outline a testing procedure, examine its reliability and assess the taxonomic significance of the results which it provided.

METHODS

Culture medium. All tests were made by using a glycerol + nitrate medium of composition (g./l.): glycerol, 30·0; $NaNO_3$, 2·0; K_2HPO_4, 1·0; $MgSO_4.7H_2O$, 0·5; KCl, 0·5; $FeSO_4.7H_2O$, 0·01; Oxoid agar no. 3, 12·0; de-ionized water 1000 ml.; after autoclaving the medium was at pH 7·0. Shinobu (1958) found that streptomycetes grew well on this medium, glycerol being a good source of carbon and $NaNO_3$ a generally available source of nitrogen. It also has the advantage of being clear when solidified, thus facilitating the reading of results of antibiotic-sensitivity tests.

Antibiotics. The antibiotics used were streptomycin sulphate (Glaxo), erythromycin (Lilly), neomycin sulphate (Boots), polymyxin B sulphate (Burroughs Wellcome), chlortetracycline hydrochloride (Cyanamid), chloramphenicol (Allen and Hanburys), viomycin sulphate (Dista), sodium novobiocin (Glaxo). Solutions of chloramphenicol were made in de-ionized water and sterilized by autoclaving. All other antibiotics were obtained as sterile powders and solutions were made in sterile water. Solutions were used immediately after their preparation to soak the filter-paper discs.

Preparation of spore suspensions. The organisms were grown on slopes of oatmeal agar (Waksman, 1961) at 25° for 14 days. Spores on the mature cultures were removed with an inoculation loop to 5 ml. sterile water. The suspensions were used within 1 hr of preparation to seed medium. Spore suspensions were shaken vigorously and

0·01 ml. samples were added to 15 ml. samples of melted medium at 45°, which was then poured into dishes and allowed to set.

Preliminary examination of testing procedure. Three methods for testing sensitivity of streptomycetes to antibiotics were examined.

(i) Antibiotics were incorporated into the culture medium and test strains were streaked on to the surface. Growth of streptomycetes on medium containing antibiotics was then compared with that on control medium without antibiotics. A major difficulty was encountered when reading the results for in many cases the growth of streptomycetes on medium containing antibiotics was uneven.

(ii) Antibiotics incorporated in Oxoid 'Multodisks' were also used. Multodisks were laid on the surface of medium seeded with streptomycetes. The results indicated that the concentrations of antibiotics in these discs were too high. Many of the antibiotics inhibited all test strains and inhibition zones were often so large that interference occurred between zones around adjacent discs.

(iii) The antibiotics listed above were incorporated into filter-paper discs. The results of this experiment indicated that it was the most satisfactory of the methods and it was selected for more detailed appraisal.

Incorporation of antibiotics into filter-paper discs. Discs of filter paper (Whatman no. 1), 5 mm. diameter, were soaked in antibiotic solutions at chosen concentrations for 2 min. The discs were then removed to sterile dishes and rapidly freeze-dried under low vacuum (0·2 torr) over P_2O_5 for 90 min. The dried discs were stored in sealed containers at 4° until use.

Selection of a suitable period of incubation. Experiments were made to select an incubation period after which clear stable inhibition zones could be obtained. Fourteen streptomycetes were tested against antibiotics in filter-paper discs. Plates were incubated at 25° for 14 days, the presence of inhibition zones being noted at 2-day intervals.

Selection of suitable concentrations of antibiotics. Experiments were made to select antibiotic concentrations which would inhibit some, but not all the streptomycetes used, thus providing results of diagnostic value. After preliminary tests with fourteen streptomycetes, the following concentrations were selected: streptomycin sulphate, 3 μg./ml.; erythromycin, 150 μg./ml.; neomycin sulphate, 3 μg./ml.; polymyxin B sulphate, 60 μg./ml.; chlortetracycline hydrochloride, 50 μg./ml.; chloramphenicol, 300 μg./ml.; viomycin sulphate, 3 μg./ml.; sodium novobiocin, 100 μg./ml. (values expressed refer to wt of salt used).

Discs soaked in antibiotic solutions at these concentrations were then tested against 76 isolates, the presence and size of inhibition zones after 7 days at 25° being noted.

Assessment of the taxonomic significance of the results. After selection of a suitable testing procedure and the standardization of the conditions for its application, the taxonomic value of the results obtained was examined. Experiments were made to compare the sensitivity patterns of strains which were considered to be identical or similar. Two cultures of each strain were grown on oatmeal agar; from these, two spore suspensions were made and used to prepare two replicate samples of seeded medium. Each replicate was tested against discs soaked in solutions of the antibiotics at chosen concentrations. Results were recorded after incubation for 7 days.

Several streptomyces species were studied by Flaig & Kutzner (1960). By reference to the colour characteristics of cultures on oatmeal agar, they recognized ten groups. Strains within a group were subgrouped on their morphological characteristics and

the results of various physiological tests, each subgroup being numbered. Several strains, which had been placed in the same subgroup and therefore considered to be identical, were selected for assessment of the taxonomic significance of antibiotic-sensitivity tests in the present work. Some named strains not studied by Flaig & Kutzner, but belonging to species included in their scheme, were also used.

In addition, several unnamed strains were used in these tests. These were isolated from a forest soil at Freshfield, Lancashire, by using the selective isolation method of Williams & Davies (1965). A preliminary grouping of these isolates was made by using a restricted number of criteria; the procedures used were chosen from those described by Shirling & Gottlieb (1966).

All colour determinations were made in conditions of standard illumination with strains grown on plates of yeast extract+malt extract agar (Pridham *et al.* 1956/57) incubated for 21 days at 25°. The colour of the spores *en masse* was determined by using the colour wheels of Tresner & Backus (1963). These consisted of colour chips arranged in seven colour series (or wheels). The chip with a colour most nearly matching that of spore mass was noted. The colour of substrate mycelium was determined, after the removal of surplus agar, by comparing the reverse side of colonies with a colour chart composed by Prauser (1964). The tab with a colour nearest to that of the dominant colour of the mycelium was noted. When soluble pigments were present, their colour was assigned to one of the following groups: red, orange, yellow, green, blue, violet. The capacity of isolates to produce a 'melanin' pigment was determined by growing them on slopes of Difco peptone iron agar. The presence or absence of a black pigment in this medium was noted after 4 days at 25°.

On the basis of these results, isolates with similar characteristics were grouped together. The antibiotic sensitivity of strains comprising several of these groups was then studied.

RESULTS

Selection of a suitable period of incubation

The results of these tests indicated that little change in the readings occurred after 7 days of incubation. Occasionally very weak inhibition zones became obscured by overgrowth of the streptomycetes during the 7-day period, but most strains needed

Table 1. *The reactions of 76 Streptomyces isolates to antibiotics in filter-paper discs*

Antibiotics	Conc. of antibiotic solutions (μg./ml.)	Isolates not inhibited (%)	Isolates with doubtful inhibition (%)	Isolates with inhibition zone* < 1 mm. (%)	Isolates with inhibition zone 1–4 mm. (%)	Isolates with inhibition zone > 4 mm. (%)
Streptomycin sulphate	3	87	0	3	9	1
Erythromycin	150	42	3	8	33	14
Neomycin sulphate	3	28	5	25	42	0
Polymyxin B sulphate	60	61	8	21	10	0
Chlortetracyline HCl	50	50	3	5	42	0
Chloramphenicol	300	60	5	1	29	5
Viomycin sulphate	3	74	0	1	25	0
Novobiocin sodium	100	16	1	1	40	42
Overall %	—	52	3	8	29	8

* Figures given are the radii of the inhibition zones.

Table 2. *Antibiotic-sensitivity spectra of Streptomyces species studied by Flaig & Kutzner (1960)*

Streptomyces species	Code numbers of Flaig & Kutzner	Sub-groups of Flaig & Kutzner	Streptomycin sulphate (3 μg./ml.)	Erythromycin (150 μg./ml.)	Neomycin sulphate (3 μg./ml.)	Polymyxin B sulphate (60 μg./ml.)	Chlortetracyline hydrochloride (50 μg./ml.)	Chloramphenicol (300 μg./ml.)	Viomycin sulphate (3 μg./ml.)	Sodium novobiocin (100 μg./ml.)
S. griseus	H26	I/7	−	−	±	−	+	−	−	++
S. griseus	H81		−	−	±	−	+	−	−	++
S. griseus	H96		−	−	±	−	+	−	−	++
S. californicus	H14	I/12a	−	−	−	−	+	−	−	++
S. purpureochromogenes	H37		−	−	−	−	+	−	−	++
S. lipmanii	H30	I/21	−	−	?	−	+	+	−	+
S. setonii	H71		−	−	+	−	+	+	−	+
S. microflavus	H32		−	−	−	−	+	+	−	+
S. annulatus	H56		−	−	−	−	+	+	−	+
S. albidus	H55		−	−	?	?	+	?	−	+
S. nigrificans	H65		−	−	?	?	+	+	−	+
S. albidoflavus	H6	I/24	−	−	+	−	+	−	−	+
S. coelicolor	H16		−	−	±	±	+	−	−	+
S. phaeochromogenes	H36	II/49	±	+	+	+	+	−	+	++
Rimosus series (Baldacci)	H109		±	±	+	+	+	−	+	++
S. xanthophaeus	B290	III/20	−	+	−	−	−	+	−	++
S. lavendulae	H29	III/21	−	++	−	−	−	+	−	+
S. lavendulae	·	NCIB 9840	−	++	−	−	−	+	−	+
S. lavendulae	·	C.B.S.	−	++	−	−	−	±	−	+
S. halstedii	H28	VII/13	−	+	+	+	−	−	−	−
S. griseolus	H25		−	+	+	+	−	−	−	−
S. reticuli	H38		−	+	+	+	−	−	−	−
*S. coelicolor**	H83	VII/79	−	+	+	±	−	−	+	−
S. tyrosinaticus	H4		−	+	+	±	−	−	+	−
Violaceus series (Baldacci)	H88		−	+	+	±	+	−	+	−
S. hirsutus†	K924	X/1	−	+	+	±	+	++	−	++
S. hirsutus†	E193	X/4	−	+	+	+	+	++	−	++
S. prasinus†	K1075	X/2	−	+	+	±	+	++	−	++
Unidentified isolate	K1029		−	+	+	?	+	?	−	++
Unidentified isolate	K1027		−	+	+	−	+	+	−	++

− = no inhibition, ? = doubtful inhibition, ± = inhibition zone < 1 mm., + = inhibition zone 1–4 mm., ++ = inhibition zone > 4 mm.

* Considered by Kutzner & Waksman (1959) to be *Streptomyces violaceoruber*.

† Identified by Hütter (1962).

this time to form clearly defined zones. Therefore, in all subsequent experiments, readings were taken after an incubation period of 7 days.

Selection of suitable concentrations of antibiotics

The results of these tests are given in Table 1. The figures indicate that the antibiotics had a differential effect on the streptomyces isolates. Results of potential diagnostic value were obtained, without reference to small differences in the sizes of inhibition zones. Analysis of the 608 streptomycete/antibiotic interactions showed that in 52% no inhibition occurred, in 45% there was inhibition and in 3% zones were too indistinct for a decision to be reached.

Table 3. *Characteristics used to define four groups of Streptomyces isolates from soil*

Group	Colour of spores (name and no. of nearest matching tab on colour wheel)*	Colour of substrate mycelium (no. of nearest matching tab on colour chart)†	Colour of soluble pigment	Production of melanin pigment
F1	4 li; brownish-grey	Coo7r	·	–
F2	5 cb; greyish-yellowish pink	Oc4r	Yellow	–
F3	3 fe to b; light brownish-grey to white	Co5b	Yellow	+
F4	5 ih; brownish-grey	Coo6s	Red-orange	+

* Tresner & Backus (1963).
† Prauser (1964).

The inhibition zones produced by the eight antibiotics did not share the same features. Sodium novobiocin gave large zones (42% over 4 mm. diam.) with distinct margins. Zones produced by polymyxin B sulphate were small but were generally stable, with a clearly defined edge. The majority of zones produced by the other antibiotics were of moderate size, between 1 and 4 mm. diameter. Those produced by streptomycin sulphate, neomycin sulphate and viomycin sulphate had clearly defined edges, but erythromycin, chlortetracycline hydrochloride and chloramphenicol often gave zones with poorly defined limits, making exact measurement difficult.

Assessment of the taxonomic significance of the results

The results obtained with isolates previously studied by Flaig & Kutzner (1960) are given in Table 2. Strains in the same subgroup generally had identical or very similar antibiotic-sensitivity spectra. Identical results were obtained for three *Streptomyces griseus* strains. *Streptomyces californicus* and *S. purpureochromogenes* had identical spectra and differed from *S. griseus* only in their reaction to neomycin sulphate; Waksman (1961) included *S. californicus* in his *griseus* series. Species in subgroup I/21 had similar spectra, but some differences in reactions to neomycin sulphate and polymixin B sulphate were observed. The reactions of the *S. lavendulae* strains and *S. xanthophaeus* differed only in the size of zone produced; *S. xanthophaeus* has many points of similarity to *S. lavendulae*, being differentiated mainly by its production of a yellow soluble pigment. The strains in subgroup VII/79 all produced

Table 4. *Antibiotic-sensitivity spectra of Streptomyces isolates from soil*

Group	Strain	Strepto-mycin sulphate (3 μg./ml.)	Erythro-mycin (150 μg./ml.)	Neomycin sulphate (3 μg./ml.)	Polymixin B sulphate (60 μg./ml.)	Chlortetra-cycline hydro-chloride (50 μg./ml.)	Chloram-phenicol (300 μg./ml.)	Viomycin sulphate (3 μg./ml.)	Novobiocin sodium (100 μg./ml.)
F1	a	±	±	+	±	−	?	±	+ +
	b	±	±	+	±	−	±	±	+ +
	c	±	±	+	±	−	?	±	+ +
	d	±	±	+	±	−	?	±	+ +
	e	±	+	+	±	−	±	±	+ +
F2	a	+ +	+	+	±	±	+	±	+ +
	b	+ +	+	+	±	±	+	±	+ +
		+ +	+	+	±	+	+	+	+ +
	d	+	+	+	±	±	+	+	+ +
	e	+ +	+	+	±	±	+	±	+ +
F3	a	−	±	+	±	−	−	?	+ +
	b	−	?	+	±	−	−	±	+ +
	c	−	?	+	±	−	−	±	+ +
	d	−	±	+	±	−	−	+	+
	e	−	±	+	±	−	−	±	+
	f	−	±	+	±	−	−	?	+ +
	g	−	?	+	±	−	−	+	+
F4	a	−	+ +	−	−	±	+	−	+ +
	b	−	+ +	−	−	±	±	−	+ +
	c	−	+ +	−	−	±	+	−	+ +
	d	−	+ +	−	−	?	±	−	+ +
	e	−	+ +	−	−	±	?	−	+ +
	f	−	+ +	−	−	±	±	−	+ +

a litmus-like pigment; the strain from Baldacci's 'violaceus' series differed from the others in its reaction to chlortetracycline hydrochloride. All strains in group X, which formed green spores, had a similar reaction. The inhibition patterns of *S. hirsutus* and *S. prasinus* were identical; these two species have many features in common and were distinguished by Hütter (1962) on the ornamentation of the surface of their spores.

The characteristics used to define four groups of Streptomyces isolates from soil are shown in Table 3. The antibiotic-sensitivity patterns of strains from each of these groups are given in Table 4. It is evident that isolates grouped on their colour characteristics and melanin reaction had very similar antibiotic-sensitivity spectra. The only differences in the reactions of strains within a group were in the sizes of zone produced. Results from the replicate tests of all strains are given in Table 5, and it is evident that replication was generally good.

Table 5. *Results from the replicate testing of Streptomyces with antibiotics in filter-paper discs*

Antibiotics	Conc. of antibiotic solutions (μg./ml.)	Replicate pairs of strains having the same reaction (%)	Replicate pairs of strains having a different reaction (%)
Streptomycin sulphate	3	100	0
Erythromycin	150	90	10
Neomycin sulphate	3	97	3
Polymyxin B sulphate	60	95	5
Chlortetracycline HCl	50	92	8
Chloramphenicol	300	97	3
Viomycin sulphate	3	97	3
Novobiocin sodium	100	95	5

DISCUSSION

Tests used for the description of microbial taxa should satisfy the following requirements. The results should be unambiguous, being recorded as 'positive' or 'negative' whenever possible. The replication of results obtained under standardized conditions should be good, the test not being too sensitive to small variations in experimental procedure. Finally, it is essential that the results obtained should have diagnostic value. The potential value of antibiotic-sensitivity patterns as a taxonomic character for streptomycetes can be assessed from the results presented here.

By using each antibiotic at a suitable concentration, results of diagnostic value were obtained without considering the exact sizes of the inhibition zones. When diagnosis is based on a positive or negative response of the test micro-organisms, rather than small differences in size of inhibition zones, good replication of results is more easily attained; many factors can influence the size of inhibition zones. Also the reaction of the streptomycetes to some of the antibiotics used resulted in zones with ill-defined edges, making accurate measurement of size difficult.

Some workers (e.g. Krassilnikov & Agre, 1964; Pridham & Lyons, 1965) studied the sensitivity of streptomycetes to antibiotics by observing their reactions when grown in the presence of organisms known to produce antibiotics. When this method is used, no control over the concentrations of the antibiotics is possible and it must be remem-

bered that some strains produce more than one antibiotic. The use of pure preparations of antibiotics would seem preferable.

The antibiotic-sensitivity patterns of streptomycetes correlated well with other taxonomic information. Strains previously grouped together by using a variety of other criteria had similar reactions to the antibiotics. Burkholder *et al.* (1954) and Okami (1956) found that sometimes strains which had been placed in the same species had different antibiotic-sensitivity patterns. This was occasionally noted in the present work, but differences were usually only in reactions to one or two antibiotics. Also it is unfortunately true that strains given the same name do not always have the same characteristics.

The results of detailed co-operative studies, reported by Gottlieb (1961) and by Küster (1961), indicated that many criteria previously used in descriptions of streptomyces species were unreliable. There is therefore a need for new ones to replace these. More reliable criteria are also needed if studies of overall similarity, using large numbers of characters, are to be successfully applied to the genus *Streptomyces* and other actinomycete genera. The results obtained here with antibiotic discs were of taxonomic value and could be included with other criteria for the characterization of species of the genus *Streptomyces*. The inclusion of other antibiotics at concentrations selected to give diagnostic results might increase the value of the results obtained.

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